A comprehensive conservation strategy for the endangered plant species *Arnica montana*

Results of a viability analysis at the population, habitat and landscape level

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Summary

In recent times, an ongoing and European wide loss of populations of the plant species *Arnica montana* is recognized. In the meantime, the species is listed as endangered and highly threatened. In South-West Germany, the ongoing decline of the species is well documented by the federal monitoring program since the 1990s. The species still occurs in distinct regions within South-West Germany and the decline of populations is recognized within each. However, until today, no efficacious solution to counteract this development is found and the loss of populations could not be stopped yet. Therefore, this thesis aimed to investigate the constitution of remaining *Arnica* populations (chapter 2 & 3), to find risk factors at the landscape and habitat level (chapter 4) and to investigate the consequences of reintroduction activities (chapter 5).

For the survey of the constitution of *A. montana* data on the genetic variation were collected by conduction microsatellite analysis with leaf material from 29 populations (**chapter 2**). The study revealed that the species don`t suffer from a general decrease of genetic diversity or the occurrence of inbreeding. In contrast, predominantly small populations rather show an excess of heterozygote individuals which means that the generative reproduction is reduced. Furthermore, the remaining populations respectively the distinct regions are highly differentiated from each other, which comes along with a reduced gene flow between the populations. Therefore, distinct regions should be regarded as distinct conservation units and conservation activities should be adapted to local conditions in order to increase the local population sizes.

In **chapter 3**, the fitness of *A. montana* was investigated. Therefore, the generative reproductive fitness of 29 populations was quantified by surveying the filling rate of seeds, the germination ability and finally the survivability of seedlings. The results showed that the overall fitness was predominantly defined by the filling rate of seeds and further influenced by the census population size. Furthermore, the filling rate was correlated positive with the extent of clonality within the populations. Hence, the determination of the reproductive and genetic fitness of *Arnica* population can be done substitutionally by the evaluation of the filling rate of seeds.

The occurrence and status of a species can be influenced by several factors on the landscape and habitat level. The first part of **chapter 4** dealt with the question whether changes in the landscape during the past 150 years can explain the loss of *Arnica* populations in South-West Germany. Therefore, historical and recent maps were analysed around the study sites concerning the amount of forest, grasslands, settlements and arable fields. The results showed that the landscape changed during the last centuries regardless of the occurrence of *A. montana*. The development rather showed regional differences, which makes it most likely that distinct regions need to be seen as distinct conservation units to improve the conservation of nutrient-poor habitats with their specific species composition. The second part of the

chapter investigated whether local habitat properties influence the occurrence respectively the population size of *Arnica* populations. Therefore, vegetation surveys and soil chemical and physical analyses were conducted at sites with and without an actual population of *A. montana*. The results revealed that the increase of nutrients led to a change in habitat conditions, which led to unfavourable conditions for the species. Due to that, the population size decreases which ends in the local extinction. In summary, the protection of nutrient-poor habitats is indispensable for *A. montana* and need to be done locally by an adapted habitat management and in general by the avoidance of habitat destruction.

Next to the application of landscape and habitat protection, conservation activities also conduct the promotion of endangered species by reintroduction. However, the exchange and artificial transport of plant material can also bring along negative aspects. The mixture of genetically differentiated or locally adapted populations can lead to an outbreeding depression, which results in the loss of fitness. Therefore, an artificial crossing experiment with *A. montana* was conducted to evaluate the risk of outbreeding depression for interpopulation crossings (**chapter 5)**. The fitness of the offspring was measured as the filling rate of the seeds. It turned out, that interpopulation crossings are in general disadvantageous for the offspring fitness compared to intrapopulation crossings, which is evidence of outbreeding depression for *A. montana*. Based on that, the mixture of distinct populations should be avoided.

The final **chapter 6** summarized the findings from the previous four main chapters. The protection of endangered plant species can only be improved when detailed investigations on the present constitution of a species (population size, reproductive fitness, genetic variation) are conducted. Such data need to be set into context with (a)biotic factors like habitat properties, landscape quantities and species-specific functional traits. Only this holistic approach gives an entire insight on the causes of risk and the necessary efforts for the landscape, habitat and local species protection can be deduced. Hence, to improve the conservation of biodiversity, conducting holistic population viability analysis for endangered and rare species is essential.

Zusammenfassung

Der fortschreitende Verlust von Populationen der Pflanzenart *Arnica montana* ist europaweit erkennbar. Die Art gilt mittlerweile als gefährdet und ihr Fortbestehen ist stark bedroht. In Südwestdeutschland wurde der Rückgang der Art im Rahmen des Artenschutzprogramms festgestellt, bei dem seit den 1990er Jahren regelmäßige Monitorings von Populationen stattfindet. In dem Gebiet kommt Arnika heutzutage in verschiedenen, geographisch voneinander getrennten Regionen vor und der Rückgang ist in jeder davon bemerkbar. Trotz verschiedener Bemühungen konnte der Rückgang der Art bis jetzt jedoch nicht verhindert werden. Die vorliegende Dissertation dient daher zunächst den aktuellen Zustand von bestehenden Arnikapopulationen in Südwestdeutschland zu erfassen (Kapitel 2 & 3). Im anschließenden Kapitel 4 wurden Gefährdungsursachen der Art auf der Landschafts- bzw. Habitatebene untersucht. Das Kapitel 5 beschäftigt sich mit den Folgen von Ansiedlungsmaßnahmen auf die Fitness der nachfolgenden Generationen.

Für die Erfassung des aktuellen Zustands von *A. montana* wurden zunächst Daten zur genetischen Variation der Art gesammelt, indem Mikrosatellitenanalysen an gesammelten Blattmaterials von 29 Populationen durchgeführt wurden (**Kapitel 2**). Die Untersuchung zeigte, dass die Art in Südwestdeutschland bisher nicht unter dem Verlust von genetischer Vielfalt oder dem Auftrennten von Inzucht leidet. Stattdessen zeigte sich vor allem in kleinen Populationen ein Überschuss an heterozygoten Individuen, dass auf eine verminderte generative Fortpflanzung hindeutet. Darüber hinaus zeigte sich eine deutliche genetische Differenzierung zwischen den verschiedenen Regionen bzw. Population, was durch einen verminderten Genfluss zwischen den Beständen im Untersuchungsgebiet erklärt werden kann. Aufgrund der Ergebnisse sollten die Regionen als separate Schutzeinheiten betrachtet werden, in denen die Schutzmaßnahen an die regionalen bzw. lokalen Gegebenheiten angepasst werden können, um die Populationsgröße zu erhöhen.

Im **Kapitel 3** wurde die Fitness von *A. montana* untersucht. Dafür wurde die generative Fortpflanzung von 29 Populationen erfasst, in dem die Füllungsrate von Saatgut, die Keimfähigkeit und die Überlebensfähigkeit von Keimlingen überprüft wurde. Die Ergebnisse zeigten, dass die Fitness hauptsächlich durch die Füllungsrate der Samen bestimmt wird, welche gleichzeitig von der Populationsgröße abhängt. Des Weiteren korreliert die Füllungsrate mit der Klonalität, d.h. dem Ausmaß an vegetativer Fortpflanzung einer Population. Somit kann durch die Bestimmung der Füllungsrate zeitgleich eine Einordnung der reproduktiven als auch genetischen Fitness erfolgen.

Das Vorkommen und der Zustand einer Art kann generell durch verschiedene äußere Faktoren auf der Landschafts- bzw. Habitatebene beeinflusst werden. In **Kapitel 4** wurde der Einfluss der Landschaftszusammensetzung bzw. deren historischen Entwicklung sowie der lokalen Habitatbedingungen auf das Vorkommen von *A. montana* untersucht. Der erste Teil des Kapitels diente

der Klärung, ob Veränderungen in der Landschaft bzw. deren Zusammensetzung während der letzten 150 Jahre den Rückgang von *A. montana* beeinflussen kann. Dafür wurde im Umkreis der Untersuchungsflächen historisches und aktuelles Kartenmaterial hinsichtlich der Fläche an Wald, Grünland, Siedlungs- und Ackerfläche analysiert. Es zeigte sich, dass sich die Landschaft über die Zeit verändert hat und nährstoffarme Lebensräume zunehmend fragmentiert in der Landschaft vorkommen. Diese Entwicklung zeigte sich unabhängig davon, ob Arnika vorkam oder nicht. Des Weiteren zeigten sich regionale Unterschiede in der Landnutzungsgeschichte wodurch die Betrachtung der Regionen als separate Schutzeinheiten unterstrichen werden konnte. Für die Erfassung der Habitatbedingungen wurden Vegetationsaufnahmen sowie bodenphysikalische und -chemische Untersuchungen durchgeführt. Die erhobenen Daten wurden mit dem Vorkommen bzw. der Populationsgröße von Arnika verglichen. Die Ergebnisse zeigten, dass sich aufgrund zunehmende Nährstoffverfügbarkeit die lokalen Habitatbedingungen geändert haben und diese nicht mehr für *A. montana* geeignet sind. Dadurch nimmt die Populationsgröße ab und endet in dem lokalen Aussterben einer Population. Somit kann zusammenfassen gesagt werden, dass der Schutz nährstoffarmer Habitate unerlässlich für den Schutz der Pflanzenart *A. montana* ist. Für den Schutz muss generell die Habitatzerstörung verhindert werden und die Flächenpflege an die Arnika-spezifischen Bedürfnisse angepasst werden.

Neben der Durchführung von Landschafts- und Flächenschutzmaßnahmen, werden einzelne Arten durch Ansiedlungsmaßnahmen unterstützt. Leider kann der künstliche Austausch von Pflanzenmaterial auch nachteilige Auswirkungen mit sich bringen. Das Vermischen von genetisch differenzierten Populationen kann zur einer Auszuchtdepression führen, was sich in einem Fitnessverlust bei den nachkommenden Generationen zeigt. Zur Abschätzung des Riskos einer Auszuchtdepression wurde ein Kreuzungsversuch durchgeführt, wobei Pflanzen aus verschiedenen Populationen gegenseitig bestäubt wurden (**Kapitel 5**). Die Fitness der Nachkommen wurde über die Füllungsrate der Samen erfasst. Die Ergebnisse zeigten, dass die Durchmischung von Populationen nachteilig für die Nachkommen ist im Vergleich zur Kreuzung von Pflanzen aus derselben Population. Dies ist der Beleg für das Auftreten von Auszuchtdepression bei Arnika und somit sollte das Durchmischen verschiedene Bestände vermieden werden.

Im letzten **Kapitel 6** wurden die Ergebnisse der vier Hauptkapitel zusammengefasst. Der Schutz gefährdeter Arten kann verbessert werden, wenn eine umfangreiche Erfassung des aktuellen Zustands (Populationsgröße, reproduktive Fitness, genetische Variation) stattfindet. Diese Daten müssen anschließend in Zusammenhang mit weiteren Faktoren wie dem Habitatzustand, Landschaftszusammensetzung und artspezifischen Eigenschaften gesetzt werden. Lediglich unter der Anwendung solcher vollumfänglichen Untersuchungen kann eine genaue Gefährdungsbeurteilung erfolgen und die Schutzmaßnahmen auf Landschafts-, Flächen- und Artebene angemessen angepasst werden.

Chapter 1

General introduction and thesis outline

Figure 1: Drawing of *Arnica montana* (Sturm, 1797-1862)

Biodiversity

According to the *Convention on Biological Diversity* (CBD), biodiversity respectively biological diversity comprises of the ecosystem, species and genetic diversity (CBD 1992). Conservation efforts should focus on the equal protection of all three levels. Genetic diversity consists of genetic variability within individuals, within populations as well as between populations. The species diversity is equivalent to the number of different taxa including all life forms on a local, regional or global scale. This type of diversity was originally established to define biodiversity and is still commonly used in ecology and nature conservation (Baur 2010). Ecosystem diversity comprises the variability of different habitats and the interaction within and between them. It further includes the interaction between different taxa and hence the amount of ecosystem services. The survey of all three levels of diversity is necessary to quantify biodiversity and hence to define e.g. biodiversity hotspots on local and regional levels or to protect species.

The need for a unique definition of biodiversity by the CBD and protection guidelines came along with the loss of biodiversity that is noticeable since long on the global scale. At last, the *Global Assessment Report on Biodiversity and Ecosystem Services* from 2019 highlighted the general loss of species in the last decades and showed that approximately 25% of species within plant and animal groups became threatened with extinction within the last 50 years (Díaz et al. 2019). Already Sala et al. (2000) pointed out that land use and climatic changes, increased nitrogen deposition and increased carbon dioxide concentration are the major drivers of this loss. Especially for species in terrestrial habitats, land use changes and increased nitrogen deposition are most harmful. The same development was shown for the status of biotope types and species in Germany (BfN 2016). Nowadays, more than two third of the occurring biotope types are threatened and furthermore the habitat quality decreases (BfN 2016). For habitats used by humans (e.g. grasslands, arable fields), this comes mainly along with eutrophication and habitat destruction (Riecken et al 2010). Both lead to changes of biotic and abiotic conditions and hence to a decrease of natural, species-rich ecosystems. This is exclusively harmful for species that are adapted to conditions of nutrient-poor habitats. In the end, populations of these species got extinct and hence the species threatened.

Extinction vortex

The mechanism causing the loss of species can be described by the term *extinction vortex (*Figure 2*)*(Frankham et al. 2012; Wilson und Primack 2019; Baur 2021). It is symbolized by a downward spiral of the status of an endangered species promoted by several biotic and abiotic impacts. First, the above-described general loss of suitable habitats leads to a decrease in species distribution

due to e.g. habitat destruction. This is predominantly negative for species growing only in habitats that are naturally rare in the landscape. In addition, the management of the landscape by humans changed in the recent decades e.g. the usage of grasslands changed from grazing to mainly mowing (Poschlod 2017). However, each land use type promotes different habitat constitutions and vegetation structures. Species adapted to a specific management type suffer a lot from changes in land use. Next to changes in management types, the productivity of open habitats increased in the last decades by artificial nutrient input (Vermeer und Berendse 1983; Matthies et al. 2004). Due to the increased fertilizer usage, nutrient availability raised and plants produced more biomass. This leads in general to a promotion of fast and high growing plant species which results in changes in the light and water availability within the habitats (Hautier et al. 2009). Such changes are predominantly harmful for low growing plants with a high light demand and these species get more likely extinct.

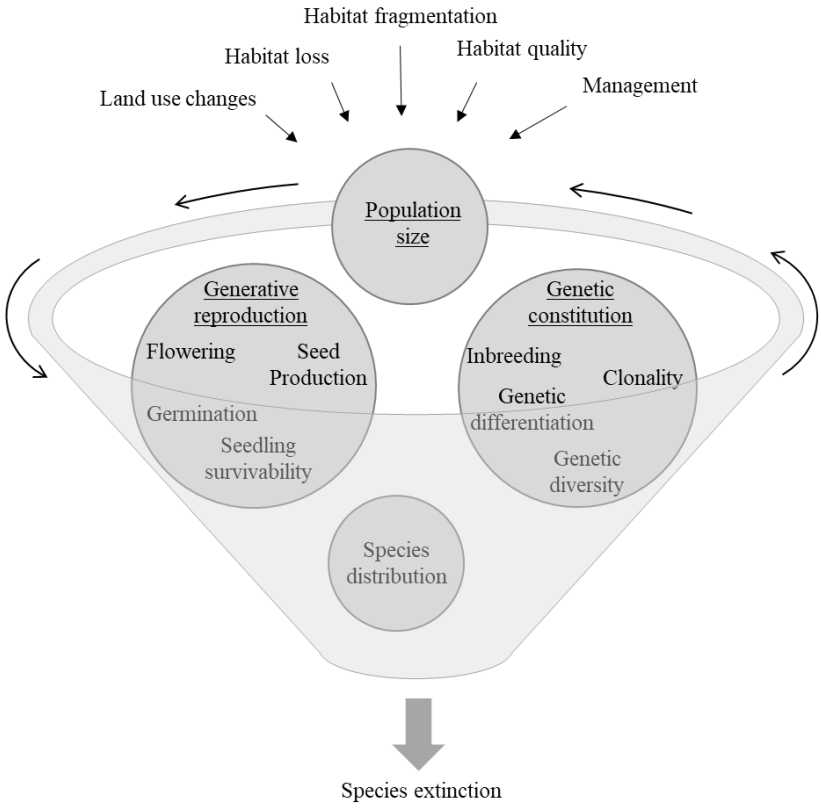


Figure 2: Scheme of *extinction vortex* for rare, endangered plant species (Wilson and Primack (2019), adapted). The viability of species is composed by several parameters of species distribution, population size, generative reproduction and genetic constitution, which all interact with each other (grey circles within the vortex). A reduced viability leads to the extinction of species. All different parameters of viability are influenced by external abiotic factors.

By the above-described reduction of species distribution respectively loss of populations, the remaining populations become isolated from each other and the gene flow between the population decreases (Ellstrand und Elam 1993; Frankham et al. 2012; Heinken und Weber 2013; Wilson und Primack 2019). Hence mating between individuals is limited to within-population mating. This increases the probability of inbreeding and ends in the loss of genetic variation. Both leads to a reduced fitness of the offspring individuals (Andersson and Waldmann 2002; Charlesworth 2003; Frankham et al. 2012). This can be seen by e.g. lower seed quality or germination and survival ability of seedlings. In the end, the generative reproduction ability decreases. Individuals with a lower genetic variation suffer in general of lower fitness, the survivability is reduced and the plants are less able to adapt e.g. to changing environmental conditions. This promotes the loss of individuals and hence the decrease of population size. Within populations with a reduced population size it was proven that the genetic variation and generative reproduction ability is decreased (Frankham 1996; Leimu et al. 2006). In summary, the constitution of a species is affected by several factors like population size, genetic diversity, generative reproduction and species distribution. All parameters are interacting with and depending on each other and hence need to be investigated in holistic conservation approaches. An example for a strongly threatened plant species is *Arnica montana*. Since decades an ongoing loss of the species distribution and decreasing populations size are reported across whole Europe but the reasons are not yet fully understood (IUCN 2021).

Study species

The natural distribution of *A. montana* reaches from Scandinavia, the Baltic States, Romania to Spain. Due to its ongoing loss, the species is nowadays listed on the H*abitats Directive of the European Union* (FFH, Appendix V) and hence the trait with plant material is restricted (EU 2013). Within in Germany, the plant species is endangered (Red List Status 3) and protected by the *federal species protection regulations* (BArtSchV) (Bundesamt für Naturschutz). Large parts of the present species distribution is located within Germany and the country has a national responsibility within the *National Biology Diversity Strategy* (BfN 2021a). In South-West Germany, within the federal state of Baden-Wuerttemberg the species is nowadays strongly endangered (Red List Status 2)(LUBW 2020a). The species is part of the federal species protection program (“Artenschutzprogramm”, ASP) since the 1990s. Within this protection program, populations are monitored by biologist regularly (LUBW). Nonetheless, in the last 25 years nearly 60% of the monitored populations of *A. montana* got extinct (Table 1).

Table 1: Status of *Arnica montana* of 84 regularly monitored populations in Baden-Wuerttemberg.

Arnica montana	Comment	n
status unknown	site data deficient	11
extinct	reasons for loss mentioned	38
extinct	reasons for loss unknwon	11
actual		24

In the monitoring reports of the federal species protection program, the reasons for extinction for most of the lost populations are well documented (Figure 3). The mentioned reasons are mainly the habitat loss by afforestation or abandonment of sites. Furthermore, the intensification of land use, improper usage and eutrophication of habitats led to unsuitable site conditions and hence the loss of *A. montana*. This shows that the species is threatened by the above-mentioned general land use changes and eutrophication. However, unfortunately for a certain number of sites, the potential reasons for the loss are unknown.

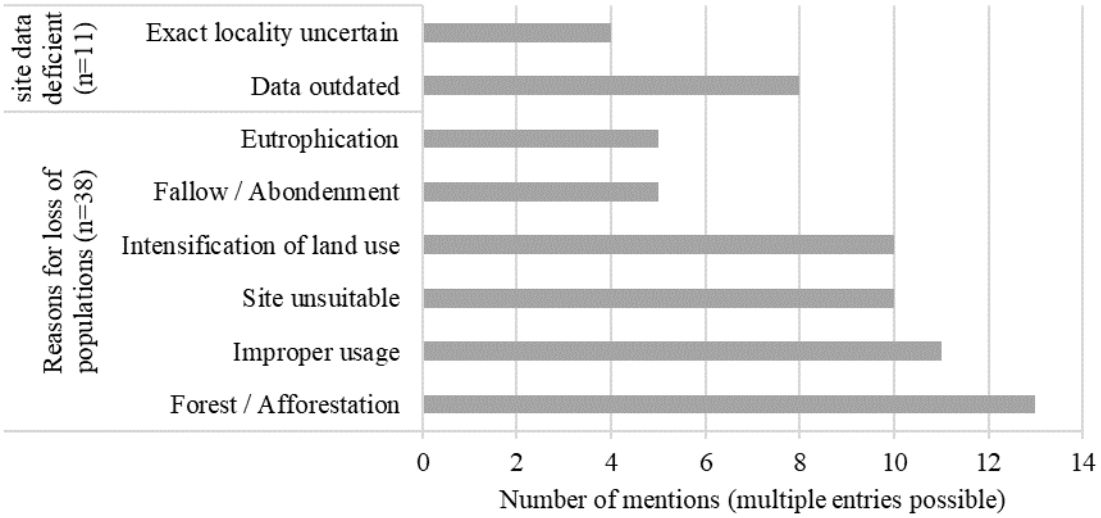


Figure 3: Reasons for local extinction of *Arnica montana* in South-West Germany, based on reports of federal species protection program.

A. montana prefers siliceous substrate with acidic soil pH (Blachnik und Zehm 2017; LUBW 2020a). It is a characteristic species for nutrient-poor, dry grass- and heathlands (e.g. *Nardetalia* grasslands). The species can occur in habitats with different moisture conditions from dry to wet (Oberdorfer et al. 2001). But it is mandatory, that it is an open habitat with high light availability near ground level. This is also reflected by an Ellenberg indicator value for light of 9, which means the species is a full light demanding plant (Ellenberg 1996). The species is known as

pasture weed and it is adapted to grazing by cattle and hence not eaten by them (Stebler und Schröter 1889).

