# GENETIC AND EPIGENETIC VARIATION OF TYPICAL GRASSLAND SPECIES – HABITAT SPECIFIC PROCESSES AND IMPLICATIONS FOR CONSERVATION

DISSERTATION ZUR ERLANGUNG DES DOKTORGRADES
DER NATURWISSENSCHAFTEN (DR. RER. NAT.)
DER FAKULTÄT FÜR BIOLOGIE UND VORKLINISCHE MEDIZIN
DER UNIVERSITÄT REGENSBURG

VORGELEGT VON

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AUS
HAMBURG

IM JAHR
2020

PROMOTIONSGESUCH WURDE EINGEREICHT AM:

20.02.2020

DIE ARBEIT WURDE ANGELEITET VON:

PROF. DR. CHRISTOPH REISCH

UNTERSCHRIFT: .....

ELLEN PAGEL

*Für meine Familie*

*„Die Natur muß gefühlt werden, wer nur sieht und abstrahirt, kann ein Menschenalter, im Lebensgedränge der glühenden Tropenwelt, Pflanzen und Thiere zergliedern, er wird die Natur zu beschreiben glauben, ihr selbst aber ewig fremd sein.“*

Alexander v. Humboldt, 1810

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## Declaration of Manuscripts

### Submitted manuscripts included in the thesis:

**Chapter Two** was submitted with the thesis' author as lead author:

**Pagel E**, Lehmair T A, Poschlod P & Reisch C. Genetic variation of typical plant species in ancient and recent oat-grass meadows: investigating the effect of land use history, landscape and vegetation structure. Under review at *Journal of Applied Ecology*.

**Chapter Three** was submitted with the thesis' author as co-lead author:

Lehmair T A, **Pagel E**, Poschlod P & Reisch C. Conservation of intraspecific diversity in litter meadows – the role of habitat age, landscape configuration and environmental factors. In revisions at *Conservation Genetics*.

**Chapter Four** was submitted with the thesis' author as lead author:

**Pagel E**, Poschlod P & Reisch C. Habitat matters – strong genetic and epigenetic differentiation among populations of *Linum catharticum* from dry and wet grasslands. In revision at *Ecology and Evolution*.

### Published and submitted manuscripts not included in the thesis:

Lehmair T A, **Pagel E**, Poschlod P & Reisch C. The impact of habitat age, landscape structure, habitat quality, and population size on the genetic variation of typical calcareous grassland plant species. Submitted to *Landscape Ecology*.

Krickl P, Gilck F, Lehmair T A, **Pagel E**, Rubanschi S, Simmel J, Straubinger C, Fischer S & Poschlod P. Die Ausstellung „Ein Leben für die Botanik – Einzigartiger Zeitzeuge des Wandels von Lebensraum- und Artenvielfalt". *Hoppea, Denkschr. Regensb. Bot. Ges*. 79 (2018): 87 – 112

# ACKNOWLEDGEMENTS

First and foremost, I would like to express my deepest gratitude to Christoph Reisch and Peter Poschlod. They gave me the opportunity to develop my skills in the “Grassland”-project and warmly welcomed me into the ‘deepest South of Germany far away from the ‘cold northern German winds’. Christoph calmly guided me through the good and the more difficult times. He supported me in developing my knowledge in molecular ecology, conservation genetics and many other things. I value his mentorship highly and am very glad he gave me so many opportunities to grow. I also want to express my gratitude to Peter, for his enthusiasm and sharing is great knowledge freely and for his feedback on the manuscripts and reports during my PhD.

Special thanks to my colleagues from the “Grassland”-project Theresa Lehmair, Cornelia Straubinger, Eva Wagner, Lina Begemann & Patrizia Krickl, who helped building the framework for this thesis. A special and deep thank you is due to Lina, who was a great and necessary emotional and professional support during the last months of my thesis.

Thanks also to Petra Schitko for guided me in the lab and providing all the materials and assistance during analysing my data. Sabine Fischer taught me the art of using ArcGIS and helped me a lot during the digitization of the endless historic maps. Thank you for your patience and answering even the silly questions. I also want to thank the Theoretical Ecology group led by Florian Hartig for their advice and help with analysing my data and R in general. All other colleagues I thank for creating such a great working environment and making me feel at home at the University of Regensburg.

Many thanks also to the Bachelor and Master students that helped with the realization of this project, either as part of their thesis or as a student helper: Simone Fabian, Lukas Feichtmeier, Christina Fischer, Helene Kleijn, Laura Krassini, Christina Manhart, Franziska Parzefall, Marco Peschke, Laila Pfättisch, Annika Schmid, Leonie Schulz, Sebastian Segieth, Jakob Speigl & Vivien Tkcocz.

My family and friends have always been there for me in every possible way and supported me through the good and the bad times of my PhD years. My parents were always the support in the background and supported my decisions and encouraged me to study in many different places and gather the experiences I made during my Bachelor, Master and PhD studies. Special gratitude is due to my late grandfather, who has first installed an interest in nature in me and build the starting block of me as a biologist and ecologist.

# SUMMARY

European semi-natural grasslands are among the most species rich habitats in the world. They were historically shaped by anthropogenic land use and developed their species and genetic variation alongside the agricultural practices of the time. The lasting effects of historic processes can be observed until today, however recent global developments are affecting this diversity in an unprecedented intensity and frequency.
The present study aims to investigate the effects of land use history, landscape structure as well as vegetation and habitat characteristics on genetic and epigenetic variation of common grassland species from different grassland habitats.

**Chapter One** introduces the scientific context the present thesis is placed in. Processes shaping biodiversity, the characteristics of the grassland ecosystems studied in this thesis and the history of grassland ecosystems in general is reviewed. Finally, grassland habitats are discussed in the context of biodiversity and conservation.

In **Chapter Two** and **Three** the influence of land use history, landscape structure and habitat quality on the genetic variation of typical grassland species from oat-grass and litter meadows was investigated. We used Amplified Fragment Length Polymorphism (AFLP) analyses to estimate genetic diversity and differentiation among populations from ancient and recent grasslands.

**Chapter Two** describes the genetic variation patterns of three typical grassland species (*Dactylis glomerata* L., s. l., *Heracleum sphondylium* L. & *Trifolium pratense* L.), studied in oat-grass meadows. Each species was influenced by different explanatory variables, but most interestingly current landscape structure and habitat quality, i.e. the amount of litter, influenced genetic diversity in this grassland habitat. Historic landscape structure and land use history were of minor interest in this comparably recently established grassland type.

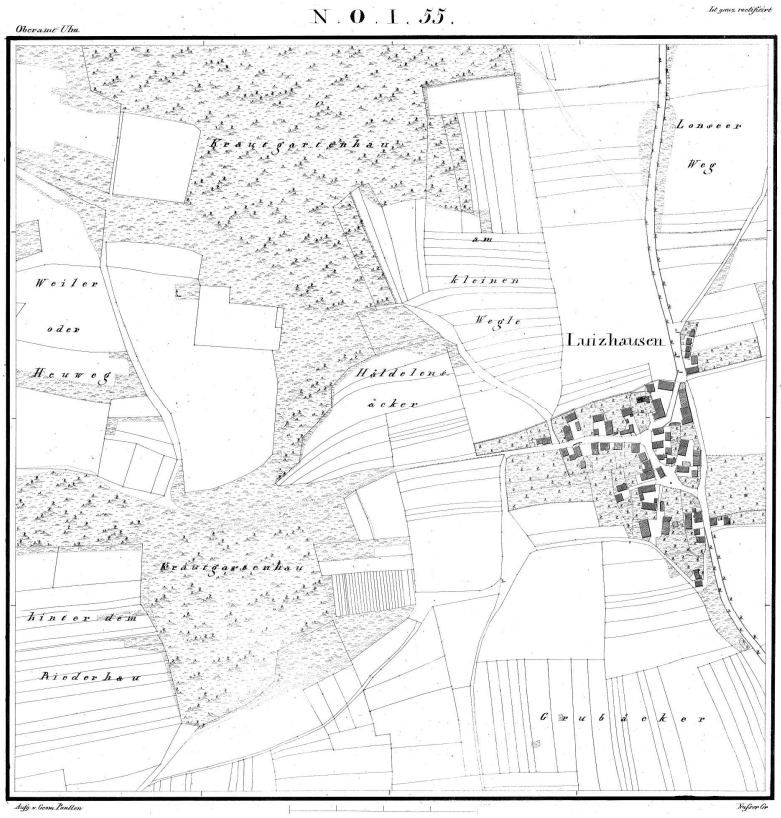
Similarly, **Chapter Three** deals with the genetic variation in litter meadow plant species (*Angelica sylvestris* L., *Filipendula ulmaria* (L.) MAXIM & *Succisa pratensis* MOENCH). Historic as well as recent gene flow patterns influenced genetic variation of the species in this ecosystem, highlighting the current importance of historic processes. However, the most important process, from a conservation point of view, was the extensive gene flow produced by seed transfer via mowing machines.

Extending the study of the intraspecific variation in grassland ecosystem to the epigenetic level, the genetic as well as epigenetic variation in *Linum catharticum* L. from different contrasting habitats is presented in **Chapter Four**. Combining AFLP and Methylation Sensitive Amplification Polymorphism (MSAP) analyses, large differences among populations from wet litter meadows and dry calcareous grasslands were observed, which could not be explained by geographic distance alone, but rather by the different local habitat conditions. This result highlights the impact of local environmental conditions on the genetic as well as the epigenetic level, which likely led to the development of different ecotypes.

In **Chapter Five** the results of the three main chapters are reviewed in the context of nature conservation. Historic as well as current gene flow processes were found to be important determinants of current genetic variation in common grassland species. Additionally, various local environmental factors contributed to the genetic as well as epigenetic variation patterns.
In conclusion, genetic diversity in different grassland ecosystems and their respective species are influenced by different historic and present processes. Thus, conservation strategies should pay tribute to historic land use practices and stochasticity, while decreasing the impact of current processes of fragmentation and habitat loss, to increase gene flow among remnants of species-rich

grasslands. Additionally, appropriate management will enhance the habitat quality, thus improving the establishment of new individuals, thereby increasing genetic variation.
Different practical concepts, such as autochthonous seed material and genetic conservation areas can play an important role in preserving genetic variation for grassland species.

# CHAPTER ONE: GENERAL INTRODUCTION



## FROM HABITATS TO WITHIN-SPECIES DIVERSITY

Biological diversity has been defined as the "variability among living organisms from all sources […], this includes diversity within species, between species and of ecosystems" by the Convention on Biological Diversity (CBD) of 1992. Thus, the CBD acknowledged three levels of biodiversity as integral parts of global biodiversity and the need to include them in global and local conservation efforts.

The different habitats all over the world provide valuable ecosystem services (Diaz et al. 2007; Lavorel 2013). Among the most important ecosystem services is the provision of habitat and resources for the plant and animal species living within them. Species can be limited to certain ecosystems and the simultaneous occurrence of other species, e.g. the marsh fritillary (*Euphydryas aurinia* ROTTEMBURG), who is dependent on his host species *Succisa pratensis* MOENCH in semi-natural grassland ecosystems (Brunbjerg et al. 2017). Additionally, natural landscapes contribute significantly to the wellbeing of humans and the more diverse the ecosystems are the stronger this effect becomes (Oteros-Rozas et al. 2018). Other important services include the regulation of ground water and carbon sequestration, provided by e.g. forest and grassland ecosystems (Hetherington and Woodward 2003; Kay et al. 2018; Wood et al. 2018; Janse et al. 2019). These processes are also important aspects in the context of climate change mitigation. Further is has been shown that species diversity can increase the quality or quantity of ecosystem services (Balvanera et al. 2006), e.g. species diversity is frequently positively correlated with productivity in grassland and forest ecosystems (Tilman et al. 2001; Fraser et al. 2015; Brun et al. 2019). Plant species diversity within green infrastructure elements, like hedges or coppice patches in a cultural landscapes, has been shown to promote biological pest control in adjacent crop species (Badenhausser et al. 2020). Thus, different plant species can perform different functions within ecosystems, highlighting the importance of species diversity.

The occurrence of a specific species in an ecosystem is dependent on local abiotic and biotic conditions. The more extreme a habitat becomes, e.g. high altitudes in alpine mountain regions, the fewer species can survive and more importantly maintain their reproductive fitness under these conditions (i.e. cold temperatures and short vegetation periods). In other words, plant species typically match their environment, by showing specific adaptations, which facilitate their fitness under the specific ecological conditions (Chase and Leibold 2003; Vellend et al. 2014).

These species-specific differences have their basis on the molecular level. The processes of mutation, selection and recombination of DNA lead to genetic variation within and among species (Rudmann-Maurer et al. 2007). Mutations can occur on the genome level (e.g. genome doubling), the chromosome level (e.g. translocation of chromosome parts) or at the DNA sequence level (e.g. nucleotide substitution) (Beebee and Rowe 2008). Via selective and neutral processes genetic variation develops and under changing conditions different genotypes can be favoured, which provide a more favourable expression of a specific trait. For example, diverse land use practices favour different alleles, leading to the differentiation of ecotypes. It has been shown that mowing led to a shift of flowering time in *Scabiosa columbaria* L., causing earlier flowering in mown compared with grazed populations and genetic differentiation among populations (Reisch and Poschlod 2009). Another study found a negative effect of intensive grassland management on the genetic diversity of *Festuca pratensis* L. (Kölliker et al. 1998).

Generally, genetic variation is influenced by similar processes as species diversity, like dispersal, colonization and extinction rate and patch size (Vellend 2005; Vellend and Geber 2005). Additionally, high genetic variation has been found to

correlate with and also to facilitate species diversity (Booth and Grime 2003; Vellend et al. 2014). However, assuming high genetic variation to persist in species rich habitats can lead to false conclusions (Taberlet et al. 2012).

Epigenetic processes, i.e. the mechanisms regulating the expression of genes in an organism (Richards et al. 2010), are enabling plant individuals or populations to quickly react to changing environmental conditions, without changing DNA sequences (Medrano et al. 2014; Gáspár et al. 2019). There are examples of poor association of genetic variation and high phenotypic diversity or habitat diversity, while genetic diversity or differentiation are low (Richards et al. 2008; Foust et al. 2016). Epigenetic diversity is increasingly explored as a mechanism explaining this phenomenon (Bossdorf et al. 2008; Robertson et al. 2017). As epigenetic modifications can be heritable (Verhoeven et al. 2010), epigenetic variation should also be considered part of global biodiversity as it forms a part of the within-species diversity.

From ecosystem diversity down to epigenetic variation, these different biotic levels form integral parts of global biodiversity. The current conditions of an ecosystems, species variation or genetic diversity are not fixed, but constantly changing. The dynamic processes that shaped the presently observed diversity are continuously changing over time. This is especially true for European grassland ecosystems, which have developed their specific variation due to various anthropogenic land use forms since the age of sedentism (Poschlod 2017). This complex history should not be neglected in the study and especially the conservation and restoration of grassland ecosystems.

## SEMI-NATURAL GRASSLANDS – HOTSPOTS OF BIODIVERSITY IN CENTRAL EUROPE

The world's most diverse ecosystems are not only found in the Earth's equatorial regions, like tropical rainforests. European grassland ecosystems are among the biodiversity hotspots with regards to their number of plant species per plot (plot size < 50 m²; Wilson et al. 2012). Along with their economic value, they also provide important ecosystem services, e.g. as food source and habitat for pollinators (Wesche et al. 2012), or by carbon sequestration (Wrage et al. 2011).

As so called semi-natural ecosystems, grasslands typically did not develop naturally, but through the influence of anthropogenic land use. Since the sedentism starting in the Neolithic Age (Dierschke and Briemle 2002; Poschlod et al. 2009a) human activity has led to the development of many diverse grassland ecosystems. Some typical grassland species were not native to central and northern Europe but were deliberately introduced from the Mediterranean region. Other species evolved from wild native plant species, along with the different land use forms applied over the centuries (Poschlod 2015). Depending on the soil properties, management system and historic land use practices, different types of grassland developed with varying species composition and diversity (Janssens et al. 1998; Dierschke and Briemle 2002).

From among those different grassland habitats, three grassland types were studied within this thesis: oat-grass meadows, litter meadows and calcareous grasslands (Fig. I). Each of these habitats has unique properties, a specific development history and their own unique species composition that evolved along with the respective land use regime.

**Figure I**: The three grassland habitats studied in this thesis within the administrative district of Tübingen. Left: Oat-grass meadow on the Swabian Alb; Middle: Litter meadow in the Württembergian Allgäu; Right: Calcareous grassland on the Swabian Alb.

Oat-grass meadows are among the most recently established grassland types. They were introduced for hay production at the beginning of the 18th century, along with the introduction of oat-grass (*Arrhenatherum elatius* (L.) J. PRESL & C. PRESL) into central Europe (Poschlod et al. 2009a; Poschlod 2017). The oat-grass meadows are defined as lowland hay meadows (Oberdorfer and Müller 1983) and belong to the union of Arrhenatherion elatioris (Mucina et al. 2016). The traditional management consists of one or two cuttings per year and low fertilization rates (Oberdorfer and Müller 1983), which provides the conditions for their specific species diversity. These meadows provide the habitat for many plant and animal species within the cultural landscape of Central Europe. Traditionally managed oat-grass meadows include 'wild' populations of agriculturally relevant species like *Dactylis glomerata* L., s. str. and *Trifolium pratense* L., where they are occurring on a broader scale of habitat and soil conditions, than in intensively managed grasslands. Thus, these populations potentially harbour genotypes adapted to their local and therefore diverse habitat conditions, providing material serviceable in plant breeding efforts for future climate conditions.

Due to the invention and application of mineral fertilizers in the 20th century, traditionally managed oat-grass meadows were often transformed into arable fields or intensively managed grasslands with up to seven cuttings per year (Kapfer 2010). This land use reduces the species diversity on these meadows dramatically (Gaujour et al. 2012) and also potentially decreases genetic diversity in the occurring species (Kölliker et al. 1998). On less profitable sites these meadows were also abandoned or afforested.

On the wet end of the grassland spectrum are the so-called litter meadows of the union Molinion caeruleae (Mucina et al. 2016). These grasslands are of very recent anthropogenic origin. During the 19th century the spread of railway tracks permitted more extensive trade even into remoter areas. As a result, in the Allgäu region the growth of cereal crops was largely abandoned, as it was more profitable to import grain and to increase animal husbandry, especially of cattle, instead. This led to a shortage of litter for stabling, leading to the establishment of litter meadows to meet this need (Poschlod and Fischer 2016; Poschlod 2017). Established on abandoned fishponds or other wet unprofitable ground the species community was artificially pushed towards tall, litter producing grasses and herbs. Extensive guidelines were written on how to best establish new litter meadows via hay transfer and seedlings and how to minimize undesired plant populations (Stebler 1898).

Typical species today are *Molinia caerulea* (L.) MOENCH, s. str. and *Filipendula ulmaria* (L.) MAXIM (Poschlod et al. 2009b). Thus, with the traditional management of cutting once in the autumn a

unique species diversity developed. Many rare or endangered species can now be found in these habitats, among them orchids, e.g. *Dactylorhiza majalis* (RCHB.) P.F.HUNT & SUMMERH. (Hedrén et al. 2001; Paun et al. 2010).
With the invention of slated floors in animal housing the litter meadows lost their importance. Consequently, they were often either abandoned or transformed into more intensively managed meadows, and the remaining litter meadows have become highly fragmented (Poschlod 2017).

Calcareous grasslands are a typical habitat on the slopes of mountainous areas like the Franconian and Swabian Alb. The sites where these grasslands developed were often unsuitable for the growth of crop species, due to their dry, nutrient poor and thin soils. Therefore, they were used for grazing of life stock, often in the form of transhumance. Large flocks of sheep and goats grazed on these sites (Poschlod and WallisDeVries 2002; WallisDeVries et al. 2002; Willerding and Poschlod 2002).
These ecosystems belong to the plant union of Bromion erecti (Mucina et al. 2016) and characteristic species are *Bromus erectus* HUDS. and *Hippocrepis comosa* L. (WallisDeVries et al. 2002; Willerding and Poschlod 2002). Today many calcareous grasslands have been either abandoned or transformed into intensively managed arable fields. The remaining sites are highly fragmented and reduced in size (Poschlod and WallisDeVries 2002). Conservation management is achieved by grazing with sheep and/or goats. However, flock sizes are much smaller, decreasing grazing pressure, while also restricting the formerly extensive gene flow among sites, by transporting seeds via endo- and epizoochory. As a consequence the species composition of calcareous grasslands is shifting towards more competitive species (Poschlod et al. 1998; Klimek et al. 2007). This habitat also belongs to one of the oldest grassland types and harbour many rare and endangered species, like *Pulsatilla vulgaris* MILL., s. l. (Korneck et al. 1996).

## TWO CENTURIES OF GRASSLAND HISTORY ON THE SWABIAN ALB AND THE WÜRTTEMBERGIAN ALLGÄU

The diversity within semi-natural grassland ecosystems is closely linked to the land use history it experienced (Poschlod et al. 2005; Karlík and Poschlod 2009; Cousins et al. 2009; Poschlod 2017). For example Aavik et al. (2008) reported management continuity as the primary determinant of plant community composition and species richness. Additionally Helm et al. (2009) found that the genetic diversity of *Briza media* L. was correlated to human population density. Historic population densities increased species and genetic diversity, while present day population densities were negatively correlated with species and genetic diversity. Therefore, when studying and conserving the biodiversity of grassland ecosystems, the land use history is of particular interest (Poschlod et al. 2009b).

The Swabian Alb and the Württembergian Allgäu have a long history of grassland management and high structural diversity and steep relief have led to the development of different grassland communities. Despite the ongoing global decline of traditionally managed grasslands (Poschlod 2017), remnants of these species rich habitats are still present within this landscape.
The Swabian Alb belongs to the low mountain range in Southern Germany, with heights up to above 1000 m above sea level. Despite the inhospitable conditions, i.e. thin and nutrient poor soils and low water availability, this area was already populated during the Neolithic age (Weller 2011).
The Württembergian Allgäu encompasses the western part of the alpine foreland and belongs to the Allgäu region in south Germany. The area includes especially the natural regions “Wetallgäuer Hügelland” and the “Bodenseebecken”. The land use practises in the Allgäu region changed along with the industrialisation and paved the way for the dominating grassland cultivation, with intensively managed silage meadows today (Kapfer 2010; Poschlod 2014).

Due to their multi-facetted land use history the Swabian Alb and the Württembergian Allgäu are therefore, well suited for studying the biodiversity of grassland ecosystems and the effects of historic land use changes. Several studies have shown an impact of historic land use and landscape structure on the species and genetic diversity in grasslands (Prentice et al. 2006; Helm et al. 2009; Rosengren et al. 2013; Münzbergová et al. 2013; Karlík and Poschlod 2019). By analysing historic maps (Tab. S1), the land use change through the centuries, and hence the habitat age, of a given site within the landscape can be reconstructed (Fig. II). Additionally, the changes in land use can be observed for specific sites as well as on the landscape scale.

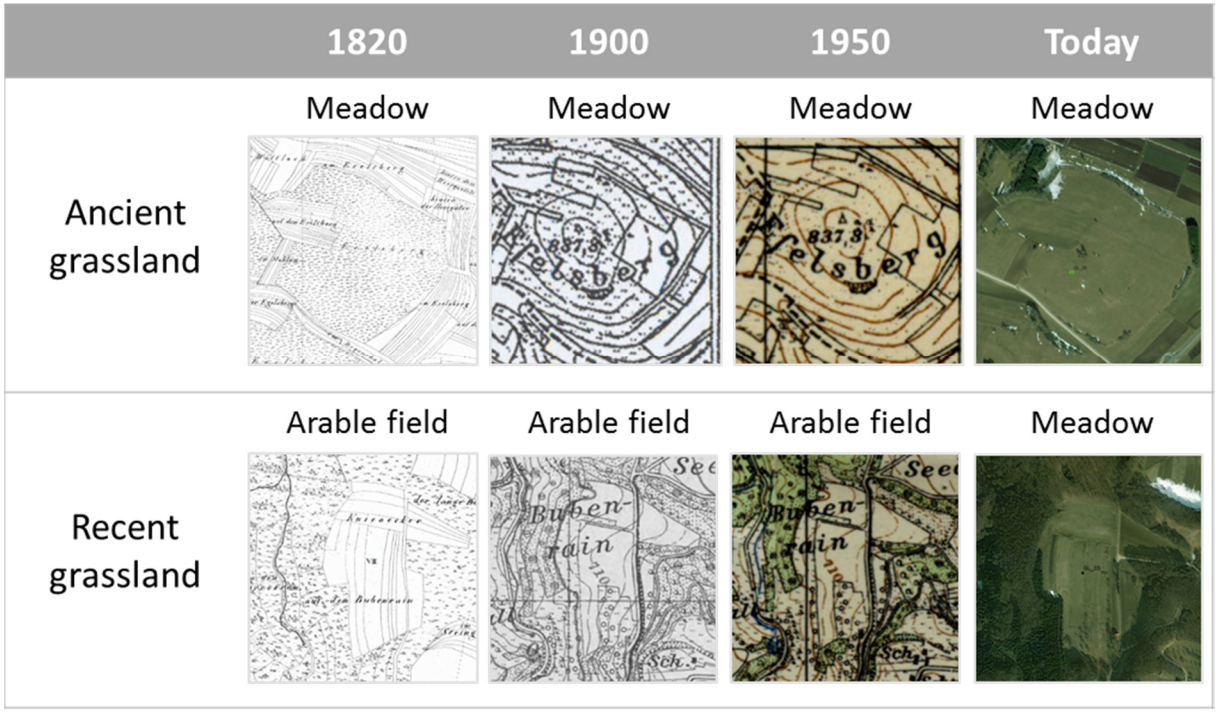



**Figure II:** Development of historic land use with the example of ancient and recent oat-grass meadows. The ancient grassland has been continuously used as grassland since before 1820, while the recent grassland was used as arable land until after WWII and then converted into grassland.

The historic landscape on the Swabian Alb and the Württembergian Allgäu has changed drastically (Fig. III). Formerly diverse mosaics of differently managed grasslands changed into a more unified landscape. Urban areas increased and especially pasture areas were abandoned or transformed to other land use forms. The earliest detailed and geo-referenceable maps are available from around 1820. These maps documented the different types of land use in practice (e.g. arable field, meadows & pasture) and the extent of urban areas. Different forms of meadow and pasture can also be distinguished. The maps from around 1820 show the landscape before or in the early stages of the industrialization. More recent comprehensive maps document the land use around World War I (1900ies) and after World War II (1950ies). Using these historic maps, the land use change over the last two centuries can be investigated and todays grasslands can be grouped into land use categories (Fig. II). So called 'ancient' sites are grasslands, which have been continuously used as grasslands since before the 1820ies, while 'recent' grasslands were developed later out of other land use forms (i.e. arable fields for oat-grass meadows and ponds for litter meadows).

This diversity of grassland habitats and their diverse land use history on the Swabian Alb and in the Allgäu make these areas ideal for studying the effects of land use history and landscape structures on genetic variation of grassland species. These studies are increasingly relevant in the context of conserving the diversity of grassland ecosystems in our cultural landscape.