Furthermore, *A. montana* is a perennial plant that belongs to the family of *Asteraceae* (Oberdorfer et al. 2001). The species can persist for long times with its long-living rhizome and can propagate vegetatively by rhizome runners (Kahmen und Poschlod 1998). In spring, ground-near rosettes with opposite leaves grow out of the rhizomes. Starting in April to May, flowering stems with lateral flower heads and opposite leaves are built. Each flowering stem builds mainly one to five flowering stems (Blachnik und Zehm 2017). The flowers are yellow to orange and the pollination by insects is mandatory (Luijten 2001). The most effective pollen transporter are syrphids, followed by bees, bugs and butterflies. The species is fully self-incompatible and at least the mating between two different individuals is necessary to produce seeds filled with an embryo (Luijten et al. 2002). In late summer, seeds are ripe and fall off the flower heads (Kahmen und Poschlod 1998). The seeds are typical *Asteraceae* achenes with a hairy pappus attached to the seeds. Seeds filled with an embryo are heavy related to the size of the pappus and the flight distance is hence limited to approximately a meter (Strykstra et al. 1998). In contrast, empty seeds are lighter and can fly for larger distances. But this doesn`t promote the propagation of the species and the dispersal ability of the species is limited. In addition, the attachment of the seeds to animal fur is bad even though the seed is covered by short, spiky hairs (Trapp et al. 2018). Already after a few minutes, the seeds fall of the fur. Hence, also the dispersal ability by grazing life stock is highly limited. After the ripe seeds are fallen off the flower heads, seed germination and establishment of seedlings takes place directly in autumn (Kahmen und Poschlod 1998). The seeds are not dormant and can`t built up a soil seed soil bank. Only a few seeds can persist the winter and need to germinate at latest in the following spring. In present times, the stage as seedling and young rosettes is very critical due to changing climatic conditions. The species can badly deal with the reduced rain fall and increased temperatures in spring and autumn (Stanik et al., 2018, 2020; LUBW 2019).

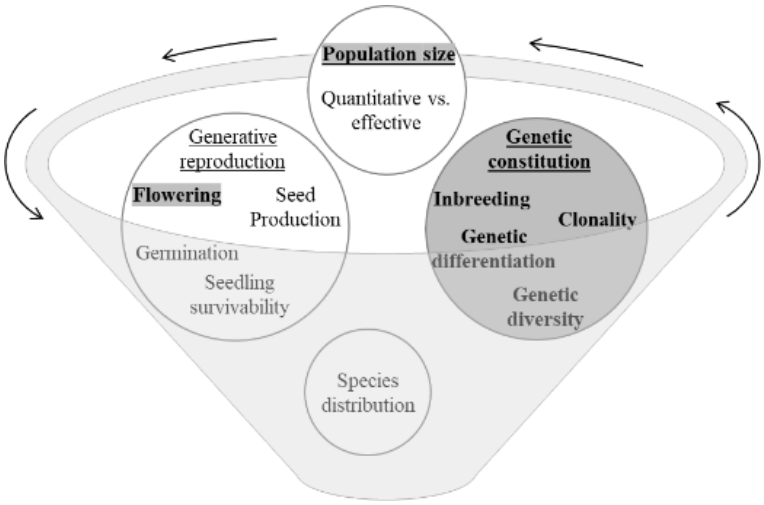
In recent times, several research projects focused on the investigation of the extinction risks of *A. montana* (Blachnik und Saller 2015a, b; Duwe et al. 2017; Van Rossum und Raspé 2018; Titze et al. 2020). All studies focused only on specific parts of the life cycle of the species respectively parts of the extinction vortex. Until now, no study included all parameters yet. The present thesis aimed to fill this gap.

Thesis outline

The thesis examines all parameters within the extinction vortex of *A. montana* in the region of South-West Germany and set them into context. First, within the chapters 2 & 3, the genetic constitution and the generative reproduction ability of the species were investigated to quantify the constitution of the species. In detail, **chapter 2** deals with the genetic constitution of remaining *Arnica* populations in South-West Germany. By applying genetic analysis, the genetic diversity within several populations was investigated and put into context with the census population size and flowering ability. Furthermore, the genetic differentiation within the study area was investigated. **Chapter 3** focused on the generative reproduction of *A. montana.* The seed quality, germination ability and survivability of seedlings from *Arnica* populations were investigated. The data were analysed in relation to the census population size, height of genetic diversity and extent of clonality within the studied populations. In **chapter 4,** sites with actual and extinct populations were compared on the level of landscape and habitat properties. By comparing the historical and recent landscape, the impact of land use changes over the last 150 years on *A. montana* was investigated. Furthermore, habitat properties were compared by conducting vegetation surveys and soil analysis. In addition, the impact of habitat properties on the population size was investigated. **Chapter 5** deals with the challenge of balancing the effects of inbreeding and outbreeding depression in case of *A. montana*. Artificial crossings between populations along a gradient of geographic respectively genetic distance were conducted to investigate their effects on the offspring fitness. In the final **chapter 6**, the findings from the previous chapters are summarized and set into context. Based on that, potential threats of *A. montana* are identified and recommendations for applied nature conservation are given.

Chapter 2

Genetic variation of *Arnica montana* – Can conservation units be derived from patterns of genetic diversity and differentiation?



Abstract

The loss of biodiversity is a large problem in recent times and is recognizable on the level of habitat, species and genetic diversity. Nowadays, endangered species often show a fragmented distribution area, populations get extinct and remaining populations decrease in size. The European plant species *Arnica montana* shows such a development and is meanwhile strongly endangered. The conducted study aimed to answer the question whether the ongoing decrease of the species in South-West Germany comes along with the loss of genetic diversity and whether the consideration of genetic differentiation can improve conservation activities. Therefore, leaf material from 29 natural *Arnica* populations were collected across South-West Germany and analysed at nine different microsatellite loci. The analysis revealed no general loss of genetic diversity but in small populations the diversity is decreased. Furthermore, these populations showed a higher extent of clonality and an excess of heterozygotes. This led to the conclusion that the species suffers from a reduced generative reproduction and the surviving of the species until today is ensured mainly by its vegetative propagation. Next to this, the remaining populations within distinct regions are highly differentiated. Hence, regions should be regarded as single conservations units and the exchange of plant material should be avoided between different populations and in particular regions. Conservation activities rather should focus on the enhancement of local population size by an adapted management and if necessary reinforcement with local plant material.

Introduction

In general, nationwide factors like changing climate conditions or increasing nitrogen deposition have a negative influence on species and their local populations (Gotelli und Ellison 2002; Thomas et al. 2004). This is further promoted by the respective management of a habitat and its surrounding because it influences the local habitat quality and the population size (Wesche et al. 2012). However, the degree of influence of these factors differs between different species. Thus, it is important that conservation activities for endangered species are adapted more species-specific and less general (Honnay und Jacquemyn 2007). Furthermore, for e.g. geographically fragmented species the definition of conservation units should be considered (Vogler und Desalle 1994; Heywood und Iriondo 2003; Jax 2006). Inside those units, conservation activities can be better adjusted to local conditions and can be more effective for species protection in the end. The survey of genetic variation can be used to define conservation units for a specific species (Frankham 2010).

Nowadays an ongoing decline of plant species occurring in nutrient-poor grasslands is recognizable and therefore many of them are represented on the red list of endangered species (Korneck et al. 1998; BfN 2016; Metzing et al. 2018). A characteristic of nutrient-poor grasslands is that they are comparable species-rich (Grime 1979; Vermeer und Berendse 1983). The occurring species are adopted to the predominantly harsh conditions in these grasslands and can deal with the reduced nutrient availability (Berendse et al. 1992). In Central Europe the amount of nutrient-poor grasslands decreased since the last century because they are strongly affected by certain land-use changes (Poschlod 2017). The intensification of agricultural land-use led to a conversion of those grasslands to profitable, intensively used grasslands with changed habitat qualities (Wesche et al. 2012). Next to that, nutrient-poor grasslands were also afforested or became fallow land when the utilization as grassland was not profitable enough. These habitat modifications due to human activities consequently led to an increasing recent fragmentation of the remaining nutrient-poor habitats in the landscape (Fahrig 2003). However, it also needs to be mentioned, that nutrient-poor grasslands never occurred nationwide and were always geographically fragmented for a certain amount. Species of these habitats evolved with this natural fragmentation. Thus, conservation activities should distinguish between natural and recent fragmentation and take it into account when conservation units are defined.

The fragmentation of a habitat results in a decreased gene flow between the occurring plant populations because either pollen or seed exchange becomes limited (Ellstrand und Elam 1993; Heinken und Weber 2013). Plant pollen is mostly transferred by wind or insects which is both reduced by fragmentation and promoted by the loss of insect biodiversity in present times

(Hallmann et al. 2017). Seeds can be dispersed either naturally e.g. by wind and water or over large distances by hay transfer by humans or due to the attachment to the fur or to endozoochory of grazing animals (Levin 1981). In the present times all these ways of exchange of genetic material is reduced because the landscape is intensively used, most grasslands are mown several times a year before seeds can ripen and animals are predominantly kept in stables.

The lack of gene flow between populations results in a genetic differentiation which is promoted by adaptations to local habitat and climate conditions by natural selection (Ellstrand und Elam 1993). The required time until a reduced gene flow is fixed on the genetic level depends on different life traits of the plants (Oostermeijer et al. 1994; Colling et al. 2002; Nybom 2004). For example, the genetic differentiation between populations of annual, self-compatible herb species is faster measurable than of long-living, clonal propagated, outcrossing species (Nybom 2004; Helm et al. 2006; Honnay und Jacquemyn 2007; Reisch und Bernhardt-Römermann 2014).

Consequently, in highly fragmented populations gene flow exists only within the remaining populations. Under these conditions, the probability of mating between closely related individuals (inbreeding) is higher for self-incompatible, outcrossing species which is further promoted by smaller population sizes with a low flowering capacity (Edmands 2007; Heinken und Weber 2013; Ottewell et al. 2016). Inbreeding leads to a reduced genetic diversity within the population, which is in general important so that populations are e.g. more adaptable to changing environmental conditions or more resistant to diseases. In the end the individual fitness of the offspring will be reduced and can be seen either by a reduced seed set, seed quality, survivability, establishment or adaptability of seedlings (Andersson und Waldmann 2002; Luijten et al. 2002; Willi et al. 2007). Hence, the population size decreases continuously and at the end populations get extinct. The investigation of the height of genetic diversity within different populations is important to see whether populations of threatened species suffer from loss of diversity or inbreeding and thus show reduced fitness.

Common activities in nature conservation to counteract the described negative effects of fragmentation, decreasing population sizes and inbreeding are reinforcement/re-stocking or reintroduction (Zippel und Lauterbach 2018). In both cases, plant material from a donor population is planted to a receiver population within the natural distribution area whereby in case of reinforcement the receiver population is not yet fully extinct. The measures correspond to a man-made reintroduction of gene flow. But in case of high genetic differentiation between the populations, the reintroduction of gene flow could result in an outbreeding depression (Edmands 2007; Barmentlo et al. 2017). This describes a reduced fitness of the offspring by mating between too distant relatives. Signs for that are a reduced seed set, seed quality, germination, seedling

survivability, establishment etc. (Luijten et al. 2002). Consequently, the long-term results are comparable to the effects of inbreeding and led to the extinction of the population respectively the species in the long-term.

All above described processes can be summarized as extinction vortex and are applicable to every (endangered) species (Matthies et al. 2004; Schlaepfer et al. 2018). The present study focused on the strongly endangered plant species *Arnica montana*. Since decades, an European-wide decline of the species is recognized (Falniowski et al. 2011; IUCN 2021) but obviously the already taken conservation activities couldn`t yet stop the extinction vortex. It aims to answer the following questions:

- **How genetically diverse are present populations of *A. montana* in South-West Germany and do populations suffer from loss of genetic diversity and/or inbreeding? Are population size and height of genetic diversity related?**
- **Are the remaining populations in Baden-Wuerttemberg, which are geographically fragmented from each other, also genetically differentiated?**
- **Can the populations in the study region be regarded as one single conservation unit or should several conservation units be defined in order to improve conservation activities?**

Material and methods

Study species

Arnica montana (Asteraceae) is a perennial, rosette-forming plant species. The species can propagate vegetatively with its long-living rhizome and thus is a clonal species (Kahmen und Poschlod 1998). For the generative propagation the flowers need to be pollinated by insects (e.g. syrphids) with pollen from another genet because *A. montana* is self-incompatible (Luijten 2001; Luijten et al. 2002). The dispersal distance of the seeds is limited due to a relatively high seed mass in relation to the size of the pappus of the achenes (Strykstra et al. 1998).

A. montana prefers siliceous bedrock with acidic soil pH and nutrient-poor grass- and heathlands with a high light availability (Kahmen und Poschlod 2000). The natural distribution area of *A. montana* is situated in Central Europe and ranges from Scandinavia, the Baltic States, Romania to Spain (Luijten et al. 2000). The species is nowadays protected by the EU habitat directives because of the progressive European-wide decline of *A. montana*.(LUBW 2020b). In the German Red List of Vascular Plants it is classified as endangered (category 3) and in certain federal states it is already defined as threatened with extinction or is even already extinct (Metzing et al. 2018).

Therefore, it is legally protected by the federal species protection regulations and several research project and applied nature conservation projects were focusing on *A. montana* in the past. The focus of the projects were either to give recommendations for species-specific management of sites (Titze et al. 2020) or to increase the local population sizes in order to harvest flowers for producing natural healing products and to commercialise them on regional markets (Blachnik und Saller 2015b).

Study sites

It has to be mentioned, that *A. montana* was never a common, widespread species because the preferred soil-acidic and nutrient-poor conditions were never widely distributed. But past floristic mappings show that *A. montana* was more widely distributed in Baden-Württemberg before 1950 (BfN and Netzwerk Phytodiversität Deutschlands 2013) and since then, an ongoing loss of populations is recognizable (LUBW 2020b) (Figure 4). Nowadays only in the southwestern part of Baden-Wuerttemberg (Southern Black Forest) the species is still widely distributed. In the rest of the state, the populations are geographically isolated from each other because of large distances between them and suitable nutrient-poor, soil-acidic grasslands are rare.

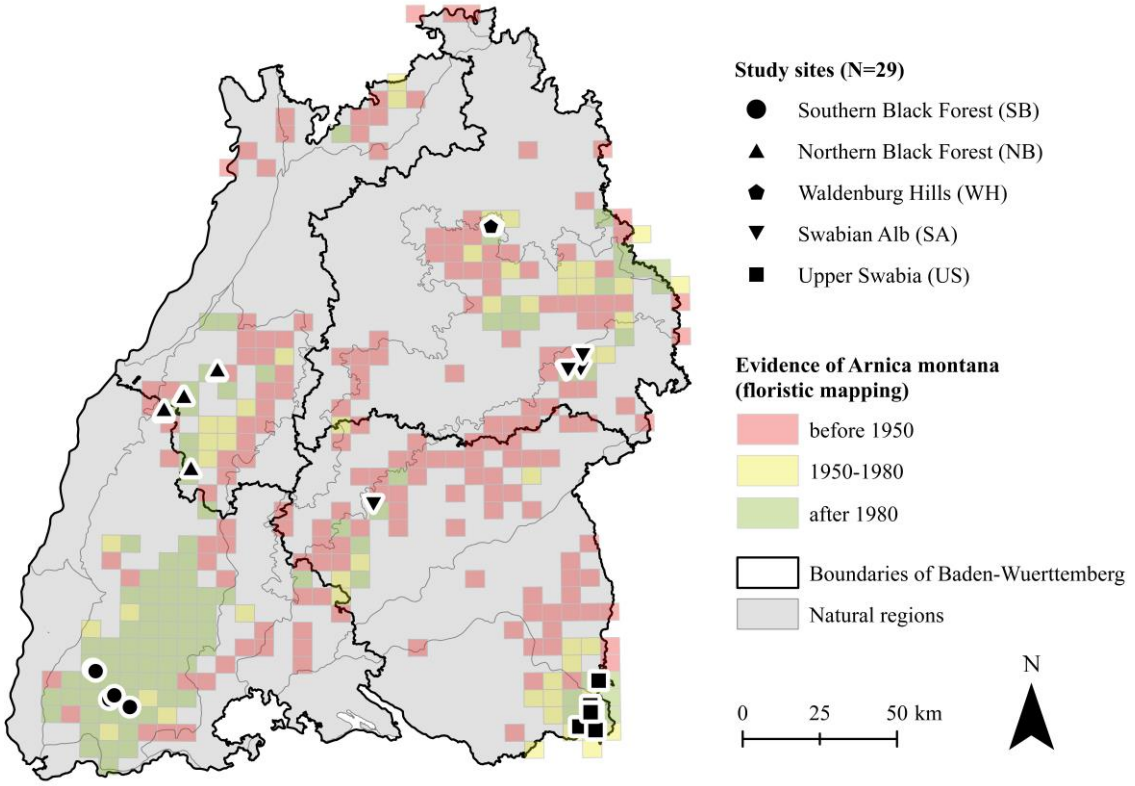


Figure 4: Geographical location of all 29 studied populations of *A. montana* in Baden-Württemberg within different regions (partial overlay).

In the present study, 29 natural populations of *A. montana* in Baden-Wuerttemberg were analysed (Figure 4, Table 2). The selected populations are distributed across the present natural range of the species in this area and can be assigned to five different regions that are based on the natural regions Southern Black Forest, Northern Black Forest, Swabian Alb, Waldenburg Hills and Upper Swabian Alb. The distance between the studied populations ranges from 0.24 to 197 km. Due to conservation issues, the coordinates of the study sites are not given in detail.

Table 2: Geographical location of the selected populations and their size as number of ramets and the percentage of flowering ramets.

Pop.	Natural Region	Altitude [m]	Number of ramets	Percentage of flowering ramets [%]
Am01	SB	1060	931079	41.10
Am02	SB	760	4508	31.32
Am03	SB	970	669672	53.25
Am04	SB	1000	728	42.99
Am05	SB	1080	5349	16.19
Am06	NB	595	650	39.54
Am07	NB	690	1253	6.15
Am08	NB	730	4021	34.74
Am09	NB	780	103	33.01
Am10	NB	753	277	16.97
Am11	NB	750	30	20.00
Am12	NB	755	3	66.67
Am13	NB	575	71	15.49
Am14	NB	665	367	60.22
Am15	WH	482	1799	6.50
Am16	WH	479	46	6.52
Am17	SA	669	4363	34.01
Am18	SA	625	229	10.48
Am19	SA	666	1041	7.97
Am20	SA	665	3231	4.92
Am21	SA	840	200	16.00
Am22	US	672	269	7.81
Am23	US	698	72	8.33
Am24	US	687	253	8.30
Am25	US	694	10	0.00
Am26	US	692	163	10.43
Am27	US	702	359	3.06
Am28	US	720	863	6.72
Am29	US	690	1538	4.68

(SB: Southern Black Forest, NB: Northern Black Forest, WH: Waldenburg Hills, SA: Swabian Alb, US: Upper Swabia)

The population size of the studied population was measured as the number of ramets, which corresponds to the number of distinguishable rosettes. Furthermore, the number of flowering

ramets was counted. Because of the ability of clonal propagation, a ramet/rosette can`t be equated with an individual. Rather, several ramets can belong to the same individual or genet. The census size of the 29 populations ranged from small populations with just three non-flowering ramets to large populations with several thousand ramets and flowering stems in the Southern Black Forest. The percentage of flowering ramets per population ranged from 0% to 66.67%.

Sampling procedure

The leaf tissue for genetic analysis was sampled in April and May 2019. Where available, 16 samples per population were collected (Table 4). In total, material from 402 ramets was sampled. A distance of at least one meter was kept between two samples in order to avoid the double sampling of the same genet due to obvious clonal propagation. The geographical position of all sampled ramets were documented with GPS (Garmin eTrex 30x). Leaf material was dried in silica gel and stored at room temperature until DNA extraction. The local nature conservation authorities permitted the collection of plant material of *A. montana*.

DNA analysis

DNA was extracted from 10-15 mg of dried leaf material by the CTAB method of Rogers and Bendich (1994) with modifications by Reisch (2007). The content of double stranded DNA was quantified with a microvolume spectrophotometer (NanoDrop One, ThermoFischer Scientific, Germany). For further analysis, every sample was diluted with water to a DNA concentration of 7.8 ng/µl.

All samples were amplified at nine different microsatellite loci developed by Van Rossum & Raspé (2018). All forward primers were marked at the 5´-end with a fluorescent dye (Biomers). In order to reduce the number of polymerase chain reactions (PCR) per sample, the microsatellite primers were combined in two separate multiplexes. In advance, the combinability of primers was tested for primer dimer formation with the application Autodimer (National Institute of Standards and Technolgy 2005). The assignment of the amplified fragments to the different loci was possible either by a different dye or by different fragment sizes.

Table 3: Studied microsatellite loci in *A. montana*, size range and number of detected alleles found in 402 samples and multiplex assignment and dye of forward primer.

Locus	Size range	Number of alleles	Multiplex	Dye
Am-AG-10	125 - 147	8	A	D4
Am-AG-4B	166 - 178	7		D3
Am-AG-1	203 - 217	7		D2
Am-AG-11	216 - 232	6		D3
Am-CT-2	252 - 272	7		D4
Am-CT-5	127 - 138	3	B	D3
Am-ATC-3	163 - 193	10		D4
Am-AG-2B	192 - 196	3		D3
Am-ATC-2	225 - 255	10		D3

All forward and reverse primers were diluted to a concentration of 10µM. For multiplex PCR all forward and reverse primers were mixed in the same ratio for the respective multiplex. Each PCR reaction contained 3.2µl DNA (7.8 ng/µl), 5.0µl 2xMastermix S (VWR), 0.8µl H_2O, 0.5µl multiplexed forward primers, 0.5µl multiplexed reverse primers. The thermocycle program for the PCR corresponds to the requirements from Van Rossum and Raspé (2018) and were done on a Master Cycler Nexus (Eppendorf, Germany). After the amplification 1µl of each PCR product were then mixed with 24.8µl of Sample Loading Solution (SLS, Beckman Coulter) and 0.2µl of CEQ Size Standard 400 (Beckman Coultier, Germany) and gel electrophoresed on an automated sequencer (GenomeLab GeXP from Beckman Coultier, method Frag-4).