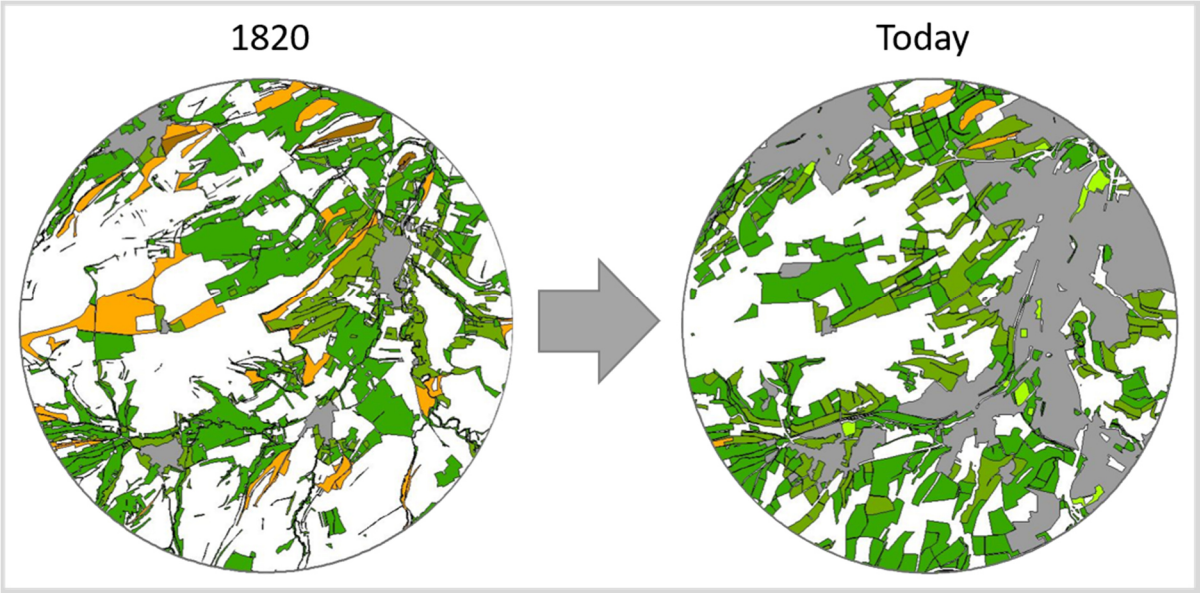



**Figure III:** Example of land use changes in the landscape across the Swabian Alb from 1820 to today (2018), (light/dark green: unwooded/wooded meadows; orange/brown: unwooded/wooded pasture; grey: urban area). Especially pastures decreased, while meadows and urban areas increased.

## CONSERVATION GENETICS AND LANDSCAPE ECOLOGY IN GRASSLAND ECOSYSTEMS

Along with the agricultural intensification during the 19$^{th}$ century, many land use practices changed, resulting in massive changes in the landscape and consequently also in grassland ecosystems (Dierschke and Briemle 2002; Sutherland 2002; Poschlod et al. 2009a). Multiple factors, like the introduction of mineral fertilizers, land use intensification, abandonment and afforestation, have led to a severe decrease in grassland biodiversity on the ecosystem, species and molecular level (Sutherland 2002; Poschlod & WallisDeVries 2002; Wesche et al. 2012; Poschlod et al. 2009; Poschlod 2015; Busch & Reisch 2015). Intensification and abandonment resulted in a loss of rare and specialist species (Hilpold et al. 2018). Increased fertilizer input and disturbance frequency have caused shifts in the species composition, of especially traditionally managed meadows, towards more tolerant species (Miller et al. 2011). More intensively managed hay and silage meadows are characterized by high nutrient input levels and mowing intensities, which led to a species poor vegetation dominated by grasses (Wesche et al. 2012). Litter meadows have lost their formerly high value and were either transformed into more productive grasslands or abandoned and have as a result decreased in abundance and quality (Wheeler 1988; Poschlod 2017). Formerly calcareous grasslands were intensively grazed by large flocks of sheep, but due to economic changes, shepherding became unprofitable. Therefore, wherever possible, pastures were transformed into arable fields or aban-

doned and left to succession (Poschlod and WallisDeVries 2002). Consequently, species-rich semi-natural grasslands have become rare and are often small and fragmented islands within an intensively managed agricultural landscape.

The negative effects of land use change are not limited to a loss in species variation. The processes of fragmentation and habitat loss have consequences on the population genetic level as well. Gene flow can be impaired by fragmentation and the loss of traditional management techniques (e.g. hay transfer), potentially decreasing genetic diversity within populations and leading to increased differentiation among them (Rudmann-Maurer et al. 2007; Aguilar et al. 2008; Franks 2010; Wellstein et al. 2013). Additionally, genetic diversity can be reduced due to random genetic drift or bottleneck events, when the gene pool of a population is suddenly diminished by stochastic environmental forces, e.g. by fire or logging (Young et al. 1996; Franks 2010).
Thus, gene flow decreases the risk of genetic impoverishment and counteracts the negative effects of founder effects and inbreeding, preventing the negative effects of inbreeding depression. Inbreeding depression can lead to the accumulation of deleterious alleles in a population and decrease overall population fitness (Amos et al. 2001; Keller 2002).

However, gene flow can also have negative consequences. When newly introduced foreign genotypes replace locally adapted alleles, genetic variation is lost by so called genetic swamping (Hufford and Mazer 2003; Byrne et al. 2011). This process can also lead to outbreeding depression. By evolving along with their specific environment populations can become genetically differentiated. When populations are strongly differentiated, the risk of outbreeding depression becomes a concern, when the introduction of new alleles and genotypes from differently adapted populations occurs. These new genotypes then potentially cause a fitness reduction in populations (Hufford and Mazer 2003; McKay et al. 2005; Ouborg et al. 2006).

As plants adapt to their specific environment, different ecotypes develop, which are genetically and phenotypically diverse. Therefore, the loss of genetic variation and locally adapted populations is relevant on several levels. As genetic variation is the basis for adaptation, high diversity will increase the probability that a population survives under changing environmental conditions. The genetic variation contained in traditionally managed, species-rich grassland ecosystems is also of relevance for the breeding of agriculturally important plants species, e.g. *Poa alpina* L. (Rudmann-Maurer et al. 2007), to meet future environmental conditions and challenges.

Preserving genetic diversity is an important concern in the conservation of declining and threatened species, due to its importance to enable species to cope with and adapt to changing environmental conditions (Ouborg et al. 2006). This concern has led to the establishment of the scientific discipline of conservation genetics, which is defined as the use of genetic variation analyses in order to reduce the risk of extinction for endangered species and to preserve the dynamic mechanisms shaping the genetic variation within and among populations (Frankham et al. 2010; Holderegger and Segelbacher 2016). Conservation strategies include in-situ conservation, as well as ex-situ strategies in botanical gardens and gene banks, both with their advantages and limitations (Gardiner et al. 2017; Nagel et al. 2019).

In modern conservation strategies plant reintroductions and population reinforcements are frequent tools to increase species and genetic variation in impoverished and degenerated ecosystems (Godefroid et al. 2011; Betz et al. 2013; Kaulfuß and Reisch 2019). In this context the origin of the plant material becomes important, due to the local adaptations of the source populations. This consideration is not only important for wild plant species, but also in agriculture. To

ensure the use of locally adapted genotypes and to maintain regional diversity, autochthonous seed origin regions for plant species were established, based on the natural regions classified for Germany (Prasse et al. 2010). These regions are used by seed manufacturers to produce local seed mixtures, to use e.g. in restoration of degraded grasslands.
The concept of autochthonous seed origin zones could be combined with so called genetic conservation areas. These areas have been recently suggested as a useful tool to conserve genetic variation within intact ecosystems (Maxted et al. 2011, 2015; Phillips et al. 2014).

## THESIS OUTLINE AND RESEARCH QUESTIONS

Global change processes have shaped biodiversity since the beginning of life on earth. Recent rapid changes can be mainly attributed to anthropogenic effects (Bonan 2008). Especially the last decades have played a major role in shaping our current ecosystems, and large losses of biodiversity has been the result. The conservation of species rich grasslands needs to consider not only the number of plant and animal species within them, but also the intraspecific variation contained in them.

In **Chapter One** the research question of this thesis is placed into the broader context of global biodiversity research and conservation. Processes influencing biodiversity, but especially intraspecific variation, are described and current and historic developments in grassland ecosystems and their implications for conservation are explored.

In **Chapter Two and Three** the genetic variation of common plant species from two different grassland habitats was investigated. The effect of land use history, changes in the landscape structure and habitat quality on the height and distribution of genetic variation was assessed. The molecular fingerprinting method of Amplified Fragment Length Polymorphism (AFLP) was used to analyse genetic variation in six different species.

Within **Chapter Two** the results of the study on typical oat-grass meadow species are presented. Three species were included in the analysis: *Dactylis glomerata*, *Heracleum sphondylium* L. and *Trifolium pratense*. One of the main driving forces for the distribution of genetic variation was habitat quality, i.e. the amount of litter present on the grasslands, which negatively affected genetic diversity in this study.

Litter meadows are a comparably young habitat (**Chapter Three**) on which *Angelica sylvestris* L., *Filipendula ulmaria* and *Succisa pratensis* occur frequently. In this habitat current management with mowing machines resulted in an admixture of the gene pool and low genetic differentiation despite strong habitat fragmentation.

In **Chapter Four** another level of intraspecific diversity was investigated. Epigenetic variation can contribute considerably to the phenotypic plasticity of populations, especially under different and temporarily variable environmental conditions. By combining AFLP analyses with Methylation Sensitive Amplification Polymorphisms (MSAP), the effect of habitat on the genetic and epigenetic variation of *Linum catharticum* L. populations was investigated. Populations from calcareous grasslands and litter meadows showed large genetic and epigenetic differentiation, explained by habitat.

Finally, in **Chapter Five** the results of the previous chapters are reviewed in the context of principal processes in grassland ecosystems and their implications for the conservation and restoration of intraspecific variation within these crucial habitats in Europe's cultural landscape. The impact of land use history, landscape structure und habitat variables are compared to other studies and the value of these results discussed in the context of

conservation management. Additionally, the results on the epigenetic variation of *L. catharticum* are discussed from a conservation point of view. Further, current concepts for the preservation of genetic variation, i.e. seed transfer zones and genetic conservation areas are discussed in the context of conservation genetics.

# CHAPTER TWO: GENETIC VARIATION IN OAT-GRASS MEADOW SPECIES

## GENETIC VARIATION OF TYPICAL PLANT SPECIES IN ANCIENT AND RECENT OAT-GRASS MEADOWS: THE EFFECT OF LAND USE HISTORY, LANDSCAPE AND VEGETATION STRUCTURE.

## ABSTRACT

Global changes in land use are threatening the diversity of many ecosystems on the intra- and interspecific level. Among these are the species-rich oat-grass meadows, which are drastically declining in quality and quantity, due to land use intensification or abandonment in recent decades. Due to their ongoing decline the remaining genetic resources of their plant species must therefore be protected. To determine the driving forces impacting genetic diversity in common oat-grass meadow species, we used data on the land use history, historic and present landscape structure as well as current vegetation and population structure.

We investigated the genetic variation of three common oat-grass meadow species (*Dactylis glomerata*, *Heracleum sphondylium* and *Trifolium pratense*). From 20 meadows we collected over 900 leaf samples and performed AFLP analyses. Additionally, we collected data on land use history and landscape structure from historic and current maps and used vegetation survey data to analyse the vegetation structure.

Our results showed average genetic diversity within the study sites, with low differentiation levels and a high gene flow among grasslands. Land use history, landscape structure and vegetation structure were found to be related to the distribution of genetic diversity in the studied species, highlighting the complex forces acting in these ecosystems, and also showing the specific impact of litter accumulation on genetic diversity.

Our results demonstrate the advantages of a multi-species approach, as it affords a wider range of conclusions. Both historic and current environmental variables influence genetic diversity in the studied species, demonstrating the importance of not neglecting the land use history of a habitat. Especially interesting is the influence of litter cover on genetic diversity, to our knowledge a relationship shown for the first time. This result highlights the importance of proper grassland management to preserve genetic diversity, as a suitable management regime of these grasslands will not only promote plant species diversity, but also genetic diversity of the species present.

Keywords: AFLP; conservation genetics; European grasslands; land use history; landscape structure; habitat quality

## INTRODUCTION

Genetic variation is of considerable relevance for all levels of biodiversity, as it is related to reproductive fitness, adaptation potential, evolutionary processes and ecosystem functioning (Amos et al. 2001; Reusch et al. 2005; Hughes et al. 2008; Banks et al. 2013). Through the study of genetic variation, spatial and temporal processes in the natural world can be explored. Additionally information on the distribution of genetic variation and its driving forces contribute to the improvement of nature conservation measures (Vellend et al. 2014).

Current global developments in land use and its detrimental effects on our ecosystems are therefore also a threat for genetic variation. Especially species rich and extensively managed European grasslands have declined drastically in recent decades, despite their relevance for species diversity and ecosystem services. Through land use intensification and abandonment these habitats are facing an ongoing decline in quality and quantity (Poschlod et al. 2005; Hejcman et al. 2013; Poschlod 2015).

Among these species-rich habitats are the oat-grass meadows, a type of lowland hay meadow of anthropogenic origin, which is characterized by e.g. *Arrhenatherum elatius*. The use of meadows for hay making was practiced on a broader scale since the Medieval Age. It has been assumed that the first meadows were established in floodplains of rivers and through the ages different forms of meadows have been established. Along with the introduction of *A. elatius* at the end of the 17$^{th}$ century, oat-grass meadows became the dominant meadow type until the second half of the 20$^{th}$ century (Poschlod et al. 2009a). The species diversity of oat-grass meadows depends on a traditional management, consisting of one or two cuttings per year and low fertilization rates (Oberdorfer and Müller 1983). However due to the invention and application of mineral fertilizers in the 20$^{th}$ century, these meadows were often transformed into intensively managed grasslands with up to seven cuttings per year (Poschlod et al. 2009a; Kapfer 2010), which reduces the species diversity on these meadows drastically (Gaujour et al. 2012) and also potentially decreases genetic diversity in the species present (Kölliker et al. 1998).

In addition to rare species, traditionally managed oat-grass meadows include 'wild' populations of agriculturally relevant species like *D. glomerata* and *T. pratense*, there occurring on a broad scale of habitat and soil conditions. Populations on these grasslands might thus harbour genotypes better adapted to local and therefore diverse habitat conditions, providing material useful in plant breeding efforts, e.g. for future climatic conditions.

As it is time and cost intensive to study a wide range of populations to find the ones of highest interest, it is of considerable value to study the factors having general impacts on the genetic variation in grasslands. Several studies have already identified groups of parameters impacting either species or genetic diversity in grasslands.

One factor frequently studied in grassland ecosystems is the land use history, often referred to as habitat age (Rosengren et al. 2013). As nearly all grassland ecosystems in Central Europe are of anthropogenic origin, the management history of these systems has an impact on genetic variation. Grasslands with a long history of traditional management practices ('ancient' sites), like grazing or hay making and transfer, show higher species and genetic diversity, due to the effects of gene flow between sites and the accumulation of different genotypes over time (Jacquemyn et al. 2004; Cousins et al. 2009). The species and genetic diversity in more recently established grasslands ('recent' sites) might thus be lower due to the shorter time available to accumulate diversity. Based on these previous studies, we expected to find differences in genetic diversity among ancient and recent sites due to their land use history.

Another frequently studied variable is the historic and present landscape structure (Jacquemyn et al. 2004; Helm et al. 2009). It has been found that for instance the area of surrounding grasslands or human settlements, may have an impact on genetic diversity in typical grassland species, especially in fragmented landscapes. Because of habitat fragmentation and thus reduced connectivity among populations, gene flow is reduced, and genetic drift or bottleneck events are possible. These processes lead to a decrease of genetic diversity within grassland patches and genetic differentiation among them. However not only connectivity can be an important landscape variable. Anthropogenic influence and disturbance, as measured by the distance to and the area of settlement in the landscape, can have an impact on genetic diversity. For example historic management intensity, based on population density, was found to be correlated to genetic diversity in *Briza media* (Helm et al. 2009). Therefore, it can be assumed that grasslands surrounded by many other grassland patches show higher genetic diversity, due to their higher connectivity. Historic settlement area and distances are thought to have an increasing effect on genetic diversity, while current anthropogenic disturbance can be expected to have rather negative impacts.

The third aspect often focused on in grassland studies is habitat quality and population structure, which is frequently observed to be correlated with genetic variation in plant species (Vellend 2005; Schleuning et al. 2009; Vellend et al. 2014). Grasslands with a high species diversity, and therefore high habitat quality, may thus also show high genetic diversity, as a result of processes working on both diversity levels (Vellend 2005). The cover of vascular plants and litter can impact the germination of seeds. It has been found that litter is acting as a seed trap and thereby reduces species diversity (Kahmen et al. 2002; Ruprecht and Szabó 2012) by reducing successful seedling establishment. Thus, the establishment of new genotypes is also reduced.

Additionally, population size is a parameter frequently associated with genetic diversity. Larger populations tend to contain higher genetic diversity, due to the decreased risk of inbreeding and genetic drift (Vergeer et al. 2003b). However the effect of population size can be overshadowed by stronger effects, e.g. of habitat quality (Vergeer et al. 2003a). Therefore, we expect to find effects of habitat quality and population structure on genetic diversity for the studied species.

Several studies focused on one or two of these groups of explanatory variables, however often with different conclusions (Helm et al. 2009; Reitalu et al. 2010; Münzbergová et al. 2013). Additionally, most studies were restricted to single species and often within a small geographical region. Most studies in this context have been conducted in grazed grasslands like calcareous grasslands and fewer studies investigated hay meadows, like the oat-grass meadows. But as the effects observed are linked to their habitat and are also species specific, it is interesting to include several species in a study design, so as to uncover variables having a more general impact, which are independent of plant species traits (e.g. pollination vector).

Therefore, we investigated these above described factors possibly impacting genetic variation in oat-grass meadow species: (i) land use history, (ii) historic and present landscape structure and (iii) current habitat quality and population structure. To investigate the importance of these three factors we analysed populations of three different grassland species (*Dactylis glomerata*, *Heracleum sphondylium* and *Trifolium pratense*) from traditionally managed oat-grass meadows on the Swabian Alb.

## METHODS

### *Study sites*

In the present study, we focused on oat-grass meadows, a comparably young grassland habitat, established for hay production at the beginning of

the 18th century with the introduction of oat-grass (*Arrhenatherum elatius)* into central Europe (Poschlod et al. 2009a; Poschlod 2017). With the invention of mineral fertilizers, these habitats were transformed either into arable land or more intensively used grasslands, with up to seven cuttings per year (Kapfer 2010), or on less profitable sites afforested or abandoned.

The studied oat-grass meadows are located on the Swabian Alb in Southwestern Germany. The Swabian Alb belongs to the low mountain range in Southern Germany, with heights up to above 1000 m above sea level. The bedrock of this area is build out of Jura limestone, formed during the Mesozoic era from coral reefs and marine sediments (Gebhardt 2008; Weller 2011).

From the currently available grasslands on the Swabian Alb 20 traditionally managed oat-grass meadows were chosen for this study (Tab. 1). To avoid sampling closely connected populations, only sites at least 1.5 km apart were included. The sampling sites closest to each other (Rechtenstein & Lauterach) were 3.02 km apart, while the greatest distance lay between Blaubeuren and Stromelsberg with 71.5 km.

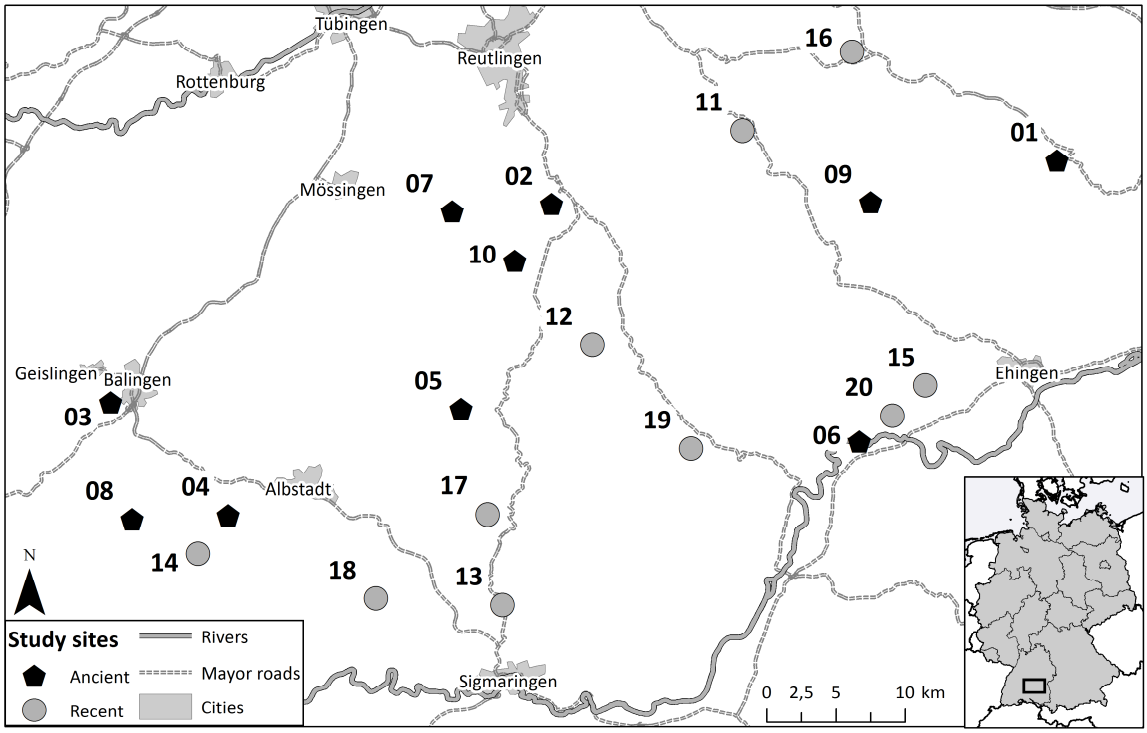



**Figure 1:** Geographic location of all 20 study sites on the Swabian Alb within the administrative district of Tübingen. Ancient grasslands are shown with a black pentagon (01-10) and recent grasslands with a grey circle (11-20).

**Table 1:** Number and names of the analysed populations, their respective age (Ancient and Recent), the geographic location they are situated in, the number of analysed individuals per species and population, as well as the estimated population size of the respective species. (Dg: *D. glomerata*, Hs: *H. sphondylium*, Tp: *T. pratense*).

| Nr. | Name | Age | Lat | Lon | *Dg* | Pop.D | *Hs* | Pop.H | *Tp* | Pop.T |
|---|---|---|---|---|---|---|---|---|---|---|
| **01** | Blaubeuren | Ancient | 48.4408 | 9.4459 | 16 | 15,759 | 16 | 685 | 16 | 32,202 |
| **02** | Gereuthau | Ancient | 48.4203 | 9.7529 | 16 | 22,744 | 16 | 11,372 | 16 | 79,606 |
| **03** | Heuberg | Ancient | 48.3028 | 9.2990 | 16 | 2,732 | 16 | 2,049 | 16 | 6,830 |
| **04** | Meßstetten | Ancient | 48.3942 | 9.2597 | 16 | 8,553 | 16 | 5,345 | 16 | 76,973 |
| **05** | Neufra | Ancient | 48.2658 | 8.8305 | 16 | 11,468 | 16 | 3,584 | 16 | 73,827 |
| **06** | Rechtenstein | Ancient | 48.1351 | 9.2107 | 16 | 51,881 | 16 | 4,150 | 16 | 103,762 |
| **07** | Sonnenbühl_1 | Ancient | 48.1685 | 8.9155 | 16 | 143,173 | 16 | 20,095 | 16 | 72,843 |
| **08** | Stromelsberg | Ancient | 48.1932 | 8.9459 | 16 | 31,380 | 16 | 28,966 | 16 | 144,829 |
| **09** | Münsingen | Ancient | 48.2752 | 9.6222 | 15 | 113,419 | 16 | 148,317 | 16 | 319,899 |
| **10** | Sonnenbühl_2 | Ancient | 48.4913 | 9.5535 | 16 | 21,812 | 16 | 4,039 | 16 | 46,048 |
| **11** | Bad Urach | Recent | 48.2616 | 9.1711 | 16 | 18,220 | 16 | 15,305 | 16 | 72,151 |
| **12** | Gammertingen | Recent | 48.2392 | 9.5579 | 16 | 45,070 | 16 | 85,477 | 16 | 132,101 |
| **13** | Sigmaringen | Recent | 48.3897 | 9.1628 | 15 | 337 | 16 | 8,415 | 16 | 15,146 |
| **14** | Luftloch | Recent | 48.1906 | 8.8516 | 16 | 1,472 | 16 | 294 | 16 | 17,075 |
| **15** | Ehingen | Recent | 48.1935 | 9.1966 | 16 | 27,411 | 12 | 685 | 16 | 40,432 |
| **16** | Römerstein | Recent | 48.1394 | 9.0882 | 16 | 45,010 | 15 | 23,371 | 16 | 57,994 |
| **17** | Veringenstadt | Recent | 48.2354 | 9.3942 | 16 | 60,783 | 16 | 3,986 | 16 | 38,862 |
| **18** | Stetten a. k. M. | Recent | 48.3942 | 9.5706 | 16 | 20,911 | 16 | 18,920 | 16 | 34,852 |
| **19** | Pfronstetten | Recent | 48.3574 | 9.2236 | 16 | 36,881 | 16 | 9,015 | 16 | 21,719 |
| **20** | Lauterach | Recent | 48.2559 | 9.5901 | 16 | 153,552 | 16 | 19,380 | 16 | 40,251 |

*Analysis of land use history, historic and present landscape structure and current habitat quality*

In a first step the land use history of the studied oat-grass meadows was accessed, with regards to their management within the last ~ 200 years. Meadows with a long consecutive use as managed grassland, dating back to the beginning of the 19$^{th}$ century, were classified as 'ancient' grasslands and were compared with meadows located on sites used as arable land until after WWII (1950ies), here called 'recent' grasslands. This classification is based on several topographic maps between 1820 and 2018 (Tab. S1). By using maps from different time points, we controlled for a continuous historic land use as meadow or arable field for the ancient and recent grasslands respectively. In total ten ancient and ten recent grasslands were included in the study design.

Secondly, we collected data concerning the historic and present landscape structure. Within a three-kilometre radius around each studied grassland, the area of managed grassland (Area.G), as well as urban areas (Area.S) were digitized, using ArcGIS (Version 10.4.1). From these areas the distance of the study sites to the distance to the next grassland and the next human settlement was calculated (Dist.G & Dist.S). The historic landscape structure was analysed based on the maps from around 1820, while the present landscape structure was inferred from the most recent topographic maps available for the area (Table S1).

Finally, to investigate the present habitat quality at the sites, vegetation surveys were conducted. At each site six surveys on 4 m² were recorded using the extended Braun-Blanquet scale (Reichelt and Wilmanns 1973), resulting in a total of 120 vegetation surveys. Within each plot the coverage of total vegetation (VP) and litter (Lit) were estimated in percent. The mean cover of vegetation and litter per study site was used in the later data analysis.

*Study species and sampling*

In our study we analysed three common grassland species, frequently occurring in oat-grass meadows: *Dactylis glomerata*, *Heracleum sphondylium* and *Trifolium pratense*.

The grass species, commonly known as orchard grass or cock's foot, *D. glomerata* is a perennial Poaceae, growing on e.g. fresh meadows, ruderal sites or semidry grasslands. This species is a widespread grass, with a high fodder value (Sebald et al. 1998). *D. glomerata* prefers good nutrient availabilities and also depends on light for seed germination (Oberdorfer et al. 2001; Rothmaler 2017).

The Apiaceae *H. sphondylium*, commonly known as hogweed, grows up to 1.50 m tall with white to yellow-greenish flowers. It occurs on fresh meadows and along ditches and roads (Rothmaler 2017). Pollination mainly occurs by flies and bees. *H. sphondylium* thrives on nutrient-rich meadows (Sebald et al. 1992), but its fodder value and grazing tolerance are low (Oberdorfer et al. 2001; Rothmaler 2017).

The legume red clover, *T. pratense*, belongs to the family of the Fabaceae and with its symbiotic bacteria acts as a soil improver. Like *D. glomerata* this species is a valuable fodder plant, used for hay production and in pastures (Sebald et al. 1992). *T. pratense* is mainly pollinated by bumblebees and is self-sterile. Red clover prefers calcareous soils with high nutrient contents and can be found in temperate regions all over the globe (Oberdorfer et al. 2001; Rothmaler 2017).

Per study site, plant leaf material from 16 individuals of each species was collected for genetic analysis, with few exceptions, when only fewer individuals were available, resulting in a total sample size of 953 individuals (Tab. 1). To prevent sampling clones or closely related individuals, leaf samples were taken at least five meters apart, with each sampling location documented via GPS (Garmin eTrex® 30x). Samples were stored in filter paper bags, dried and stored on silica gel at room temperature until DNA extraction.

Additionally, at each site the population size per species was estimated. Within 10 to 15 randomly placed 1 m²-plots (depending on grassland size) the occurrence of each species was counted. From the mean number of individuals over all plots, multiplied with the present habitat size, the population size of each species was calculated (Tab. 1). For two populations of *H. sphondylium* no individuals occurred in the plots, although the species has been collected on the site. Therefore, as an approximation for the calculation of the population size, we assumed one individual occurring in one plot.

*Molecular analyses*

Amplified fragment length polymorphisms (AFLP) are a quick and easy tool to estimate the genetic variation of a given species. As no prior sequence knowledge is required, this method provides a cost-effective way to analyses multiple species in a short time framework (Vos et al. 1995).

From the dried leaf material, DNA was extracted following the CTAB protocol by Rogers & Bendich (1994) with modifications by Reisch (2007). Extracts were diluted with water to 7.8 ng/µL and used for AFLP analysis, carried out in accordance with the protocol provided by Beckman Coulter as previously described by Reisch (2008).

To prepare the double stranded DNA adapters, equal volumes of both single strands of EcoRI and MseI adaptors (Biomers) were mixed in a 0.2 mL reaction vessel and heated for 5 minutes at 95 °C, followed by 10 minutes at 25 °C. A combined step

of DNA restriction and adapter ligation took place by adding 3.6 µL mixture containing 2.5 U EcoRI (Thermo Scientific), 2.5 U MseI (Thermo Scientific), 0.1 µM EcoRI and 1 µM MseI adapter pair, 0.5 U T4 Ligase with its corresponding buffer (Thermo Scientific), 0.05 M NaCl and 0.5 µg BSA (BioLabs/NBA) to 6.4 µL of genomic DNA with a concentration of 7.8 ng/µL. Samples were then incubated at 37 °C for 2 h, followed by a final enzyme denaturation step at 70 °C for 15 minutes. The obtained restriction-ligation products were then diluted 10-fold using 1x TE buffer for DNA (20 mM Tris-HCL, pH 8.0; 0.1 mM EDTA, pH 8.0).

Pre-selective DNA amplification was carried out by adding 1 µL diluted DNA of the restriction-ligation product, pre-selective EcoRI and MseI primers (Biomers), including a single selective nucleotide (EcoRI-A and MseI-C) to an AFLP Core Mix (PeqLab) containing 1x Buffer S, 0.2 mM dNTPs and 1.25 U Taq-Polymerase. PCR was performed in a 5 µL reaction volume with an initial step at 94 °C for 2 minutes, followed by 30 cycles of 20 s denaturation at 94 °C, 30 s annealing at 56 °C and 2 minutes elongation at 72 °C, concluding with 60 °C for 30 minutes for complete extension, finally cooling down to 4 °C. After the PCR step the samples were diluted 20-fold with 1x TE buffer for DNA.

For each study species three primer combinations were chosen (Tab. S2), after screening 36 primer combinations for eight randomly chosen individuals per species, for the subsequent selective PCR step. Each primer includes three selective nucleotides and each Eco-RI-primer was labelled with a fluorescent dye for fragment detection (Beckman dye D2, D3 & D4). For this PCR step 0.75 µL diluted pre-selective product was added to an AFLP Core Mix (1x Buffer S, 0.2 mM dNTP's, 1.25 U Taq-Polymerase, PeqLab), 0.25 µM selective EcoRI (Biomers) and 0.25 µM MseI (Biomers) primers, resulting in a total reaction volume of 5 µL. The used PCR parameters were 2 min at 94 °C, 10 cycles of 20 s denaturation at 95 °C, annealing for 30 s at 66 °C and 2 min elongation at 72 °C, while annealing temperature was reduced every subsequent step by 1 °C. This touch-down cycles were then followed by additional 25 cycles of 20 s denaturation at 94 °C, 30 s annealing at 56 °C and 2 min elongation at 72 °C, completed by a following 30 min step at 60 °C and a final cool down to 4 °C.

The obtained selective PCR products were diluted 2-fold (D2) and 5-fold (D4) with 1x TE buffer for DNA, while D3 was not diluted. Samples were pooled by mixing 5 µL of each selective PCR product of a given sample and adding them to a mixture of 2 µL sodium acetate (3 M, pH 5.2), 2 µL Na2EDTA (100 mM, pH 8) and 1 µL glycogen (Roche) in a 1.5 mL tube. The DNA was precipitated by adding 60 µL of 96 % ethanol (-20 °C) and immediate shaking. Pellets were obtained by 20 min centrifugation at 12700 g at 4 °C, the supernatant was poured off, following a pellet washing step by adding 200 µL 76 % ethanol (- 20 °C) and again centrifugalized at the above-mentioned conditions. Samples were then vacuum dried in a concentrator (Eppendorf). Dried DNA pellets were re-dissolved in a mixture of 24.8 µL Sample Loading Solution (SLS, Beckman Coulter) and 0.2 µL CEQ Size Standard 400 (Beckman Coulter). Thus, prepared samples were separated by capillary gel electrophoresis using an automated sequencer (GenomeLab GeXP, Beckman Coulter). Results were surveyed using the GeXP software (Beckman Coulter), exported as synthetic gel files (.crv) and analysed using the software Bionumerics 4.6 (Applied Maths, Kortrijk, Belgium). Only those fragments in the computed gels that showed intense and distinct bands were used for further analyses. Samples yielding unclear or weak band patterns, or obviously representing PCR artefacts, were repeated once. In total 318, 315 and 320 samples of each respective species were used for subsequent statistical analyses (Tab. 1). Band scoring resulted in 185 fragments for *D. glomerata*, 184 for *H. sphondylium* and 163 for *T. pratense*.

The reproducibility of the AFLP analysis was tested by calculating the genotyping error rate (Bonin et al. 2004). The analysis of 10 % of all studied samples (32 individuals per species) was replicated using the same DNA extracts. Fragments were scored and the percentage of diverging fragments per individual calculated. Following this analysis, a genotyping error rate of 2.96 % for *D. glomerata*, of 3.65 % for *H. sphondylium* and of 5.23 % for *T. pratense* was determined.

*Data analysis*

To evaluate the differences between historic and present landscape and habitat quality, Dunn's test was performed in R using the PMCMR package (Pohlert 2014), checking for significant de- or increases in the observed variables.

Using the AFLP fragment data, a binary matrix was created, representing the presence and absence of the respective fragments for each studied individual. Based on this 0/1 matrix, genetic variation within populations of each species was calculated using the software PopGene 32 (Yeh et al. 1997). This program allows, amongst others, the calculation of Nei's Gene Diversity ($H=1-\Sigma (p_i)^2$).

Additionally, a hierarchical Analysis of Molecular Variance (AMOVA) (Excoffier et al. 1992), based on pairwise Euclidian distances between samples, was calculated applying GenAlEx 6.5 (Peakall and Smouse 2012), to analyses the genetic variation within and among all populations and among populations from ancient and recent grasslands. Based on the genetic distance values ($\phi_{PT}$ values), produced by the AMOVA, and the geographic distance among populations, a Mantel test was performed with 999 permutations (Mantel 1967) in the GenAlEx software, to test for correlations of genetic and geographic distances.

Following Lynch & Milligan (1994) genetic distances among populations were calculated as Nei's distance (Ds) with non-uniform prior distribution of allele frequencies in the software AFLP-surv (Vekemans 2002). Using the thus obtained values a consensus neighbour-net-graph was generated with the program Splitstree 4.14.6 (Huson and Bryant 2006).

A Bayesian cluster analysis was performed with the software Structure (Version 2.3.4) (Pritchard et al. 2000, 2010) separately for all three species, to investigate the population structure in the present data set and assign individuals into groups. The most likely number of groups was calculated using 100,000 Markov Chain Monte Carlo simulations with a burn-in period of 10,000 iterations in an admixture model. Analyses for the predefined value of K were run 20 times per K=1-21. To summarize the results the web tool 'Structure Harvester' (Earl and vonHoldt 2012) was used. For each species the model, which gave persistent results for multiple runs and the highest ΔK value, was used to infer the best estimate of K following the method of Evanno et al. (2005).

Finally, to investigate the effects of the various environmental factors multivariate linear models were run in RStudio 1.1.423 (RStudio Team 2016), separately for each studied species and additionally for the mean diversity over all analysed species ('Mean-model').

Before constructing the full model, Pearson correlation coefficients were calculated for all explanatory variables (Tab. S2). From the full model, containing the historic and present total grassland and settlement area, historic and present distances to next grassland and settlement, land use history, population size, as well as vegetation and litter cover, an AIC based backward selection procedure was used to identify the model best fitting the data, using the '*AICc*' method from the '*MuMIn*' R-package (Burnham and Anderson 2004). To account for the difference in scale of the predictor variables used in the models, the function "scale()" was used on all variables except land use history.

## RESULTS

*Historic and present landscape structure*

Around the study sites settlement area increased to six times its previous extent from 1820 to the present day. With this expansion the distance of the study sites to the next urban area decreased also (Tab. 2). However recent grasslands were located significantly closer to present settlement areas, than historic grasslands (Fig. 2a, $p = 0.028$). Total area of grassland increased around all study sites within the observed period, however the increase was only significant for historic grasslands (Tab. 2, Fig. 2b, $p < 0.01$). As the total grassland area increased, the distance between the study sites and the closest located grassland decreased between 1820 and 2018 (Tab. 2).

*Current habitat quality and population size*

The vegetation surveys showed that the sites are covered by around 90 % of vegetation, ancient and recent grasslands did not differ significantly. Litter cover showed an overall mean of 2.2 % and also did not vary significantly among ancient and recent grasslands (Tab. 2). The vegetation and litter cover were negatively correlated, the higher the overall vegetation cover, the lower the litter cover (Tab. S2).

The size of the studied populations varied greatly among sites. In all species population sizes ranged between a few hundred individuals up to several hundred thousand. We found no difference in population size between ancient and recent grasslands in any of the studied species (Tab. 1).

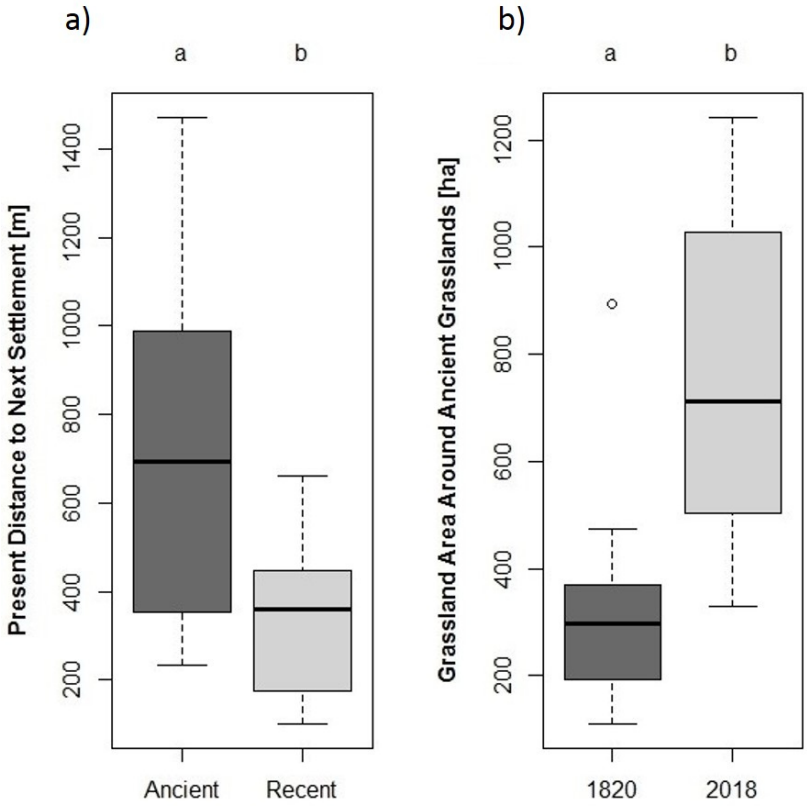



**Figure 2:** Boxplots showing the development of the landscape structure from 1820 to 2018, **(a)** Present distance to the next settlement or city separated for the two age categories of the investigated grasslands. A Dunne test revealed ancient grasslands to be located further away from settlements than recent grasslands ($p = 0.028$). **(b)** Grassland area [ha] around ancient grasslands in 1820 and at present. The amount of grassland around ancient grasslands increased significantly ($p < 0.01$).

**Table 2:** Results of the environmental structure of the study sites, with the distances to the next grassland (Dist.G) and settlement (Dist.S) [m], area of grassland (Area.G) and settlement cover (Area.S) [ha] from present (2018) and historic (1820) maps, as well as overall vegetation (VP) and litter (Lit) cover on the respective study sites [%], separated for ancient and recent grasslands. For each variable the mean within ancient and recent grasslands and the overall mean is given.

| Nr. | Age | Dist.S 2018 | Dist.S 1820 | Dist.G 2018 | Dist.G 1820 | Area.S 2018 | Area.S 1820 | Area.G 2018 | Area.G 1820 | VP | Lit |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **01** | Ancient | 234.6 | 802.9 | 90.3 | 36.6 | 215.9 | 44.2 | 328.2 | 243.2 | 88.3 | 2.2 |
| **02** | Ancient | 987.8 | 1740.9 | 166.6 | 136.8 | 211.5 | 17.8 | 504.3 | 111.1 | 91.7 | 1.2 |
| **03** | Ancient | 730.7 | 1221.1 | 25.7 | 51.3 | 758.5 | 83.9 | 980.8 | 893.8 | 89.5 | 2.8 |
| **04** | Ancient | 888.7 | 960.7 | 6.0 | 161.6 | 538.3 | 33.2 | 1241.2 | 353.3 | 88.7 | 1.3 |
| **05** | Ancient | 260.1 | 379.1 | 98.8 | 81.0 | 182.4 | 30.7 | 339.3 | 364.7 | 75.8 | 6.7 |
| **06** | Ancient | 352.8 | 285.2 | 34.8 | 43.3 | 199.4 | 66.3 | 533.2 | 369.5 | 93.3 | 2.2 |
| **07** | Ancient | 610.0 | 1087.9 | 66.4 | 498.7 | 226.6 | 29.6 | 1026.3 | 194.1 | 85.8 | 5.2 |
| **08** | Ancient | 658.5 | 634.8 | 307.0 | 44.0 | 160.1 | 31.3 | 1045.3 | 474.1 | 79.2 | 2.2 |
| **09** | Ancient | 1470.5 | 1867.4 | 152.3 | 156.3 | 127.1 | 30.5 | 523.5 | 241.7 | 96.0 | 0.3 |
| **10** | Ancient | 446.2 | 506.6 | 37.1 | 97.9 | 104.7 | 52.4 | 890.2 | 336.5 | 94.3 | 0.2 |
| **Mean Ancient** | | **664.0** | **948.7** | **98.5** | **130.7** | **272.4** | **42.0** | **741.2** | **358.2** | **88.3** | **2.4** |
| **SD** | | 361.3 | 514.3 | 85.9 | 130.7 | 197.8 | 19.1 | 313.4 | 203.9 | 6.2 | 2.0 |
| **11** | Recent | 274.6 | 697.0 | 61.3 | 217.8 | 120.4 | 22.1 | 733.6 | 285.8 | 85.8 | 2.3 |
| **12** | Recent | 660.8 | 1233.2 | 160.8 | 353.0 | 86.9 | 24.0 | 519.9 | 178.8 | 86.7 | 1.2 |
| **13** | Recent | 175.4 | 457.8 | 18.9 | 129.8 | 99.9 | 26.5 | 291.6 | 527.2 | 84.2 | 3.5 |
| **14** | Recent | 455.0 | 708.8 | 117.4 | 230.0 | 352.0 | 35.9 | 1334.3 | 407.6 | 88.3 | 1.0 |
| **15** | Recent | 344.2 | 296.5 | 85.5 | 296.5 | 58.8 | 18.2 | 413.5 | 168.1 | 90.8 | 3.3 |
| **16** | Recent | 374.4 | 1171.9 | 27.3 | 73.6 | 140.2 | 25.9 | 918.7 | 1074.5 | 97.5 | 1.0 |
| **17** | Recent | 432.7 | 1542.7 | 11.9 | 150.7 | 152.1 | 30.8 | 310.4 | 298.5 | 93.2 | 2.0 |
| **18** | Recent | 173.3 | 509.4 | 5.7 | 39.7 | 398.6 | 51.5 | 701.6 | 224.4 | 93.3 | 2.0 |
| **19** | Recent | 101.3 | 373.1 | 11.4 | 69.6 | 76.7 | 29.5 | 424.4 | 202.1 | 96.3 | 1.2 |
| **20** | Recent | 1272.0 | 1999.6 | 128.8 | 468.3 | 156.3 | 10.2 | 506.5 | 190.2 | 85.8 | 1.7 |
| **Mean Recent** | | **426.4** | **899.0** | **62.9** | **202.9** | **164.2** | **27.5** | **615.4** | **355.7** | **90.2** | **1.9** |
| **SD** | | 321.9 | 536.2 | 54.0 | 131.1 | 110.3 | 10.4 | 304.3 | 263.0 | 4.5 | 0.9 |
| **Overall Mean** | | **545.2** | **923.8** | **80.7** | **166.8** | **218.3** | **34.7** | **678.3** | **357.0** | **89.2** | **2.2** |
| **SD** | | 362.2 | 526.0 | 73.9 | 135.8 | 169.1 | 17.0 | 315.2 | 235.3 | 5.5 | 1.5 |

**Table 3:** Genetic diversity of all three species given as Nei's gene diversity, followed by the mean diversity over all species for each site. The mean of each age category is given with the respective standard deviation, as well as the over-all mean of all populations.

| | | Nei's Gene Diversity [H] | | | |
|---|---|---|---|---|---|
| **Nr.** | **Age** | ***Dac glo*** | ***Her sph*** | ***Tri pra*** | ***Mean*** |
| **01** | Ancient | 0.274 | 0.170 | 0.220 | 0.221 |
| **02** | Ancient | 0.274 | 0.173 | 0.224 | 0.224 |
| **03** | Ancient | 0.258 | 0.157 | 0.257 | 0.224 |
| **04** | Ancient | 0.243 | 0.182 | 0.229 | 0.218 |
| **05** | Ancient | 0.235 | 0.169 | 0.212 | 0.206 |
| **06** | Ancient | 0.260 | 0.170 | 0.237 | 0.222 |
| **07** | Ancient | 0.244 | 0.160 | 0.214 | 0.206 |
| **08** | Ancient | 0.258 | 0.178 | 0.201 | 0.212 |
| **09** | Ancient | 0.255 | 0.193 | 0.214 | 0.221 |
| **10** | Ancient | 0.251 | 0.210 | 0.192 | 0.218 |
| **Mean Ancient** | | **0.255** | **0.176** | **0.220** | **0.217** |
| **SD** | | 0.012 | 0.015 | 0.017 | 0.007 |
| **11** | Recent | 0.242 | 0.161 | 0.224 | 0.209 |
| **12** | Recent | 0.257 | 0.168 | 0.224 | 0.216 |
| **13** | Recent | 0.250 | 0.149 | 0.247 | 0.215 |
| **14** | Recent | 0.260 | 0.179 | 0.210 | 0.216 |
| **15** | Recent | 0.246 | 0.166 | 0.224 | 0.212 |
| **16** | Recent | 0.244 | 0.170 | 0.212 | 0.209 |
| **17** | Recent | 0.241 | 0.159 | 0.213 | 0.204 |
| **18** | Recent | 0.237 | 0.198 | 0.218 | 0.218 |
| **19** | Recent | 0.247 | 0.177 | 0.215 | 0.213 |
| **20** | Recent | 0.257 | 0.185 | 0.221 | 0.221 |
| **Mean Recent** | | **0.248** | **0.171** | **0.221** | **0.213** |
| **SD** | | 0.007 | 0.013 | 0.010 | 0.005 |
| **Overall Mean** | | **0.252** | **0.174** | **0.220** | **0.215** |
| **SD** | | 0.011 | 0.014 | 0.014 | 0.006 |

**Table 4:** Results of the Three-Level AMOVA given as the genetic variation among ancient and recent populations, as well as among all and within the respective populations of the studied species. Levels of significance are based on 999 iteration steps.

| | | df | SS | MS | % | ϕPT |
|---|---|---|---|---|---|---|
| ***D. glomerata*** | Among ancient and recent | 1 | 36.2 | 36.2 | 0 | 0.022 *** |
| | Among populations | 18 | 521.9 | 28.8 | 2 | |
| | Within populations | 298 | 6512.8 | 21.9 | 98 | |
| ***H. sphondylium*** | Among ancient and recent | 1 | 20.9 | 20.9 | 0 | 0.046 *** |
| | Among populations | 18 | 449.7 | 25.0 | 5 | |
| | Within populations | 295 | 4106.4 | 13.9 | 95 | |
| ***T. pratense*** | Among ancient and recent | 1 | 22.1 | 22.1 | 0 | 0.029 *** |
| | Among populations | 18 | 446.5 | 24.8 | 3 | |
| | Within populations | 300 | 4971.8 | 16.6 | 97 | |

Signif. Code: p < 0.001 ***

df, degree of freedom; SS, sum of squares; MS, mean squares
%, proportion of genetic variation, ΦPT, indicator for genetic differentiation among populations

*Genetic diversity and differentiation*

The Poaceae *D. glomerata* showed a mean genetic diversity of 0.252. The herbaceous species *H. sphondylium* showed a mean Nei's gene diversity of 0.174, while the legume *T. pratense* showed a mean diversity of 0.220 (Tab. 3). Nei's gene diversity did not differ among historic and recent populations. The AMOVA showed only low levels of differentiation among populations and no differentiation between ancient and recent populations, while most variation could be found within populations (Tab. 4).

The Mantel-test revealed no geographical pattern in neither of the three plant species (*Dac glo*: $R^2$ = 0.0051, *p* = 0.240; *Her sph*: $R^2$ = 0.0051, *p* = 0.223; *Tri pra*: $R^2$ = 0.0006, *p* = 0.387), further supporting the results of the AMOVA.

The generated neighbour-net graphs showed all grasslands intermixing well and frequently, irrespective of their land use history. The different species showed no similar pattern in the construction of the neighbour-nets (Fig. S1-S3).

*Multivariate Analysis*

For each species, as well as the 'Mean'- model, a significant linear model could be found, but the final models, calculated with the AICc method, differed among species (Tab. 5). Genetic diversity in *D. glomerata* was influenced by the litter cover (Lit) on the grassland, with increasing litter cover leading to lower levels of genetic diversity. Additionally, in this model, land use history (Age) was revealed to have a significant association with genetic diversity. In the model populations of *D. glomerata* on historic grasslands showed higher genetic variation than populations sampled from recent grasslands. This model explained 29.4% of the observed variation. The model for *H. sphondylium* included only one significant variable. Genetic diversity of this species was positively related to the present distance to the next settlement (Dist.S_2018). This association explained 18.8 % of the observed variation.

The model for *T. pratense* explained 42.4 % of the observed variation with a positive association between genetic diversity and present settlement

area (Area.S_2018), as well as a negative association with present grassland area (Area.G_2018).
In the 'Mean-model', incorporating the mean diversity of all studied species, associations were found for three explanatory variables: historic settlement area (Area.S_1820), land use history (Age) and litter cover (Lit), explaining 55.4 % of the observed variation. Land use history and litter cover had a negative association with mean genetic diversity, while Area.S_1820 showed a positive association (Fig. 3 a-c).

**Table 5:** Linear models for Nei's gene diversity for each species and the mean diversity with the spatial and vegetation structure of the study sites. For each model the explanatory variables remaining in the final model, the degrees of freedom (df), the sign of the association with the response variable (+/-), the t-value and its significance (p-value) is shown. The overall adjusted $R^2$ and overall p-value is given for each model.

| Species | Expl. Variable | df | +/- | *t*-value | *p*-value |
|---|---|---|---|---|---|
| ***D. glomerata*** | Age | 1 | - | -2.370 | 0.029 * |
| | Lit | 1 | - | -2.623 | 0.018 * |
| | Error | 17 | | | |
| Adj. $R^2$ = 0.294 $p$ = 0.02 | | | | | |
| ***H. sphondylium*** | Dist.S_2018 | 1 | + | 2.326 | 0.032 * |
| | Error | 18 | | | |
| Adj. $R^2$ = 0.188 $p$ = 0.03 | | | | | |
| ***T. pratense*** | Area.S_2018 | 1 | + | 3.889 | 0.001 * |
| | Area.G_2018 | 1 | - | -2.902 | 0.009 * |
| | Error | 17 | | | |
| Adj. $R^2$ = 0.424 $p$ = 0.004 | | | | | |
| ***Mean*** | Area.S_1820 | 1 | + | 2.481 | 0.024 * |
| | Age | 1 | - | -2.508 | 0.023 * |
| | Lit | 1 | - | -3.864 | 0.001 * |
| | Error | 16 | | | |
| Adj. $R^2$ = 0.554 $p$ = 0.001 | | | | | |

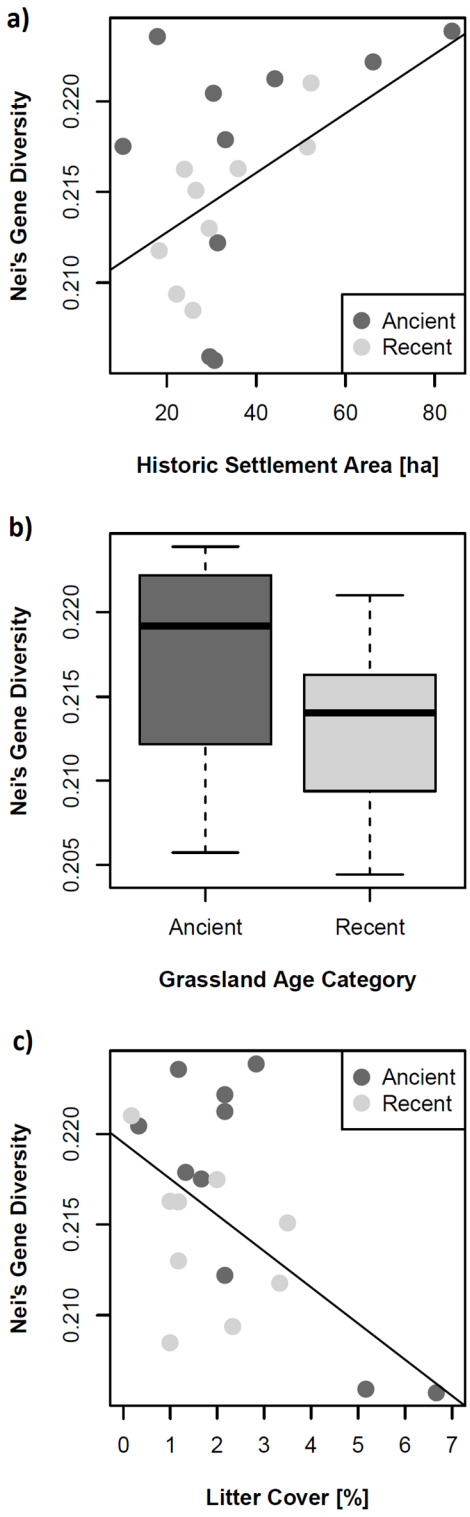



**Figure 3:** Visualization of the significant influencing variable on mean genetic diversity in the 'Mean' -model ($R^2$ = 0554). **(a)** The association of mean genetic diversity with historic settlement area (p = 0.02), **(b)** with habitat age (p = 0.02) and **(c)** with percentage of litter cover on the studied grasslands (p = 0.001). Ancient grasslands are indicated in dark-grey and recent ones in light-grey.