Data analysis

Genetic diversity

The results from the capillary gel electrophoresis were exported as synthetic gel files in order to detect the length of the amplified fragments with the software BioNumerics (Applied Maths, Version 7.6.3). With the results of the fragment sizing, genetic diversity within each population was calculated using GenAlEx (Version 6.51b2) as the average number of alleles per locus (N_a), number of private alleles (A_p), percentage polymorphic loci (PPL), Shannon-Index (I), observed and unbiased expected heterozygosity (H_O, uH_e) and inbreeding coefficient (F_{IS}) (Peakall und Smouse 2012). Furthermore, the number of multilocus genotypes (MLG) was calculated, whereas two samples were considered as the same MLG when they showed the same alleles at all loci. Based on that, the extent of clonality (C) was calculated as $C = 1-^{(MLG\ -1)}/_{(N-1)}$, where N is the number of sampled ramets per population. Genetic diversity among study regions was compared using non-parametric tests because data of each region were not normally distributed and included a different number of populations. Moreover, correlation between genetic diversity and altitude

respectively the population size (number of ramets, percentage of flowering rosettes) was tested with spearman rank correlation test. All statistical analysis were done in R (Version 4.0.1).

Genetic differentiation

A Bayesian cluster analysis was calculated with Structure (version 2.3.4.) in order to assign the samples to genetic clusters (Pritchard et al. 2000). The analysis was done using the admixture model with following settings: length of burn-in period 10.000, number of Markov Chain Monte Carlo replications 100.000, 20 iterations for K = 1-30. The online-tool Structure Harvester was used to summarize the results from the Baysian cluster analysis. Following the method of Evanno et al. (2005) the most likely number of clusters was determined by the highest ΔK value. GenAlEx was used to calculate hierarchical analysis of molecular variance (AMOVA) (Peakall und Smouse 2012). For all samples, a three-level AMOVA was calculated considering the regional and population affiliation of each sample. Furthermore, separate two-level AMOVAs were run to calculate the genetic differentiation among populations within study regions. Pairwise correlation between the genetic (F_{ST}) and geographic distance was tested using the Mantel test in GenAlEx (999 permutations) in order to identify a potential pattern of isolation by distance. This was done once for all samples and second for the samples separated due to the regions.

Results

A total of 55 alleles was detected with three to ten alleles per locus (Table 3). 25 samples across all populations showed a three-allelic pattern at locus Am-AG-4B 25 which is not normal for a diploid species (Kantartzi 2013). Thus, no genotyping at this locus was possible and it was therefore excluded from the further analysis.

Genetic diversity

The number of different alleles ranged between 1.38 and 4.63 alleles per loci and population (Table 4). The regional comparison showed a significant lower number of alleles in the region Upper Swabia and Northern Black Forest compared to the populations located in the Waldenburg Hills (Table 5). In six populations private alleles were detected. The private alleles belonged to different loci and the concerned populations are not located in a specific region. In the most populations all loci were polymorphic. Seven different populations (Am11, Am12, Am13, Am18, Am22, Am25, Am28) showed a reduced number of polymorphic loci (37.5 – 87.5%). With only three of the eight loci, population Am13 showed the lowest percentage of polymorphic loci.

Table 4: Genetic diversity of *A. montana* measured at eight microsatellite loci for 29 populations.

Pop.	Region	N	N_a	A_P	PPL	I	H_o	uH_e	F_{IS}	MLG	C
Am01	SB	16	3.63	0	100	1.04	0.500	0.596	0.13	16	0
Am02	SB	16	3.88	1	100	1.02	0.563	0.564	-0.03	16	0
Am03	SB	16	3.75	1	100	0.89	0.539	0.488	-0.14	16	0
Am04	SB	16	3.50	0	100	0.91	0.494	0.517	0.01	16	0
Am05	SB	16	3.75	1	100	0.94	0.469	0.557	0.13	16	0
Am06	NB	16	3.38	0	100	0.83	0.504	0.481	-0.08	15	6.67
Am07	NB	16	3.75	0	100	1.01	0.520	0.574	0.07	16	0
Am08	NB	16	4.00	0	100	0.95	0.570	0.527	-0.12	16	0
Am09	NB	16	3.88	0	100	0.94	0.469	0.510	0.05	14	13.33
Am10	NB	9	3.63	0	100	0.98	0.514	0.564	0.03	7	25.00
Am11	NB	8	2.25	0	87.5	0.66	0.469	0.450	-0.11	3	71.43
Am12	NB	3	1.63	0	62.5	0.43	0.625	0.375	-1.00	1	100.00
Am13	NB	10	1.38	0	37.5	0.26	0.375	0.197	-1.00	1	100.00
Am14	NB	10	3.00	1	100	0.81	0.588	0.483	-0.28	9	11.11
Am15	WH	16	4.00	0	100	1.05	0.641	0.586	-0.13	16	0
Am16	WH	16	4.13	0	100	1.00	0.485	0.547	0.08	16	0
Am17	SA	16	3.75	0	100	1.07	0.648	0.607	-0.10	16	0
Am18	SA	5	2.13	0	87.5	0.61	0.575	0.436	-0.46	2	75.00
Am19	SA	16	3.75	0	100	0.98	0.531	0.538	-0.02	15	6.67
Am20	SA	16	4.63	2	100	1.09	0.609	0.584	-0.08	16	0
Am21	SA	11	3.88	0	100	1.08	0.557	0.619	0.06	10	10.00
Am22	US	16	2.00	0	75	0.55	0.344	0.368	0.03	16	0
Am23	US	16	3.50	1	100	0.82	0.484	0.473	-0.06	15	6.67
Am24	US	16	2.88	0	100	0.83	0.531	0.511	-0.07	16	0
Am25	US	10	2.88	0	87.5	0.72	0.463	0.432	-0.13	10	0
Am26	US	16	3.25	0	100	0.86	0.547	0.504	-0.12	16	0
Am27	US	16	3.38	0	100	0.94	0.563	0.547	-0.06	16	0
Am28	US	16	2.75	0	87.5	0.69	0.422	0.419	-0.04	16	0
Am29	US	16	2.63	0	100	0.66	0.359	0.416	0.11	16	0

N: number of samples, N_a: mean number of detected alleles per loci, A_p: number of private alleles, PPL: percentage of polymorphic loci [%], I: Shannon-Index, H_o: observed heterozygosity, uH_e: unbiased expected heterozygosity, F_{IS}: inbreeding coefficient, MLG: number of multilocus genotypes, C: percentage of clonality [%].

Unbiased expected heterozygosity ranged from 0.20 to 0.62 whereas observed heterozygosity was a bit higher and ranged from 0.34 and 0.65. Values for observed heterozygosity didn`t differ significantly between the regions. Shannon-Index ranged from 0.26 to 1.09 and differed significantly between the regions. Similar to recent analysis the region Upper Swabia showed significantly low values. The inbreeding coefficient in populations Am12, Am13 and Am18 was very low in comparison to the other populations. In eleven of the 29 populations ramets with the same MLG were detected within the population. The sampled ramets from population Am12 and Am13 had all the same MLG. Thus, the proportion of clonality ranged from 0 to 100% and

showed the significantly highest values in the region Northern Black Forest compared to the populations in Upper Swabia and Southern Black Forest (Table 5).

Table 5: Mean genetic diversity (±standard error) of *A. montana* in five different regions in Baden-Wuerttemberg (SB: South Black Forst, NB: Northern Black Forest, WH: Waldenburg Hills, SW: Swabian Alb, US: Upper Swabia).

	SB			NB			WH			SA			US			p
N_a	3.70	±0.06	ab	2.99	±0.33	b	4.06	±0.06	a	3.63	±0.41	ab	2.91	±0.17	b	0.02
A_P	0.6	±0.24		0.11	±0.11		0	±0.00		0.4	±0.00		0.13	±0.13		0.25
PPL	100	±0.00		87.5	±7.51		100	±0.00		97.5	±2.50		93.75	±3.34		0.49
I	0.96	±0.03	a	0.76	±0.09	ab	1.02	±0.03	a	0.97	±0.09	ab	0.76	±0.04	b	0.03
H_o	0.510	±0.02		0.51	±0.02		0.56	±0.08		0.58	±0.02		0.46	±0.03		0.09
uH_e	0.540	±0.02	a	0.46	±0.04	ab	0.57	±0.02	ab	0.56	±0.03	a	0.46	±0.02	b	0.03
F_{IS}	0.02	±0.05		-0.27	±0.14		-0.02	±0.11		-0.12	±0.09		-0.04	±0.03		0.54
MLG	16	±0.00	a	9.11	±2.13	b	16	±0.00	ab	11.8	±2.69	ab	15.13	±0.74	a	0.02
C	0	±0.00	a	36.39	±14.02	b	0	±0.00	ab	18.33	±14.3	ab	0.83	±0.83	a	0.01

N_a: mean number of detected alleles per loci, A_p: number of private alleles, PPL: percentage of polymorphic loci [%], I: Shannon-Index, H_o: observed heterozygosity, uH_e: unbiased expected heterozygosity, F_{IS}: inbreeding coefficient, MLG: number of multilocus genotypes, C: percentage of clonality [%], p: significance level of Kruskal-Wallis-test, small letters indicate significant differences based on pairwise Mann-Whitney-U-test.

The non-parametric correlation test between the genetic diversity and the altitude above sea level didn`t show any significant correlations as well as the correlations with the percentage of flowering ramets ($p > 0.05$, Table 7 in appendix). In contrast, the number of ramets per side showed a significant influence on genetic diversity. The more ramets there were at a side, the higher was the allelic richness, percentage of polymorphic loci, Shannon-Index and number of multilocus genotypes (Figure 5). Furthermore, the extent of clonality was significantly higher in smaller populations.

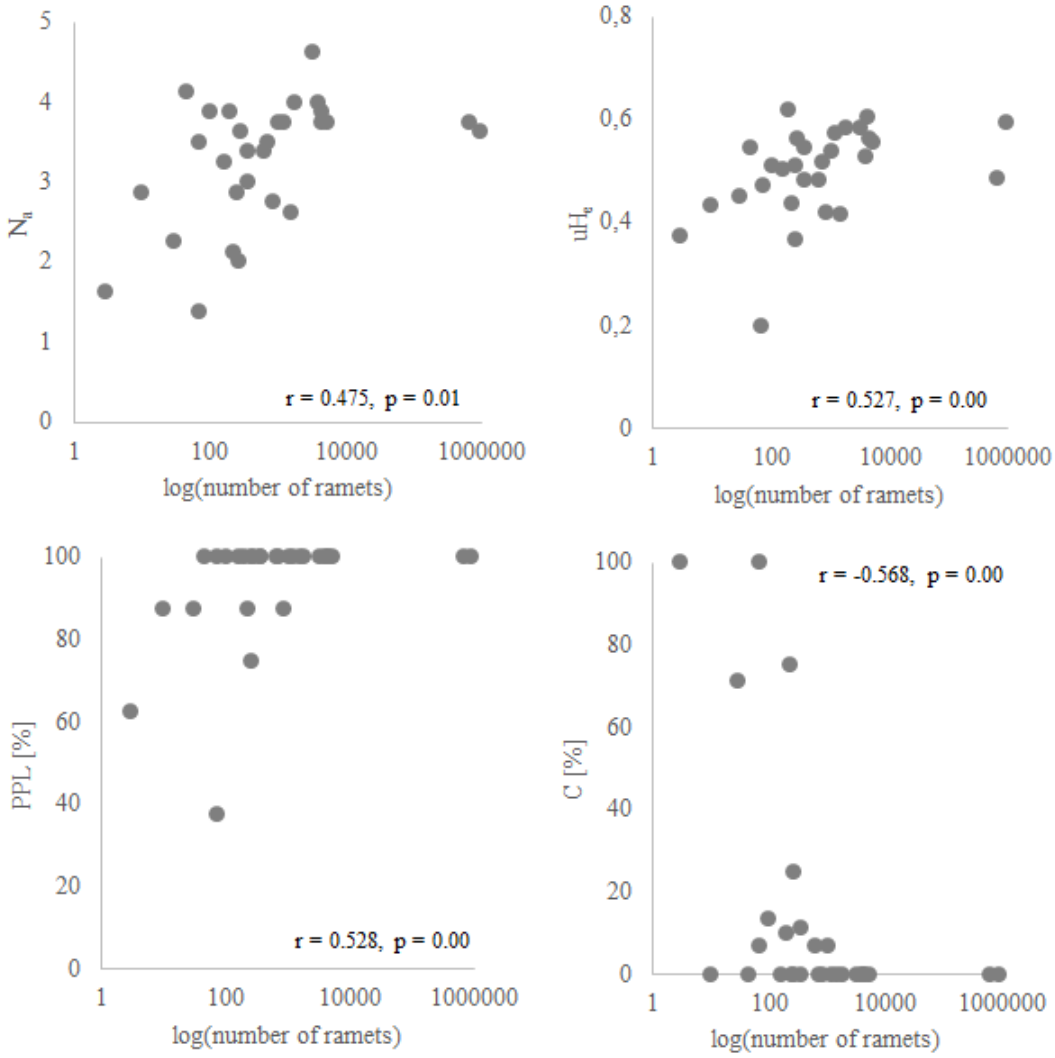


Figure 5: Relationship between population size (number of ramets, log-transformed) and allelic richness (N_a), unbiased expected heterozygosity (uH_e), percentage of polymorphic loci (PPL) and clonality (C). (r: Spearman-rank correlation coefficient, p: significance level).

Genetic differentiation

Structure analysis revealed that the 402 samples are assigned to two genetic clusters (Figure 6). One cluster included the ramets from Southern Black Forest and Upper Swabia. Furthermore, population Am13 located in the Northern Black Forest was with an amount of approximately 85% related to this cluster. The second cluster included the remaining populations from Northern Black Forest, Waldenburg Hills and Swabian Alb. Thus, the two clusters show a geographical separation into a northern and a southern group (Figure 9 in appendix). The results of the three-level AMOVA proved that the two clusters revealed by the Baysian cluster analysis are significantly differentiated from each other. 10.8% (F_{RT}) of the variance could be explained by the assignment to the two clusters.

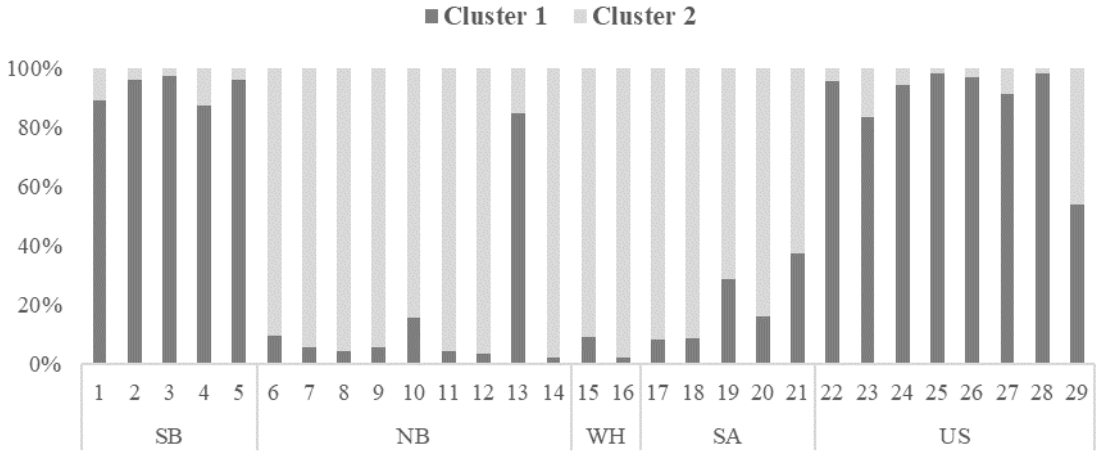


Figure 6: Results of the Baysian Cluster Analysis show the assignment of the 29 studied populations of *A. montana* to the two genetic clusters.

In all AMOVAs the majority of genetic variation was detected within populations (F_{ST} = 75.6 – 94.0%). The assignment of the samples to the different regions explained 9.1% (F_{RT}) of the total genetic variance. The differentiation into the two clusters based on Bayesian analysis could explain 10.8% of variance. The results of both three-level AMOVAs showed that the populations inside each cluster respectively each region are differentiated from each other (F_{SR} = 12.6% / 13.6%).

The results of the two-level AMOVAs revealed that genetic differentiation was lower in the regions Waldenburg Hills/Swabian Alb (6.0%) and Southern Black Forest (7.0%) compared to the region Northern Black Forest (17%) and Uppper Swabia (20.5%). All variance components of both three- and two-level AMOVAs were statistically significant ($p<0.001$).

Table 6: Results of analysis of molecular variance (AMVOA) within and among the 29 studied populations of *A. montana* and the study regions or clusters from Bayesian cluster analysis.

			df	SS	MS	Est. Var.	% Var.	p
Three-level AMOVA with clusters (K=2)								
	Among cluster	F_{RT}	1	130.395	130.395	0.293	**10.8%**	0.001
	Among populations	F_{SR}	27	331.017	12.260	0.370	**13.6%**	0.001
	Within populations	F_{IS}	775	1590.819	2.053	2.053	**75.6%**	0.001
Three-level AMOVA with regions								
	Among regions	F_{RT}	5	206.347	41.269	0.238	**9.1%**	0.001
	Among populations	F_{SR}	23	255.066	11.090	0.330	**12.6%**	0.001
	Within populations	F_{IS}	775	1590.819	2.053	2.053	**78.3%**	0.001
Two-Level AMOVA within regions								
SB	Among populations	F_{ST}	4	29.963	7.491	0.165	**7.0%**	0.001
	Within populations	F_{IS}	155	341.594	2.204	2.204	**93.0%**	
NB	Among populations	F_{ST}	8	89.246	11.156	0.405	**17.1%**	0.001
	Within populations	F_{IS}	199	391.471	1.967	1.967	**82.9%**	
WH + SW	Among populations	F_{ST}	6	37.517	6.253	0.147	**6.0%**	0.001
	Within populations	F_{IS}	185	423.436	2.289	2.289	**94.0%**	
US	Among populations	F_{ST}	7	113.857	16.265	0.474	**20.5%**	0.001
	Within populations	F_{IS}	236	434.319	1.840	1.840	**79.5%**	

df: degrees of freedom, SS: sum of squares, MS: quadratic mean, Est. Var: estimated variance, %Var.: percentage of variance, p: level of significance based on 999 replications, SB: Southern Black Forest, NB: Northern Black Forest, WH: Waldenburg Hills, SW: Swabian Alb, US: Upper Swabia.

The Mantel test for isolation by distance across all samples showed a positive and significant correlation between genetic and geographic distance ($r = 0.280$, $p = 0.01$, Figure 7). Inside the different regions a positive correlation between genetic and geographic distance was found within the region Upper Swabia ($r = 0.598$, $p = 0.037$). For the other regions the test was not significant ($p > 0.05$) (Table 8 in appendix).

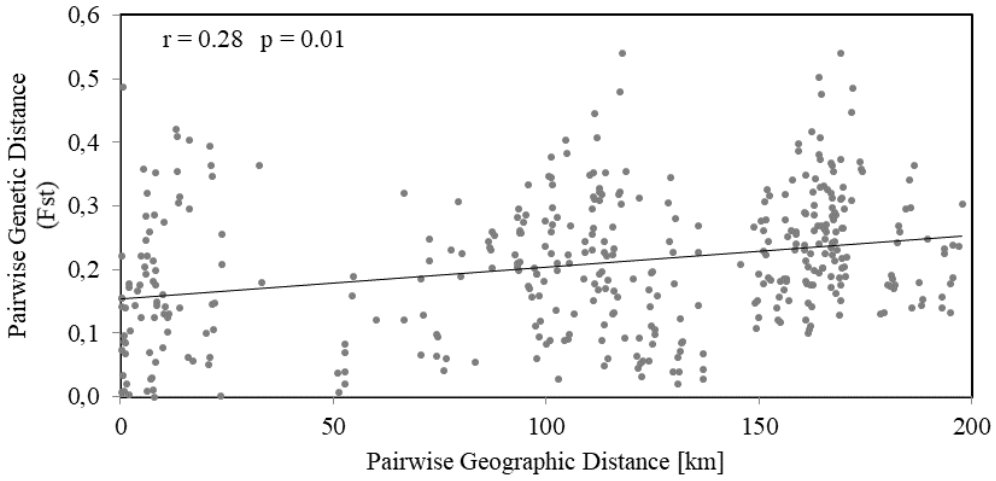


Figure 7: Correlation of pairwise genetic and geographic distance of 29 populations of *A. montana* (402 samples) in Baden-Württemberg (Mantel-Test).

Discussion

Genetic diversity in correlation to population size

The genetic diversity of *A. montana* measured inside the majority of the studied populations is typical for long-living, self-incompatible plant species (Nybom 2004). Furthermore, the range of observed and expected heterozygosity is comparable to genetic studies on *A. montana* in Central Europe (Kahmen und Poschlod 2000; Maurice et al. 2016; Duwe et al. 2017; Van Rossum und Raspé 2018). Consequently until now no actual, general loss of genetic diversity is recognizable in Baden-Wuerttemberg in contrast to *Arnica* populations at the edge of its natural distribution area (Vera et al. 2020). This underlines the importance of the protection in South-West Germany respectively Central Europe. Furthermore, it supports that Germany respectively the federal states have to maintain and improve the conservation activities to protect the species in order to counteract the present decline of the species.

However, genetic diversity and clonality were clearly correlated with population size which has similarly been observed in previous studies of *A. montana* (Kahmen und Poschlod 2000; Maurice et al. 2016; Van Rossum und Raspé 2018) and is a well-known phenomenon (Lammi et al. 1999; Van Rossum und Triest 2003; Bachmann und Hensen 2007; Szczecińska et al. 2016; Busch und Reisch 2016; Duwe et al. 2018; Ismail et al. 2018). In most studies, the population size of clonal species is described as the number of flowering stems. Nevertheless, the results of the present study are comparable to them because in the actual study the number of ramets and number of flowering stems were correlated significantly. Furthermore, the number of ramets might be the

better indicator for population size of clonal species because the amount of flowering stems from *A. montana* might differ between years and depends more e.g. on annual climatic conditions (Sugier et al. 2013; Titze et al. 2020). In contrast to other studies on *A. montana* (e.g. Duwe et al. 2017) no influence of altitude on genetic diversity could be detected which can be explained by the rather small altitudinal and geographical range of the study.