## DISCUSSION

### *Genetic variation in oat-grass meadow species*

The observed genetic diversity among and within populations varied only slightly in the three studied species. Overall *D. glomerata*, *H. sphondylium* and *T. pratense* showed moderate levels of genetic diversity, within the range observed for species with similar life history traits (Reisch and Bernhardt-Römermann 2014) and on a level comparable to other studies (Kölliker et al. 2003; Last et al. 2014). As expected, due to its wind pollination, *D. glomerata* showed the highest genetic diversity, while the insect pollinated *H. sphondylium* and *T. pratense* showed lower values. For all three species the highest genetic diversity was found in populations on ancient grasslands.

Genetic differentiation between populations was low and we did not detect any geographical or population structure within the study area in any of the three species. As the distribution of the investigated species is not limited to oat-grass meadows, but extents also to road margins, hedges and other grassland types (Rothmaler 2017), there are many possibilities of gene flow among populations. Pollen of *D. glomerata* have been shown to be transported by wind as far as 10 km (Willerding and Poschlod 2002), explaining the low genetic differentiation observed here. Honey bees were found to forage along long distances (up to 9.5 km), providing long-distance pollen dispersal for insect pollinated species (Beekman and Ratnieks 2000). Additionally oat-grass meadows are a comparably young habitat, with a well-documented history of seed transfer events, re-sowing as a measure to increase productivity and seed trade within Europe (Kauter 2001; Hejcman et al. 2013; Poschlod 2017), explaining the low spatial-genetic structure observed here.

### *Effect of land use history*

We found land use history to be associated with genetic diversity in *D. glomerata* and in the

'Mean-model'. Ancient meadows, continually used as grassland for over 200 years, showed overall higher genetic diversity in the studied plant populations, while populations on meadows established on arable fields after WWII showed overall lower genetic diversity values.

An impact of land use history has previously been shown for plant species diversity in European grasslands (Cousins et al. 2009; Reitalu et al. 2010), as well as on the genetic level for typical grassland species (Jacquemyn et al. 2004; Rosengren et al. 2013). For example Rosengren et al. (2013) found a positive relationship between habitat age and the genetic diversity in the moss *Homalothecium lutescens* (HEDW.) H. ROB. explained by the effects of grazing continuity on the ancient grassland patches. Jacquemyn et al. (2004) found allele frequencies to be related to habitat age in *Primula elatior* (L.) HILL, with younger populations showing lower genetic differentiation explained by historic landscape changes.

Our findings for typical oat-grass meadow species, can be explained by the long tradition of sowing and re-sowing practices on historic meadows. Due to the repeated introduction of new genotypes, genetic diversity may be accumulated in ancient grasslands, giving them a higher genetic diversity compared to recent grasslands at present. Recent meadows were established on arable fields within the last 60 years and thus experienced fewer introductions, resulting in lower accumulated genetic diversity. Additionally, the management practices changed simultaneously with the establishment of the recent meadows in the middle of the 20$^{th}$ century, with modern agricultural machines and more intensive fertilization leading to a more homogenous land use (Poschlod 2017). This modern land use practices at the beginning of the meadow establishment, decreased small scale variations in the disturbance regime, thus providing less opportunities for establishment of new genotypes as well as genetic differentiation.

*Effect of historic and present landscape structure*

The landscape structure on the Swabian Alb has changed dramatically during the last 200 years. Urban areas increased enormously, leading to a decrease in the distance of the studied grasslands to the next settlement, suggesting a larger human impact on these ecosystems (Frey et al. 2016). Additionally, the total grassland cover in the study area increased and with it the connectivity of grassland patches. As the distribution of our studied plant species is not limited to extensively used grasslands, we assume them to be potentially present on most of the grasslands in this area. We found significant associations between present landscape configuration and genetic diversity in *H. sphondylium* and *T. pratense*, while in the 'Mean-model' diversity was associated with historic landscape structure.

Similar associations were also found in other studies focused on the influence of historic and present landscape configuration on genetic diversity (Helm et al. 2009; Münzbergová et al. 2013; Reisch et al. 2017). Helm et al. (2009) found that present landscape structure was an important predictor for genetic variation in *Briza media*, where grasslands with a high connectivity index also exhibited high genetic diversity. Additionally, they found a negative correlation of genetic diversity with current human population density. Similarly, we found a positive association between genetic diversity in *H. sphondylium* and present distance to the next human settlement, meaning that populations that are less influenced by human disturbance exhibit higher genetic diversity. This relationship might best be explained by the mowing sensitivity of hogweed (Dierschke and Briemle 2002), with mowing frequency, at least in traditionally managed grasslands, likely decreasing with increased distance to the next settlement area.

Contrastingly genetic diversity in *T. pratense* was negatively associated with present grassland area and positively with present settlement area. The negative relationship with grassland area can be explained by considering that the topographic

maps do not include information on the management intensity of the investigated grasslands. The areas in the surrounding of our studied grasslands will include intensively managed grasslands, sown with industrial seed mixtures, which likely do not include different genotypes. Thus, through gene flow the grasslands which are surrounded by a larger proportion of intensively managed grasslands will over time decrease in genetic diversity.

Human settlement area, in other words disturbance intensity (Helm et al. 2009), has a positive effect on genetic diversity through neutral and selection-driven processes (Banks et al. 2013). Reisch et al. (2017) found historic landscape configuration to be more important for genetic variation in typical calcareous grassland species than present habitat conditions.
Similarly, in our 'Mean-model' historic settlement area is positively associated with mean genetic diversity, supporting the assumption that historic human impact (e.g. mowing and sowing events) contributed to the build-up of current genetic diversity, as mentioned above.

*Effect of present habitat quality and population size*

The multivariate linear models revealed a negative association of litter cover on genetic diversity in *D. glomerata* and in the 'Mean-model'. The higher the cover of litter on a grassland, the lower the observed genetic diversity.
To our knowledge, a relationship between plant litter cover and genetic variation has not been previously reported in any published studies. Most studies investigating the effect of litter accumulation on grasslands have focused on plant species variation, seed germination and seedling establishment (Schleuning et al. 2009; Ruprecht et al. 2010; Ruprecht and Szabó 2012). As litter prevents the establishment of new individuals, the effect on genetic diversity is obvious. Even though our studied populations did not show signs of genetic impoverishment, the consequences of genetic drift, due to seeds trapped in litter, will on the long term decrease genetic diversity and lead to a loss in reproductive fitness. This finding also has important implications for the conservation and restoration of grasslands. Proper management practices that reduce litter accumulation, will not only improve species richness, as shown by e.g. Ruprecht et al. (2010), but also genetic variation of the species present on the grassland.

Finally, we did not find an effect of population size on genetic diversity, even though this relationship has often been found in other species (Vergeer et al. 2003a; Honnay and Jacquemyn 2007). This result can be explained by the large populations of the studied species observed here and the high gene flow, as discussed above.

*Conclusion*

The results of our study highlight the possibilities a multi-species approach affords. By including several species and a range of different explanatory variables we could show that, while individual species are mainly influenced by present landscape and vegetation structure, the analysis over all species showed the importance of historic landscape structure and land use history. Thus, we conclude that by using an integrated approach, historic developments can be better accounted for.
Interestingly, the litter cover present on the grassland had one of the strongest impacts, a relationship not shown before. As litter cover is also negatively correlated with species diversity, this result highlights the importance of proper grassland management to maintain species as well as genetic diversity.

# CHAPTER THREE: GENETIC VARIATION IN LITTER MEADOW SPECIES

## GENETIC VARIATION OF LITTER MEADOW SPECIES REFLECTS GENE FLOW BY HAY TRANSFER AND MOWING WITH AGRICULTURAL MACHINES

## ABSTRACT

Litter meadows, historically established for litter production, are species-rich and diverse ecosystems. These meadows drastically declined during the last decades along with decreasing litter use in modern livestock housing. The aim of our study was to identify the drivers of genetic variation in litter meadow species. Therefore, we tested whether genetic diversity and differentiation depend on habitat age, landscape structure, habitat quality, and/or population size.

We analysed 892 individuals of *Angelica sylvestris*, *Filipendula ulmaria*, and *Succisa pratensis* from 20 litter meadows across the Allgäu in Baden-Württemberg (Germany) using AFLP analyses.

All study species showed moderate levels of genetic diversity, while genetic differentiation among populations was low. Neither genetic diversity nor differentiation were clearly driven by habitat age. However, landscape structure, habitat quality as well as population size revealed different impacts on the genetic diversity of our study species. Historic and present landscape structures shaped the genetic diversity patterns of *A. sylvestris* and *F. ulmaria*. The genetic diversity of *F. ulmaria* populations was, moreover, influenced by the local habitat quality. *S. pratensis* populations seemed to be affected only by population size.

All explanatory variables represent past as well as present gene flow patterns by anthropogenic land use. Therefore, we assume that genetic diversity and differentiation were shaped by both historical creation of litter meadows via hay transfer and present mowing management with agricultural machines. These land use practices caused and still cause gene flow among populations in the declining habitats.

Keywords: AFLP; conservation; genetic variation; litter meadow; management; semi-natural grassland

## INTRODUCTION

Litter meadows constitute valuable habitats for many specialised, rare, and endangered plant and animal species (Wheeler 1988). Therefore, these semi-natural grasslands belong to the most species-rich ecosystems in Central Europe (Kull and Zobel 1991) and represent key areas for biodiversity conservation in agricultural landscapes, despite their comparably short land use history and limited spatial distribution.

According to Poschlod (2017), the construction of railway lines opened up the Alpine foreland region at the end of the 19$^{th}$ century. Agricultural crops were imported, and subsistence farming efforts became redundant. Farming practices consequently changed from laborious cultivation of arable fields to more efficient grassland management for livestock farming. During this time, straw, used as bedding in stables, became scarce. Therefore, litter meadows were established, either transforming fodder meadows or by mowing large wet- and peatlands. Whereas sowing and/or planting of litter plants were recommended for the establishment in drained ponds or peat-mined areas, Stebler (1898) described four management treatments for the conversion of fodder meadows into litter meadows without ploughing: (i) late cutting over several years, (ii) waiver of fertilization, (iii) irrigation, and (iv) resowing seeds or planting seedlings. Moreover, litter meadows were established by hayseed application (Müller 1752). During the 1960s, litter meadow cultivation became redundant due to massive land use changes (Poschlod 2017). Slatted floors gained more relevance in animal husbandry and thus, liquid manure replaced solid manure as preferred fertilizer. Furthermore, mineral fertilizer became comparably cheap, leading to a transformation of unproductive litter meadows into more productive fodder meadows.

Nowadays, remaining litter meadows are threatened by land use intensification, abandonment, and habitat fragmentation (Billeter et al. 2002). Habitat fragmentation limits pollen and seed exchange, restricting gene flow among populations (Schmitt 1983; Steffan-Dewenter and Tscharntke 1999; Willerding and Poschlod 2002; Honnay et al. 2006) and increasing, therefore, the likelihood of inbreeding depression, the accumulation of deleterious mutations, and the extent of genetic drift (Young et al. 1996; Picó and Van Groenendael 2007). Consequently increased genetic differentiation and reduced genetic diversity (Barrett and Kohn 1991; McKay et al. 2005), may lower individual plant fitness and thus, increase their extinction risk (Ellstrand and Elam 1993; Young et al. 1996). Hence, the knowledge about potential impact factors on genetic variation patterns becomes highly relevant to protect genetic variation, as a fundamental level of biodiversity (May 1994).

Due to an outstanding land use history, litter meadows could be found either on historically old ('ancient') or historically young ('recent') sites. In this study, ancient sites were wet grasslands at least since the 1820ies, while recent sites were artificially created on drained ponds during the 1900s. High gene flow at the time of establishment and afterwards may lead to comparable levels of genetic variation among populations on sites with different habitat age (Vandepitte et al. 2010). Nevertheless, the number and origin of colonists (Wade and McCauley 1988; Whitlock and McCauley 1990) as well as the rate of gene flow and selection after colonization (Dlugosch and Parker 2008) drive genetic variation patterns of recent populations. These populations may, therefore, show both reduced genetic variation due to bottlenecks and increased divergence among populations by selection (Wade and McCauley 1988; Dlugosch and Parker 2008). Previous studies observed already comparatively decreased genetic variation levels within and among populations on recent sites (Jacquemyn et al. 2004; Dlugosch and Parker 2008; Ramakrishnan et al. 2010). Hence, we expected an impact of habitat age on the genetic variation of typical litter meadow species.

Over the past century, biodiversity decline was mainly induced by habitat loss at local, regional, and global scales (Balmford et al. 2005). Small populations, suffering from disrupted mutualistic interactions with pollinators or seed dispersers (Tscharntke and Brandl 2004), show enhanced extinction rates due to increased levels of inbreeding, loss of genetic variation through genetic erosion, fitness decline, and loss of evolutionary adaptation potential (Young et al. 1996; Adriaens et al. 2006). Nevertheless, rescue effects may lead to increased colonisation and reduced extinction rates in highly connected sites (Brown and Kodric-Brown 1977). We hypothesize, therefore, an impact of habitat size and connectivity on genetic variation. Moreover, gene flow, seed dispersal and establishment are influenced by land use patterns (Reitalu et al. 2010; Purschke et al. 2012) representing further determinants for gene flow and genetic variation in today's fragmented landscapes. Populations are sometimes affected more by historic than by present landscape configurations due to a time lag in a specie's response (Adriaens et al. 2006). Hence, we included historic as well as present landscape structures in our analyses.

Abandonment and missing biomass removal led to deteriorated habitat conditions in litter meadows. Moss and/or litter layers build-up and act as seed traps (Ruprecht and Szabó 2012), while increased vegetation height causes ground shadowing (Jensen and Gutekunst 2003). Germination as well as establishment of seedlings are consequently restrained (Maas 1988; Špačková and Lepš 2004; Poschlod and Biewer 2005). Populations may decrease in size and a decline of genetic variation becomes more likely (Billeter et al. 2002). Therefore, we hypothesized an impact of habitat quality on the genetic variation of common litter meadow species.

In modern fragmented landscapes, remaining litter meadows are often small, fragmented, and isolated. Populations on these sites are comparatively small and more vulnerable to demographic and environmental stochasticity, despite intact vegetation structure (Hooftman et al. 2003). These populations may suffer from reduced probabilities of gene flow, increased genetic drift, and enhanced levels of inbreeding (Van Treuren et al. 2005; Aguilar et al. 2008). They may, therefore, show lower genetic variability, reduced generative (Schmidt and Jensen 2000) as well as vegetative performance (de Jong and Klinkhamer 1994), and face a higher risk of extinction (Spielman et al. 2004; Ouborg et al. 2006). Various studies observed already a positive relationship between population size and genetic variation (LEIMU et al. 2006). Therefore, we would expect a positive impact of population size on genetic variation as well.

A range of studies already investigated the impact of habitat age, historic and present landscape structure, habitat quality, and population size on genetic variation in dry grassland habitats (e.g. Prentice et al. 2006; Schmidt et al. 2009; Baessler et al. 2010; Rosengren et al. 2013; Reisch et al. 2017). Nevertheless, studies concerning wet grassland habitats, such as litter meadows, are still scarce.

Therefore, we analysed the genetic variation of three widespread litter meadow species using amplified fragment length polymorphism (AFLP) analyses. We chose the mainly insect-pollinated perennials *Angelica sylvestris*, *Filipendula ulmaria*, and *Succisa pratensis* (Kühn et al. 2004) as study species. We ranked linear regression models according to AICc values to shed light on the relative importance of environmental factors on genetic variation patterns of the studied litter meadow species. Hence, the land use history and thus, the habitat age of the studied litter meadows was reconstructed using historical cadastral maps from different points in time. Moreover, historic and present landscape structures including distance to the nearest settlement, area size, total area of surrounding wet grasslands, and connectivity were quantified on the basis of historic (1820ies) and present (2018) cadastral maps. Local habitat quality was investigated with regards to vegetation cover

data and population size. Applying these methods we aimed at answering the following questions: (i) What is the impact of habitat age on genetic diversity? Are populations of different habitat age genetically differentiated? (ii) Is genetic diversity influenced by historic and/or present landscape structure? (iii) How is genetic diversity shaped by current habitat quality and/or population size?

## METHODS

*Study design*

In our study, we analysed the genetic variation of three typical litter meadow species: *Angelica sylvestris* (Apiaceae; 2n = 22), *Succisa pratensis* (Dipsacaceae; 2n = 18), and *Filipendula ulmaria* (Rosaceae; 2n = 14). *A. sylvestris* and *S. pratensis* flower between July and September, while *F. ulmaria* is flowering from June to August. All study species are perennials with a mixed mating system, showing insect (e.g. bees, syrphids, wasps, beetles) as well as self-pollination (Kühn et al. 2004). We selected 20 litter meadows distributed across the Allgäu in south-west Germany to study the effect of various environmental factors on genetic variation (Fig. 4, Tab. S4). The study region is characterized by a temperate climate with precipitation between 900 and 1600 mm/year and annual temperatures from 5.5 to 7.5 °C.

The land use history of the litter meadows was reconstructed with historical cadastral maps from three different points in time (1820ies, 1910/1920ies, and 1950ies) to investigate the impact of habitat age on genetic variation (Tab. S1). We identified eleven sites as historically old ('ancient'), which have been wet grasslands since before the 1820ies, and nine sites as historically young ('recent'), which developed from ponds during the 1900s, applying the software ArcGIS® 10.3.1 (Esri, Redlands, CA, USA).

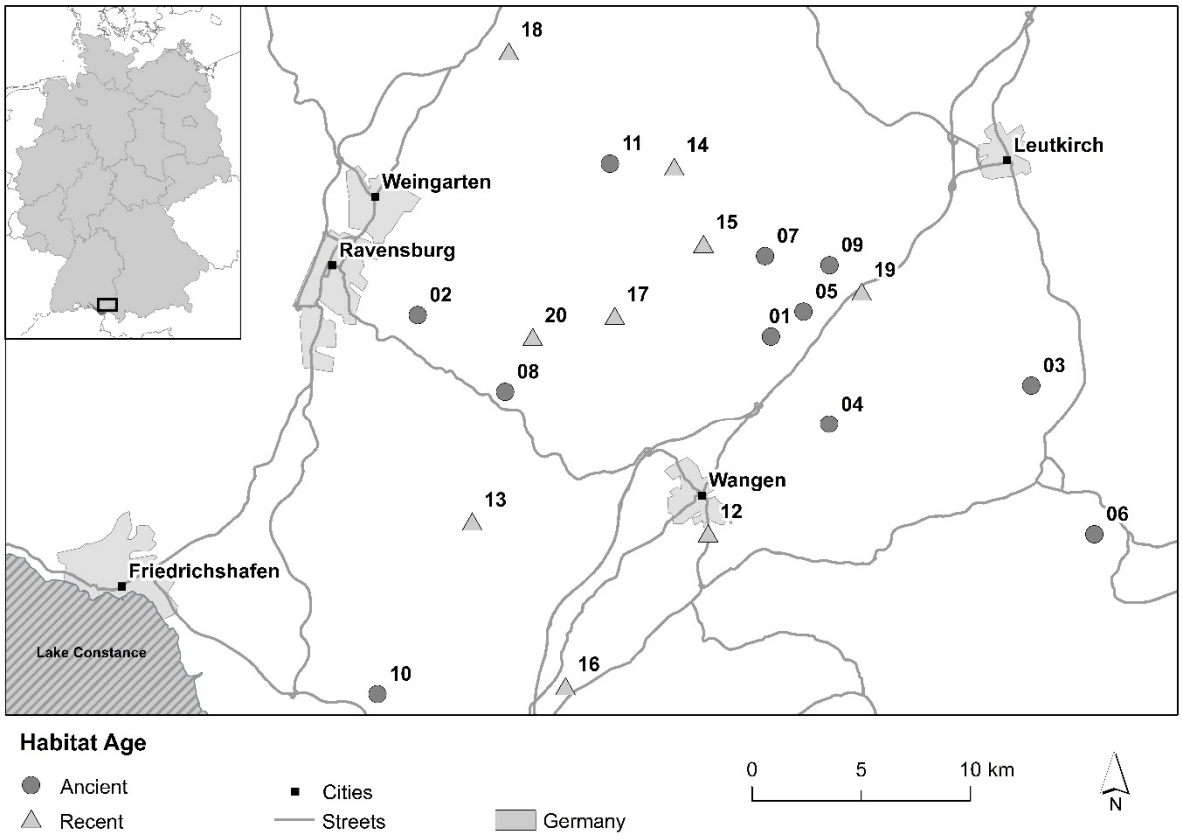



**Figure 4:** Geographic position and habitat age of the analysed populations of *A. sylvestris, F. ulmaria,* and *S. pratensis* in the Allgäu region. Ancient sites are shown with a circle (01-11) and recent grasslands with a triangle (12-20).

In a next step, we digitized the oldest cadastral maps available for the area (1820ies) as well as current topographical maps (2018) in a 3 km radius around each study site. Following landscape structures were chosen as potential explanatory variables for genetic diversity (Tab. S5): historic and present distance to the nearest settlement, historic and present total area of wet grasslands within each circle, and size of each study site. Moreover, we calculated historic and present connectivity according to Hanski (1994) as $S_i = \sum_{j \neq I} \exp(-\alpha d_{ij}) A_j$, where $S_i$ is the connectivity of the patch i, $d_{ij}$ is the distance (km) between patches i and j, $A_j$ is the area (ha) of the patch j, and α is the parameter of the exponential distribution setting the influence of distance on connectivity (Helm et al. 2006). Following Lindborg and Eriksson (2004) and Reitalu et al. (2010) α was set to one and not weighted by the dispersal abilities of the plant species in the community.

The cover of vascular plants, mosses, litter, and open soil were incorporated from vegetation surveys to examine the impact of the local habitat quality on genetic diversity (Tab. S6). Furthermore, we aimed to test the influence of the population size on genetic diversity. The population size of each species was, therefore, determined by counting the number of individuals in 10 to 15 1 $m^2$ plots per study site. The average number of individuals per square meter was then multiplied with the present area size (Reisch et al. 2018). For those study sites, where no individual could be found within investigated plots although plant material was collected, the total number of individuals was set from 0 to 1 before multiplying (Tab. S6).

We sampled 16 individuals per population and species for molecular analyses to display more than 90 % of the total genetic diversity (Leipold et al. 2020). The fresh leaf material was frozen in plastic bags in liquid nitrogen and stored at -20 °C until DNA extraction.

*Molecular analyses*

The DNA extraction was carried out following the CTAB protocol from Rogers and Bendich (1994) modified by Reisch (2007). The DNA quality and concentration were determined with a spectrophotometer. Afterwards, the DNA samples were diluted to the same level of 7.8 ng DNA per µl $H_2O$. We chose the analysis of amplified fragment length polymorphism (AFLP; Vos et al., 1995) for the analysis of the genetic variation within populations. The AFLP analyses were performed following the standardized protocol of Beckmann Coulter (Bylebyl et al. 2008). After screening 36 primer combinations per species, three species specific primer combinations were chosen for the selective amplification (Tab. S2). The automated sequencer GeXP (Beckmann Coulter) was used to separate the fluorescence-labelled DNA fragments by capillary gel electrophoresis. Virtual gels were analysed manually using the software Bionumerics 4.6 (Applied Maths, Kortrijk, Belgium). Only strong and clearly defined fragments were taken into account for further analyses, while samples without clear banding pattern, due to unsuccessful AFLP, were repeated or ultimately excluded.

A genotyping error rate was determined to ensure the reproducibility of the AFLP analyses (Bonin et al. 2004). Therefore, 10 % of all investigated samples were analysed twice. The percentage of fragments showing differences between original and replicate lay at 3.61 % (*A. sylvestris*), 5.36 % (*F. ulmaria*), and 4.93 % (*S. pratensis*).

*Statistical analyses*

The presence or absence of bands per particular fragment and individual was transformed into binary (0/1) matrices in Bionumerics 4.6. Based on these matrices we calculated the genetic diversity within each population in Popgene 32 (Yeh et al. 1997) as Nei's gene diversity (GD) $H = 1 - \sum(p_i)^2$, with $p_i$ representing the allele frequency.

A Kruskal-Wallis test with a post-hoc-Dunn's test (Dinno, 2015) and following Bonferroni p-adjustment (Bland and Altman 1995) was calculated in R to compare Nei's gene diversity on species level (RStudio Team 2016). We further tested the dependence of Nei's gene diversity on habitat age.

Hierarchical analyses of molecular variance (AMOVA) based on pairwise Euclidian distances between samples were calculated using the software GenAlEx 6.41 (Peakall and Smouse 2012). Hence, we analysed the genetic variation within and among populations as well as among populations on ancient and recent sites.

We computed Mantel tests with 999 permutations (Mantel 1967) to display correlations of geographic and genetic distances ($\Phi_{PT}$ values calculated in the AMOVA) among populations.

Correlation tests (Pearson correlation coefficients) were conducted to test for intercorrelations among explanatory variables (ii – xiii) (Tab. S7). Wilcoxon-Mann-Whitney tests displayed possible differences between past and present landscape variables (Tab. S8).

We formulated full linear regression models for each species in R Studio 1.1.423 (RStudio Team 2016) describing the variation of Nei's gene diversity in association to the scaled explanatory variables: (i) habitat age (not scaled), (ii) area size, (iii) historic and (iv) present total area of wet meadows, (v) historic and (vi) present distance to nearest settlement, and (vii) historic and (viii) present connectivity, which were described above. Further data about the coverage of (ix) vascular plants, (x) mosses, (xi) litter, (xii) open soil, and (xiii) population size were included in these models. We ranked all possible linear models according to AICc values (Akaike Information Criterion corrected for small sample sizes) to detect the models with the highest information content (Burnham and Anderson 2004).

## RESULTS

### *Genetic diversity and differentiation*

All studied species revealed similar levels of genetic diversity (Fig. 5). The mean genetic diversity of *A. sylvestris* populations lay at 0.216, ranging between 0.193 and 0.244. Similar values were found for *F. ulmaria*, whose mean genetic diversity was 0.216, with a minimum of 0.184 and a maximum of 0.248. Mean genetic diversity of *S. pratensis* was slightly lower with 0.210, varying from 0.167 to 0.242 (Tab. 6).

Overall genetic differentiation among populations was low. The differentiation found among populations was estimated at 4 % for *A. sylvestris* and at 5 % for *S. pratense*. *F. ulmaria* showed the highest differentiation rate with 8 % (Tab. 7). However, the AMOVAs showed no genetic differentiation among populations from ancient and recent sites.

Mantel tests revealed no significant correlation between genetic and geographic distances in either species (*A. sylvestris*: r = 0.0527, p = 0.052; *F. ulmaria*: r = 0.0003, p = 0.423; *S. pratense*: r = 0.0026, p = 0.334). Therefore, the studied populations are not likely to be isolated by distance.

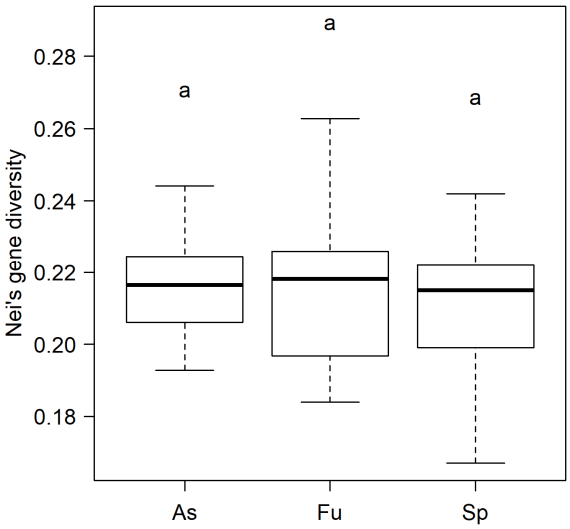



**Figure 5:** Nei's gene diversity of *A. sylvestris* (As), *F. ulmaria* (Fu), and *S. pratensis* (Sp)

**Table 6:** Number (No.), name (Population), and habitat age of the analysed populations. Also specified is the number of investigated individuals (N) and Nei's gene diversity per population of *A. sylvestris* (As), *F. ulmaria* (Fu), and *S. pratensis* (Sp).

| | | | N | | | Nei's Gene Diversity | | |
|---|---|---|---|---|---|---|---|---|
| **No.** | **Population** | **Age** | **As** | **Fu** | **Sp** | **As** | **Fu** | **Sp** |
| **01** | Arrisried | Ancient | 16 | 16 | 16 | 0.218 | 0.248 | 0.215 |
| **02** | Schlier | Ancient | 16 | 16 | 16 | 0.203 | 0.187 | 0.205 |
| **03** | Schwanden | Ancient | - | 16 | 16 | - | 0.220 | 0.215 |
| **04** | Ratzenried | Ancient | 16 | 15 | 16 | 0.216 | 0.220 | 0.242 |
| **05** | Liebenried | Ancient | 16 | 16 | 16 | 0.226 | 0.205 | 0.209 |
| **06** | Argen | Ancient | 16 | 16 | 16 | 0.212 | 0.193 | 0.188 |
| **07** | Kißlegg | Ancient | 15 | 16 | 16 | 0.203 | 0.209 | 0.231 |
| **08** | Rotheidlen | Ancient | 15 | 16 | 16 | 0.244 | 0.195 | 0.207 |
| **09** | Bremberg | Ancient | 16 | 16 | 16 | 0.229 | 0.227 | 0.218 |
| **10** | Nitzenweiler | Ancient | 16 | 16 | 16 | 0.193 | 0.198 | 0.179 |
| **11** | Wolfegg | Ancient | 16 | 16 | - | 0.233 | 0.236 | - |
| **12** | Wangen im Allgäu | Recent | 16 | 16 | 16 | 0.198 | 0.221 | 0.199 |
| **13** | Hinteressach | Recent | 16 | 16 | 16 | 0.217 | 0.263 | 0.220 |
| **14** | Wolfegg | Recent | 16 | 16 | 16 | 0.217 | 0.225 | 0.167 |
| **15** | Rotenbach | Recent | 15 | 16 | 16 | 0.207 | 0.246 | 0.230 |
| **16** | Hüttenweiler | Recent | 16 | 16 | 16 | 0.206 | 0.184 | 0.231 |
| **17** | Vogt | Recent | 16 | 16 | 16 | 0.223 | 0.213 | 0.222 |
| **18** | Gwigg | Recent | 16 | 16 | 16 | 0.213 | 0.216 | 0.194 |
| **19** | Sigrazhofen | Recent | 16 | 16 | - | 0.223 | 0.190 | - |
| **20** | Edensbach | Recent | 16 | 16 | - | 0.233 | 0.222 | - |
| **Mean** | | | | | | **0.216** | **0.216** | **0.210** |
| **SD** | | | | | | ± 0.003 | ± 0.005 | ± 0.004 |

**Table 7:** Genetic variation per species among populations on ancient and recent sites (habitat age), among and within studied populations detected by AMOVA. Levels of significance are based on 999 iteration steps.

| Species | AMOVA | df | SS | MS | Est. Var. | % | $\Phi_{PT}$ | |
|---|---|---|---|---|---|---|---|---|
| ***A. sylvestris*** | Among ancient and recent | 1 | 19.63 | 19.63 | 0.00 | 0 | 0.040 | *** |
| | Among populations | 17 | 463.21 | 27.25 | 0.71 | 4 | | |
| | Within populations | 282 | 4514.20 | 16.01 | 16.01 | 96 | | |
| ***F. ulmaria*** | Among ancient and recent | 1 | 53.73 | 53.73 | 0.04 | 0 | 0.077 | *** |
| | Among populations | 18 | 866.09 | 48.12 | 1.71 | 8 | | |
| | Within populations | 299 | 6242.00 | 20.88 | 20.88 | 92 | | |
| ***S. pratensis*** | Among ancient and recent | 1 | 26.27 | 26.27 | 0.00 | 0 | 0.053 | *** |
| | Among populations | 15 | 393.22 | 26.21 | 0.77 | 5 | | |
| | Within populations | 255 | 3539.81 | 13.88 | 13.88 | 95 | | |

Signif. Code: p < 0.001 ***

df, degree of freedom; SS, sum of squares; MS, mean squares; Est. Var., estimated variation;
%, proportion of genetic variation, ΦPT, indicator for genetic differentiation among populations

Overall genetic differentiation among populations was low. The differentiation found among populations was estimated at 4 % for *A. sylvestris* and at 5 % for *S. pratense*. *F. ulmaria* showed the highest differentiation rate with 8 % (Tab. 7). However, the AMOVAs showed no genetic differentiation among populations from ancient and recent sites.

Mantel tests revealed no significant correlation between genetic and geographic distances in either species (*A. sylvestris*: r = 0.0527, p = 0.052; *F. ulmaria*: r = 0.0003, p = 0.423; *S. pratense*: r = 0.0026, p = 0.334). Therefore, the studied populations are not likely to be isolated by distance.

*Linear regression models*

The AICc model selection generated significant models for all studied species (Table 8 a-c). The model for *A. sylvestris* only included a negative association with the present area size, indicating a decrease of genetic diversity with increasing meadow area (Table 8 a). Genetic diversity in *S. pratensis* was negatively affected by population size (Table 8 c), explaining 21.51 % of the observed variation. For *F. ulmaria* the model revealed more than one connection with the explanatory variables included (Table 8 b). Present connectivity was the most important variable negatively influencing current genetic diversity, while historic connectivity was positively associated. Present distance to the next settlement and present total area of wet meadows were positively related to genetic diversity in this species. Habitat age was also a significant predictor for genetic diversity, indicating a tendency for recent meadows to show higher genetic diversity levels. Both moss and vascular plant cover were positively associated with genetic diversity of *F. ulmaria*. Overall, the model accounted for 75.37 % of the observed variation.

**Table. 8:** Linear models explaining genetic diversity of *A. sylvestris* (a), *F. ulmaria* (b) and *S. pratensis* (c) populations in litter meadows. The effect size of the association with the response variable (Estimate), the standard error (SE), and the p-value are given for each of the variables within the models.

**(a) *A. sylvestris***

| | | **Estimate** | **SE** | **p-value** | |
|---|---|---|---|---|---|
| | (Intercept) | 0.216 | 0.005 | < 0.001 | *** |
| **Response Variable** | **Explanatory variable** | | | | |
| Nei's Gene Diversity | Area_S | -0.007 | 0.005 | 0.019 | * |
| Residual Standard Error: 0.01155 On 17 Degrees of Freedom | | | | | |
| Multiple R-Squared: 0.283, Adjusted R-Squared: 0.2408 | | | | | |
| F-Statistic: 6.71 On 1 And 17 Df, P-Value: 0.01905 | | | | | |

**(b) *F. ulmaria***

| | | **Estimate** | **SE** | **p-value** | |
|---|---|---|---|---|---|
| | (Intercept) | 0.208 | 0.007 | < 0.001 | *** |
| **Response Variable** | **Explanatory variable** | | | | |
| Nei's Gene Diversity | Age_recent | 0.019 | 0.010 | 0.004 | ** |
| | Area_2018 | 0.023 | 0.009 | < 0.001 | *** |
| | Con_2018 | -0.029 | 0.009 | < 0.001 | *** |
| | Dist_2018 | 0.011 | 0.006 | 0.002 | ** |
| | Con_1820 | 0.010 | 0.006 | 0.005 | ** |
| | Moss | 0.010 | 0.006 | 0.009 | ** |
| | VP | 0.006 | 0.005 | 0.042 | * |
| Residual Standard Error: 0.01074 On 12 Degrees of Freedom | | | | | |
| Multiple R-Squared: 0.8444, Adjusted R-Squared: 0.7537 | | | | | |
| F-Statistic: 9.304 On 7 And 12 Df, P-Value: 0.0004949 | | | | | |

**(c) *S. pratensis***

| | | **Estimate** | **SE** | **p-value** | |
|---|---|---|---|---|---|
| | (Intercept) | 0.210 | 0.008 | < 0.001 | *** |
| **Response Variable** | **Explanatory variable** | | | | |
| Nei's Gene Diversity | Population size | -0.010 | 0.009 | 0.035 | * |
| Residual Standard Error: 0.01768 On 15 Degrees of Freedom | | | | | |
| Multiple R-Squared: 0.2642, Adjusted R-Squared: 0.2151 | | | | | |
| F-Statistic: 5.385 On 1 And 15 Df, P-Value: 0.03481 | | | | | |

Signif. codes: < 0.001 ***; 0.001 ≤ p < 0.01 **; 0.01 ≤ p < 0.05 *; ≥ 0.05 n.s.

Area_S, Area Size; Area_2018, present total area of wet meadows [ha]; Dist_2018, present distances to the nearest settlement [km]; Con_1820/Con_2018, historic and present connectivity; Moss, moss cover [%]; VP, vascular plant cover [%]

## DISCUSSION

### *Genetic diversity and differentiation*

We observed similar values of genetic variation within and among populations of all study species. The genetic diversity of these species slightly exceeded the values expected for insect pollinated species (Reisch and Bernhardt-Römermann 2014). Genetic differentiation among populations was generally low, with *F. ulmaria* showing the highest differentiation. Spatial isolation did not play a major role for population differentiation.

Previous studies have shown that seeds are well transported among meadows via mowing machines (Strykstra et al. 1997). The litter meadows investigated here are typically mown by only few conservation managers once in the autumn (personal communication), enhancing gene flow by seed exchange among sites. Additionally, the occurrence of the study species is not strictly limited to litter meadows (Oberdorfer et al. 2001) and they are pollinated by a diverse group of insects (Kühn et al. 2004), providing many opportunities for gene flow by pollinators among sites.

Other studies on genetic diversity and differentiation of the species analysed here are scarce. Only the effect of inbreeding and population size on the genetic variation of *S. pratensis* was already studied using allozyme electrophoresis (Vergeer et al. 2003a). Therefore, the genetic variation observed in these species is not directly comparable with other studies.

### *Effect of habitat age on genetic variation*

Levels of genetic diversity in all three study species were similar among populations on ancient and recent sites. Additionally, habitat age revealed no significant impact on genetic diversity in *A. sylvestris* and *S. pratensis* in the linear regression models. This result stands in contrast to the studies of Jacquemyn et al. (2004) and Rosengren et al. (2013), who observed a comparatively lower genetic diversity on recent sites, e.g. in the moss species *Homalothecium lutescens*. However, historic management practices of sowing, hay and seedling transfer for the establishment and maintenance of litter meadows (Poschlod and Fischer 2016; Poschlod 2017) likely supported high levels of gene flow among ancient and recent sites. Moreover, all study species are pollinated by numerous different insects (Kühn et al. 2004) increasing the levels of gene flow among sites. Thus, gene flow by pollinators and seed dispersal at the time of founding and afterwards might reduce the effects of habitat age (Vandepitte et al. 2010).

Habitat age was a significant predictor for genetic diversity patterns of *F. ulmaria,* revealing a tendency of more recent sites to show higher diversity values. However, the variable 'habitat age' was possibly included by the model selection algorithm to correct for the overestimation of historic connectivity, which is significantly lower today. Therefore, we conclude that habitat age generally had no impact on genetic diversity of our study species.

Furthermore, we observed no significant differentiation among populations concerning habitat age. The practice of litter meadow establishment and traditional management practices ensured high levels of gene flow in the past. Today, seeds are still comparatively well transported via mowing machines among litter meadows (Strykstra et al. 1997). These land use practices supported and still support relatively high levels of gene flow, preventing genetic differentiation among populations on ancient and recent sites.

### *Effects of landscape structure on genetic diversity*

Genetic diversity in *A. sylvestris* was negatively associated with the area of the respective litter meadow, indicating larger meadows to comprise lower genetic diversity. Larger habitats are expected to sustain larger populations and thus, also higher genetic diversity (Ouborg et al. 2006). In the case of *A. sylvestris* neither genetic diversity nor habitat size correlated with population

size. *A. sylvestris* might colonize microsites instead of whole meadows due to variable local habitat conditions and is also not limited to litter meadows as habitat, which might falsify the impact of population size. Furthermore, habitat size was determined via topographic maps leading to a potential over- or underestimation of litter meadows' habitat size. Therefore, we assume no or only a weak impact of habitat size on the genetic diversity of *A. sylvestris* populations.

Historic and present landscape structures revealed the greatest impact on the genetic diversity of *F. ulmaria* populations. The total present area of wet meadows, historic and present connectivity, and the present distance to the next settlement were associated with genetic diversity levels. All these factors have previously been shown to influence genetic diversity in grassland species (Jacquemyn et al. 2004; Reitalu et al. 2010; Münzbergová et al. 2013).

Genetic diversity in *F. ulmaria* increased with the present area of wet meadows around the studied populations. A large patch size and a high proportion of habitats within a geographic region is frequently found to increase genetic diversity by improving patch connectivity via pollinators or other gene flow vectors (Ouborg et al. 2006; Prentice et al. 2006). Gene flow among closely located patches decreases the effects of inbreeding and genetic drift and thus, maintains high genetic diversity (Aguilar et al. 2008).

We found a positive impact of historic connectivity on the genetic diversity in *F. ulmaria* complying with the findings of Münzbergová et al. (2013), who observed a positive effect of historic habitat connectivity on genetic diversity of *S. pratensis.* In the past, traditional management of litter meadows included frequent sowing or transplanting of plant material to increase the vegetation cover of desired litter producing species (Poschlod 2017). These management practices, which may have positively affected undesired species as well, maintained high gene flow levels across the whole region. High connectivity among sites may increase colonization and reduce extinction rates, explaining the positive effect of historic connectivity on the genetic diversity of *F. ulmaria*.

However, present connectivity revealed an opposite effect on the genetic diversity in *F. ulmaria*. The cultivation of litter meadows became redundant during the last decades and thus, remaining species-rich litter meadows within the study region are managed by only few conservation managers today (personal communication). Moreover, seeds of all study species are fully developed during mowing season in late autumn (Poschlod et al. 2003) and are likely to be transported well via mowing machines (Strykstra et al. 1997), creating 'too much' gene flow among populations. Exceptionally high levels of gene flow may induce an impoverishment of the local gene pool due to 'genetic 'swamping' and thus, cause a negative impact of present habitat connectivity on genetic diversity in *F. ulmaria*.

The present distance to the nearest settlement revealed a positive impact on the genetic diversity of *F. ulmaria*. It is generally accepted that anthropogenic disturbance levels decrease with increasing distance to the next settlement. Since comparatively low levels of man-made disturbance led to an increase of both species and genetic diversity (Frey et al. 2016), genetic diversity levels in *F. ulmaria* increased with rising distance to the nearest settlement.

*Effect of habitat quality and population size*

Habitat quality parameters and population size explained genetic diversity patterns in the studied species. The genetic diversity of *F. ulmaria* was positively associated with moss and vascular plant cover. In a vegetation unit, the frequent abundance of mosses and vascular plants is expected to decrease germination and establishment of plant species (Špačková et al. 1998; Poschlod and Biewer 2005; Drake et al. 2018). However, in wet grassland habitats mosses can act as safe sites for germination (Wang et al.

2012) by retaining seeds (Freestone 2006), producing more stable habitat conditions, and protecting seedlings from harsh climatic conditions (Donath and Eckstein 2010; Lemke et al. 2015). Similarly, grass tussocks can also retain seeds and facilitate germination, especially in wet environments (Wang et al. 2012). A high coverage of moss and vascular plants may, therefore, facilitate the germination and establishment of *F. ulmaria* in litter meadows and consequently increase genetic diversity levels.

Correlations between population size and genetic diversity are expected to be positive, with larger populations maintaining more genotypes (Vergeer et al. 2003a; Ouborg et al. 2006). However, the genetic diversity of *S. pratensis* decreased with increasing population size. Grassland plant species with long life cycles, slow intrinsic dynamics, and comparatively large population size may occur as remnant populations in modern landscapes (Maurer et al. 2003). Piqueray et al. (2011) observed, moreover, that historic habitat configurations may often affect the present occurrence of a species, indicating a time lag between habitat loss, fragmentation, and their consequences on genetic diversity (Helm et al. 2006). Therefore, previous studies predicted a delayed response of genetic diversity to habitat fragmentation (Honnay et al. 2007). Additionally, *S. pratensis* is a more specialised and less widespread species than *A. sylvestris* and *F. ulmaria*. The Pearson correlation revealed a negative impact of moss cover on the population size of *S. pratensis* and, moreover, a negative relationship between the cover of moss and open soil. Therefore, we hypothesise that *S. pratensis* depends on open soil for successful germination and establishment. Hence, genetic diversity levels were low, despite high population sizes, due to a potential extinction debt and/or missing niches for germination and establishment.

*Conclusion*

Our study revealed significant and species-specific impacts of landscape structure, habitat quality, and population size on genetic diversity. While the influence of habitat size on genetic diversity in *A. sylv*estris remained unclear, *F. ulmaria* populations were significantly driven by the distance to the nearest settlement, the total area of litter meadows, and their connectivity. Moreover, the cover of mosses and vascular plants showed a significant impact on the genetic diversity of *F. ulmaria* populations. The genetic diversity of *S. pratensis* populations was affected in two ways: directly by population size and indirectly by the cover of moss.

Abandonment of traditional land use practices changed the abundance and local habitat quality of semi-natural litter meadows during the last decades. Additionally, the practice of litter meadow establishment, traditional and also current management practices, caused and still cause man-made gene flow among litter meadows. Thus, historic and present landscape structures as well as local habitat quality turned out as key variables driving genetic variation patterns of typical litter meadow species.

Hence, the future conservation of these species rich habitats should pay reference to historic as well as present processes to ensure the maintenance of litter meadows in our cultural landscape. Different mowing machines should be used in a rotating system to ensure moderate levels of gene flow and thus, counteract an impoverishment of the gene pool by genetic ‘swamping’. Furthermore, traditional management practices should be supported to promote appropriate germination niches.

# CHAPTER FOUR: GENETIC AND EPIGENETIC VARIATION IN *LINUM CATHARTICUM*

## HABITAT MATTERS – STRONG GENETIC AND EPIGENETIC DIFFERENTIATION AMONG POPULATIONS OF *LINUM CATHARTICUM* FROM DRY AND WET GRASSLANDS

## ABSTRACT

Plant species differ in their ecological amplitude, with some species occurring in very different habitats under strongly differentiated environmental conditions. We were interested to what extent the occurrence of *Linum catharticum* in dry calcareous grasslands (Bromion) and wet litter meadows (Molinion), two habitats on opposing ends concerning e.g. moisture level, is reflected on the genetic and epigenetic level.

Using AFLP and MSAP analyses we studied the genetic and epigenetic variation of *L. catharticum* from calcareous grasslands and litter meadows. From each habitat we sampled five populations with 16 individuals per site.

We observed lower genetic than epigenetic diversity, but considerable differentiation among habitats, which was stronger on the genetic than the epigenetic level. Additionally, we observed a strong correlation of genetic and epigenetic distance, irrespective of geographic distance. The dataset included a large portion of fragments exclusively found in populations from one or the other habitat. Some epigenetic fragments even occurred in different methylation states depending on the habitat.

We conclude that environmental effects act on both the genetic and epigenetic level, producing the clear differentiation among populations from calcareous grasslands and litter meadows. These results may also point into the direction of ecotype formation in this species.

Keywords: calcareous grasslands; DNA methylation; ecotypes; epigenetics; litter meadows; *Linum catharticum*; habitat differentiation

## INTRODUCTION

Through the wide variety of our global ecosystems, plant species experience an extraordinary range of environmental conditions among and within habitats (Schulz et al. 2014). Some species, often referred to as habitat specialists, are limited to very specific habitat conditions, like salt marsh or alpine species. This specialization is often considered as a limited niche breadth. Others are known as habitat generalists and have a broader ecological amplitude, enabling them to occur under different habitat conditions (Devictor et al. 2010). The study of habitat specialization and the concept of the ecological niche has been widely under discussion and different concepts have been described (Chase and Leibold 2003; Devictor et al. 2010). It has been proposed that the occurrence of habitat specialists is governed mainly by environmental processes, while the distribution of generalist species is determined more by dispersal processes (Pandit et al. 2009). However, it is likely that intraspecific variation plays a key role in the ability of plant species to grow under specific conditions. Evolutionary mechanisms have led to the adaptation of plant species to varying conditions and phenotypic plasticity enables plant individuals to cope with rapid changes. Many of these processes depend on genetic variation and evolutionary mechanisms, but there is also growing evidence that epigenetic processes play a major role in the response of plant individuals and populations to different or changing environmental conditions, especially on short time scales (Medrano et al. 2014; Gáspár et al. 2019).

Different epigenetic mechanisms, like histone modifications, small RNAs and DNA methylation, can cause stable alterations in gene expression, while DNA sequences remain unchanged (Bossdorf et al. 2008; Verhoeven et al. 2010; Schulz et al. 2013). The best studied mechanism to date is DNA methylation, which frequently occurs at CG sites in promotor regions in the DNA sequence. The addition of a methyl group to the cytosine molecule often leads to gene silencing, but can also activate gene expression (Bossdorf et al. 2008; Schulz et al. 2013). DNA methylation can be caused via genetic control, environmental influences or by spontaneous epimutations (Richards et al. 2017). Epigenetic modifications can be stably inherited across generations (Gáspár et al. 2019), thus transmitting favourable phenotypic variation to the offspring generations. Epigenetic variation is often correlated with genetic diversity, but several studies also found independent epigenetic variation, which was explained by environmental conditions rather than genetic variation (Riddle and Richards 2002; Bossdorf et al. 2008; Herrera and Bazaga 2010; Richards et al. 2010; Herrera et al. 2014; Medrano et al. 2014). By the environmental selection of stable epigenetic variants with increased fitness, correlated genetic selection can be guided. However the magnitude of this potential in wild populations has yet to be studied more intensively (Herrera and Bazaga 2010).

In recent years, studies of natural populations have been added to the studies of model organisms, like *Arabidopsis thaliana* (L.) HEYNH. or crop species (Heer et al. 2018), broadening our understanding of epigenetic processes under natural conditions. Most recent studies focussed on perennial plant species, e.g. clonal *Populus tremuloides* MICHX. stands (Ahn et al. 2017), salt marsh perennials (Foust et al. 2016), or a typical grassland species like *Plantago lanceolata* L. (Gáspár et al. 2019). All these studies found epigenetic differences, which could at least partially be attributed to differences in their local environmental conditions (flooding frequency and intensity, habitat openness & herbivory). These results suggest that epigenetic variation is indeed an important mechanism for plants to cope with rapid changes in their environment. However, data from species with really broad ecological amplitudes concerning soil physical or chemical parameters, exceeding the differences mentioned above are, to our best knowledge, still missing.

Epigenetic variation has also great potential and significance in nature conservation and restoration. Local adaptation of plant population might be not fixed genetically, but (partially) epigenetically, thus adding another concern to exchange of plant material among sites. The origin of plant material for restoration of plant populations or

habitats is important, as local adaptations may not be advantageous in new surroundings (McKay et al. 2005; Bucharova et al. 2017). In Germany seed transfer zones have been established to prevent the mixing of regionally adapted material (Durka et al. 2017). However, species with a broad ecological niche can occur on different habitats and thus be differently adapted within these zones, which may not be visible by the genetic fingerprint alone. Different populations of a generalist species can contain specialized individuals that represent only a part of the broader populations ecological niche (Araújo et al. 2011).

In this study we were interested in the annual plant species *Linum catharticum*. This species occurs on a broad range of habitats, reaching from dry to wet grasslands, making it a typical indicator for periodically wet or dry conditions in dry and wet grasslands respectively. Changes in water availability are a key aspect of global change dynamics and have profound effects on phenological and physiological plant processes (Reyer et al. 2013). Therefore, the study of a species growing on both ends of the moisture spectrum in temperature European grasslands will provide some valuable insights into plant response possibilities under future weather extremes. To investigate the genetic and epigenetic variation of *L. catharticum* under natural, but divergent habitat conditions, we included populations from dry calcareous grasslands and wet litter meadows in our data set. Calcareous grasslands are semi-natural habitats, which are dependent on grazing to maintain their specific species composition (WallisDeVries et al. 2002). They are generally characterized by dry and nutrient poor soils (Dierschke and Briemle 2002). The vegetation is dominated by e.g. *Bromus erectus* (WallisDeVries et al. 2002; Willerding and Poschlod 2002) and belongs to the union of Bromion erecti (Mucina et al. 2016). Litter meadows are dominated by species like *Molinia caerulea* (Poschlod et al. 2009b) and these ecosystems are characterized by nutrient poor and wet soils (Dierschke and Briemle 2002; Poschlod 2017). The vegetation belongs to the union Molinion caeruleae (Mucina et al. 2016). Today calcareous grasslands are typically grazed (e.g. sheep and goats), while litter meadows are mown once in the autumn, both as part of conservation management practices. The two grassland types provide habitat for many endangered plant and animal species, like the spring pasqueflower *Pulsatilla vernalis* (L.) MILL. (Betz et al. 2013) or the marsh fritillary *Euphydryas aurinia* (Brunbjerg et al. 2017) and are threatened by habitat fragmentation, intensification and abandonment (Abt 1991; Poschlod and WallisDeVries 2002).