The inbreeding coefficient F_{IS} was smaller than zero in most populations and correlated with population size. This implements an heterozygote excess in those populations which is a sign for reduced generative reproduction within the populations (Reed und Frankham 2003; Ottewell et al. 2016). The absence of evidence of inbreeding is due to the full self-incompatibility of *A.* montana (Luijten et al. 1996). Clonal species like *A. montana* can avoid a reduced ability of sexual propagation by vegetative growth and are therefore able to persist for a certain time (Nybom 2004; Helm et al. 2006). But unfortunately, they are then unable to evolve and adapt to changing conditions which is necessary in times of changing climate conditions. In particular because it was already proven that the surviving rate and fitness of *A. montana* is and will be influenced negatively by the present and upcoming climate conditions (Vikane et al. 2019; Stanik et al. 2020, 2021). It is therefore important to increase the population size to improve the amount and possibility of generative reproduction.

Differentiation between populations and regions

Bayesian cluster analysis as well as the AMOVAs showed a clear and high differentiation among the actual populations of *A. montana* and the study regions in South-West Germany. The separation into a northern and a southern group of populations respectively different geographical regions underlines the results of Duwe et al. (2017) where the same pattern was already shown along a longer north-south distance across Central Europe. Furthermore, the Mantel test revealed a general pattern of isolation by distance. The strong differentiation values among populations (between and among regions/clusters) clearly indicates a long-lasting lack of gene flow among clusters, regions and populations (Ottewell et al. 2016). It is therefore most likely, that the measurable genetic differentiation is a result of natural fragmentation which is promoted by recent anthropogenic fragmentation and loss of populations (Plenk et al. 2019). Generally, the temporal gap between fragmentation and genetic differentiation is even larger in clonal species, which means that clonal species can buffer human impact to a certain degree (Nybom 2004).

Normally, gene flow between plant populations requires the exchange of seeds and/or pollen (Levin 1981). The high differentiation value reveal that both seems to be reduced in the study area. A natural exchange of seeds of *A. montana* between populations can´t take place due to the

reduced natural dispersal distance of the species (Strykstra et al. 1998) and its reduced ability to attach to the fur of grazing animals e.g. sheeps (Szczecińska et al. 2016; Trapp et al. 2018). Both can be explained by the heavy seed mass in relation to the small size of the pappus and the relatively short hairs on the seed surface. *A. montana* is insect-pollinated whereby syrphid, bees and butterflies are the most effective pollen transporter (Luijten 2001). Most of the actual *A. montana* populations are either surrounded by dense and high growing forests, intensive used landscape or have a distance larger than one kilometre from each other what makes an effective pollen transfer by insects more difficult (Potts et al. 2010). The decreasing biomass of insects in general has an additional negative impact on the gene flow between self-incompatible plant species (Hallmann et al. 2017).

Different levels of differentiation within study regions can be explained by the different history of land-use in the different regions. The main distribution area of *A. montana* is located in the Southern Black Forest which is characterized since centuries by large and closely located grasslands and thus gene flow e.g. by pollen exchange is possible (see chapter 5). The differentiation of the populations in the northeastern part of Baden-Wurttemberg (Swabian Alb, Waldenburg Hills) is also rather low although the geographical distance between the sites is large. In this area the amount of grassland was relatively high and was just reduced during the last decades due to either an intensification of land-use or afforestation. Thus, the fragmentation is comparably young and is not yet fixed genetically.

The region of the Northern Black Forest is since centuries a forest-dominated region. Thus, the grasslands with *A. montana* are fragmented from each other since long-time. Therefore, even closely located populations are fragmented since long time and local genotypes could evolve which led to a high genetic differentiation. In Upper Swabia, *A. montana* grows predominantly on litter meadows. Those grasslands are man-made and were rare in former times, were than promoted in the last century and are nowadays threatened e.g. due to land use intensification (Poschlod 2017). Therefore, those habitats are isolated from each other since ever and the genetic differentiation among the populations of *A. montana* as insect-pollinated and clonal propagated species could evolve. This low gene flow is further promoted by the low number of flowering ramets and thus reduced pollen ability in the populations in this area (Araki et al. 2007).

Genetic variation of plant species can give hints on the history of post glacial recolonization (e.g. Schmitt et al. 2002; Slovák et al. 2012; Meindl et al. 2016). In the present study the height of diversity or appearance of private alleles in the differentiated clusters and regions didn`t differ and therefore no glacial refugia can be defined like it was possible for e.g. *Meum athamanticum* in that region (Huck et al. 2009, 2012). Due to the results, it`s more likely that *A. montana* had

several glacial refugia in Europe. This is even more likely because the habitats were the species can occur have an extremely large environmental range (Maurice et al. 2012; Duwe et al. 2017; Stanik et al. 2018; Sugier et al. 2019). In order to reconstruct the postglacial recolonization a broader and denser sampling across the whole distribution area in Europe would be needed.

Conclusions with respect to conservation units and future activities

The small differences in genetic diversity between the regions/clusters makes it most likely that the protection of all populations respectively regions is important for the protection of *A. montana*. The high genetic differentiation between the geographical distinct regions reveals that distinct regions should be regarded as single conservation units (Vogler und Desalle 1994; Heywood und Iriondo 2003; Frankham 2010, 2015; Ottewell et al. 2016). Inside the units the management of the populations need to be adapted to the local site conditions and thus can differ between the units. The differentiation into units is supported by the different land-use history of the regions in Baden-Wuerttemberg.

Furthermore, especially in small populations the effects of missing generative reproduction is measurable. Thus, the first step of protection should be to maximize the population size at each site as much as possible to enable effective pollination inside each population to avoid the excess of heterozygotes (Ottewell et al. 2016). Therefore, small populations could be supported by re-stocking. For that, seeds from the same populations should be cultivated and then planted as young rosettes back to the locality (Sugier et al. 2013). Due to present changing climatic and habitat conditions the natural establishment and fitness of seedling of *A. montana* is reduced and planting rosettes is more efficient (Stanik et al. 2018, 2020; Hollmann et al. 2020). Furthermore, in order to protect isolated populations with locally evolved genotypes from extinction, it is important to adapt the habitat conditions to the localities and the management to the former time when the locally occurring genotypes evolved (Reckinger et al. 2010; Wesche et al. 2012).

Due to the high values of differentiation, an artificial mixture of populations of *A. montana* should be avoided (Ottewell et al. 2016) or just take place when information on ecology and genetic variation of the species is investigated in advanced (Pegtel 1998). Otherwise the crossing between genetically differentiated individuals can lead to outbreeding depression (Montalvo und Ellstrand 2001; Luijten et al. 2002; Frankham 2015; Bucharova et al. 2017). The offspring will suffer from reduced fitness by e.g. reduced seed set, survivability or adaptability. In long-living species the negative effects of outbreeding depression often is just recognizable after several generations and not directly after the introduction of foreign plant material (Fenster und Dudash 1994). If the introduction is unavoidable, the environmental conditions at the donor and receiver population

should be as comparable as possible and the genetic differentiation should be as low as possible (Van Rossum und Raspé 2018; Bucharova et al. 2019).

Appendix

Table 7: Relationship between genetic diversity indices and altitude, number of rosettes and percentage of flowering ramets (r: spearman rank correlation coefficient).

Spearman-Rank-Correlation	**Altitude**		**Number of ramets**		**Percentage of flowering ramets**	
	r	p	r	p	r	p
N_a	0.098	0.61	**0.475**	**0.01**	-0.01	0.96
A_{Privat}	0.149	0.44	0.359	0.06	0.153	0.43
PPL	0.13	0.50	**0.528**	**0.00**	0.063	0.75
I	0.113	0.56	**0.537**	**0.00**	-0.061	0.76
H_o	-0.146	0.45	0.256	0.18	0.25	0.19
uH_e	0.17	0.38	**0.527**	**0.00**	0.007	0.97
F_{IS}	0.347	0.07	0.272	0.15	-0.212	0.27
MLG	0.083	0.67	**0.654**	**0.00**	-0.261	0.17
C	-0.075	0.70	**-0.568**	**0.00**	0.365	0.05

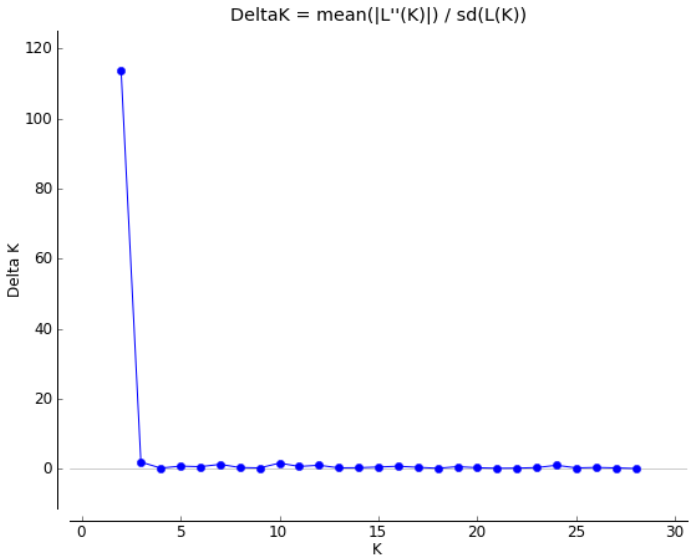


Figure 8: Results of Bayesian Cluster Analysis: Delta K in relation to the number of tested groups K= 1-30.

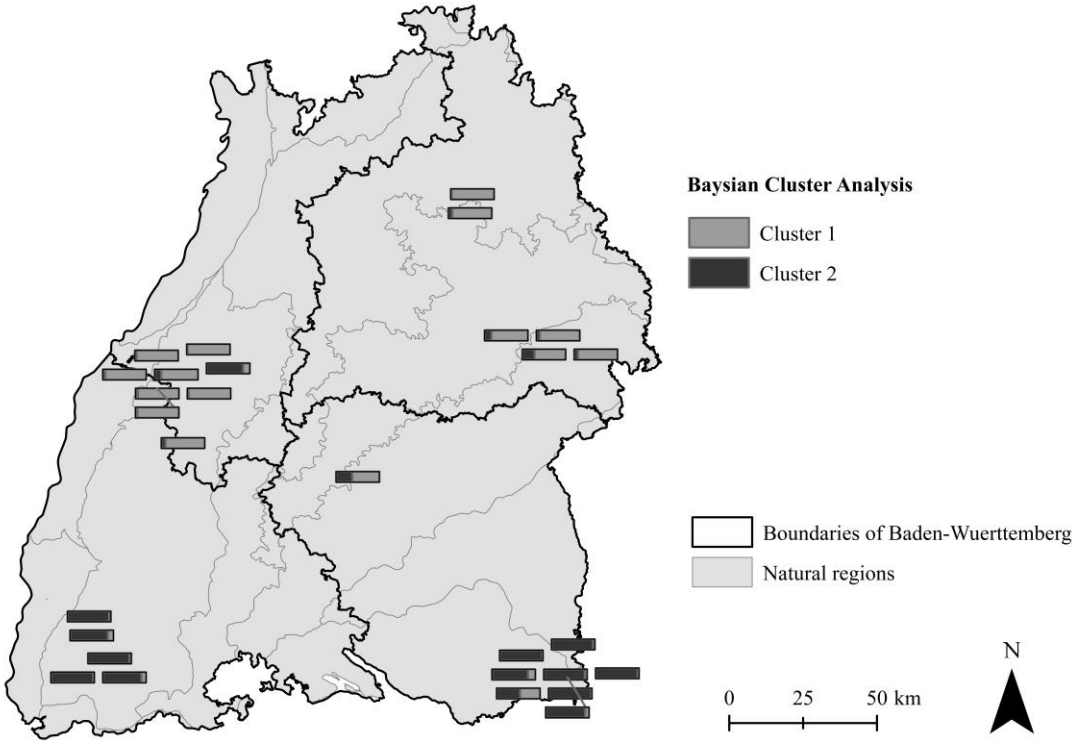


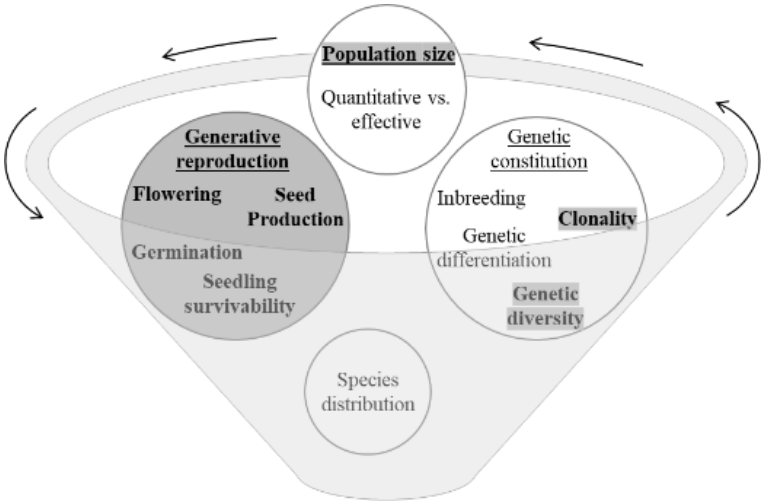
Figure 9: Results of Bayesian Cluster Analysis shows two genetic clusters in 29 studied populations of *A. montana* in Baden-Wuerttemberg.

Table 8: Results of mantel test for all samples and inside different regions (r: correlation coefficient, p: significance level).

Region	r	p
SB	0.392	0.101
NB	0.170	0.201
WH+SA	0.040	0.352
US	0.598	**0.037**
all samples	0.280	**0.010**

Chapter 3

Population size and extent of clonality determine generative reproductive fitness of *Arnica montana*



Abstract

A major aim of nature conservation is to preserve and improve the fitness of a species to increase long term survivability. The fitness of species can be defined at different levels, e.g. the genetic or reproductive fitness. The first describes the level of genetic diversity. The latter is the assemblage of the ability to produce seeds that can germinate, establish and grow up to flowering plants. The present study focused on both levels and first asked the question how large the reproductive fitness of the strongly endangered plant species *Arnica montana* in South-West Germany is. Based on this it was then analysed whether the reproductive fitness is correlated with genetic diversity or census population size. For the evaluation of reproductive fitness, seeds from 29 natural populations were collected. The filling rate was determined via X-ray examination as well as germination ability and survivability tests under controlled conditions were conducted. The results showed that the reproductive fitness mainly was influenced by the census population size and the filling rate of seeds. This in turn correlated with the extent of clonality within the populations. Hence, the fitness of *Arnica montana* can easily be determined by monitoring the filling rate of seeds which gives at the same time an insight in the clonal structure of the populations.

Introduction

The protection of biodiversity is one of the main aims of nature conservation, which was agreed internationally within the Convention on Biological Diversity (CBD) and entered into force in the early 1990ies (CBD 1992). Thus, conservation laws and regulations on the global, European and national level were passed in order to protect species (BfN 2021b). Nevertheless, the size of many plant populations is decreasing continuously and populations respectively species get extinct (BfN 2016; Metzing et al. 2018). It is a well-studied pattern that small populations are more likely to be threatened to extinction and it is mainly explained by a reduced ability to reproduce generatively and hence reduced fitness (e.g. Brys et al. 2004a; Leimu et al. 2006; de Vere et al. 2009; Busch und Reisch 2016). Various parts of a plants´ life cycle can contribute to the fitness, which in turn also depends on the population size or genetic constitution.

The life cycle of clonal growing, perennial plant species combines generative reproduction with vegetative propagation (Grime 1979; Barrett 2015) (Figure 10). The latter includes the propagation by e.g. buds or runners or in minor cases by asexual production of seeds (apomixes). It enables individuals to persist for long time and to propagate without investing energy on the building of flowers or seeds. Plants descending from vegetative propagation don`t differ in morphology and hence plants barely can be assigned to a distinct genet in the field. Thus for clonal growing plant species, the determination of population size is difficult and often overrated (Reisch et al. 2018). But this can be corrected by genetic analysis. Especially for outbreeding species, clonal growth can be disadvantageous. Due to the clonality, the same genet can build several inflorescences that are spatially distributed. Hence, it becomes more likely that the same genotype mate (Eriksson 1993). This increases the risk of inbreeding and hence a loss of fitness.

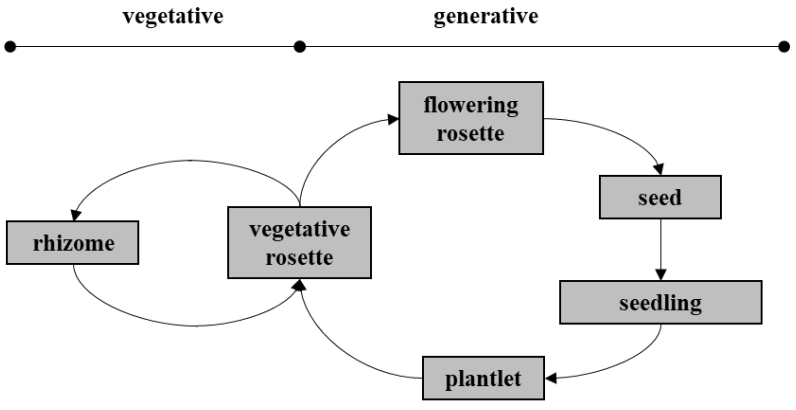


Figure 10: Life cycle of clonal plant species (e.g. *Arnica montana*) combining vegetative and generative propagation (Grime 1979; Kahmen und Poschlod 1998).

In general, the generative reproduction of a plant comprises pollination, seed production, seed dispersal, germination, establishment and survival of seedlings and all contribute to the generative fitness of a plant species (Grime 1979; Kahmen und Poschlod 1998). The influence of population size on each part of the generative life cycle differs due to species-specific life history traits such as e.g. self-compatibility, germination niche or seed longevity and thus need to be investigated separately for different plant species.

One advantage of generative or sexual reproduction is the mating of different genotypes by transferring pollen from one plant to another (Zhang und Zhang 2007). This allows the rearrangement and mixing of genes which leads to the building of new genotypes that can cope (better) with e.g. changing conditions. Those changes can refer to general climatic changes, increase or decrease of nutrient supply or changing management conditions. Thus, sexual reproduction increases the amount of genetic diversity, which allows the species to adapt to changing environmental conditions and to buffer the threat of extinction (Ellstrand und Elam 1993).

Besides successful pollination and production of seeds with well-developed embryo, it is important that seeds are able to germinate and seedlings can establish and survive for the long-term perspective. The seeds of *A. montana* are not dormant and have to germinate directly after the seed ripeness in autumn or at the latest in the following spring (Schwabe 1990; Bakker et al. 1996; Kahmen und Poschlod 1998). Thus, they are unable to build a persistent soil seed bank. Hence, the time for germination and seedling establishment is rather short for *A. montana* in nature and therefore a critical stage in the life cycle (James et al. 2011). If either the germination or the survival ability is reduced, the generative fitness will be reduced notably which is disadvantage for *Arnica*.

In nature conservation, species protection programs were instituted to increase the constitution of threatened species like *A. montana*. Until now, the main effort to protect the species was to quantify the countable population size. In case of reduced population size, the management of the site is adapted. But unfortunately, the generative reproduction ability is rarely taken into account. In order to improve the efforts of nature conservation, different parts of the generative part of the life cycle of *A. montana* were investigated within this study in order to answer following questions:

- **Does population size of *A. montana* differ between populations respectively different geographical regions within South-West Germany?**
- **Is generative fitness of *A. montana* correlated with population size, genetic diversity or extent of clonality and how can nature conservation improve the fitness?**

Material and methods

Study species

Arnica montana (Asteraceae) mainly grows in nutrient-poor grasslands and heathlands on siliceous and soil-acidic bedrock (Oberdorfer et al. 2001; Blachnik und Zehm 2017; LUBW 2020a). It is a rosette-forming herb with cross-opposite leaves and along the flowering stems, the leaves are arranged opposite. Each flowering stem builds at the end on average one to five flower heads with up to 100 single flowers each. The flowering period starts in June and lasts until end of July respectively beginning of August. The species is mainly insect-pollinated and self-incompatible (Luijten 2001; Luijten et al. 2002). Directly after the ripening of the seeds, the achenes fall off the mother plant (Kahmen und Poschlod 1998). In general, the ability to disperse over long distances by e.g. wind is reduced due to a high weight to size ratio of the achenes (Strykstra et al. 1998). Next to the generative reproduction, the species can propagate clonally by rhizome runners (Kahmen und Poschlod 1998).

Study sites

Twenty-nine populations of *A. montana* were selected across the natural distribution range in Baden-Wuerttemberg (Figure 11, Table 13 in appendix). The populations are separated into different geographical regions that are based on the natural regions Southern/Northern Black Forest, Waldenburg Hills, Swabian Alb and Upper Swabia. Genetic analysis proved that this separation is fixed on the genetic level and the regions are highly differentiated from each other. (see chapter 2). This is due to a lack of gene flow between the populations since long time.

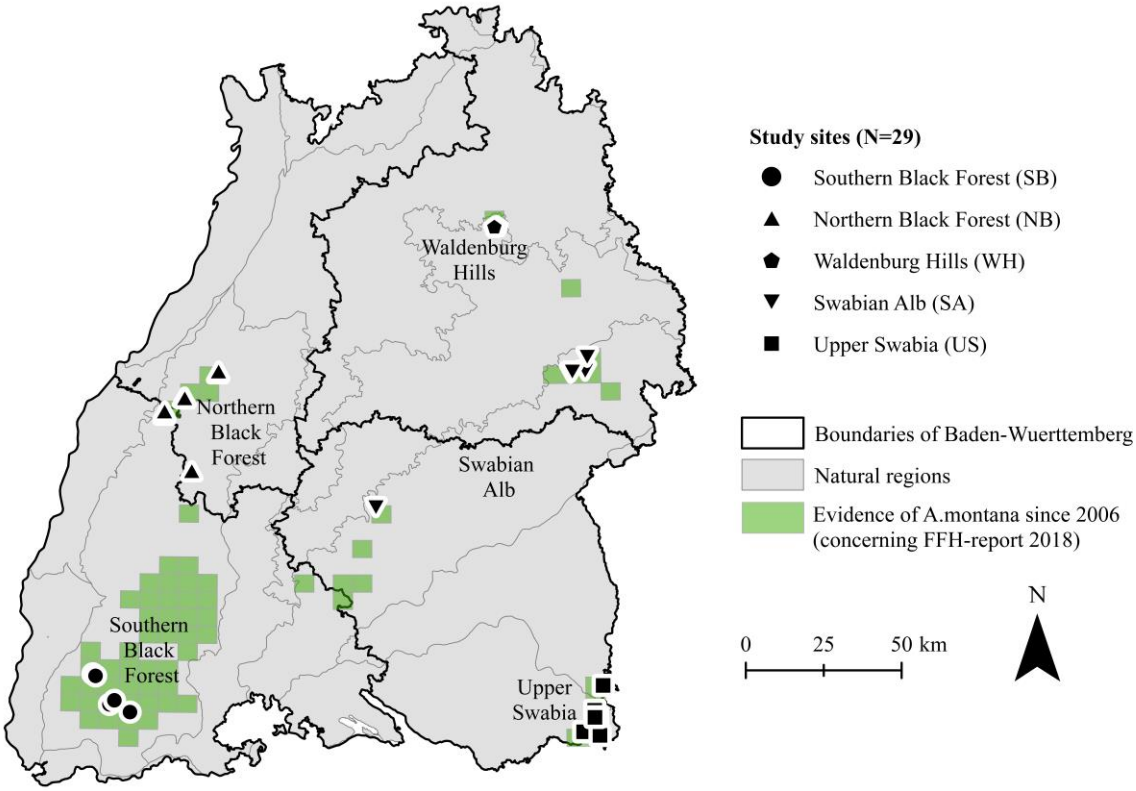


Figure 11: Geographical location of 29 studied populations of *A. montana* in Baden-Wuerttemberg within different regions across the natural distribution of the species (partial overlay).