We used Amplified Fragment Length Polymorphisms (AFLP) and Methylation Sensitive Amplification Polymorphisms (MSAP) to investigate the genetic and epigenetic structure of these populations. The use of dominant markers is a powerful, quick and easy tool to study non-model species without large reference data bases and gives stable results on the genetic and epigenetic structure of populations (Schulz et al. 2013; Lele et al. 2018). These methods provide a first step towards understanding the role of genetic and epigenetic variation in natural populations of *L. catharticum*. By these means we aimed at investigating one of the basic ecological questions (Bossdorf et al. 2008), whether populations from the two different habitats showed differences in their genetic and epigenetic variation and how this variation is structured among the populations. We hypothesised that epigenetic variation would show a clear pattern across all populations, while genetic variation within and among populations would be expected to be comparably low.

## METHODS

### *Study species*

*Linum catharticum* occurs as an annual or biennial herb (Ebel and Mühlberg 1987; Hensen 2008) and is common within the study region, but locally populations are decreasing due to land use intensification or meadow afforestation (Sebald et al. 1992). Its shoots reach between 5 and 30 cm in height, with small white-yellow flowers, blooming from June to July (Sebald et al. 1992; Oberdorfer et al. 2001; Rothmaler 2017). Pollination occurs by small dipterous insects (Düll and Kutzelnigg

2005), but seeds are frequently produces via selfing (Knuth 1898; Lundgren et al. 2013). The small sticky seeds are dispersed by animals or by hayseed (litter meadows; Poschlod & Biewer, 2005) and also form a long lived seed bank (> 100 years) (Milberg 1994; Fischer et al. 1996; Poschlod et al. 1998), but seedling mortality is considered high (Bradshaw and Doody 1978). *L. catharticum* mainly occurs on calcareous substrate with a wide range of moisture levels (Sebald et al. 1992; Oberdorfer et al. 2001).

*Study sites and sampling*

To investigate the genetic and epigenetic differences due to habitat, we collected plant samples from two habitats on opposing ends of the moisture spectrum: calcareous grasslands and litter meadows. In a previous study Lehmair et al. (unpublished) analysed the genetic variation of *L. catharticum* from 19 calcareous grasslands across the Swabian Alb and found intermediate levels of genetic diversity and low levels of genetic differentiation among populations. We did not detect any indication for isolation by distance, despite the considerable maximum distance of 85 km among populations. Some of these calcareous grasslands were therefore included in the present study and their population genetic and epigenetic diversity compared with those from populations originating from litter meadows in the Allgäu region.

The differences among calcareous grasslands and litter meadows are manifesting on the biotic and abiotic level. The vegetation structure of the two studied habitats differs in their grass, legume and herb cover. While calcareous grasslands are more dominated by herbaceous species, grasses dominate the vegetation cover in litter meadows (Tab. S9). The Ellenberg Indicator values were used to describe the abiotic conditions at the site in combination with basic soil analysis. The two habitats showed large differences in water and nutrient availability. While calcareous grasslands show dry conditions, litter meadows are in wet conditions. Additionally, litter meadows showed more acidic soils than calcareous grasslands. The same pattern is visible for the nutrient availability, calcareous grasslands are less limited in their available nutrients than litter meadows (Tab. S10), however both habitats would still be considered nutrient poor compared to other grassland types.

Study sites were selected, based on the availability of sufficient numbers of plant individuals and five sites were chosen for each habitat, within similar distances from each other. Five populations of *L. catharticum* were located on calcareous grasslands, which were compared with five populations from the more wet litter meadows (Tab. 9, Fig. S5). At each study site 16 plant samples were collected (min. distance between samples: 5 meters) in individual plastic bags and frozen in liquid nitrogen. Sampling took place during the early phase of flowering, so samples were from comparable life stages. The samples were stored in the lab at -20°C until DNA extraction.

*Molecular analyses*

From the frozen plant material, DNA was extracted using the CTAB protocol by Rogers & Bendich (1994), modified by Reisch (2007). DNA extracts were diluted with water to a standardized concentration (7.8 ng/µL) and used for further analysis. In a first step samples were analysed using Amplified Fragment Length Polymorphisms (AFLP) according to the protocol by Reisch (2008) adapted from Vos et al. (1995). The same DNA samples were then used in a Methylation Sensitive Amplified Polymorphism analysis (MSAP), as described by Salmon *et al.* (2008) and Schulz *et al.* (2013). This procedure is based on the same protocol as the AFLP analysis, but uses each sample in two separate reactions, starting with the DNA restriction step. Instead of *MseI* which is used for AFLP analysis, two other restriction enzymes, which are so called isoschizomers (*HpaII* and *MspI*), are paired with *EcoRI*. Due to their specific methylation sensitivity they allow for differentiation between different methylation states within their restriction site. (For further methodological details see (Schulz et al.

2013). Prior to both AFLP and MSAP analysis suitable primer combinations were screened and for each marker type three combinations chosen for this analysis (Tab. S2).

Individuals that resulted in unclear band patterns were repeated once and then omitted from the final data set, thus resulting in 80 samples for the calcareous grasslands and 70 samples from litter meadows.

For both the AFLP and MSAP analyses a genotyping error rate following the procedure of Bonin et al. (2004) was estimated by repeating the analysis of 10% of the studied individuals (16 individuals) and comparing resulting band patterns. This procedure resulted in a genotyping error rate of 2.52 % in the AFLP analysis and of 1.38 % in the MSAP analysis. We therefore conclude that the observed differences among individuals and populations are due to actually present molecular differences and not due to methodical errors.

**Table 9:** Number and names of the analysed populations, their respective habitat, the geographic location they are situated in, as well as the number of analysed individuals per population.

| Nr | Name | Habitat | Lat | Lon | N |
|---|---|---|---|---|---|
| **C1** | Bichishausen | Calcareous grassland | 48.3349 | 9.5013 | 16 |
| **C2** | Justingen | Calcareous grassland | 48.4034 | 9.6905 | 16 |
| **C3** | Büchelesberg | Calcareous grassland | 48.3080 | 9.7247 | 16 |
| **C4** | Hohenstein | Calcareous grassland | 48.3202 | 9.3159 | 16 |
| **C5** | Gomadingen | Calcareous grassland | 48.3911 | 9.3770 | 15 |
| | | | | | |
| **L1** | Arrisried | Litter meadow | 47.7536 | 9.8787 | 10 |
| **L2** | Weitershofen | Litter meadow | 47.8169 | 9.8996 | 13 |
| **L3** | Argen | Litter meadow | 47.6702 | 10.0746 | 16 |
| **L4** | Rotheidlen | Litter meadow | 47.7320 | 9.7161 | 16 |
| **L5** | Nitzenweiler | Litter meadow | 47.6077 | 9.6367 | 15 |

*Data analysis*

AFLP and MSAP fragment data were analysed separately, using the software Bionumerics 7.6.3 (Applied Maths) to create a binary matrix for each dataset, representing the presence and absence of a given fragment for each individual. The AFLP dataset included 158 fragments, while the MSAP dataset comprised 337 fragments. The 0/1 matrix of the MSAP data set was then scored for unmethylated (Epi_u), methylated (Epi_m) and hemimethylated (Epi_h) epiloci using the scoring method 'Mixed-Scoring 2' as proposed by Schulz et al. (2013), using the R-script 'MSAP_calc'. This scoring procedure then resulted in a binary matrix for each epiloci type (302 Epi_u loci, 282 Epi_m loci and 222 Epi_h loci). Using the implemented PCoA Analysis in the 'MSAP_calc' all four datasets were analysed thus.

For all four binary matrixes (AFLP, Epi_u, Epi_m, Epi_h) genetic variation within populations, expressed by Nei's Gene Diversity ($H=1-\Sigma (p_i)^2$) was calculated using PopGene 32 (Yeh et al. 1997). To test for differences among populations from different habitats in the height of their genetic diversity we used a t-test.

To explore genetic and epigenetic differentiation, a hierarchical Analysis of Molecular Variance (AMOVA) (Excoffier et al. 1992), based on pairwise Euclidian distances between samples, was calculated applying GenAlEx 6.5 (Peakall and Smouse 2012). The two habitats were used as regions in this analysis.

To study the population structure a Bayesian cluster analysis was performed with Structure 2.3.4 (Pritchard et al. 2010) separately for all four marker types. To calculate the most likely number of groups we used a burn-in of 10 000 iterations and 100 000 MCMC simulations with K set between 1 and 12. For each K analysis was run 20 times. The web tool 'Structure Harvester' (Earl and vonHoldt 2012) was used to summarize the results. Following the method of Evanno *et al.*, (2005) we used the highest ΔK value to determine the best estimate of K.

The use of simple and partial Mantel tests has been discussed in the literature as being appropriate for these kinds of studies. Therefore we based our correlation analyses on the example of Lele *et al.*, (2018) and used simple and partial Mantel tests as well as multiple matrix regressions with randomization (MMRR) analyses. Based on the genetic and epigenetic distance values ($\phi_{PT}$ values), produced by the AMOVA, and the Euclidean geographic distance among populations we performed simple Mantel tests using GenAlEx and partial Mantel tests using the 'vegan' package (Oksanen et al. 2019), to test for the correlations of genetic and epigenetic distance with geographic distance and the respective other (epi)genetic distances. Additionally, analogous to Lele *et al.*, (2018) we used the MMRR function provided by Wang, (2013) using 9 999 permutations, to test for correlations of genetic and geographic distances, while controlling for epigenetic distance, as partial mantel tests have been attributed with some drawbacks (Wang 2013; Lele et al. 2018).
All analyses were conducted in R Studio 1.1.423 (RStudio Team 2016), if not otherwise specified.

## RESULTS

### *Genetic and epigenetic diversity and structure*

Genetic diversity was generally low, with an average over all populations of 0.078 and did not differ significantly among habitats. Epigenetic diversity for unmethylated and methylated epiloci differed significantly among habitats, with populations from litter meadows showing lower genetic and epigenetic diversity, than those from calcareous grasslands. Diversity for hemimethylated loci was even lower than genetic diversity and also did not differ significantly among habitats (Fig. 6, Tab. S11). Most hemimethylated loci occurred in less than five individuals and thus can only play a minor role in our dataset.

The datasets showed many fragments to be specific for one of the two habitats. The AFLP dataset included 33 fragments private to calcareous grasslands, while 25 fragments were only found in individuals from litter meadows. The MSAP dataset also showed large numbers of epiloci exclusively found in populations from one or the other habitat (CG: Epi_u 62, Epi_m 85, LM: Epi_u 44, Epi_m 31).

Additionally, we checked for epiloci, which were primarily found as methylated in one habitat, while it occurred primarily unmethylated in the other and vice versa. We in total found 13 of these fragments, of which seven were methylated in litter meadow populations and unmethylated in calcareous grasslands.

The other six fragments showed methylation in calcareous grasslands, while they appeared primarily unmethylated in litter meadow populations. We did not include the private fragments for Epi_h, as most fragments occurred only in few individuals and therefore are of minor importance for this dataset.

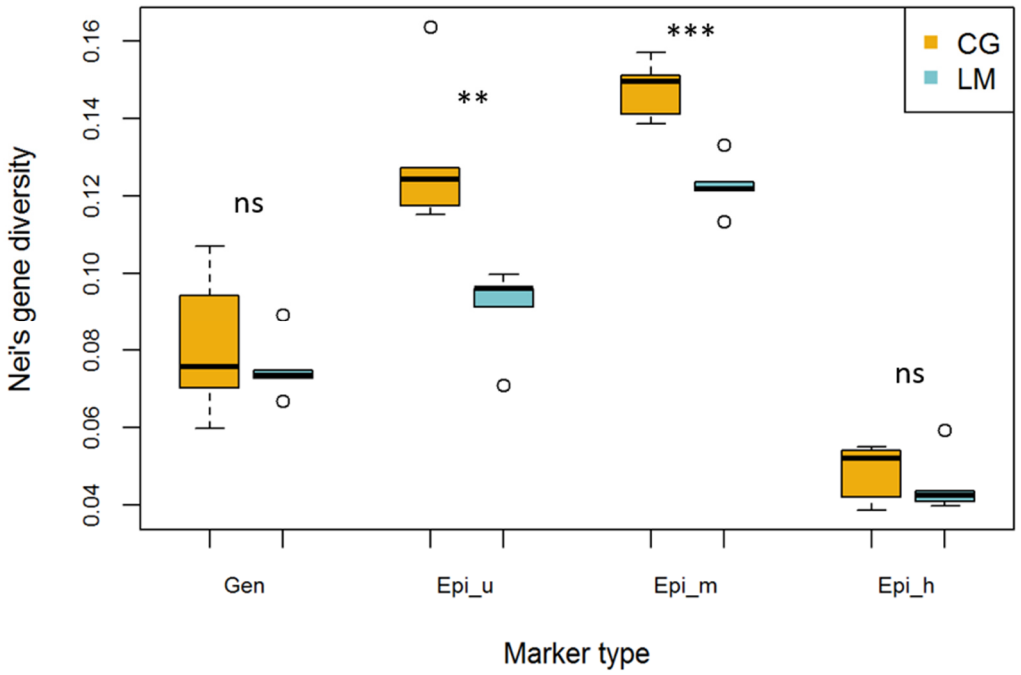



**Figure 6:** Nei's gene diversity separated by marker type, in orange the calcareous grasslands and in blue the litter meadows. The diversity for Epi_u and Epi_m is significantly different among habitats (Epi_u: p = < 0.01, t-value = 3.795; Epi_m: p = < 0.001, t-value = 5.354).

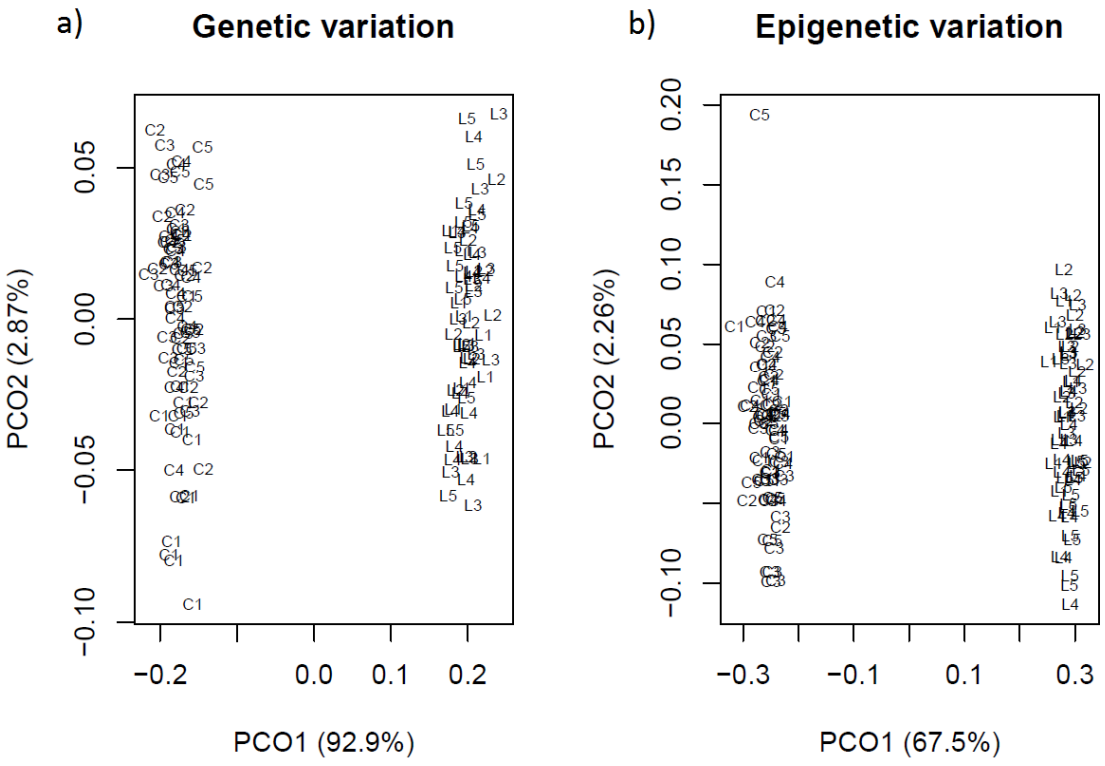



**Figure 7:** Principal Coordinate Analysis of the genetic dataset (a) and epigenetic dataset (b). The two habitats (C – calcareous grasslands, L – litter meadows) are clearly separated in two groups, on the genetic and epigenetic level.

Differentiation among habitats was generally very high. Genetic differentiation among habitats was 80 %. Epigenetic differentiation was lower among habitats than genetic differentiation (Epi_u: 62 %, Epi_m: 57 %). For Epi_h the majority of variation was found within populations (81 %), while 16 % were found among habitats (Tab. 10).

These results are further illustrated by the Principal coordinate analysis, which showed the two habitats as clearly separated, both on the genetic (Fig. 7 a) and epigenetic level (Fig. 7 b). The first axis explained 92.9 % of the variation within the genetic dataset, while the first axis for the epigenetic dataset explained 67.5 %. The second axis explained around two percent of the variation in both datasets, showing the high within habitat similarity of the populations. The Bayesian cluster analysis gave K = 2 as the most likely number of groups for all marker types, which represented the two habitats (Fig. S6), additionally supporting the above described results.

**Table 10:** Results of the Three-Level AMOVAs given as the genetic variation among habitats, among and within the respective populations for each marker type. (*** indicates a p-value of below 0.05).

| Marker Type | df | SS | MS | % | ϕpt-statistics |
|---|---|---|---|---|---|
| ***Genetic variation for Gen*** | | | | | |
| Among habitats | 1 | 2249.23 | 2249.23 | 80 | 0.835 *** |
| Among pops | 8 | 200.58 | 25.07 | 3 | |
| Within pops | 140 | 859.38 | 6.14 | 17 | |
| ***Epigenetic variation for Epi_u*** | | | | | |
| Among habitats | 1 | 2677.77 | 2677.77 | 62 | 0.663 *** |
| Among pops | 8 | 467.34 | 58.417 | 5 | |
| Within pops | 140 | 2682.282 | 19.16 | 34 | |
| ***Epigenetic variation for Epi_m*** | | | | | |
| Among habitats | 1 | 2275.00 | 2275.00 | 57 | 0.573 *** |
| Among pops | 8 | 380.01 | 47.50 | 3 | |
| Within pops | 140 | 2859.50 | 20.43 | 39 | |
| ***Epigenetic variation for Epi_h*** | | | | | |
| Among habitats | 1 | 145.75 | 145.75 | 16 | 0.194 *** |
| Among pops | 8 | 122.04 | 15.26 | 4 | |
| Within pops | 40 | 1257.79 | 8.98 | 81 | |

Signif. code: p < 0.001 ***

df, degree of freedom; ss, sum of squares; ms, mean squares; %, proportion of genetic variation;
ϕpt, indicator for genetic differentiation among populations

Simple Mantel tests showed strong and significant correlations between genetic and epigenetic distances, as well as of all marker types with geographic distance (Tab. 11). Partial Mantel test and the MMRR analysis showed, that the correlations between genetic and epigenetic distances was much more pronounced than the impact of geographic distance for unmethylated and methylated epiloci. For the correlation of genetic distance with epigenetic distance of methylated loci,

the correlation with geographic distance was even not significant, even though they were strongly correlated in the simple Mantel test. For hemimethylated loci the correlation with genetic distance was not as strong and correlation with geographic distance played a more pronounced role (Fig. 8 & Tab. 12).

**Table 11:** Results of the Mantel tests conducted for genetic (Gen) and epigenetic (Epi_u, Epi_m, Epi_h) distance with geographic distance (Geo) and the respective other (epi)genetic distances. Above diagonal is the $R^2$-value and the respective p-value below the diagonal.

| | **Gen** | **Epi_u** | **Epi_m** | **Epi_h** | **Geo** |
|---|---|---|---|---|---|
| **Gen** | x | 0.944 | 0.964 | 0.858 | 0.892 |
| **Epi_u** | 0.006 | x | 0.986 | 0.915 | 0.905 |
| **Epi_m** | 0.001 | 0.001 | x | 0.915 | 0.921 |
| **Epi_h** | 0.006 | 0.001 | 0.001 | x | 0.883 |
| **Geo** | 0.003 | 0.001 | 0.002 | 0.001 | x |

**Table 12:** Summary of the multiple matrix regression analysis with randomization (MMRR) relating the genetic distance matrix with geographic and epigenetic distance matrices.

| | | | | **Linear Predictor Matrices** | | | |
|---|---|---|---|---|---|---|---|
| | | **Overall Regression** | | **Geographical Distance** | | **Epigenetic Distance** | |
| **Differentiation Matrix** | **Epigenetic Matrix Used** | F | *p* | Coeff. | *p* | Coeff. | *p* |
| Gen | Epi_u | 381.237 | 0.0011 | 0.0024 | 0.024 | 0.967 | 0.0018 |
| Gen | Epi_m | 570.13 | < 0.001 | 0.0003 | 0.75 | 1.247 | 0.0014 |
| Gen | Epi_h | 184.42 | < 0.001 | 0.0016 | 0.0006 | 0.080 | 0.0251 |

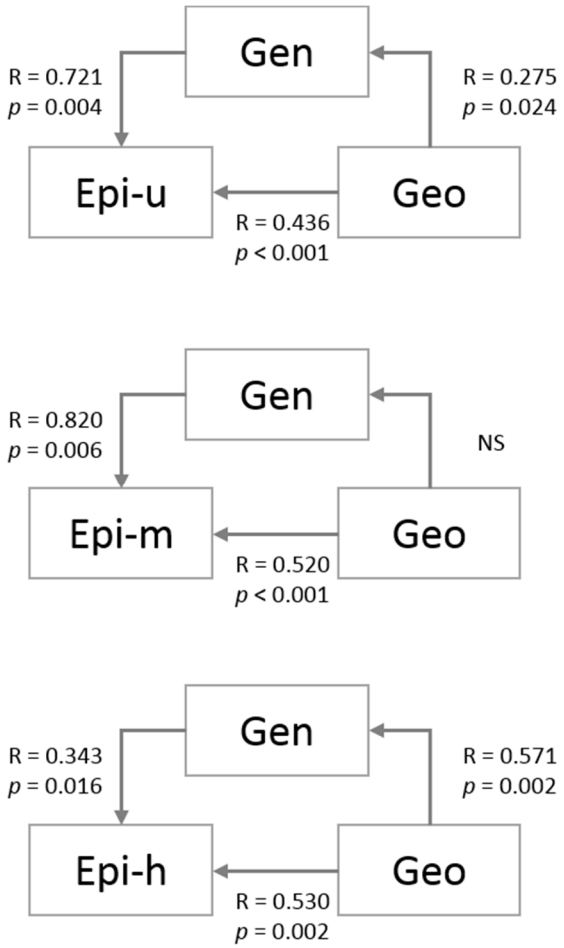



**Figure 8**: Genetic and epigenetic correlations to variation in geographic distance using partial Mantel tests with the Euclidian genetic and epigenetic distance matrices and geographical distance matrices across all populations. The correlations between genetic and epigenetic distance were calculated in separate partial Mantel tests. (Gen = genetic variation, Epi_u = epigenetic variation of unmethylated epiloci, Epi_m = epigenetic variation of methylated epiloci, Epi_h = epigenetic variation of hemimethylated epiloci, Geo = geographical distance, NS = not significant, r = correlation coefficient and respective p-value).

## DISCUSSION

It has been frequently reported in recent years, that epigenetic mechanisms may contribute significantly to the adaptive potential of plant individuals in natural populations (Bossdorf et al. 2008; Richards et al. 2010) and several studies have found evidence for habitat or environment specific epigenetic differences among populations (Lira-Medeiros et al. 2010; Richards et al. 2012; Ahn et al. 2017).

In our study we found comparably low levels of genetic diversity within populations. With a mean Nei's gene diversity of 0.078 over all populations, genetic diversity was lower than we would have expected, even for a mainly self-pollinated species (Reisch and Bernhardt-Römermann 2014). In the previous study of *L. catharticum* from 19 calcareous grasslands, mean genetic diversity was estimated at 0.155 (Lehmair *et al.*, unpublished), which is more in accordance with the values expected from rather common species. However, the present dataset includes many private fragments, only present in populations from one or the other habitat type, which decreases the estimated genetic diversity. By excluding the private fragments, not present in the respective dataset, diversity values would increase. The epigenetic datasets also included many private fragments. However, epigenetic diversity for unmethylated and methylated epiloci was still higher than genetic diversity. This finding is consistent with the results of other studies (Lira-Medeiros et al. 2010; Richards et al. 2012; Wang et al. 2019), which frequently also found higher epigenetic than genetic diversity in their studied non-model species. The low genetic diversity in these studies can be explained also by their study design of choosing e.g. clonal species. As a mainly selfing species (Knuth 1898; Lundgren et al. 2013) *L. catharticum* recombination events can be considered as rare, thus explaining the low genetic diversity. Epigenetic diversity is influenced by environmental conditions and spontaneous epimutations can also contribute to the observed higher epigenetic than genetic diversity (Wang et al. 2019). Similar to Schulz *et al.*, (2014) we found hemimethylated epiloci to play a marginal role in our dataset and therefore will exclude these epiloci from further discussion.

Genetic and epigenetic differentiation was high among populations from the two different habitats. Even though genetic diversity was lower

than epigenetic diversity, differentiation was higher on the genetic than on the epigenetic level. In the previous study on *L. catharticum* from calcareous grasslands across the Swabian Alb (max distance among sites: 85 km) variation among populations was estimated at 8 % (Lehmair *et al*., unpublished). These findings present tremendous differences to the levels of differentiation found among populations from the two different habitats. This indicates that geographic distance alone cannot explain the strong genetic and epigenetic differentiation found here among populations from different habitats, with maximum distances of 95 km. The maximum geographic distance among sites is similar to our previous study, however differentiation was much higher in the present study. Both the MMRR analysis and the partial Mantel tests showed that the correlation of genetic and epigenetic variation was quite marked and that the influence of geographic distance was of minor importance. These results are comparable to Lele *et al.*, (2018), who also found a strong correlation of the genetic and epigenetic variation and low correlations with geographic distance in *Vitex negundo var. heterophylla* L. (Chinese chastetree), even though the maximum distance among populations was 150 km, spanning a wider geographical range than even our study. Additionally Lele *et al.*, (2018) also found correlations between epigenetic variation and phenotypic diversity in their study, however local adaptation to divergent habitat conditions were mainly attributed to genetic variation.

In our dataset genetic and epigenetic fingerprints were strongly associated with the habitat of origin. A large fraction of fragments was private to one of the habitats. Populations from calcareous grasslands showed higher genetic as well as epigenetic diversity, which for methylated and unmethylated loci was also statistically significant. Additionally, we found a number of MSAP fragments present in both habitats, but which had different methylation states in each of them (e.g. methylated in calcareous grassland populations and unmethylated in litter meadow populations). Environmental disturbances are expected to be higher in calcareous grasslands, with multiple stress factors, e.g. water limitation, grazing and trampling, nutrient limitations, contributing to a generally more heterogeneous habitat (WallisDeVries et al. 2002), also expressed by the soil conditions of the studied grasslands. In litter meadows there is typically only one major disturbance event, i.e. mowing during autumn (Kapfer 1995). These environmental differences can explain the divergence on the genetic and epigenetic level found here and also the higher epigenetic variation found in populations of calcareous grasslands.