The present main distribution area of *A. montana* is located in the Southern Black Forest where the existing populations are still widespread (LUBW 2020a). In the rest of the federal state number and size of the populations has decreased over the past decades and the species is strongly endangered nowadays. The altitude of the sites ranged from 479 and 1080 m above sea level (Table 13 in appendix). The exact coordinates of the study sites are not given due to the high conservation status of the species.

Population size

The size of every study population was estimated as total number of rosettes (TR) and total number of flowering rosettes (FR). Based on that, the proportion of flowering rosettes (PFR) was calculated as PFR = $^{FR}/_{TR}$. Furthermore, for each site the area (A) covered with *A. montana* was documented with GPS in the field (GPS eTrex 30) and afterwards the density of rosettes (DTR = $^{TR}/_{A}$) respectively the density of flowering rosettes (DFR= $^{FR}/_{A}$) was calculated.

Genetic diversity

For the analysis of genetic diversity leaf material of all study populations were collected. If available, leaves from 16 different rosettes were collected inside each population with a minimum distance of one meter. The leaves were stored separately in filter bags and dried with silica gel. From each sample, 10-15mg of dried leaf material was used for the extraction of the DNA which was done by the method of Rogers and Bendich (1994) and modified by Reisch (2007). Afterwards the content of double stranded DNA was measured with a microvolume spectrophotometer NanoDrop One (ThermoFischer Scientific, Germany) and diluted with water to a concentration of 7.8 ng/µl. This working solution were used to amplify fragments at nine different microsatellite loci developed by Van Rossum and Raspé (2018) for *A. montana*. The number of polymerase chain reactions per sample (PCR) could be reduced by combining the microsatellite primes in two multiplexes (A: AG-10, AG-4B, AG-1, AG-11, CT-2, B: CT-5, ATC-3, AG-2B, ATC-2). The amplified fragments could be assigned to the different loci because of different fluorescent dyes or fragment size. Each PCR contained 3.2µl DNA (7.8 ng/µl), 5.0µl 2xMastermix S (VWR), 0.8µl H_2O, 0.5µl multiplexed forward primers (10 µM), 0.5µl multiplexed reverse primers (10µM). The chosen conditions for the thermocycle program for the PCR corresponded to the specifications from Van Rossum and Raspé (2018). 1µl of the PCR product was mixed with 24.8µl of Sampling Loading Solution (SLS, Beckman Coulter) and 0.2µl of CEQ Size Standard 400 (Beckman Coultier, Germany) and analysed on an automated sequencer (GenomeLab GeXP from Beckman Coultier, method Frag-4). The size of fragments were detected with BioNumerics (Applied Maths, Version 7.6.3) and genetic diversity within each population was calculated with GenAlEx (Version 6.51b2). For the analysis with GenAlEx, loci AG-4B was not considered because it showed a three-allelic pattern for some samples which should appear in a diploid species. The level of genetic diversity in every population was described by Shannon´s Information Index (I), observed heterozygosity (H_o) and inbreeding index (F_{IS}). The extent of clonality (C) of a population was calculated as the ratio between samples with the same genotype at all loci (MLG) and the number of analysed samples (N) ($C = 1 - {}^{MLG-1}/_{N-1}$).

Generative fitness

Seed quality

Complete flower heads with ripe seeds were collected from mid of June to beginning of July 2018. If possible, ten flower heads were sampled randomly within each population, with a distance of at least five meters between the respective plants. In small populations less flower heads were sampled to avoid negative effects on the population (ENSCONET 2009). The seeds from each

flower head were stored separately in paper bags and were kept dry, dark and at room temperature until further treatments.

In July 2018, the number of seeds per flower heads was counted (N). Afterwards the seeds were x-rayed with a Faxitron X-Ray MX-20 (Faxitron Bioptics, LLC, USA) with 18kV for 10 seconds (Figure 12). The number of seeds with a well-built embryo was detected (N_{fertil}) and the filling rate calculated as $^{Nfertil}/_{N}$. For further analysis, empty seeds were separated from filled seeds. The filled seeds from the same population were then combined and mixed carefully.

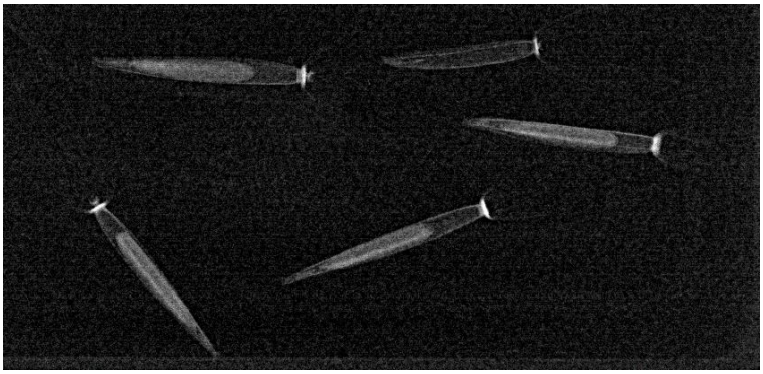


Figure 12: X-Ray image of four seeds of *A. montana* filled with developed embryo and one empty seed (top right).

Germination ability test

The germination ability test was done separately for each population. Therefore, a maximum of 50 filled seeds was randomly selected from every population and the pappus had been removed from all seeds. The seed length from every seed was measured and the mean length per population calculated. The seeds were then divided into five equal portions and from each portion, the seed weight was measured. Based on that, the mean mass per seed was calculated for every population.

Then, seeds from each portion were placed in a single petri dish on two layers of filter paper and watered with deionized water. The petri dishes were placed in a climate chamber (Rumed, type 1301, Rubarth Apperate GmbH, Germany) with an artificial day/night-rhythm of 14h light and 10h darkness and a day temperature of 22°C respectively night temperature of 14°C. These settings correspond to the conditions in nature and showed good germination results in previous studies (Lang et al. 2014; Leipold et al. 2019). The petri dishes were controlled twice a week for germinated seeds and watered if necessary. After the three weeks, the germination ability test was finished because the seeds either had germinated or were classified as not germinable if the seeds were soft by pressing with a pair of forceps. The germination rate for every petri dish was

calculated as ratio between the number of germinated seeds and used seeds. The mean germination rate per population was the average of the five replicates.

Survivability of seedling and fitness

Germinated seeds were placed single in a 4 x 6 cell tray filled with a mixture of acidic cultivation substrate (pH 3.8, Alpenflor) and acidic sand (Silex). The mixture contained 140 litres of substrate and 25 kg of sand. The plants were cultivated outside in the botanical garden of the University of Regensburg and were watered either with rainwater or deionised water. After three months of cultivation, the seedlings became too big for the cell trays and were repotted to single plant pots (diameter 13cm) with the same soil-sand-mixture. During the winter period (November to March) the pots were placed in unheated concrete enclosures with removable glass cover in order to prevent the rhizomes from cold snaps. The plants were regularly checked for viability until April 2021. The ratio between the survived plants and the number of planted seedlings expresses the survival rate. The total generative fitness for each population was calculated as the product of mean filling rate, mean germination rate and survival rate.

Data analysis

All statistical analysis were run with R (Version 4.0.1). The normal distribution of data in general and data within the regions were tested with Shapiro-Wilk-Test in advance. In case of normally distributed data, one-way ANOVAs with Games-Howell post hoc tests were used to test for regional differences, because homogeneity of variances was not given. In case of non-normally distributed data, non-parametric tests were used for group comparison. The relationship of generative fitness with the population size and the genetic diversity was tested by non-parametric Spearmann rank correlation.

Results

Population size

The total number of rosettes at the 29 studied sites of *A. montana* ranged from three to several hundred thousand rosettes per site (Table 9). The highest numbers of rosettes were counted in the region of the Southern Black Forest and its regional mean differed significantly form the other regions (Table 10). The number of rosettes didn`t increase with altitude ($r = 0.204$, $p = 0.289$) but with populated area ($r = 0.895$, $p = 0.000$).

Table 9: Population size of 29 populations of *Arnica montana* in Baden-Wuerttemberg.

Pop.	Region	TR	FR	PFR [%]	Area [m²]	DTR [m^{-2}]	DFR [m^{-2}]
Am01	SB	931079	382635	41.10	39858	23.36	9.60
Am02	SB	4508	1412	31.32	7427	0.61	0.19
Am03	SB	669672	356590	53.25	21327	31.40	16.72
Am04	SB	728	313	42.99	304	2.40	1.03
Am05	SB	5349	866	16.19	8048	0.66	0.11
Am06	NB	650	257	39.54	206	3.15	1.24
Am07	NB	1253	77	6.15	343	3.65	0.22
Am08	NB	4021	1397	34.74	9455	0.43	0.15
Am09	NB	103	34	33.01	84	1.23	0.41
Am10	NB	277	47	16.97	191	1.45	0.25
Am11	NB	30	6	20.00	39	0.77	0.15
Am12	NB	3	2	66.67	0.03	120.00	80.00
Am13	NB	71	11	15.49	25	2.84	0.44
Am14	NB	367	221	60.22	25	14.68	8.84
Am15	WH	1799	117	6.50	479	3.76	0.24
Am16	WH	46	3	6.52	37	1.24	0.08
Am17	SA	4363	1484	34.01	434	10.06	3.42
Am18	SA	229	24	10.48	2	134.71	14.12
Am19	SA	1041	83	7.97	92	11.32	0.90
Am20	SA	3231	159	4.92	876	3.69	0.18
Am21	SA	200	32	16.00	46	4.38	0.70
Am22	US	269	21	7.81	135	1.99	0.16
Am23	US	72	6	8.33	15	4.82	0.40
Am24	US	253	21	8.30	78	3.23	0.27
Am25	US	10	0	0.00	28	0.36	0.00
Am26	US	163	17	10.43	165	0.99	0.10
Am27	US	359	11	3.06	370	0.97	0.03
Am28	US	863	58	6.72	218	3.96	0.27
Am29	US	1538	72	4.68	233	6.59	0.31

TR: total number of rosettes, FR: number of flowering rosettes, PFR: percentage of flowering rosettes, DTR: density of rosettes, DFR: density of flowering rosettes.

The size of the area populated by *A. montana* ranged from 0.03m² (Am12) to nearly 4 ha (Am01). No significant differences between the study regions concerning the size of the area populated with *A. montana* were observed, but the area tended to be the largest in the region Southern Black Forest ($p = 0.054$). The density of rosettes and flowering stems per site ranged from less than one rosette respectively one flowering stem per square meter to very dense growing populations like Am12 with 120 rosettes /m² or Am18 with 135 rosettes / m². However, differences between the study regions concerning in density were not significant.

Table 10: Mean population size (± standard error) of *A. montana* in five different geographical regions in Baden-Wuerttemberg (SB: South Black Forst, NB: Northern Black Forest, WH: Waldenburg Hills, SW: Swabian Alb, US: Upper Swabia).

Region	TR		FR		PFR [%]		Area [m²]	DTR [m⁻²]	DFR [m⁻²]
SB	322267 ± 199517	a	148363 ± 90419	a	36.97 ± 6.25	a	15393 ± 6996	11.69 ± 6.54	5.53 ± 3.32
NB	753 ± 430	b	228 ± 149	bc	32.53 ± 6.84	a	1152 ± 1039	16.47 ± 13.02	10.19 ± 8.78
WH	923 ± 877	ab	60 ± 57	abc	6.51 ± 0.01	abc	258 ± 221	2.5 ± 1.26	0.16 ± 0.08
SA	1813 ± 843	b	356 ± 283	b	14.68 ± 5.16	ab	290 ± 165	32.83 ± 25.51	3.86 ± 2.62
US	441 ± 182	b	26 ± 9	c	6.17 ± 1.2	c	155 ± 42	2.86 ± 0.77	0.19 ± 0.05
p	0.03		0.008		0.002		0.054	0.295	0.103

TR: total number of rosettes, FR: number of rosettes with flowering rosettes, PFR: percentage of flowering rosettes, DTR: density of rosettes, DFR: density of flowering rosettes, p: significance of Kruskal-Wallis-Test, small letters indicate significant differences based on pairwise Mann-Whitney-U-tests.

The proportion of flowering rosettes varied between 0 and 66.67%. The proportion of flowering rosettes in the region Southern (36.97%) and Northern Black Forest (32.53 %) were significantly higher than in the other regions (Table 10, Figure 13). In the populations on the Swabian Alb, on average 14.68% of the rosettes produced a flowering stem. Lowest flowering percentage was counted in populations inside the region Upper Swabia (6.17 %). The percentage of flowering rosettes correlated significantly with the altitude above sea level ($r = 0.425$, $p = 0.021$).

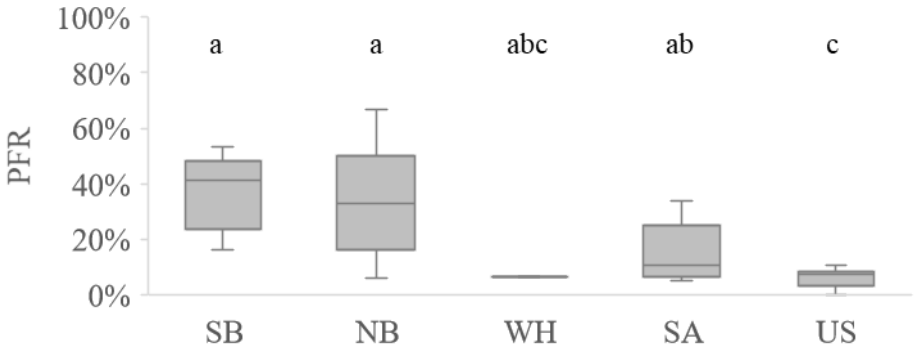


Figure 13: Mean percentage of flowering rosettes (PFR) in regional comparison. Significant differences between regions are marked by different letters (pairwise Mann-Whitney-U-test, $p<0.05$). (SB: Southern Black Forest, NB: Northern Black Forest, WH: Waldenburg Hills, SA: Swabian Alb, US: Upper Swabia).

Generative fitness

The generative fitness of *A. montana* was analysed for only 23 of the studied 29 populations. In four of the six omitted populations, no seeds of *A. montana* could be collected in 2018 because either all seeds were already fallen off the flower heads (Am13), the sites were mown or grazed at the time of seed collection (Am09, Am10) or no flower stems were produced (Am25). In the collected flower heads of population Am27 and Am12, no respectively only one filled seed was found and therefore neither the germination nor the survival test was done.

Table 11: Generative fitness of 29 studied populations of *A. montana* in Baden-Wuerttemberg.

Pop	Region	Filling rate [%]	Seed mass [mg]	Seed length [mm]	Germination rate [%]	Survival rate [%]	Fitness [%]
Am01	SB	60.59	1.26	6.12	92.00	89.13	49.68
Am02	SB	81.42	1.26	6.07	94.00	91.49	70.02
Am03	SB	64.80	1.30	5.97	98.00	81.63	51.84
Am04	SB	82.20	1.83	6.69	96.00	87.50	69.05
Am05	SB	45.00	1.10	5.49	96.30	88.46	38.33
Am06	NB	66.64	0.96	5.27	98.00	69.39	45.32
Am07	NB	27.74	1.26	6.43	94.00	57.45	14.98
Am08	NB	36.00	1.17	6.25	98.00	79.59	28.08
Am09	NB	NA	NA	NA	NA	NA	NA
Am10	NB	NA	NA	NA	NA	NA	NA
Am11	NB	20.40	1.57	6.31	100.00	62.00	12.65
Am12	NB	1.08	NA	NA	NA	NA	NA
Am13	NB	NA	NA	NA	NA	NA	NA
Am14	NB	43.29	1.20	6.06	92.00	65.22	25.98
Am15	WH	74.60	1.50	6.75	98.00	93.88	68.63
Am16	WH	79.03	1.82	6.84	100.00	92.00	72.71
Am17	SA	76.58	1.49	7.08	98.00	83.67	62.79
Am18	SA	19.89	1.20	6.09	88.00	63.64	11.14
Am19	SA	8.55	0.66	5.39	48.00	50.00	2.05
Am20	SA	39.55	0.77	5.90	82.00	56.10	18.19
Am21	SA	74.15	1.82	6.49	100.00	76.00	56.35
Am22	US	10.43	1.52	6.14	88.00	72.73	6.68
Am23	US	5.11	0.99	4.61	100.00	77.78	3.97
Am24	US	85.00	1.18	5.84	98.00	95.92	79.90
Am25	US	NA	NA	NA	NA	NA	NA
Am26	US	91.42	1.16	5.50	98.00	93.88	84.11
Am27	US	0.00	NA	NA	NA	NA	NA
Am28	US	68.76	1.12	5.70	100.00	90.00	61.88
Am29	US	69.41	1.11	6.10	98.00	71.43	48.59

In total, 17,711 seeds of *A. montana* were collected with an average filling rate of 49.2% per flower head. The filling rate of the populations ranged from 0 to 91.42% (Table 11). The rate tended to be higher in the populations of the Southern Black Forest and the Waldenburg Hills (Table 14 in appendix). The average mass and length of the filled seeds used for the germination

test varied per population between 0.66 to 1.82 mg respectively between 4.61 to 7.08 mm per seed. Both mass and length didn`t significantly differ between the regions.

The germination ability of filled seeds was on average 93.66% for all populations, except population Am19 that showed a reduced germination rate (48.00%). The average survivability of the planted seedlings after 2.5 years of cultivation was 78.21%. The survivability of the seedlings from the region Swabian Alb (65.88%) was significantly lower compared to the populations from region Waldenburg Hills (92.94%). The populations from the other regions showed intermediate values (72.28 – 87.64%) (Table 14 in appendix).

The generative fitness ranged between 1.08 and 84.11% and the one-way ANOVA showed a significant regional influence (p = 0.023) (Figure 14). The fitness of populations in the Northern Black Forest (14.23%) was significantly lower than in the Southern Black Forest (55.78%) or the Waldenburg Hills (70.67%). Populations from the Swabian Alb (30.1%) and Upper Swabia (35.64%) varied inside the regions and did not differ significantly from the other regions.

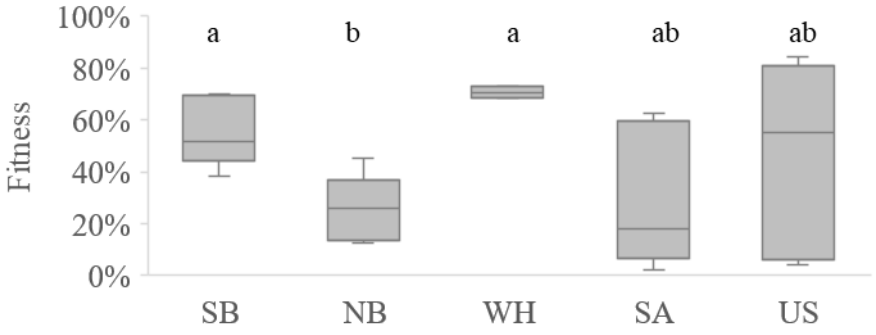


Figure 14: Generative fitness calculated as product of filling, germination and survival rate per population in regional comparison (ANOVA: F = 3.949, p = 0.023). Significant differences between regions are marked by different letters (post-hoc Games-Howell, p<0.05; regions: SB: Southern Black Forest, NB: Northern Black Forest, WH: Waldenburg Hills, SA: Swabian Alb, US: Upper Swabia).

Correlation between generative fitness, population size and genetic diversity

Correlation analysis showed that fitness was strongly correlated with filling rate of the seeds (r = 0.98, p = 0.000) and survival rate (r = 0.62, p = 0.001) (Table 12, Figure 15). In addition, filling and survival rate were correlated (r = 0.54, p = 0.006). Furthermore, germination rate increased with survival rate (r = 0.45, p = 0.028). However, seed mass and length were not correlated with other variables of generative fitness. But seed length and seed mass correlated highly significant with each other, which means that larger seed were heavier (r = 0.72, p = 0.000).

Table 12: Correlation between variables of generative fitness of 23 populations of *A. montana*. Above the triangle correlation coefficients r (Spearmann rank) are given, below triangle the level of significance p are given (significant correlations written in bold).

r / p	Filling rate	Mass	Length	Germination rate	Survival rate	Fitness
Filling rate		0.16	0.29	0.19	**0.54**	**0.98**
Mass	0.465		**0.72**	0.30	0.35	0.19
Length	0.173	**0**		0.06	0.04	0.30
Germination rate	0.379	0.152	0.776		**0.45**	0.24
Survival rate	**0.006**	0.09	0.858	**0.028**		**0.62**
Fitness	**0**	0.383	0.159	0.262	**0.001**	

The network in figure 15 shows the significant correlations between generative fitness, population size and genetic diversity (non-significant correlations are not shown). The overall generative fitness of *A. montana* increased with population size (number of rosettes and flowering stems) and populated area. Population size was not significantly correlated with filling rate, germination rate or survival rate.

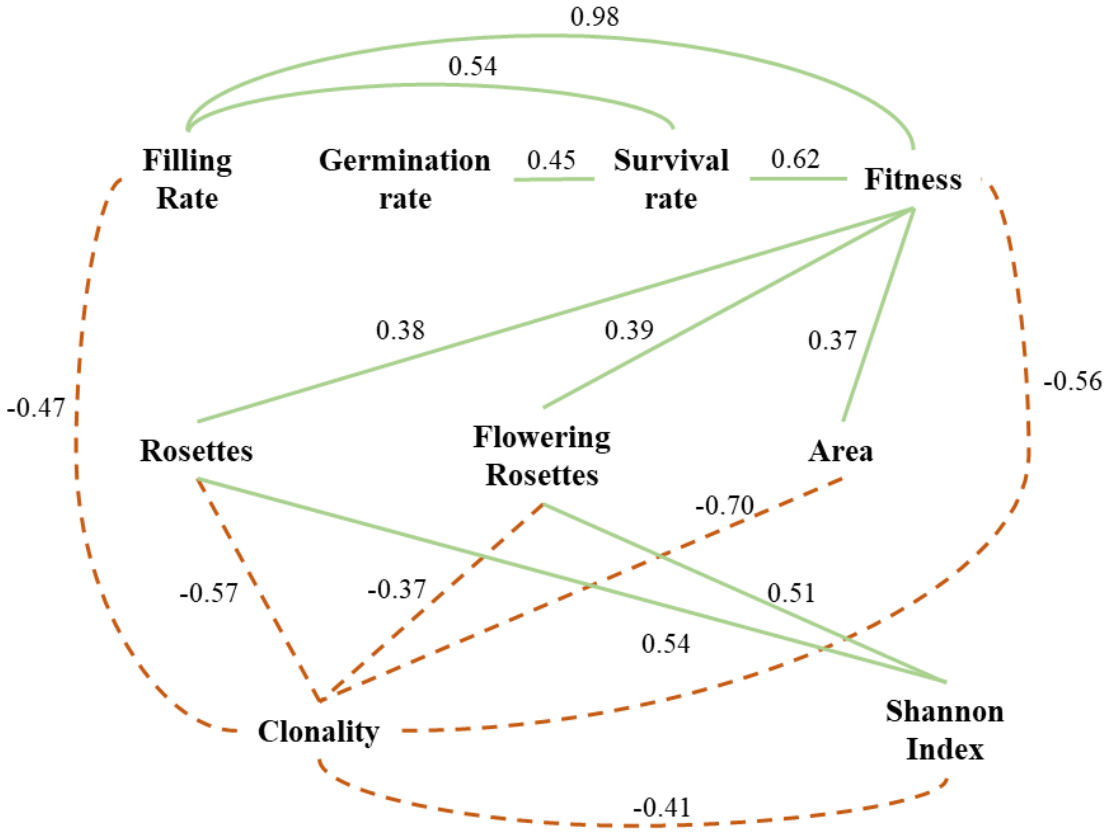


Figure 15: Significant correlations between generative fitness parameters, populations size (number of rosettes, number of flowering rosettes and overgrown area) and amount of clonality of 23 populations of *Arnica montana* in Baden-Wuerttemberg (numbers indicate correlations coefficients of Spearmann rank correlation test, green solid line indicates positive correlation, red dashed lines indicates negative correlation).

At sites with a small population size the amount of clonality increased significantly. At the same time, clonality had a negative influence on the filling rate of the seeds ($r = -0.47$, $p = 0.019$) and fitness ($r = -0.56$, $p = 0.002$). Genetic diversity expressed by Shannon-Index was not correlated with generative fitness but positively with population size.

Discussion

Population size in context with regional differentiation

The survey of the population size proved that the main distribution area of *A. montana* is located in the Southern Black Forest. The sites in this region, where the species grows, are large and thus high number of rosettes could establish. In the rest of the study area, the nutrient-poor and soil-acidic habitats where *A. montana* occurs declined continuously due to intensifying land-use and fertilizer input and thus the population size decreased (LUBW 2020c). Hence decreasing habitat size has a negative effect on the population size because the species-specific habitat conditions are not given and rosettes can not grow or establish (Vergeer et al. 2003).

A. montana is a perennial plant species that overwinters as rhizome in the soil and in the following spring, leaves and flowering stems grow out of it (Blachnik und Zehm 2017; LUBW 2020a). The winter period is necessary to introduce the hibernation and the warmer climate in spring is necessary to start the vegetation and flowering period. In the study area, the temperature and precipitation differ naturally in average and course of the year between the geographical regions (LUBW 2021). Furthermore, due to recent climate changes, it became less likely that the winter temperature is below zero degrees and the probability of snow layer decreased. These changes are more recognizable on lower altitudes compared to higher elevated regions. Therefore, it is very likely that the recognized decreased percentage of flowering rosettes in lower altitudes as well as regional differences is due to different regional climatic conditions (Giménez-Benavides et al. 2007; Zhang und Zhang 2007). These climatic differences can`t be influenced by local conservation activities and hence the percentage of flowering rosettes can only be compared within regions but not within a larger scale (Vogler und Desalle 1994; Jax 2006).

Generative fitness in context with population size and genetic diversity

The generative fitness of the studied populations correlated stronger with the filling rate of the seeds than with the germination and survival rate. The latter was higher across all populations compared to the filling rate and didn`t correlate with the population size or genetic diversity. Germination and survival ability were studied under controlled conditions in climate chambers

respectively botanical garden with continuous water supply for seeds and seedlings and without concurrence. In nature, drought and high respectively dense growing vegetation reduces the survivability of seedling of *A. montana* (Kahmen und Poschlod 1998; Hollmann et al. 2020; Stanik et al. 2020, 2021). Thus, it is very likely that the total generative fitness in nature is even lower than calculated in this study, which can be reinforced by the fact that no seedlings were recorded in the field during the study (personal observations). It further promotes that the germination of seeds and establishment of seedlings will be an even more critical stage in natural life cycle for *A. montana* in future due to recent changes in climatic conditions, which results in drier summers (Koch et al. 2021).

The total generative fitness of studied populations of *A. montana* increased within larger population sizes, which in turn were genetically more diverse and included more genotypes that were different. Large population sizes have a positive effect on the fitness of several plant species (Vergeer et al. 2003; Leimu et al. 2006; Musche et al. 2008; de Vere et al. 2009; Busch und Reisch 2016). Thus, conservation measures should improve the local population size by e.g. adapted management.

Blachnik und Saller (2015) found that populations with less than 1000 rosettes of *A. montana* showed a reduced fertility. In the present study, this value cannot be supported. Populations with a size between 100 and 1000 rosettes showed both high and low proportions of fertile seeds. Instead, the amount of fertile seeds correlated with the extent of clonality. This is a typical pattern for clonal species that are self-incompatible (Brys et al. 2004b; Leimu et al. 2006). Thus, the number of different genotypes (also known as effective population size) is more important for the fitness respectively the number of fertile seeds than the pure number of rosettes and flowering stems (Rossetto et al. 2004) and should be taken more into account during population monitoring. In general, the extent of clonality can only be clarified by genetic analysis because rosettes descending from generative or vegetative propagation are not distinguishable in the field. On the other hand, analysis regarding the generative fitness are time-consuming and seeds are irreversible used for e.g. germination tests. In the present study the extent of clonality correlated negatively with the filling rate of collected seeds and the total generative fitness. The two latter are positively correlated with each other in turn. Hence, the filling rate of seeds from a population of *A. montana* can be used as a fast detection method for generative fitness and at the same time for the extent of clonality. For *A. montana* the determination between filled and empty seeds is feasible in the field because filled seeds are black and hard, whereas empty seeds are bright and compressible (Kahmen und Poschlod 1998; Luijten 2001; Titze et al. 2020). Thus, it is easy to survey the filling rate in the context of monitoring activities and conservation measures. To achieve a more

objective and thus reliable classification, the seeds can also be x-rayed in the lab, which is as well a quick and gentle determination (Tausch et al. 2012). Both methods have the advantage, that the seeds are not used up, are available for further scientific or conservation purposes, and give information on the extent of clonality inside the surveyed population.

Conclusions

The study showed that larger population sizes are beneficial for the generative and genetic fitness of *A. montana*. Therefore, conservation measures should counteract the habitat loss and increase the amount and quality of nutrient-poor, soil-acidic habitats to increase the area where *A. montana* can grow. In larger populations the extent of clonality is reduced and mating between different genotypes is more likely. The proportion of filled seeds can be used as a proxy for the generative fitness and at the same time for the determination of the extant of clonality within the population.

Appendix

Table 13: Regional location, altitude above sea level and genetic diversity of 29 studied populations of *A. montana* in Baden-Wuerttemberg.

Population	Region	Altitude [m]	I	Ho	F_{IS}	C [%]
Am01	SB	1060	1.04	0.50	0.13	0.00
Am02	SB	760	1.02	0.56	-0.03	0.00
Am03	SB	970	0.89	0.54	-0.14	0.00
Am04	SB	1000	0.91	0.49	0.01	0.00
Am05	SB	1080	0.94	0.47	0.13	0.00
Am06	NB	595	0.83	0.50	-0.08	6.67
Am07	NB	690	1.01	0.52	0.07	0.00
Am08	NB	730	0.95	0.57	-0.12	0.00
Am09	NB	780	0.94	0.47	0.05	13.33
Am10	NB	753	0.98	0.51	0.03	25.00
Am11	NB	750	0.66	0.47	-0.11	71.43
Am12	NB	755	0.43	0.63	-1.00	100.00
Am13	NB	575	0.26	0.38	-1.00	100.00
Am14	NB	665	0.81	0.59	-0.28	11.11
Am15	WH	482	1.05	0.64	-0.13	0.00
Am16	WH	479	1.00	0.49	0.08	0.00
Am17	SA	669	1.07	0.65	-0.10	0.00
Am18	SA	625	0.61	0.58	-0.46	75.00
Am19	SA	666	0.98	0.53	-0.02	6.67
Am20	SA	665	1.09	0.61	-0.08	0.00
Am21	SA	840	1.08	0.56	0.06	10.00
Am22	US	672	0.55	0.34	0.03	0.00
Am23	US	698	0.82	0.48	-0.06	6.67
Am24	US	687	0.83	0.53	-0.07	0.00
Am25	US	694	0.72	0.46	-0.13	0.00
Am26	US	692	0.86	0.55	-0.12	0.00
Am27	US	702	0.94	0.56	-0.06	0.00
Am28	US	720	0.69	0.42	-0.04	0.00
Am29	US	690	0.66	0.36	0.11	0.00

SB: Southern Black Forest, NB: Northern Black Forest, WH: Waldenburg Hills, SA: Swabian Alb, US: Upper Swabia, I: Shannon-Index, Ho: oberserved heterozygosity, F_{IS}: inbreeding-index, C [%]: clonality.

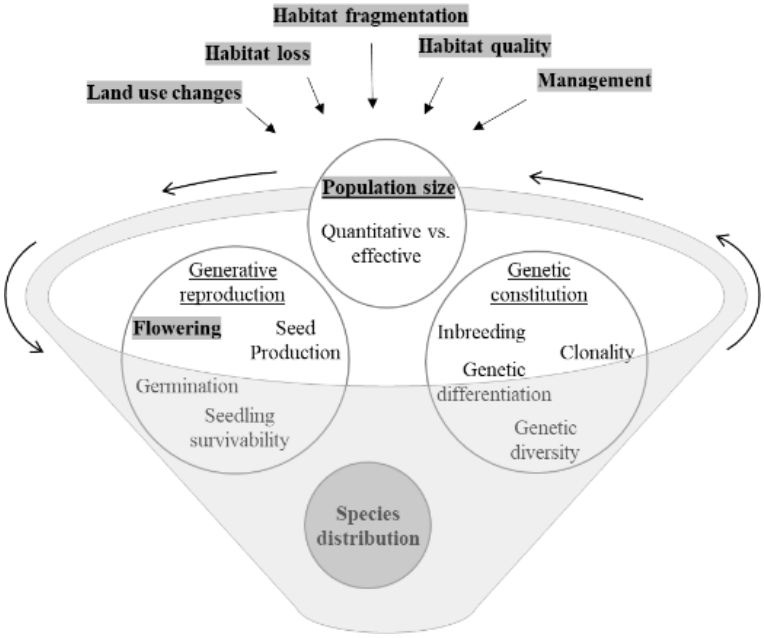
Table 14: Mean generative reproductive fitness of 23 populations of *A. montana* in five geographical regions in Baden-Wuerttemberg.

Region	Filling rate [%]	Seed mass [mg]	Seed length [mm]	Germination rate [%]	Survival rate [%]		Fitness [%]	
SB	66.8 ± 6.96	1.35 ± 0.12	6.07 ± 0.19	95.26 ± 1.03	87.64 ± 1.64	ab	55.78 ± 6.07	a
NB	32.53 ± 9.04	1.36 ± 0.15	6.02 ± 0.18	97 ± 1.34	72.28 ± 6.34	ab	14.23 ± 5.37	b
WH	76.82 ± 2.22	1.66 ± 0.16	6.79 ± 0.04	99 ± 1	92.94 ± 0.94	a	70.67 ± 2.04	a
SA	43.74 ± 13.83	1.19 ± 0.22	6.19 ± 0.28	83.2 ± 9.39	65.88 ± 6.21	b	30.1 ± 12.34	ab
US	47.16 ± 15.19	1.18 ± 0.07	5.65 ± 0.23	97 ± 1.84	83.62 ± 4.47	ab	35.64 ± 13.06	ab
p	0.254	0.444	0.119	0.165	**0.023**		**0.025**	

p: significance of one-way ANOVA, small letters indicate significant differences based on post-hoc test (Games-Howell).

Chapter 4

Habitat properties affect the occurrence and population size of the strongly endangered plant species *Arnica montana*



Abstract

Arnica montana is a strongly endangered European plant species occurring in rare nutrient-poor and soil-acidic habitats. In order to improve the conservation status of the species, efforts on the landscape, habitat and population level can be implemented but each have a different impact on the success of the species´ protection. The conducted study focused on the question whether the landscape respectively habitat properties have an influence on the occurrence and the population size of *Arnica montana*. For the investigations on the landscape level, a comparison of the historic and recent landscape around sites with actual (n=29) and extinct (n=11) *Arnica* populations was conducted in South-West Germany. The results revealed a general change in the landscape during the past 150 years and an increasing dominance of forest which led to an ongoing isolation of remaining populations. The survey of local habitat properties showed that nutrient availability increased at sites where *Arnica* got extinct or populations were small. This led to changes in the local habitat conditions like a reduced light availability at ground level due to more high and dense growing grass species. In summary, the protection of nutrient-poor habitats is essential for the protection of *A. montana* and has to be conducted by landscape and habitat protection. Therefore, the further destruction of the habitats needs to be avoided as well as the restoration and persistence of nutrient-poor conditions need to be implemented by an adapted management.

Introduction

In recent times, the loss of biodiversity is a visible pattern worldwide and takes place at the level of ecosystem, species and genetic diversity (Sala et al. 2000). It is mainly assigned to man-made changes in land-use, habitat destruction, increased nitrogen deposition and carbon dioxide concentration (Baur 2010). Central European grassland are strongly affected by these changes and are therefore today of unfavourable quality (BfN 2020). Especially nutrient-poor grasslands suffer from the increasing nutrient input (e.g. nitrogen deposition, increased fertilizer usage) and intensification of human land-use. (Matthies et al. 2004; Wörz und Thiv 2015; BfN 2016; Poschlod 2017; Stanik et al. 2018; Peppler-Lisbach et al. 2020). Originally, nutrient-poor grasslands are species-rich habitats (Löbel et al. 2006). The species occurring in these grasslands are adapted to specific habitat conditions such as vegetation gaps and high light availability (Huber et al. 2017). The human eutrophication of the grasslands especially favours especially competitive, fast and high growing grass species and the amount of aboveground biomass increases (Hautier et al. 2009). This reduces the availability of light and causes the loss of many typical species requiring light for e.g. reproduction. Decreasing species richness in formerly nutrient poor grasslands can hence be ascribed to a denser vegetation structure and increased competition with species promoted by higher nutrient availability.

Besides the decreasing quality of nutrient-poor habitats, their pure destruction is the reason for the loss of many species. Unprofitable habitats like nutrient-poor grasslands were formerly abandoned or also afforested to improve the profitability which also ends in the loss of occurring species (Buse et al. 2015; Poschlod 2017). Thus, the remaining nutrient-poor habitats of sufficient quality are fragmented in the recent landscape. This led to an isolation of the remaining populations (Jongman 2002; Fahrig 2003). By the structural fragmentation of populations, the gene flow between them is reduced which leads to increased genetic differentiation and loss of genetic diversity after a certain time. The effects of fragmentation on genetic variation differs between species and depends on the specific life history traits of the species in particular concerning e.g. pollination mechanisms, self-compatibility, dispersal ability, persistence in recent vegetation and soil seed bank (Kolb und Diekmann 2005). Within isolated populations, the loss of genetic diversity is the main driver for loss of adaptability to environmental changes and thus loss of fitness. This ends in the decrease of population size and promotes the local extinction of a species (Zieverink und Hachmöller 2003; Krauss et al. 2010). The ongoing decrease of population size with loss of genetic diversity and fitness, that is promoted by external circumstances, is named extinction vortex and is common for rare and fragmented species (Frankham et al. 2017).

In order to counteract the loss of biodiversity, recent nature conservation strategies comprise different approaches to protect species (Baur 2021). A first possibility is the pure species protection that aims to improve the constitution of species by e.g. implementing a species-specific management but is on the side very cost-intensive (Heywood und Iriondo 2003). Moreover, especially in nutrient-poor habitats slight changes in the management to protect a specific endangered species can be negative for another one. Thus, species protection always has the duty to define the subject of interest which not necessarily leads to general habitat conservation (Simberloff 1998). A second possibility is the protection of habitats and its specific properties, which is mainly implemented by defining conservation areas. This strategy focuses on the improvement of the quality of endangered, nutrient-poor habitats due to which a variety of species can be protected. As described before, general and large-scale drivers like habitat destruction or atmospheric nitrogen deposition can influence the quality of habitats negatively (Fischer und Lindenmayer 2007). Thus, as a third possibility, concepts for landscape protection are implemented in nature conservation. This approach aims to preserve whole landscapes with all varieties of landscape structures but still allows the usage of natural resources. Hence, landscape destruction is forbidden and the reduction of fertilizer usage mandatory to reduce the general eutrophication. In this way, threatened habitats and their specific species composition can be protected from extinction. This enables the protection of ecosystems, but it is rarely adaptable to a specific species.

A popular example for a European plant species that suffers from ongoing extinction is *Arnica montana*. The major threat of this plant species is the loss of appropriate habitats (Blachnik und Zehm 2017; Stanik et al. 2018; LUBW 2020a). The species needs acidic-soil and nutrient-poor grassland habitats, which are one of the most endangered habitats in Germany (BfN 2016). It is most likely, that the general eutrophication decreases the habitat quality and promotes the extinction of the species. The general increase of atmospheric nitrogen and sulfate (SOx) deposition led to an increase of nutrient availability and increase of soil acidity (Fennema 1992). Nowadays, the species is part of species protection programs and topic of several research projects (Blachnik und Saller 2015a, b; Duwe et al. 2017; Titze et al. 2020). Aims of these projects were to protect the species from further decline. But unfortunately, the loss couldn`t yet be stopped. Most studies on the species investigated the impact of the local habitat quality on population size of *A. montana* but the influence of landscape structures on the occurrence of the species have never been analysed in detail. To close this gap, this study focused on the following questions:

- **Can the actual occurrence of *A. montana* be explained by changes in the landscape during the last 150 years?**
- **What`s the impact of local habitat properties on the occurrence and the population size respectively structure of *A. montana*?**
- **How can landscape, habitat and species protection be improved to protect *A. montana* from further extinction?**

Material and methods

Study species

The herbal plant species *Arnica montana* is a rosette-forming, perennial *Asteraceae* and used as medicinal plant since long (Seemann et al. 2010; Poschlod und Heilmann 2018). The leaves are arranged opposite in the basal rosette as well as along the flowering stems (Blachnik und Zehm 2017; LUBW 2020a). The flowering period of the yellow-orange flowers lasts from May to August. The flowers are mandatory pollinated by insects and the most effective pollinators are syrphids, bees, bugs and butterflies (Luijten 2001). Furthermore, the species is self-incompatible and must be pollinated by pollen from another individual to produce fertile seeds. Directly after the pollination, seeds are built as achenes with a white pappus. Their dispersal ability is low. Wind dispersal is strongly reduced (less than one meter) because the seeds are too heavy in relation to the size of the pappus (Strykstra et al. 1998). Furthermore, the ability to attach to the fur of animals is low (Trapp et al. 2018). The seed bank is transient and the ripe seeds need to germinate directly after dispersal in late summer or autumn (Bakker et al. 1996; Kahmen und Poschlod 1998). Next to the generative reproduction, *A. montana* can also propagate vegetatively. The rhizome builds close to the soil surface horizontal offshoots from which further rosettes can grow. If full-grown rosettes are detached from the rhizome, they can take root again. *A. montana* is a typical species of open grassland habitats with a high light availability and moderate moisture and temperature requirements (Ellenberg 1996; LUBW 2020a). Furthermore, the species prefers acidic and nutrient-poor soils. It occurs on a wide ecological range of habitats concerning moisture. It can grow either on dry *Nardus* grasslands or on wet litter meadows on organic substrate.

The natural distribution area of *A. montana* is in Central Europe and ranges from Scandinavia to Spain. In all European countries an ongoing decline of populations is observed since decades (IUCN 2021). Thus, the species is protected on the European level by the appendix V of the habitat directives (Blachnik und Zehm 2017; LUBW 2020a). Due to the strong decline in Germany, the species has a Red List Status of 3, which means that it is threatened (Metzing et al. 2018) and is nowadays protected by the federal species protection regulations. Furthermore, it

became a species of federal responsibility because the centre of the European distribution area is in Germany (BfN 2021a).

Study area

For the present study, 29 sites with an actual occurrence of *A. montana* were selected across the actual distribution area of the species in South-West Germany (Figure 16, Table 20 in appendix). Further 11 sites were selected, where the species got extinct in recent times. Due to former floristic mappings, it was proven that the species earlier occurred on these eleven sites and detailed coordinates were available. Sites were not selected when obvious habitat destruction at a site (e.g. afforestation or intensive agricultural usage) was responsible for the loss of a local *Arnica* population. Such sites with unknown reasons for the loss of *A. montana* are rare. Thus, it was not possible to equate the number of study sites with and without an actual occurrence of the species. Due to conservation issues, the exact coordinates of the study sites are not given.