An influence of environmental conditions on epigenetic variation was found in several studies (Verhoeven et al. 2010; Richards et al. 2012; Medrano et al. 2014; Foust et al. 2016). Verhoeven *et al.*, (2010) found an increase in epigenetic variation within stress treatments in *Taraxacum officinale* WEBER ex WIGG., especially in an herbivory and pathogen defence trigger treatment. Under high simulated herbivory or pathogen pressure epigenetic variation increased. Herbivory by large animals is an important stressor associated with calcareous grasslands, which is not present in litter meadows in an equal way. Additionally *L. catharticum* populations have been found to decrease in fitness parameters under high trampling intensities (Bradshaw and Doody 1978). This suggests that the observed differences in genetic and epigenetic diversity and differentiation and also the marker specific differences can be logically attributed not only to geographic distance but maybe more importantly to environmental differences among the habitats.

Some studies have found environmental processes to influence genetic and epigenetic diversity independently (Verhoeven et al. 2010), but many studies also found a strong correlation of genetic and epigenetic diversity (Herrera and Bazaga 2010; Shan et al. 2012; Schulz et al. 2014; Dubin et al. 2015; Kawakatsu et al. 2016;

Robertson et al. 2017; Wang et al. 2019). The genetic and epigenetic variation in this study was strongly correlated, suggesting parallel processes, both on the genetic, and thus evolutionary, level and on the epigenetic level, suggesting different epigenotypes or ecotypes in the different habitats.

The extent to which these differences are visible on differently expressed genes and phenotypes would need to be assessed via furthermore detailed studies, e.g. with common garden and crossing experiments. More advanced molecular tools might help to determine which genes are differently regulated within the two habitats. Different subspecies have been proposed for *L. catharticum*, based on differences in morphology, but scientific evidence for the parallel occurrence of the two described subspecies in the study region is not available (Sebald et al. 1992). The strong genetic differentiation could be cautiously interpreted as an indication of different ecotypes in the different ecosystems.

As Herrera & Bazaga, (2010) already discussed, to understand the potential of epigenetic variation in adaptive and evolutionary processes, we need more studies in natural contexts. We therefore conclude that the results presented here give some indications on the magnitude of differences possible within a species and a comparably restricted geographical setting and provides a starting point for future research.

# CHAPTER FIVE: 'GO WITH THE FLOW' – GENERAL DISCUSSION, CONCLUSION & PERSPECTIVES

## GENERAL DISCUSSION, CONCLUSION AND PERSPECTIVES

Grasslands perform valuable ecosystem services and have been recognized as important biodiversity hotspots by the European Union (European Commission 2013) through the inclusion into the habitats directive (German: FFH-Richtlinie). The unique biodiversity of these ecosystems has been intensively studied in recent decades, investigating the historic development and the causes for current decline (Brys et al. 2005; Helm et al. 2006; Poschlod and Baumann 2010; Hejcman et al. 2013), as well as the processes shaping the current diversity (Lindborg and Eriksson 2004; Rasran et al. 2007; Aguilar et al. 2008), to improve the conservation of grassland biodiversity.

The studies presented in this thesis show that diverse factors influenced the within-species variation in different grassland species, depending on habitat of origin and species characteristics. Historic as well as current landscape structure influenced genetic variation in oat-grass meadow species as well as litter meadow species. Gene flow, both historic and current, turned out as a key factor shaping genetic variation in litter meadow populations. Oat-grass meadow plant populations were not only shaped by gene flow, but the habitat quality and thus germination niches played a significant role on shaping genetic variation in these species.
Between different grassland types, the same species can show markedly different genetic variation as well as epigenetic fingerprints. The strong differentiation between populations from different habitats observed in *Linum catharticum* can best be explained by the different habitat conditions, causing ecotype formation.

## EFFECT OF LAND USE HISTORY, LANDSCAPE VARIABLES AND HABITAT QUALITY

The persistent effect of land use history on grassland biodiversity has been emphasised by several studies. Especially in calcareous grasslands there have been multiple studies identifying effects of land use history on species diversity and community composition (Gustavsson et al. 2007; Aavik et al. 2008; Karlík and Poschlod 2009; Reitalu et al. 2010). Historic land use and landscape structure can explain current species diversity and composition, when current landscape does not seem to affect them. Thus, some studies concluded, that these grasslands might be suffering from extinction debt and will thus lose their currently high diversity in the future (Helm et al. 2006; Jackson and Sax 2010; Piqueray et al. 2011). Other studies even aimed at identifying species indicators for different land use histories, i.e. species, whose occurrence indicates a long history of grazing or agricultural use (Karlík and Poschlod 2019).

The effect of land use history of and landscape structure on genetic diversity has been studied for different species. While some studies found an effect of human population density (Helm et al. 2009), others found effects of land use history or habitat age (Jacquemyn et al. 2004; Rosengren et al. 2013). Investigating the genetic variation of *Briza media* in grasslands with species extinction debt, Helm et al. (2009) found that human impact and habitat fragmentation were associated with a decrease in genetic variation and concluded that species loss might be preceded by a loss of genetic diversity.

However, most of these studies were limited to single species and mainly focussed on dry calcareous grasslands. Instead, the studies described in Chapter Two and Three, focussed on two less studied grassland habitats. The influence of land use history, landscape structure and habitat quality on the genetic variation was compared among several species. By thus combining the analysis of several plant species from the same grassland sites, it was demonstrated the response of genetic variation to the different investigated explanatory variables is species and habitat dependent. Therefore, the results fit in well with previous studies, most of which also found species- and habitat dependent effects of different landscape or land use history variables. However, most of

the effects of land use history and landscape structure on genetic variation can be attributed to gene flow, through either historic or current gene flow patterns.

Historic land use often provided extensive gene flow among grassland sites through different vectors, e.g. direct seeding, hay transfer or epi- and endozoochory. These gene flow processes were disrupted with the overall agricultural intensification, induced by the invention of mineral fertilizers, which resulted in a massive habitat loss and fragmentation of species-rich grasslands (Poschlod et al. 2005).

Additionally, current habitat quality, i.e. vegetation and litter cover, was found to be an important determinant of genetic variation in oatgrass meadow and litter meadow species. These results are also reflected in other studies, who found negative, but also positive effects of moss and litter cover on germination and establishment of new plant individuals and thus also on the genetic diversity (Špačková et al. 1998; Jeschke and Kiehl 2008; Ruprecht et al. 2010; Ruprecht and Szabó 2012; Drake et al. 2018). These results emphasize the importance of habitat quality. When habitat conditions deteriorate, e.g. due to insufficient management, gene flow alone cannot maintain or increase species and genetic diversity, when germination and establishment of new individuals are restricted by insufficient habitat quality. Therefore, by recognizing the historic management applied by farmers to different grassland types and thereby increasing habitat quality as well as gene flow among grasslands, the success of conservation management could be improved.

### EPIGENETIC VARIATION IN THE VIEW OF GRASSLAND CONSERVATION

The study of epigenetic variation triggers new exciting questions in biodiversity research. The different epigenetic mechanisms, i.e. DNA methylation, histone modifications and small RNAs (Wendel and Rapp 2005), are not only relevant on the level of molecular pathways in model organisms, but also in the more applied view of ecologists, who are interested in explaining the patterns observed in the field. As Bossdorf et al. (2008) emphasised, ecologists can use epigenetic patterns in natural populations to understand the mechanisms underlying natural variation in ecologically important traits. Additionally, epigenetic variation may also be important in the response of plant species, populations and individuals to global change processes (Richards et al. 2017). Epigenetic variation has been found to increase population resilience to long term drought stress (Heer et al. 2018), or to increase productivity and stability of plant populations (Latzel et al. 2013). Thus, the study of epigenetic patterns in natural populations and ecosystems provides an interesting and important new field of research in the context of nature conservation.

Even though the results presented in Chapter Four do not permit interpretations on the level of genetic adaptation, the results still show enormous genetic and epigenetic differentiation among populations of *Linum catharticum* from calcareous grasslands and litter meadows. The two habitats are characterized by a large difference in the local ecological conditions, i.e. water availability and pH, making it highly likely that the genetic and epigenetic patterns are matched by the environmental patterns. Thus, the observed differences on the molecular level may also be important from a conservation point of view. Global change processes, e.g. increased drought and eutrophication (Zavaleta et al. 2003; Ahuja et al. 2010), will alter the environmental conditions of many ecosystems, including the here studied grasslands. The ability of species to adapt to rapidly changing environmental conditions will be crucial for population's survival under future climatic and environmental conditions. Therefore, epigenetic variation and its influence on the phenotypic variation, will likely contribute to the survival of populations under stress.

Furthermore, epigenetic variation has been proposed as a factor contributing to the invasion success of Japanese knotweed, *Reynoutria japonica* HOUTT. (Richards et al. 2008, 2012). Invasive species are a major threat to native ecosystems and their species. Many studies on invasive species report low genetic variation due to bottlenecks and founder effects, therefore, genetic variation alone cannot explain the invasion success of these species (Ouborg et al. 2006; Dlugosch and Parker 2008; Henry et al. 2009). Thus, studies on epigenetic variation of these species, might improve the understanding which factors make a species invasive, even under low genetic diversity.

## CONSERVATION GENETICS IN EUROPEAN GRASSLANDS

Grassland ecosystems have declined rapidly in recent decades (Klimek et al. 2007) and the remnants of species-rich traditionally used grasslands often have to be managed from a conservation perspective to maintain their species diversity. Depending on the land use history, i.e. the traditional management that led to the development of a specific grassland type, the appropriate conservation management varies among habitats. However, strict conservation management policies also create new challenges. For example fixed mowing dates for hay meadows promote the occurrence of toxic weed species like *Colchicum autumnale* L. (Jung et al. 2012), thus making the hay useless for use in animal husbandry. This example demonstrates that current conservation management can yet be improved to better preserve the biodiversity of grassland ecosystems.

Conservation management typically focusses on preserving and promoting the typical species composition of a given grassland type. However, the genetic variation contained in plant populations from traditionally managed grasslands is an important concern. Genetic variation is crucial for the adaptation of species to changing climatic and environmental conditions. Additionally, these grasslands also contain 'wild' populations of agriculturally relevant species like *Trifolium pratense*, which might be useful in future breeding efforts. Thus, the preservation of genetic variation in grassland ecosystems has become a focus point in nature conservation strategies.

To maintain local adaptations and avoid outbreeding depression the use of autochthonous seed material for the (re)establishment or reinforcement of grassland ecosystems has been proposed as a suitable conservation measure (Vander Mijnsbrugge et al. 2010; Jørgensen et al. 2016). As genetic differentiation among the populations studied in this thesis were comparably low, the results would support the transport of seed material within the limited geographical region of the Swabian Alb and the Württembergian Allgäu, with a low probability of outbreeding depression for the studied species.

However, the overall practicability of using autochthonous seed material has been called into question (McKay et al. 2005). While some studies find a poor fit of the autochthonous seed origin zones with the actual geographical differentiation patterns of wild populations (Listl et al. 2017), other studies report a relatively good fit (Durka et al. 2017), supporting the current practice. Kaulfuß and Reisch (2019) found strong genetic differentiation among natural and restored populations of two common grassland species, due to different ploidy levels. Also the seeding method plays an important role in the success of grassland restoration via seeds (Montalvo et al. 2002; Yurkonis et al. 2010; Kiehl et al. 2010).

A major concern, in the use of autochthonous seed material from commercial growers, is added by the rapid evolution that can take place during the propagation of grassland seeds, especially in short lived and selfing species (Nagel et al. 2019). Thus, the aim of seed origin zones to preserve local adaptations cannot be attained with certainty. These points show that the use of autochthonous seed material cannot be the only strategy to

maintain genetic variation and local adaptations in grassland ecosystems.

### GENETIC CONSERVATION AREAS

To address this issue, in recent years the idea of genetic conservation areas has gained popularity, especially for so called 'crop wild relatives' (CWR) (Maxted et al. 2011, 2015; Frese 2014; Phillips et al. 2014). Genetic conservation areas are established to actively preserve genetic variation of a target species under in-situ conditions (Maxted et al. 2011), thus preserving local adaptations and genotypes. Current procedure suggest the use of protected areas as preferable sites for genetic conservation areas, due to greater sustainability and efficacy (Maxted et al. 2011; Frese 2014). Target species of current efforts to establish genetic conservation areas focus on socio-economic relevant species, like fodder and forage crops, medicinal plants as well as crop wild relatives (Maxted et al. 2007). However, the concept of conservation areas targeting genetic variation could also be applied to grassland species, thus preserving intraspecific variation and local adaptations of these important ecosystems under natural conditions.

Focussing conservation efforts on specific populations of a given plant species will have genetic consequences (Neel and Cummings 2003) and therefore the selection process is important. The captured genetic variation will differ depending on the number of populations included (Whitlock et al. 2016; Leipold et al. 2020). The higher genetic diversity and differentiation among the populations the more genetic conservation areas would be needed within a minimal setting to also incorporate rare alleles. Based on the moderately high levels of genetic diversity and low genetic differentiation found in the species studied within the scope of this thesis, applying the method proposed by Whitlock et al. (2016) would potentially lead to only few populations needed per species to represent most of the genetic diversity within the region. The selection of the specific sites could then be based on other factors, e.g. conservation status as proposed by Maxted et al. (2011) and Frese (2014). Other determining factors could be the habitat quality or historic and present landscape structure, thereby including also the results discussed above. Thus, genetic conservation areas could be established for the studied species based on genetic data.

However, even within protected areas species diversity has been shown to decrease (Leuschner et al. 2013; Hallmann et al. 2017), due to inadequate management and negative influences of agriculture within the landscape. These findings highlight the importance of proper management also for genetic conservation areas. Additionally, as described in this thesis genetic variation is dynamic and (at least for the species studied here) largely shaped by gene flow and habitat quality. Therefore, the restoration of well-connected and traditionally managed grasslands within the cultural landscape as well as the reduction of the negative impact of intensive agriculture, combined with genetic conservation areas, could contribute significantly to the preservation and maintenance of genetic variation in grassland species.

## FUTURE PERSPECTIVES

The genetic variation of common grassland species from oat-grass and litter meadows has been shaped by different factors, both historically and currently. However, each studied species showed different responses, showing a species-specific effect of land use history, historic and present landscape structure and habitat quality. But most effects can be attributed to gene flow effects and habitat quality. Therefore, current processes, including ongoing habitat loss and fragmentation will likely have a lasting effect on genetic variation in grassland species.

However, by applying the knowledge on land use history and historic gene flow patterns to improve current gene flow and increase habitat quality, current conservation management success could be enhanced. Additionally, the use of modern concepts like autochthonous seed mate-

rial for grassland restoration and the establishment of genetic conservation areas can contribute significantly to the preservation of genetic variation of grassland species within the European cultural landscape.

The results presented in this thesis build a basis for future research in this area. Through the combination of several study species from the same grassland sites (Chapter Two & Three), deeper insights into general processes could be obtained. These results could be further improved by adding less common species to the dataset and by extending the study region to uncover general processes. To study local adaptations and the effects of environmental variables the use of different molecular tools, i.e. next-generation sequencing methods would be advisable (Holderegger and Segelbacher 2016).

The fourth chapter introduced a rather new field of grassland biodiversity research. The patterns observed in *L. catharticum* could be further supported by analysing other species that occur on both habitats, e.g. *Briza media*. The study design could be further improved by attempting a geographically more even study design and by using common garden experiments or translocation studies to study the phenotypic differences among populations from different habitats and to test the heritability of the methylation patterns and adaptability of these populations under different conditions.

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## Unpublished References:

Lehmair T A, Pagel E, Poschlod P, Reisch C, (unpublished) The impact of habitat age, landscape structure, habitat quality, and population size on the genetic variation of typical calcareous grassland plant species. Submitted at Mol Ecol.

# SUPPLEMENTARY MATERIAL

**Supplementary Material for all Chapters**

**Table S1:** The different maps used for land use history reconstruction and digitization with their respective years of origin, name and source, including reference scale when available.

| Year | Name | Sources |
|---|---|---|
| **1819 - 69** | Land Surveys of the Kingdom of Württemberg and principality of Hohenzollern | Kohler, K. 1858. Die Landesvermessung des Königreichs Württemberg in wissenschaftlicher, technischer und geschichtlicher Beziehung. Cotta. (1:2,500)<br>Landesarchiv Baden-Württemberg, Gemarkungspläne Hohenzollern (1:2,500)<br>http://www.landesarchiv-bw.de |
| **1875-76** | Land Surveys of the grand duchy of Baden | Landesarchiv Baden-Württemberg. Flurkarten des Königreichs Baden. http://www.landesarchiv-bw.de (1:10,000) |
| **1808- 64** | Historical cadastral maps of Bavaria | BayernAtlas. https://geoportal.bayern.de/bayernatlas |
| **1857** | Historical cadastral maps of Vorarlberg (Austria) | http://vogis.cnv.at/atlas3/init.aspx?karte=basiskarten_und_bilder |
| **1910 - 1920ies** | Topographic Maps of the Kingdom of Württemberg | SLUB (Sächsische Landesbibliothek - Staats- und Universitätsbibliothek Dresden). 2018. Topographische Karten (Meßtischblätter) Deutschland 1870-1943. http://www.deutschefotothek.de/cms/kartenforum-sachsen-messtischblaetter.xml. (1:25,000) |
| **1950ies** | Allied Nations Topographic Maps | Ritz, M. 2018. Landeskartenarchiv.de. https://www.landkartenarchiv.de/deutschland_topographischekarten.php. (1:25,000) |
| **2017 - 18** | Current topographic maps | Landesamt für Geoinformation und Landentwicklung Baden-Württemberg (LGL). (1:25,000) |

**Table S2:** The primer combinations used for each species with the respective fluorescent dye in the AFLP and MSAP analyses. D2-primer products were diluted two-fold and D4-primer products were diluted five-fold.

| Chapter | Species | D2 (black) | D3 (green) | D4 (blue) |
|---|---|---|---|---|
| **Oat-grass meadows Chapter Two** | *D. glomerata* | M-CAA/E-ACC | M-CAT/E-AGG | M-CAT/E-ACT |
| | *H. sphondylium* | M-CAT/E-ACC | M-CAA/E-AAG | M-CTC/E-ACA |
| | *T. pratense* | M-CAA/E-AAC | M-CAA/E-AAG | M-CAC/E-ACA |
| **Litter meadows Chapter Three** | *A. sylvestris* | M-CTC/E-ACC | M-CAC/E-ACG | M-CTC/E-ACA |
| | *F. ulmaria* | M-CAA/E-AAC | M-CAA/E-AAG | M-CAT/E-ACT |
| | *S. pratensis* | M-CAC/E-ACC | M-CTC/E-ACG | M-CTC/E-ACT |
| **Epigenetics Chapter Four** | *L. catharticum* - AFLP | M-CTC/E-AAC | M-CTA/E-AGG | M-CAA/E-ACA |
| | *L. catharticum* - MSAP | H/M-TCCA/E-AAC | H/M-AAT/E-AAG | H/M-TCAA/E-ACT |

## Supplementary Material for Chapter Two

**Table S3:** Significant ($p < 0.05$) inter-correlations (Pearson correlation coefficients) between the explanatory variables used in the oat-grass meadow study.

| | Dist.S 2018 | Dist.G 2018 | Dist.S 1820 | Dist.G 1820 | Area.S 1820 | Area.S 2018 | Area.G 1820 | Area.G 2018 | VP | Lit |
|---|---|---|---|---|---|---|---|---|---|---|
| **Dist.S_2018** | 1 | | | | | | | | | |
| **Dist.G_2018** | 0.45 | 1 | | | | | | | | |
| **Dist.S_1820** | 0.82 | | 1 | | | | | | | |
| **Dist.G_1820** | | | | 1 | | | | | | |
| **Area.S_1820** | | | | - 0.54 | 1 | | | | | |
| **Area.S_2018** | | | | | 0.68 | 1 | | | | |
| **Area.G_1820** | | | | | | | 1 | | | |
| **Area.G_2018** | | | | | | 0.54 | | 1 | | |
| **VP** | | - 0.46 | | | | | | | 1 | |
| **Lit** | | | | | | | | | - 0.68 | 1 |

Dist.S_1820/Dist.S_2018, historic and present distances to the nearest settlement [km]
Dist.G_1820/Dist.G_2018, historic and present distances to the nearest grassland [km]
Area.S_1820/Area.S_2018, historic and present settlement area [ha]
Area.G_1820/Area.G_2018, historic and present grassland area [ha]
VP, vascular plant cover [%]
Lit, litter cover [%]

**Figure S1:** Consensus Neighbour-Net of the *Dactylis glomerata* populations, ancient (1-10) and recent (11-20) sites are well intermixed.

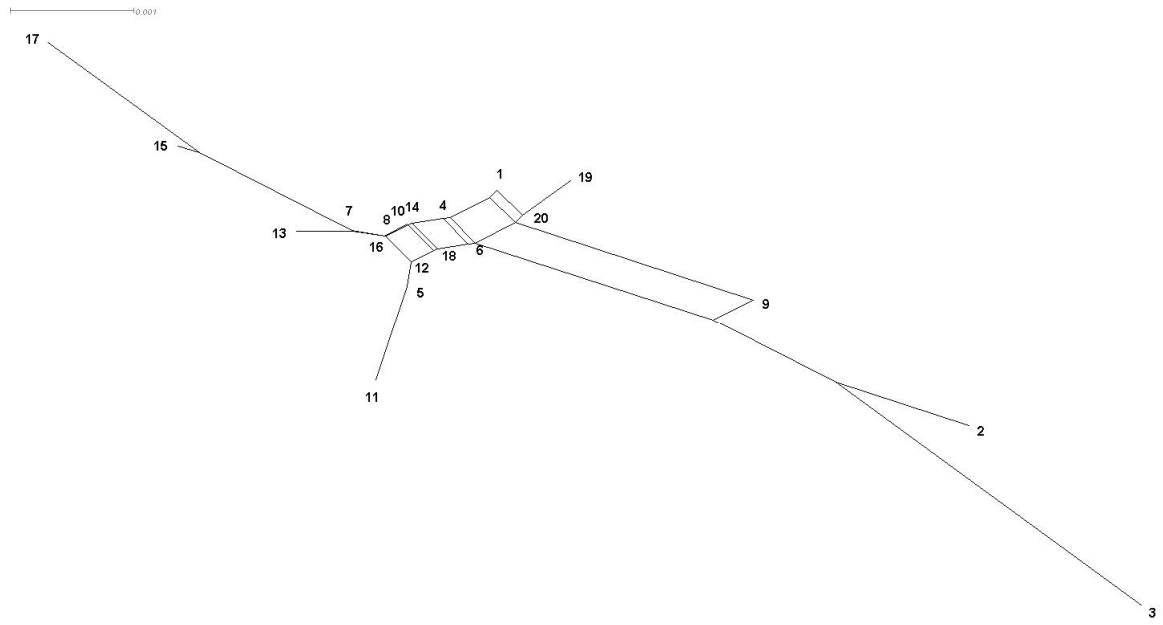



**Figure S2:** Consensus Neighbour-Net of the *Heracleum sphondylium* populations, ancient (1-10) and recent (11-20) sites are well intermixed.

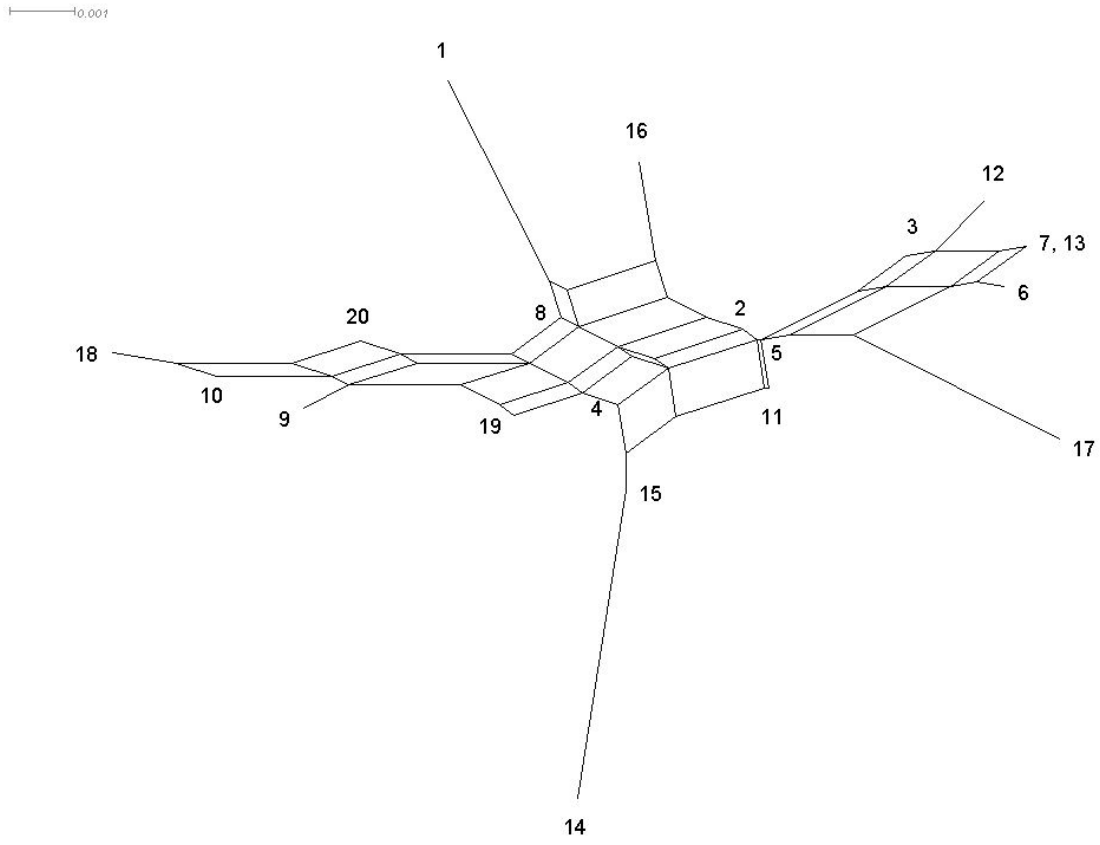


**Figure S3:** Consensus Neighbour-Net of the *Trifolium pratense* populations, ancient (1-10) and recent (11-20) sites are well intermixed.