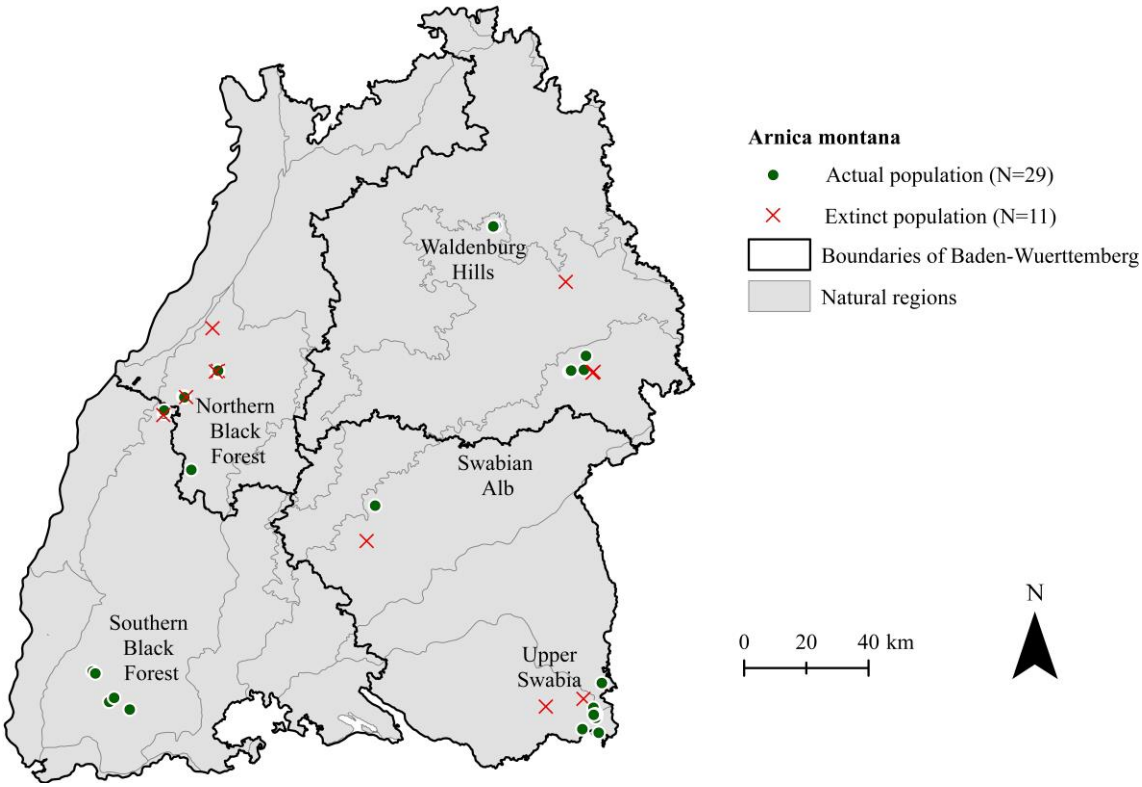


Figure 16: Geographical location of 40 studied sites with an actual (N=29) or extinct (N=11) population of *A. montana* in Baden-Wuerttemberg within different regions across the natural distribution of the species (partial overlay possible).

In Baden-Wuerttemberg, *A. montana* occurs in several geographically separated natural regions (Southern/Northern Black Forest, Waldenburg Hills, Swabian Alb, Upper Swabia). The habitat

of all sites located in the region Upper Swabia and one in Northern Black Forest (in total 11 sites) are mown litter meadows on organic substrate, whereas the habitat of the other 29 sites are dry grasslands on mineral substrate that are either mown or grazed (Table 20 in appendix). In total, 14 of the 40 study sites are in nature conservation areas and 31 are protected by the European habitat directives (FFH).

Reconstruction of historic and recent landscape structure

Data on the historical and recent landscape structure around all 40 study sites were collected by digitalising maps from mid-19th century and today. For the sites in the western part of the area, historical cadastral maps from the former territory of Baden (1857-1935, "Badische Gemarkungspläne 1:10.000", *www2.landesarchiv-bw.de/ofs21/olf/struktur.php?bestand=14217*) were used and for the other study sites cadastral maps from the former territory of Wuerttemberg (1822-1866, "Historische Landesaufnahme 1:.2.500", *www2.landesarchiv-bw.de/ofs21/olf/struktur.php?bestand=50259*). A topographical map of Baden-Wurttemberg from the year 2014 was used as source to analyse the present landscape structure.

For the landscape analysis, a circle with a radius of three kilometres was drawn around each study site. Within these circles, the total area of grassland, forest, arable field and settlements was digitalised using ArcGIS Desktop (ESRI, Version 10.4.1). Unfortunately, it was not possible to distinguish between different kinds of grassland (e.g. intensive/extensive used grassland) because the scale of the different maps varied and thus needed to be unified to grassland. Subsequently the cover of each landscape type in percent was calculated regarding the area of the circle.

Recent habitat properties

At all sites, the recent habitat properties were surveyed in implemented permanent plots (5m x 5m) in 2018. The position of the plots referred to the occurrence of *A. montana* at the sites. In case of an actual, homogeneous distribution of the species, the plot was placed in the centre of the population respectively the site. For heterogeneous populations with occasional occurrence of *A. montana*, the plot was placed decentral at the site but it was mandatory, that the species occurred in the plot. For the sites where *A. montana* got extinct, the plot was placed in the centre of the former population.

Within each plot, the vegetation was surveyed by applying the extended Braun-Blanquet scale (Reichelt und Wilmanns 1973) in June/July 2018 during the flowering period of *A. montana*. Next to the abundance of all plant species, the total coverage of grasses, herbs, litter, mosses, and bare

soil were recorded in percent. Furthermore, the mean height of grasses and herbs was calculated by measuring it five times per plot.

In September 2018, soil samples for chemical analysis were collected within each plot (VDLUFA 1997). In case of an actual occurrence of *A. montana* the samples were taken close to the rosettes but without affecting the plants. Within each plot, five separate soil samples were taken with a soil sampler (Puerckhauer, diameter 2cm) from a depth of 2-15cm samples and were mixed. Directly after the sampling, the soil was dried by 40°C and sieved (2mm) to remove organic contamination like roots and dry leaves. The total amount of carbon and nitrogen in the samples was measured by using an elemental analyser (NC 250CHN, Carlo Erba Reagents, Germany). Soil reaction was measured in a suspension of dry soil (10g) and either distilled water (25ml) to get the active soil acidity (pH (H_2O)) or 0.01M $CaCl_2$ for the potential soil acidity (pH ($CaCl_2$)) (Karlik und Kolb 2007). In both cases the pH was measured with a universal pH meter (SenTix41 electrode, WTW, Weilheim, Germany) one hour after mixing soil and solution. Additionally, the conductivity was measured in a suspension of soil and distilled water (1:5) with the LF240 electrode (WTW, Weilheim, Germany). From further 5g of dried soil, an extract with calcium acetate lactate (CAL) was done, from which the amount of phosphorous was detected photometrically (UV 1 spectrophotometer, ThermoSpectronic, USA). An atomic absorption spectrometer (SolarSpektrometer, Thermo Elemental, USA) was used to determine the amount of potassium (K), manganese (Mn), aluminium (Al), magnesium (Mg) and iron (Fe) in each extract.

The soil depth was measured five times within each plot and then the mean value per plot was calculated. Furthermore, the water holding capacity (WHC) was investigated. Thus, a soil core ($100cm^3$) was taken with a metal cylinder from each plot and watered for 24 hours by placing it on saturated filter paper. The saturated soil was weighted, subsequently dried at 100 °C and weighted again when completely dried. The difference between the two weights describes the amount of water that is available for plants and is given in percentage relation to the dry weight of the soil.

Population size and structure

At the 29 sites with an actual occurrence of *A. montana* the population size of the species within the permanent plots was reported. Therefore, the total number of rosettes (TR) and of flowering rosettes were counted. Based on that, the percentage of flowering rosettes (PFR) could be calculated. Furthermore, the plots were divided into 625 sub-squares with an equal grid size of 20cm x 20cm. For each sub-square the absence/presence of *A. montana* was mentioned. Based on

the percentage amount of sub-squares with *A. montana* the distribution of the species within the plots was calculated as following: DIST = $^{\text{Number of plots with } A.\ montana}/_{625}$ x 100%. High values for distribution mean a more homogenous occurrence of the species, whereas small values are prominent for more clustered populations.

Statistical analysis

All data concerning the landscape structure were analysed in order to compare the sites with and without an actual population of *A. montana* concerning their historical and recent landscape composition. The data within the groups were not normally distributed and thus non-parametric tests (Wilcoxon text, Mann-Whitney U-test) were conducted.

In a second analysis the historical and recent landscape of all 40 sites were compared regardless of the actual occurrence of *A. montana* to see general changes in the landscape structure over time. The historic and recent landscape structure was compared within the different distribution regions in Baden-Wuerttemberg with non-parametric Wilcoxon rang tests.

For the analysis of the vegetation surveys, the Braun-Blanquet values were transformed back to mean cover values. Based on that, the weighted Ellenberg indicator values (EIV) concerning light, temperature, continentality, moisture, reaction and nutrient for each plot were calculated (Ellenberg 1996) as well as the mean forage value, mowing, grazing and trampling tolerance based on species-specific data from the BIOFLOR database (Briemle et al. 2002; Klotz et al. 2002). For the subsequent comparison of the sites with an actual and an extinct population of *A. montana*, the non-parametric Mann-Whitney U-test was applied. Multivariate analysis methods were conducted to visualize the plant species composition at the sites. In case of a non-linearity along the first aces a detrended correspondence analysis (DCA) was applied. If the length of the gradient of the first aces was smaller than 2.5 the method of principal component analysis (PCA) was used. For the 29 sites with an actual occurrence of *A. montana* the influence of the habitat quality on the population size was tested by non-parametric Spearman-Rho correlation tests. All statistical analysis were run with R (Version 4.0.1).

Results

Landscape analysis

The landscape analysis revealed that the structures around the study sides changed regardless of the present occurrence of *A. montana.* Around both types of sites, the amount of forest increased (Table 15). Forest was the dominant landscape structure in the mid-19th century (53.67%) and is still in present times (65.47%). The cover of human settlements changed also over time. In former times, settlements covered on average 0.48% of the mapped area and it increased to 3.50%. The growth of settlements over time was furthermore significant if only sites with respectively without an actual population of *A. montana* were considered in the analysis. The cover of arable fields significantly decreased the most over time compared to other landscape structures on sites with as well as without *A. montana*. In contrast to all that, the average cover of grassland did not significantly differ over time (historic: 26.66%, recent: 24.93%). This was detected regardless whether *A. montana* actually occurs at a site or not.

Table 15: Historic and recent landscape structure around sites with actual (N=29) and extinct (N=11) occurrence of *A. montana* (p: significance of paired Wilcoxon rang test).

	Actual (N = 29)					Extinct (N = 11)				
	Historic		Recent			Historic		Recent		
	Mean	±SE	Mean	±SE	p	Mean	±SE	Mean	±SE	p
Grassland	25.67	2.615	25.58	4.360	0.75	18.35	4.233	23.22	7.040	0.48
Forest	52.28	4.832	64.51	4.631	**0.00**	57.32	8.062	68.00	7.604	**0.01**
Arable field	18.43	3.354	6.08	2.356	**0.00**	18.85	4.989	4.94	2.106	**0.00**
Settlement	0.46	0.063	3.44	0.612	**0.00**	0.52	0.116	3.65	0.790	**0.00**
Rest	1.32	0.203	0.35	0.103	**0.00**	0.74	0.171	0.17	0.078	**0.01**

The landscape analysis within the different natural regions showed that the changes in the landscape over time happened within all regions (Table 16). But the proportion of the cover of different landscape structures showed regional differences. Apart from the region Waldenburg Hills, the percentage cover of forest respectively settlement increased whereas the cover of arable fields decreased.

Table 16: Historic and recent landscape structures (grassland, forest, arable fields, settlement) around 40 sites in Baden-Wuerttemberg and within distinct regions (SB: Southern Black Forest, NB: Northern Black Forest, WH: Waldenburg Hills, SA: Swabian Alb, US: Upper Swabia; p: significance of paired Wilcoxon rang test between historic and recent landscape).

			Historic		**Recent**				
		N	Mean	±SE	Mean	±SE	p		
Grassland	Total	40	23.66	2.257	24.93	3.663	0.90		
	SB	5	40.46	3.916	28.64	3.692	**0.04**		↘
	NB	15	11.41	2.541	8.91	1.477	0.46		
	WH	2	20.46	0.340	10.11	0.225	0.18		
	SA	8	22.36	3.811	11.08	4.028	**0.04**		↘
	US	10	35.31	2.154	61.16	1.735	**0.01**	↗	
Forest	Total	40	53.67	4.107	65.47	3.912	**0.00**	↗	
	SB	5	55.07	3.579	69.48	3.982	**0.04**	↗	
	NB	15	75.35	4.226	87.12	2.987	**0.01**	↗	
	WH	2	67.04	0.205	76.58	0.250	0.18		
	SA	8	51.56	7.201	63.38	5.571	**0.01**	↗	
	US	10	19.44	1.607	30.43	1.731	**0.01**	↗	
Arable Fields	Total	40	18.54	2.759	5.77	1.791	**0.00**		↘
	SB	5	3.52	0.475	0.11	0.069	**0.04**		↘
	NB	15	5.56	1.823	1.85	1.507	**0.00**		↘
	WH	2	10.42	0.115	10.38	0.115	0.16		
	SA	8	25.03	5.110	21.92	5.574	1.00		
	US	10	41.97	1.008	0.61	0.137	**0.01**		↘
Settlement	Total	40	0.48	0.055	3.50	0.489	**0.00**	↗	
	SB	5	0.38	0.061	1.70	0.335	**0.04**	↗	
	NB	15	0.39	0.080	2.02	0.277	**0.00**	↗	
	WH	2	0.32	0.005	2.23	0.285	0.18		
	SA	8	0.23	0.057	3.58	1.037	**0.01**	↗	
	US	10	0.89	0.085	6.80	1.216	**0.01**	↗	
Rest	Total	40	1.16	0.159	0.30	0.078	**0.00**		↘
	SB	5	0.55	0.172	0.01	0.005	**0.04**		↘
	NB	15	0.59	0.138	0.08	0.029	**0.00**		↘
	WH	2	1.77	0.020	0.72	0.085	0.18		
	SA	8	0.97	0.215	0.03	0.010	**0.02**		↘
	US	10	2.34	0.342	0.90	0.202	**0.01**		↘

In the region Upper Swabia (US), forest had the lowest percentage cover in the historical (19.44%) and recent (30.43 %) landscape. In mid-19th century, arable fields (41.97%) and grassland (35.31%) characterized this region. Over time the landscape became more grassland dominated (61.16%) and arable field disappeared (0.61%).

In contrast to that, the study sites in the Northern Black Forest (NB) were always surrounded by the highest amounts of forest (historical: 75.35%, recent: 87.12%). The usage as grassland and arable fields in this area was hardly represented. In former and present times, the landscape within the regions Southern Black Forest (SB) and Swabian Alb (SA) were a mixture of forest and grasslands. On the Swabian Alb, arable fields were and still is a well-represented form of land

use. However, the percentage cover of grassland decreased significantly over time. The lost grassland area was meanwhile mainly replaced by forest and human settlements.

Habitat properties

At the 40 study sites, a total of 206 different plant species were detected. The maximum number of species per plot was 35. The average number of species didn`t differ between sites with (N=23.1) and without (N=24.0) *A. montana*. At all sites grasses and herbs dominated the vegetation and only few tree and shrub species were recorded. The multivariate analysis revealed large differences in the species composition between sites on organic and mineral substrate (Figure 19 in appendix). Thus, the sites on the different substrates were analysed separately.

Sites on mineral substrate

The cover of the recorded vegetation layers didn`t significantly differ between sites with and without *A. montana* on mineral substrate (Table 17, full list in appendix in Table 23). However, the cover of mosses and grasses tended to be higher at sites without *A. montana*, whereas the cover of herbs was lower. Furthermore, the cover of litter (23.78%) was nearly twice as high as at sites with an actual population of *A. montana* (12.55%). In general, the sites with *A. montana* showed a lower vegetation height.

Table 17: Significant differences between sites with an actual (N=20) and an extinct (N=9) population of *A montana* on mineral substrate concerning vegetation structure, weighted Ellenberg indicator values and soil chemistry (p: significance of Mann Whitney-U test; complete list in appendix).

		actual	extinct	p
Number of sites		20	9	-
Altitude	[m]	749.70 ± 38	608.67 ± 50.47	**0.03**
Litter Cover	[%]	12.55 ± 4.51	23.78 ± 6.28	**0.04**
Height of Grasses	[cm]	65.51 ± 2.8	84.00 ± 4.56	**0.00**
EIV Light		7.38 ± 0.08	6.65 ± 0.15	**0.00**
EIV Reaction		3.47 ± 0.24	4.53 ± 0.41	**0.02**
EIV Nutrient		2.46 ± 0.13	3.68 ± 0.29	**0.00**
Mowing tolerance		4.53 ± 0.21	5.57 ± 0.38	**0.02**
Fe	[mg/kg]	13.72 ± 1.46	8.39 ± 1.47	**0.02**
pH ($CaCl_2$)		3.87 ± 0.06	4.20 ± 0.15	**0.03**

The lightness for herbs and grasses was higher at sites with an actual population of *A. montana*. Furthermore, the weighted Ellenberg indicator values for soil reaction and nutrient were in comparison to sites without *A. montana* lower. In addition, the mowing tolerance of the vegetation of sites with an extinct population was higher (5.57) than at sites with an actual occurrence (4.53).

The soil chemical and physical analysis didn`t show high differences between the two groups. Only the iron content and the potential soil acidity differed significantly. Furthermore, the soil depth tended to be deeper on sites without *A. montana*.

The multivariate analysis of sites on mineral substrate revealed a clear differentiation in the plant species composition between sites with and without *A. montana* along the first axis (Figure 17). Higher cover of the grasses *Alopecurus pratensis*, *Dactylis glomerata* and *Holcus lanatus* characterized the sites with an extinct population. Furthermore, *Rancunculus repens* was more presented on these sites. In contrast, *Molinia caerulea* was more typical for sites with *A. montana*. The vegetation of the sites with a former population was dominated by species with a higher mowing tolerance, higher forage value and higher Ellenberg indicator value for nutrients. In addition, the concentration of manganese and magnesium was larger on these sites.

In the ordination graph, the study sites were separated along the first axis according to the local occurrence of *A. montana*. Sites with larger *Arnica* populations were located in the centre of the first axis. Thus, the ecological gradient along the first axis respectively the species composition at the sites couldn`t explain the size of current *Arnica* populations.

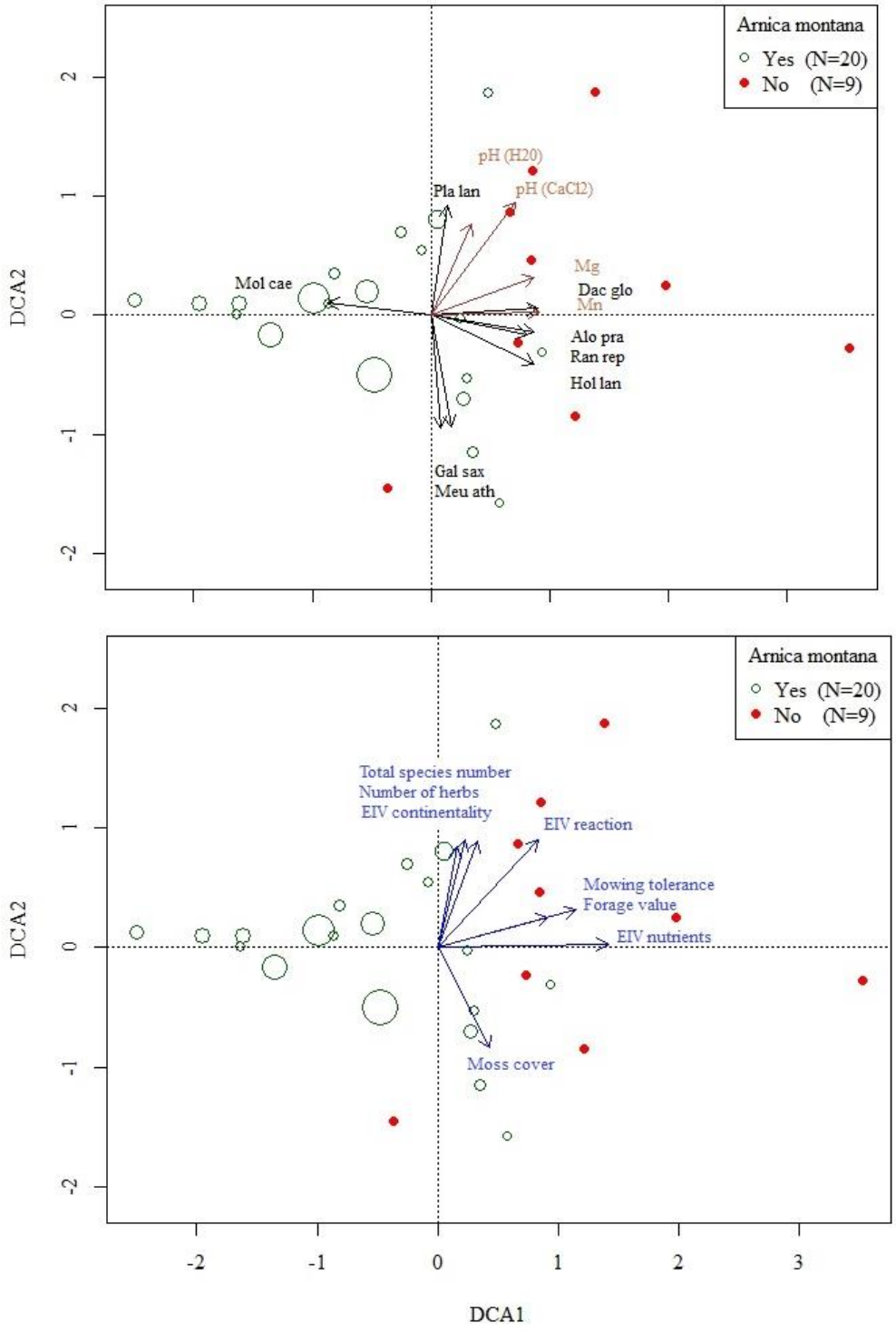


Figure 17: DCA of plant species composition of 29 sites on mineral substrate with significant correlations ($r^2 > 0.3$) with species occurrence and soil chemical parameters (above) and vegetation structure parameters (below). Size of points represent number of vegetative population size of *A. montana* (length of gradient = 6.0258, Mol cae = *Molinia caerulea*, Pla lan = *Plantago lanceolata*, Dac glo = *Dactylis glomerata*, Alo pra = *Alopecurus pratensis*, Ran rep = *Ranunculus repens*, Hol lan = *Holcus lanatus*, Gal sax = *Galium saxatile*, Meu ath = *Meum athamanticum*).