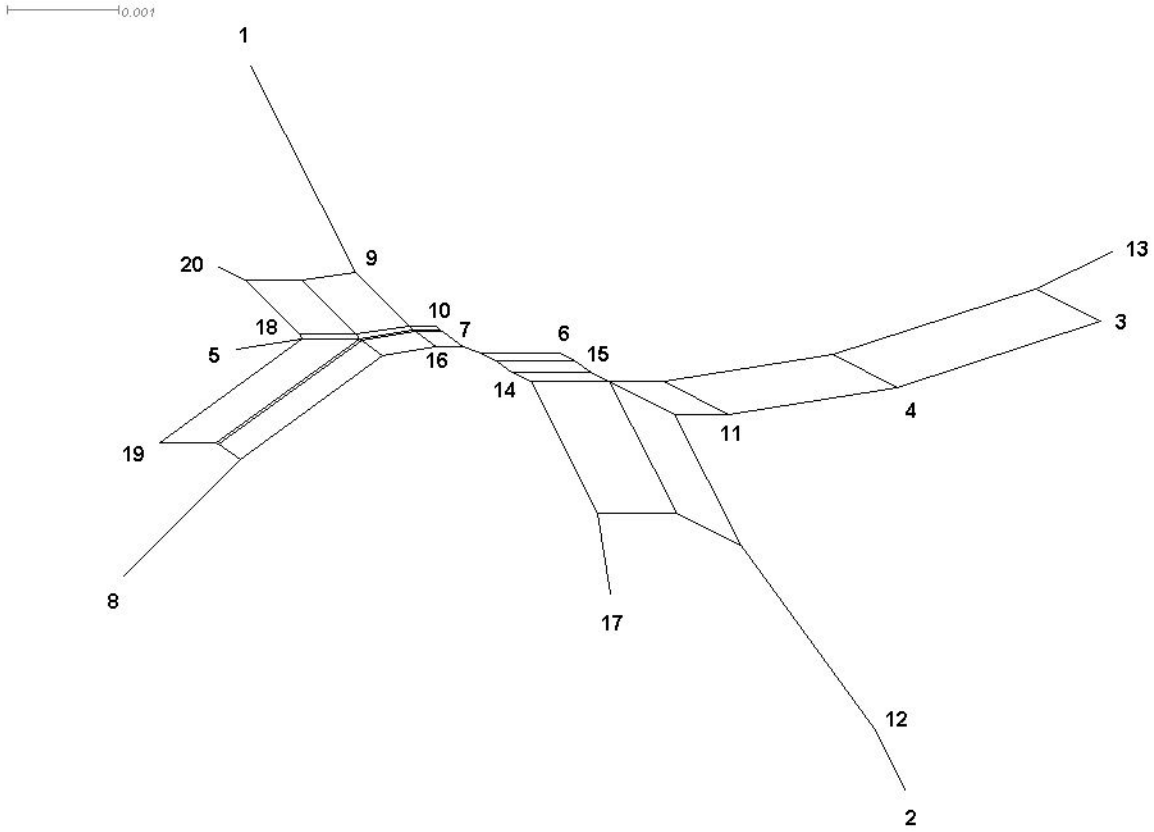
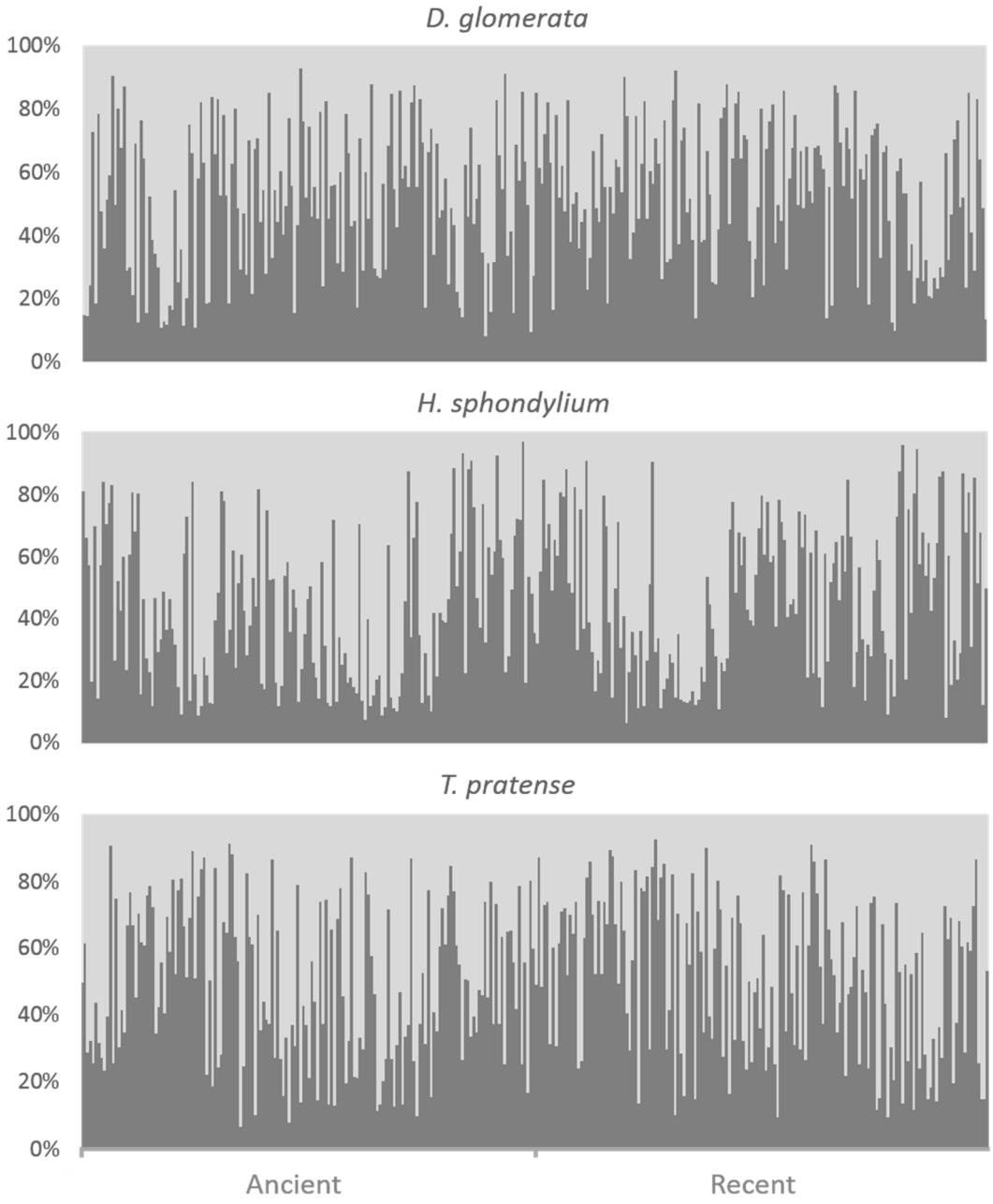


**Figure S4:** Results of the Bayesian cluster analysis for *D. glomerata*, *H. sphondylium* and *T. pratense*. Populations were not assigned to a specific group. For neither species the individuals were assorted into distinct groups. Shown is the graph for K = 2, as the most likely K in all species.



**Supplementary Material for Chapter Three**

**Table S4:** Number (No.), name (Population), and position (WGS84) of the analysed populations.

| No. | Population | Lat. (N) | Lon. (E) |
|---|---|---|---|
| **01** | Arrisried | 47° 45' 07'' | 9° 52' 06'' |
| **02** | Schlier | 47° 45' 09'' | 9° 39' 08'' |
| **03** | Schwanden | 47° 43' 12'' | 10° 2' 11'' |
| **04** | Ratzenried | 47° 43' 15'' | 9° 54' 14'' |
| **05** | Liebenried | 47° 45' 16'' | 9° 53' 15'' |
| **06** | Argen | 47° 40' 18'' | 10° 4' 17'' |
| **07** | Kißlegg | 47° 47' 19'' | 9° 52' 18'' |
| **08** | Rotheidlen | 47° 43' 20'' | 9° 42' 19'' |
| **09** | Bremberg | 47° 46' 21'' | 9° 54' 20'' |
| **10** | Nitzenweiler | 47° 36' 23'' | 9° 38' 22'' |
| **11** | Wolfegg | 47° 49' 25'' | 9° 46' 24'' |
| **12** | Wangen im Allgäu | 47° 40' 08'' | 9° 50' 07'' |
| **13** | Hinteressach | 47° 40' 10'' | 9° 41' 09'' |
| **14** | Wolfegg | 47° 49' 11'' | 9° 49' 10'' |
| **15** | Rotenbach | 47° 47' 13'' | 9° 50' 12'' |
| **16** | Hüttenweiler | 47° 36' 14'' | 9° 45' 13'' |
| **17** | Vogt | 47° 45' 17'' | 9° 47' 16'' |
| **18** | Gwigg | 47° 52' 22'' | 9° 43' 21'' |
| **19** | Sigrazhofen | 47° 46' 24'' | 9° 56' 23'' |
| **20** | Edensbach | 47° 45' 26'' | 9° 43' 25'' |

**Table S5**: Historic and present landscape structure of and around the analysed study sites.

| No. | Area_1820 | Dist_1820 | Con_1820 | Area_S | Area_2018 | Dist_2018 | Con_2018 |
|---|---|---|---|---|---|---|---|
| **01** | 144.063 | 0.415 | 161.712 | 3.769 | 15.219 | 0.255 | 7.706 |
| **02** | 55.109 | 0.816 | 22.880 | 7.345 | 25.040 | 0.562 | 14.042 |
| **03** | 101.816 | 0.321 | 19.601 | 1.487 | 183.627 | 0.286 | 27.944 |
| **04** | 45.189 | 0.239 | 20.868 | 0.354 | 19.146 | 0.449 | 3.625 |
| **05** | 146.429 | 0.229 | 31.732 | 2.204 | 24.568 | 0.231 | 6.621 |
| **06** | 141.307 | 0.344 | 45.371 | 3.590 | 30.241 | 0.470 | 18.393 |
| **07** | 97.989 | 0.264 | 29.213 | 2.530 | 21.987 | 0.298 | 7.324 |
| **08** | 39.178 | 0.364 | 10.741 | 1.091 | 32.398 | 0.223 | 11.161 |
| **09** | 103.673 | 0.336 | 47.789 | 2.396 | 41.218 | 0.276 | 9.369 |
| **10** | 108.528 | 0.328 | 32.125 | 2.470 | 75.885 | 0.308 | 21.152 |
| **11** | 69.767 | 0.347 | 10.567 | 1.817 | 6.463 | 0.346 | 2.112 |
| **12** | 109.420 | 0.322 | 28.842 | 3.696 | 33.390 | 0.303 | 12.750 |
| **13** | 94.742 | 0.525 | 13.116 | 0.637 | 49.780 | 0.498 | 13.882 |
| **14** | 60.067 | 0.498 | 7.130 | 3.658 | 12.674 | 0.507 | 4.738 |
| **15** | 111.016 | 0.409 | 19.837 | 7.237 | 26.164 | 0.396 | 11.030 |
| **16** | 203.294 | 0.127 | 29.672 | 7.562 | 45.781 | 0.132 | 17.924 |
| **17** | 29.230 | 0.322 | 3.123 | 1.748 | 37.518 | 0.277 | 9.394 |
| **18** | 114.972 | 1.178 | 38.507 | 3.308 | 28.965 | 0.682 | 17.332 |
| **19** | 95.027 | 0.319 | 36.660 | 0.908 | 37.024 | 0.298 | 15.482 |
| **20** | 55.344 | 0.311 | 20.797 | 1.315 | 54.622 | 0.309 | 13.524 |
| **Mean** | **96.308** | **0.401** | **31.514** | **2.956** | **40.086** | **0.355** | **12.275** |
| **SD** | ± 9.60 | ± 0.05 | ± 7.38 | ± 0.49 | ± 8.35 | ± 0.03 | ± 1.42 |

Area_S, Area Size [ha]
Area_1820/Area_2018, historic and present total area of wet meadows [ha]
Dist_1820/Dist_2018, historic and present distances to the nearest settlement [km]
Con_1820/Con_2018, historic and present connectivity

**Table S6:** Habitat quality of the analysed study sites as well as population size per species and investigated populations.

| | Habitat Quality | | | | Population Size | | |
|---|---|---|---|---|---|---|---|
| **No.** | **VP** | **Moss** | **Lit** | **O-Soil** | **Ang syl** | **Fil ulm** | **Suc pra** |
| **01** | 73.0 | 69.0 | 9.6 | 1.0 | 50,252.1 | 2,512.6 | 40,201.7 |
| **02** | 87.0 | 36.0 | 23.0 | 2.2 | 4,896.8 | 142,006.9 | 53,864.7 |
| **03** | 77.0 | 78.0 | 2.6 | 0.6 | 0.0 | 4,957.8 | 991.6 |
| **04** | 86.0 | 34.0 | 9.8 | 0.4 | 354.1 | 18,415.5 | 10,978.4 |
| **05** | 84.0 | 67.0 | 12.6 | 0.0 | 5,878.0 | 74,944.9 | 1,469.5 |
| **06** | 76.0 | 59.0 | 10.0 | 2.2 | 150,776.7 | 222,575.2 | 59,832.0 |
| **07** | 71.0 | 56.0 | 7.2 | 1.8 | 31,625.2 | 168,667.5 | 2,108.3 |
| **08** | 79.5 | 62.0 | 11.1 | 2.6 | 11,635.1 | 37,087.0 | 13,089.5 |
| **09** | 87.0 | 55.5 | 3.0 | 3.1 | 4,791.0 | 62,283.3 | 7,985.0 |
| **10** | 80.0 | 71.0 | 1.8 | 1.6 | 67,503.2 | 306,234.2 | 13,171.4 |
| **11** | 81.0 | 63.0 | 2.2 | 3.8 | 23,618.9 | 350,650.1 | 0.0 |
| **12** | 84.0 | 72.0 | 18.4 | 0.0 | 61,595.6 | 359,718.5 | 24,638.3 |
| **13** | 87.0 | 78.0 | 3.4 | 0.6 | 14,439.0 | 33,549.4 | 1,698.7 |
| **14** | 75.0 | 16.0 | 30.0 | 6.6 | 33,529.3 | 198,127.6 | 85,347.3 |
| **15** | 94.0 | 58.0 | 10.6 | 2.0 | 50,661.5 | 260,544.9 | 36,186.8 |
| **16** | 80.0 | 61.0 | 7.2 | 3.2 | 20,164.3 | 151,232.1 | 25,205.3 |
| **17** | 81.0 | 36.0 | 12.0 | 7.8 | 75,751.6 | 48,947.2 | 3,496.2 |
| **18** | 76.0 | 66.0 | 7.8 | 1.0 | 11,027.6 | 114,686.9 | 2,205.5 |
| **19** | 67.5 | 63.3 | 4.5 | 5.7 | 5,296.6 | 27,239.8 | 0.0 |
| **20** | 83.0 | 72.0 | 58.0 | 0.0 | 876.4 | 81,503.5 | 0.0 |
| **Mean** | **80.5** | **58.6** | **12.2** | **2.3** | **31233.7** | **133294.2** | **19123.5** |
| **SD** | ± 1.4 | ± 3.6 | ± 2.9 | ± 0.5 | ± 8282.6 | ± 25866.5 | ± 5423.1 |

VP, cover of vascular plants [%]; Moss, cover of mosses [%]; Lit, cover of litter [%]; O-Soil, cover of open soil [%]; Ang syl/Fil ulm/Suc pra, population size of *A. sylvestris*, *F. ulmaria*, and *S. pratense*

**Table S7**: Significant ($p < 0.05$) correlations (Pearson correlation coefficients) between the explanatory variables used in the linear models.

| | Landscape Structure | | | | | | | Habitat Quality | | | | Population Size | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Area_1820 | Dist_1820 | Con_1820 | Area_2018 | Dist_2018 | Con_2018 | Area_S | VP | Moss | Lit | O-Soil | Ang syl | Fil ulm | Suc pra |
| **Landscape Structure** | | | | | | | | | | | | | | |
| Area_1820 | 1 | | | | | | | | | | | | | |
| Dist_1820 | | 1 | | | | | | | | | | | | |
| Con_1820 | 0.47 | | 1 | | | | | | | | | | | |
| Area_2018 | | | | 1 | | | | | | | | | | |
| Dist_2018 | | 0.82 | | | 1 | | | | | | | | | |
| Con_2018 | | | | 0.78 | | 1 | | | | | | | | |
| Area_S | 0.45 | | | | | | 1 | | | | | | | |
| **Habitat Quality** | | | | | | | | | | | | | | |
| VP | | | | | | | | 1 | | | | | | |
| Moss | 0.46 | | | | | 0.50 | | | 1 | | | | | |
| Lit | | | | | | | | | | 1 | | | | |
| O-Soil | | | | | | | | | -0.60 | | 1 | | | |
| **Population Size** | | | | | | | | | | | | | | |
| Ang syl | | | | | | | | | | | | 1 | | |
| Fil ulm | | | | | | | | | | | | 0.46 | 1 | |
| Suc pra | | | | | | | 0.53 | | -0.53 | | | | | 1 |

Area_S, Area Size [ha]; Area_1820/Area_2018, historic and present total area of wet meadows [ha];
Dist_1820/Dist_2018, historic and present distances to the nearest settlement [km];
Con_1820/Con_2018, historic and present connectivity;
VP, cover of vascular plants [%]; Moss, cover of mosses [%];
Lit, cover of litter [%]; O-Soil, cover of open soil [%];
Ang Syl/Fil Ulm/Suc Pra, population size of *A. sylvestris*, *F. ulmaria*, and *S. pratense*

**Table S8**: Significant ($p < 0.05$) differences between historic (1820) and present (2018) landscape variables.

| Landscape Structure | Mean | SE | p-Value | |
|---|---|---|---|---|
| **Area_1820** | 96.31 | 42.93 | <0.001 | *** |
| **Area_2018** | 40.09 | 37.34 | | |
| **Dist_1820** | 0.40 | 0.23 | 0.383 | n.s. |
| **Dist_2018** | 0.36 | 0.13 | | |
| **Con_1820** | 31.51 | 33.00 | <0.001 | *** |
| **Con_2018** | 12.28 | 6.36 | | |

Signif. codes: $p < 0.001$ ***; $p \geq 0.05$ n.s.

Area_1820/Area_2018, historic and present total area of wet meadows [Ha]
Dist_1820/Dist_2018, historic and present distances to the nearest settlement [Km]
Con_1820/Con_2018, historic and present connectivity

**Supplementary Material for Chapter Four**

**Table S9:** Vegetation Structure represented by the different percentage covers of vascular plants (VP), mosses (Moss), plant litter (Lit), open soil (O-Soil), grasses (Grass), legumes (Leg) and herbaceous species (Herb) for all study sites. Estimates were calculated from five plots (2x2 m) per study site. The given p-value is based on t-tests.

| ID | VP | Moss | Lit | O-Soil | Grass | Leg | Herb |
|---|---|---|---|---|---|---|---|
| **C1** | 79.0 | 43.0 | 3.2 | 7.6 | 28.0 | 2.0 | 51.0 |
| **C2** | 82.0 | 81.0 | 3.6 | 0.4 | 34.0 | 5.8 | 42.2 |
| **C3** | 79.0 | 57.0 | 10.0 | 7.4 | 32.8 | 12.0 | 34.2 |
| **C4** | 83.0 | 78.0 | 5.0 | 1.0 | 39.0 | 3.4 | 40.6 |
| **C5** | 88.0 | 88.0 | 9.4 | 0.5 | 31.0 | 6.6 | 50.4 |
| | | | | | | | |
| **L1** | 73.0 | 69.0 | 9.6 | 1.0 | 55.0 | 0.0 | 17.0 |
| **L2** | 79.0 | 43.0 | 22.0 | 1.6 | 63.0 | 0.0 | 16.0 |
| **L3** | 76.0 | 59.0 | 10.0 | 2.2 | 40.0 | 3.0 | 34.0 |
| **L4** | 79.5 | 62.0 | 11.1 | 2.6 | 52.5 | 1.2 | 26.5 |
| **L5** | 80.0 | 71.0 | 1.8 | 1.6 | 41.0 | 1.2 | 38.0 |
| | | | | | | | |
| **p - value** | n.s. | n.s. | n.s. | n.s. | 0.006 | 0.027 | 0.013 |

**Table S10:** Soil characteristics of the studied grasslands expressed as the water holding capacity (WHC) [%], the pH measured in $CaCl_2$, phosphorous content [g/kg], potassium content [g/kg] and Carbon/Nitrogen ratio (C/N). Additionally, the Ellenberg Indicator values for soil moisture (F), nutrient availability (N) and soil reaction (R) is given. The given p-value is based on t-tests.

| ID | WHC | pH | P | K | C/N | F | N | R |
|---|---|---|---|---|---|---|---|---|
| **C1** | 53.08 | 7.10 | 10.92 | 115.28 | 19.84 | 4.10 | 4.26 | 7.73 |
| **C2** | 92.26 | 5.78 | 16.78 | 111.04 | 13.92 | 4.50 | 4.49 | 7.65 |
| **C3** | 63.32 | 7.08 | 31.36 | 195.16 | 28.88 | 4.28 | 4.63 | 7.75 |
| **C4** | 104.50 | 6.96 | 31.00 | 176.88 | 20.58 | 4.42 | 4.72 | 7.75 |
| **C5** | 85.70 | 6.76 | 12.20 | 53.84 | 17.46 | 4.40 | 4.41 | 7.59 |
| | | | | | | | | |
| **L1** | 80.15 | 5.21 | 43.38 | 70.86 | 17.64 | 7.61 | 2.87 | 6.18 |
| **L2** | 71.13 | 4.09 | 20.90 | 54.27 | 15.74 | 6.94 | 2.45 | 4.87 |
| **L3** | 87.98 | 5.32 | 24.94 | 44.66 | 12.17 | 7.40 | 3.01 | 5.52 |
| **L4** | 82.94 | 5.51 | 33.14 | 84.27 | 14.97 | 7.25 | 2.61 | 6.82 |
| **L5** | 77.60 | 5.09 | 28.59 | 21.50 | 14.43 | 7.68 | 2.84 | 6.78 |
| | | | | | | | | |
| **p - value** | n.s. | 0.001 | n.s. | 0.026 | n.s. | < 0.001 | < 0.001 | 0.002 |

**Table S11:** Genetic diversity of all studied populations given as Nei's gene diversity for all obtained marker types (Gen, Epi_u, Epi_m, Epi_h) and the respective mean per habitat and over all populations. The p-value for the t-test is given (* significant).

| Nr. | Habitat | Gen | Epi_u | Epi_m | Epi_h |
|---|---|---|---|---|---|
| **C1** | CG | 0.094 | 0.115 | 0.139 | 0.042 |
| **C2** | CG | 0.107 | 0.127 | 0.157 | 0.052 |
| **C3** | CG | 0.060 | 0.124 | 0.151 | 0.055 |
| **C4** | CG | 0.070 | 0.118 | 0.141 | 0.039 |
| **C5** | CG | 0.076 | 0.164 | 0.150 | 0.054 |
| | **Mean all CG** | **0.081** | **0.130** | **0.148** | **0.048** |
| | SD | 0.017 | 0.018 | 0.007 | 0.007 |
| **L1** | LM | 0.073 | 0.091 | 0.091 | 0.044 |
| **L2** | LM | 0.089 | 0.096 | 0.096 | 0.041 |
| **L3** | LM | 0.075 | 0.071 | 0.071 | 0.042 |
| **L4** | LM | 0.067 | 0.100 | 0.100 | 0.059 |
| **L5** | LM | 0.073 | 0.096 | 0.096 | 0.040 |
| | **Mean all LM** | **0.075** | **0.091** | **0.123** | **0.045** |
| | SD | 0.007 | 0.010 | 0.006 | 0.007 |
| **Mean Over All Pop.** | | **0.078** | **0.110** | **0.135** | **0.047** |
| Standard Deviation | | 0.013 | 0.024 | 0.014 | 0.007 |
| **p-Value** | | 0.545 | 0.0078* | 0.0007* | 0.540 |

**Figure S5:** Map of all study sites used in Chapter Four. Calcareous grasslands (C1-C5) were located on the Swabian Alb and litter meadows (L1-L5) in the Allgäu region.

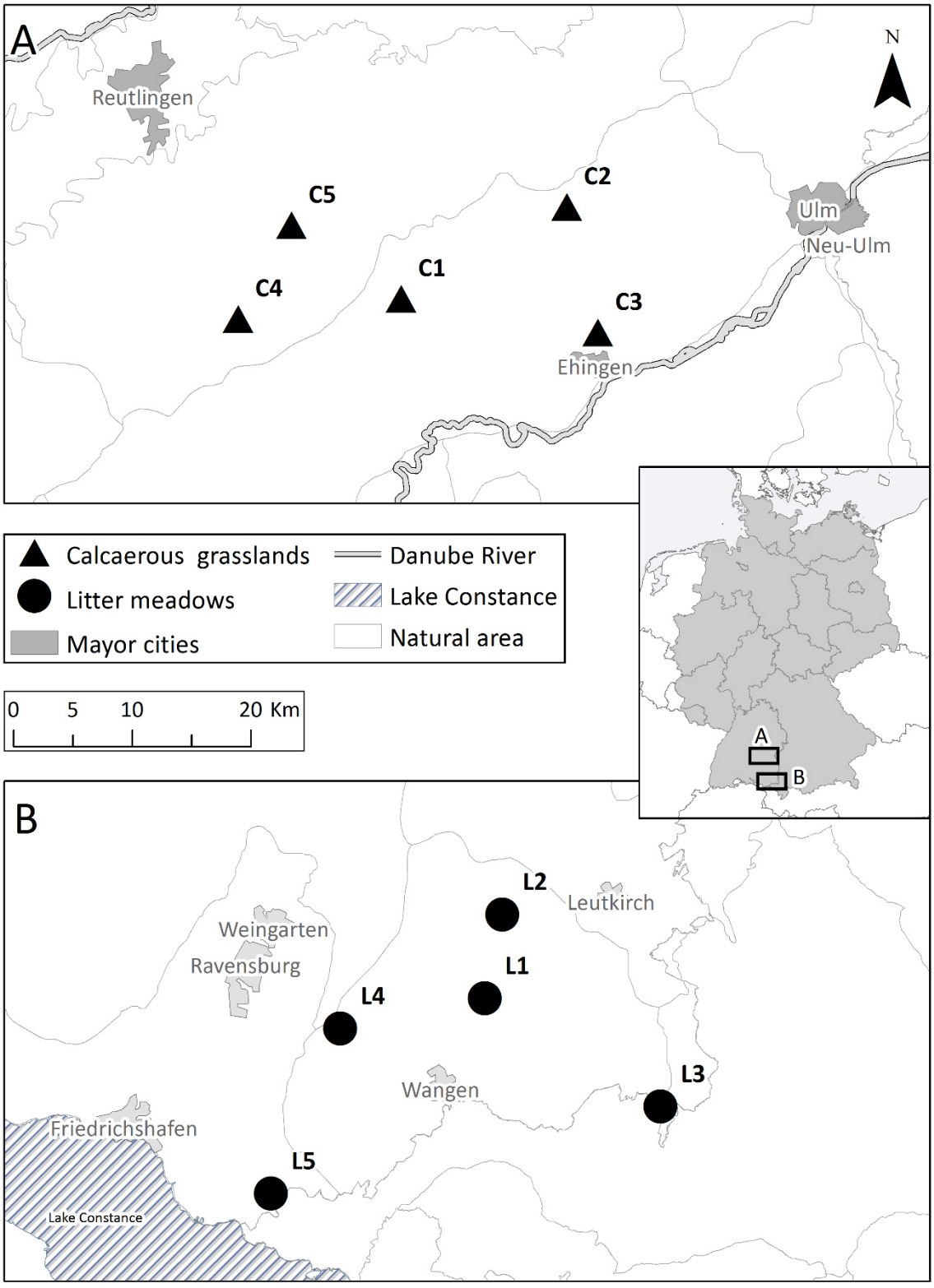


**Figure S6:** Results of the Structure Analyses for the genetic (Gen) and epigenetic (Epi_u, Epi_m, Epi_h) datasets. Populations were clearly sorted according to their habitat of origin. (Dark-grey: CG, Light-grey: LM).