Sites on organic substrate

The comparison of sites with and without an actual population on organic substrate revealed three significant differences between the two groups (Table 18, full list in appendix in Table 23). The mean weighted Ellenberg indicator value concerning temperature and the mean conductivity of the organic soil was higher at sites with an extinct population. Furthermore, the grazing tolerance of the species growing at sites with *A. montana* was higher. The vegetation structure at the two sites with extincted *Arnica* populations tended to have more tree and shrub species and the cover of herbs was lower.

Table 18: Significant differences between sites with an actual (N=9) and an extinct (N=2) population of *A montana* on organic substrate concerning vegetation structure, weighted Ellenberg indicator values and soil chemistry (p: significance of Mann Whitney-U test; complete list in appendix).

	actual		extinct		p
Number of sites	9		2		-
EIV Temperature	4.50	± 0.14	5.57	± 0.24	**0.03**
Grazing tolerance	3.84	± 0.12	3.14	± 0.2	**0.03**
Conductivity [µS/cm]	138.67	± 21.13	300.50	± 50.5	**0.03**

The ordination graph of the mutlivariate analysis of the plant species composition of all eleven sites on organic substrate revealed large differences in species composition on the sites Am06 and Am23. Thus, these two sites were left out for the further multivariate analysis. The remaining seven sites with an actual population of *A. montana* were located close to each other in the ordination diagramm (Figure 18). The two sites without *A. montana* were placed far from them and thus their plant species compoisition differed a lot. Am39 was for example characterized by signifant higher abundance of *Carex nigra* and Am40 by the signficant lack of *Vaccinium oxycoccus*. Furthermore, the two sites were characterized by a lower number and cover of grass species and a lower soil acidity.

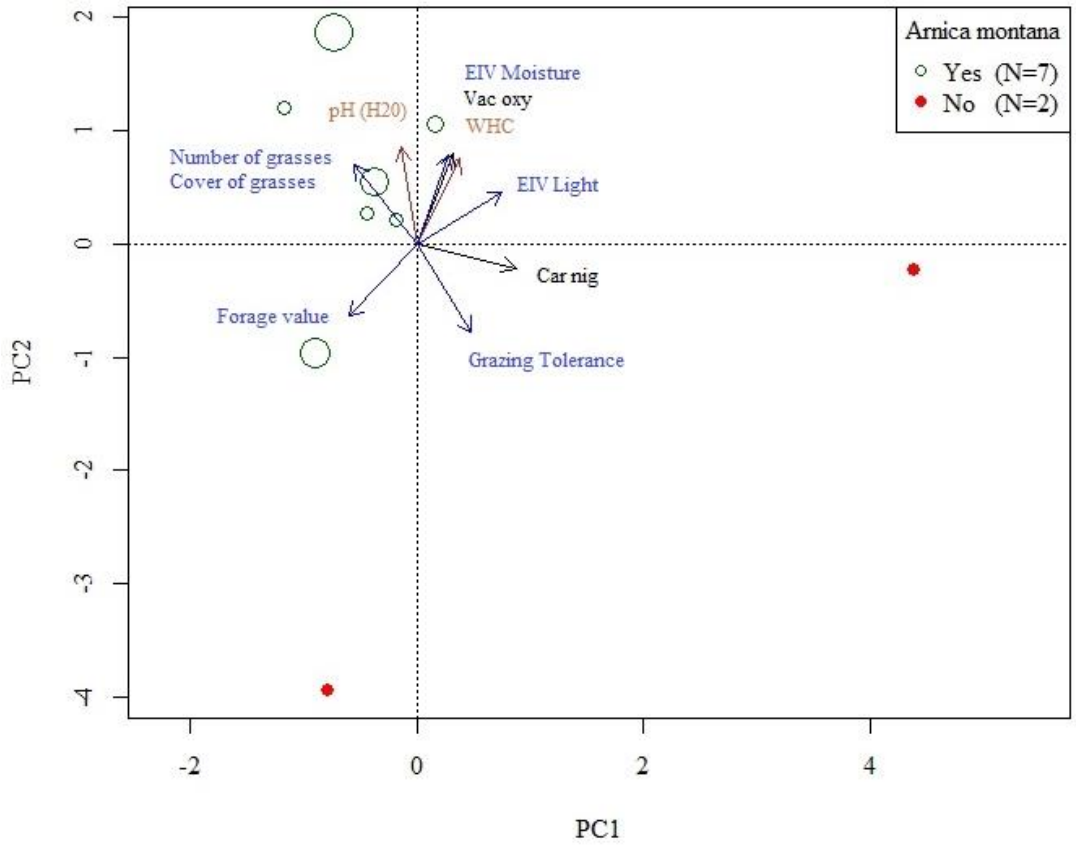


Figure 18: PCA of plant species composition of 9 sites on organic substrate with significant correlations ($r^2 > 0.3$) with species occurrence (black) soil chemical parameters (brown) and vegetation structure parameters (blue). Size of points represent number of vegetative population size of *A. montana* (PC1: 23.16%, PC2: 17.49%, Vac oxy = Vaccinium oxycoccus, Car nig = Carex nigra).

Influence of habitat property on population size and structure

The number of arnica rosettes within the 29 permanent plots ranged from 2 to 1105. The distribution of the rosettes within the plot were either occasionally or homogenous if *A. montana* was detected within more than 40% of the grid cells. The calculated values for the percentage of flowering rosettes were between 0.0% and 66.7% and was significantly correlated with the altitude of the study sites (Table 19).

Table 19: Pairwise correlations coefficients between total number of rosettes (TR), percentage of flowering rosettes (PFR) and distribution (DIST) of *Arnica montana* within a 25m²-square and local habitat property (vegetation cover, species number, weighted Ellenberg indicator value, soil chemistry/physic) (significant levels: ***: p<0.001, **: p<0.005, *: p<0.05).

		Total (N=29)		
		TR	PFR	DIST
Altitude	[m]	-0.10	**0.38** **	-0.04
Vegetation Cover				
Gras	[%]	0.14	-0.37	0.16
Herb	[%]	0.01	0.23	0.02
Litter	[%]	-0.19	**-0.64** ***	-0.18
Moss	[%]	**-0.39** *	-0.24	-0.35
Bare soil	[%]	0.09	**0.38** *	0.03
Number of species				
Grases		0.26	-0.03	**0.39** *
Herbs		-0.05	**0.64** ***	0.00
Weighted Ellenberg Indicator Value				
Light		0.19	0.10	0.21
Temperature		-0.28	-0.14	-0.25
Continentality		0,07	-0.11	0.15
Moisture		-0.09	**-0.60** ***	0.00
Reaction		-0.24	0.33	-0.18
Nutrient		-0.15	**0.64** ***	-0.16
Mowing tolerance		-0.25	**0.60** ***	-0.26
Grazing tolerance		-0.11	**0.52** **	-0.10
Trampling tolerance		-0.11	**0.45** *	-0.09
Forage value		-0.31	**0.60** ***	-0.36
Soil chemistry/physic				
Soil depth	[cm]	-0.23	**-0.54** **	-0.12
WHC	[Gew.-%]	0.03	**-0.43** *	0.11
C	[%]	0.08	**-0.46** *	0.09
N	[%]	0.15	-0.27	0.18
C/N		-0.13	**-0.62** ***	-0.17
P	[mg/kg]	0.25	-0.34	0.23
K	[mg/kg]	0.09	-0.36	0.22
Mg	[mg/kg]	0.03	0.09	0.05
Mn	[mg/kg]	-0.31	**0.51** **	**-0.38** *
Fe	[mg/kg]	-0.09	-0.16	0.01
Al	[mg/kg]	**0.44** *	0.15	0.37
pH (H_2O)		-0.06	0.10	0.11
pH ($CaCl_2$)		-0.31	0.04	-0.12
Conductivity	[µS/cm]	0.11	-0.34	0.15

At study sites on mineral substrate, the correlation analysis revealed that the number of rosettes and the distribution within the plot decreased with higher moss cover (Table 22 in appendix). In addition, the mowing tolerance and the forage value was lower at sites with large and homogenous populations. Furthermore, the content of aluminium and manganese in the soil was significantly higher respectively lower for these sites. Litter influenced the flowering ability of the rosettes significantly negative, whereas at sites with an herb-rich plant composition the percentage of flowering rosettes was higher. Besides this, the carbon to nitrogen ratio had a negative impact on the building of flowering stems whereas magnesium promoted it.

At sites on organic substrate, the number of arnica rosettes decreased with higher soil moisture (Table 22 in appendix). Furthermore, the percentage of flowering rosettes was higher on sites with higher weighted forage values and lower content of potassium in the soil.

Discussion

Impact of landscape history

Regardless of the present occurrence of the study species *A. montana*, changes in the landscape structure in South-West Germany during the last 150 years were clearly recognizable. Changes in the landscape appeared naturally since the development of the Central Europe landscape after the last glacial period and are connected to the development of species richness (Divíšek et al. 2020). Several studies revealed that present species and genetic diversity is often linked to the historical landscape structure instead of the current landscape and thus visualize pattern of extinction debt (e.g. Cousins und Eriksson 2002; Lindborg und Eriksson 2004; Reisch et al. 2017). This led to the conclusion, that knowledge on landscape history should be taken more into account in nature conservation to protect habitats and species (Kuussaari et al. 2009).

The landscape analysis conducted in this study, illustrated the general changes of the landscape within the last 150 years but no specific impact of these changes on the present occurrence of *A. montana*. The increase of forest and human settlement respectively the decrease of arable fields since the mid-19th century took place around all sites regardless of the present occurrence of the species. The lack of influence of landscape changes during the last 150 years on the local extinction of a species seems to be typical for species growing within threatened habitats (Rovzar et al. 2016). On the one hand, these species-specific habitats (e.g. soil-acidic, nutrient-poor grasslands) naturally occurred in low amount and are detected hardly on either historical or actual cadastral maps. Hence, it is difficult to see the real decrease of these habitats by large-scale landscape analysis (Löbel et al. 2006; Pacha und Petit 2008; Wesche et al. 2012). Furthermore,

these habitats are naturally rare and the loss of a specific population or site may not be obvious for long times. This phenomenon is known as extinction debt and a common problem for biodiversity conservation because ongoing recent changes in the landscape are not directly connectable to the loss of a population (Kuussaari et al. 2009). The time needed until the delayed effect of habitat loss or reduced connectivity is recognizable is even higher for perennial, clonal growing species like *A. montana* (Helm et al. 2006). These species can persist vegetatively for years without the need of generative reproduction if the habitat is not seriously destroyed by e.g., afforestation. The recent landscape changes rather promote the fragmentation of the actual populations which then later on results in an ongoing loss of species (Krauss et al. 2010; Scherreiks et al. 2022). Hence, a landscape analysis can reveal general changes in the landscape structure but further analysis on local habitat properties are necessary to apply the findings for the protection of specific species (Löbel et al. 2006; Pacha und Petit 2008; Wesche et al. 2012).

According to the results, the changes in landscape structure were different between geographical regions where *A. montana* occurs in South-West Germany. The historic landscape in the Southern Black Forest was a mosaic of forest and grassland. The topography of the region favoured forestry and grazing of grasslands since centuries (Henkner et al. 2018). Thus, since long time nutrient-poor grassland habitats (e.g. suitable for *A. montana*) are widely spread in this region and large populations could establish there. In particular, because of unprofitability, the former grazing of the pastures became rarer and the grasslands were recently either afforested or the usage intensified. Within the region Northern Black Forest, grassland was in contrast always a rare habitat type and became even less common due to recent afforestation. The region was historically a forest-dominated region with long history of forestry (Rösch 2009) and grasslands have always been more isolated. The landscape on the Swabian Alb changed from a mosaic of grasslands, arable field and forest to a forest-dominated region with fragmented grasslands. In former times, extensively used and grazed grasslands in this area were connected naturally and by grazing livestock (Mattern et al., 1992; Steffan-Dewenter & Tscharntke, 2002). Due to the intensification of agricultural land-use, the amount of extensively used grasslands however decreased. Besides this, the major bedrock in this area is Jurassic underground and karst with basic soil reaction (Gwinner 1976). Thus, the soil-acidic grasslands are naturally very rare on the Swabian Alb. The study sites in the region Upper Swabia were less affected by afforestation and the region is still grassland-dominated. This seems in the first view positive, because more possible area for grassland species is available. But the landscape analysis do not show that since decades the land use in the area was intensified and nutrient-poor habitats like litter-meadows became rare in this region (Poschlod 2017).

Thus, within all regions the historic development of landscape structures was connected to natural differences in topography and climatic conditions which resulted in a different human usage of the landscape since centuries (LUBW 2019). To improve the protection of the different landscapes with their variety of habitats and species, these natural differences in landscape history on a regional scale should be considered.

Influence of habitat properties

The survey of the habitat properties revealed that the nutrient content in the soil was higher at study sites where *A. montana* got extinct than on sites with an actual population. Furthermore, the nutrient content was higher at sites with small number of rosettes and a heterogeneous distribution. At sites on mineral substrate, the increased nutrient content was indicated by a higher Ellenberg indicator value for nutrient, forage value, vegetation and in contrast a lower carbon to nitrogen ratio. At sites on organic substrate, the supply of exchangeable chemical elements and thus nutrients was higher indicated by a higher total conductivity. Higher nutrient content leads to an increase of competitive and fast growing grass species with an negative effect on *A. montana* (Pegtel 1994; Jurkiewicz et al. 2010). In the present study, those species were e.g. *Dactylis glomerata, Deschampsia flexuosa, Alopecurus pratensis* or *Holcus lanatus*. These were more abundant at sites where *A. montana* disappeared. The increasing biomass of especially grasses reduces the light availability in the understory of grassland communities (Hautier et al. 2009). Especially for light-preferring species with a leaf-rosette close to the soil-surface like *A. montana* these changes are negative because the rate of photosynthesis is reduced due to the reduced light availability. Hence, the results of the study underlines that a major threat of (endangered) plant species is the eutrophication of ecosystems and the resulting changes in the vegetation structure. This is even worse in case of nutrient-poor habitats (Marrs 1993; Wesche et al. 2012; Wörz und Thiv 2015; Peppler-Lisbach et al. 2020). Furthermore, the results of the present study underline the findings of previous studies concerning the threat of *A. montana* (Maurice et al. 2012; Blachnik und Saller 2015a; Stanik et al. 2018; Sugier et al. 2019; Hollmann et al. 2020; LUBW 2020a).

In contrast to the negative impact of nutrients on the occurrence and population size of *A. montana*, a positive effect of higher nutrient content on the flowering ability was detected. Especially magnesium content increased the percentage of flowering rosettes at the study sites and promotes the findings of Meyer-Berge (1991) and Sugier et al. (2019) who showed a positive effect of nutrient content (e.g. phosphorus) on the flowering ability of *A. montana*. The building of flowers and seeds for generative reproduction are in general cost-intensive for plants and thus

normally reduced in nutrient-poor habitats (Johnson et al. 2017). Plant species are adapted to nutrient-poor conditions and reproduce therefore often clonally and generative reproduction is not mandatory every year. Due to the eutrophication of nutrient-poor habitats and the increased availability of certain elements, the building of flowers is more often enabled and further the building of seeds. But in case of *A. montana* this is unfortunately just a short-term positive effect. Hollmann et al. (2020) showed in a greenhouse experiment that the seedling survivability of *A. montana* is reduced by higher nutrient availability, because it leads to a higher and denser vegetation structure and ends in unsuitable conditions for the seedlings. Furthermore, the root and shoot growth of *A. montana* is reduced when the content of several elements at the same time increases (Kroeze et al. 1989). This means that populations with a high proportion of flowering stems don`t automatically have a higher and successful generative reproduction.

At sites on organic substrate, the number of rosettes decreased with higher soil moisture. Higher soil moisture leads to anaerobe soil conditions, which can be negative for rhizomes of plant species that are not adapted to this conditions (Stanik et al. 2021). Litter meadows are naturally wet habitats. But the moisture can differ within sites and on drier parts *Nardus* grasslands established. This was further promoted humans by drainage (Vermeer und Berendse 1983; Blachnik und Zehm 2017; LUBW 2020a). In general, present nature conservation activities counteract these unnatural drainages and rise the water level e.g. to reduce the carbon dioxide (Leifeld et al. 2011). Following, the moisture rises within the sites and the microhabitats for *A. montana* disappear. However, it should be considered, that drier areas within the litter meadow should still remain to enable the occurrence of *A. montana*.

The vegetation composition at the study sites included mowing tolerant species, which fits to the circumstance that most study sites are managed by mowing nowadays and species are adapted to this management (Gaujour et al. 2012). The abundance of mowing tolerant species was higher at sites with small *Arnica* population or even without an actual occurrence. Since long, *A. montana* is known as weed on grazed pastures and it is avoided by cattle (Stebler und Schröter 1889). Hence, the species and its life cycle are adapted to grazing. This led to the conclusion that mowing is not the best type of management for sites with *Arnica* populations and cattle should rather graze them.

Conclusions with respect to nature conservation

The results of the study presented here show that the protection of endangered plant species like *A. montana* could be improved when knowledge on landscape history, habitat properties and

demands of specific species is combined. The development of recent landscape structures needs to be viewed with local topographical respectively climatic conditions and thus with the regional landscape history. Further afforestation, abandonment and eutrophication of unprofitable, nutrient-poor grassland habitats should be avoided to stop further habitat loss respectively fragmentation of the remaining nutrient-poor habitats. An adapted management (e.g. combined mowing and grazing suggested by Stanik et al. (2018)) can counteract the nutrient input to nutrient-poor, soil-acidic habitats. Cattle formerly grazed the habitats where *A. montana* naturally occurs and the life history traits of *A. montana* are adapted to this. Thus, this type of grassland usage should be applied more to manage such habitats. The local vegetation structure must meet the requirements of *A. montana* as uncompetitive, light loving species and seeds need open soil for germination and establishment. Thus, the abundance of dominant grass species need to be reduced by e.g. scarifying the grasslands in early spring or late autumn respectively not during the vegetation period of *A. montana* (Kahmen und Poschlod 1998; Stanik et al. 2018). A positive side effect would be the simultaneous reduction of mosses and litter. Both effects the generative reproduction of *Arnica* populations negatively because seeds can`t germinate respectively seedlings can`t establish on dense litter or moss cover (Ruprecht et al. 2010; Ruprecht und Szabó 2012). Furthermore, the flowering ability of populations shouldn`t be used to quantify the quality of populations, because it alone does not enable successful generative reproduction.

Appendix

Table 20: Selected study sites with an actual (N=29) and extinct (N=11) population of *A. montana* with regional affiliation, substrate type, status of habitat protection concerning European habitat directives (FFH) and nature conservation area (NSG).

Study site	Natural region	Substrate	FFH	NSG	A. montana	Altitude (m a.s.l.)
Am01	SB	mineral	yes	yes	actual	1060
Am02	SB	mineral	yes	yes	actual	760
Am03	SB	mineral	yes	yes	actual	970
Am04	SB	mineral	yes	yes	actual	1000
Am05	SB	mineral	yes	yes	actual	1080
Am06	NB	organic	yes	no	actual	595
Am07	NB	mineral	yes	no	actual	690
Am08	NB	mineral	yes	no	actual	730
Am09	NB	mineral	yes	no	actual	780
Am10	NB	mineral	yes	no	actual	753
Am11	NB	mineral	yes	no	actual	750
Am12	NB	mineral	yes	no	actual	755
Am13	NB	mineral	yes	no	actual	575
Am14	NB	mineral	yes	no	actual	665
Am15	WH	mineral	yes	yes	actual	482
Am16	WH	mineral	yes	yes	actual	479
Am17	SA	mineral	yes	yes	actual	669
Am18	SA	mineral	yes	yes	actual	625
Am19	SA	mineral	yes	yes	actual	666
Am20	SA	mineral	yes	yes	actual	665
Am21	SA	mineral	yes	no	actual	840
Am22	US	organic	no	no	actual	672
Am23	US	organic	yes	yes	actual	698
Am24	US	organic	yes	no	actual	687
Am25	US	organic	yes	no	actual	694
Am26	US	organic	yes	no	actual	692
Am27	US	organic	no	no	actual	702
Am28	US	organic	no	no	actual	720
Am29	US	organic	yes	yes	actual	690
Am30	NB	mineral	yes	no	extinct	608
Am31	NB	mineral	yes	no	extinct	735
Am32	NB	mineral	yes	no	extinct	505
Am33	NB	mineral	yes	no	extinct	650
Am34	NB	mineral	yes	yes	extinct	392
Am35	NB	mineral	no	no	extinct	423
Am36	SA	mineral	no	no	extinct	645
Am37	SA	mineral	no	no	extinct	645
Am38	SA	mineral	no	no	extinct	875
Am39	US	organic	no	no	extinct	652
Am40	US	organic	no	no	extinct	713

Table 21: Historic and recent landscape structures around sites with actual (N=29) and extinct (N=11) occurrence of *A. montana* (p: significance of pairwise comparison by Mann-Whitney U-test).

		Actual (N = 29)		Extinct (N = 11)		
		Mean	±SE	Mean	±SE	p
Grassland	Historic	25.67	2.615	18.35	4.233	0.23
	Recent	25.58	4.360	23.22	7.040	0.94
Forest	Historic	52.28	4.832	57.32	8.062	0.46
	Recent	64.51	4.631	68.00	7.604	0.56
Arable field	Historic	18.43	3.354	18.85	4.989	0.64
	Recent	6.08	2.356	4.94	2.106	0.46
Settlement	Historic	0.46	0.063	0.52	0.116	0.66
	Recent	3.44	0.612	3.65	0.790	0.58
Rest	Historic	1.32	0.203	0.74	0.171	0.08
	Recent	0.35	0.103	0.17	0.078	0.34

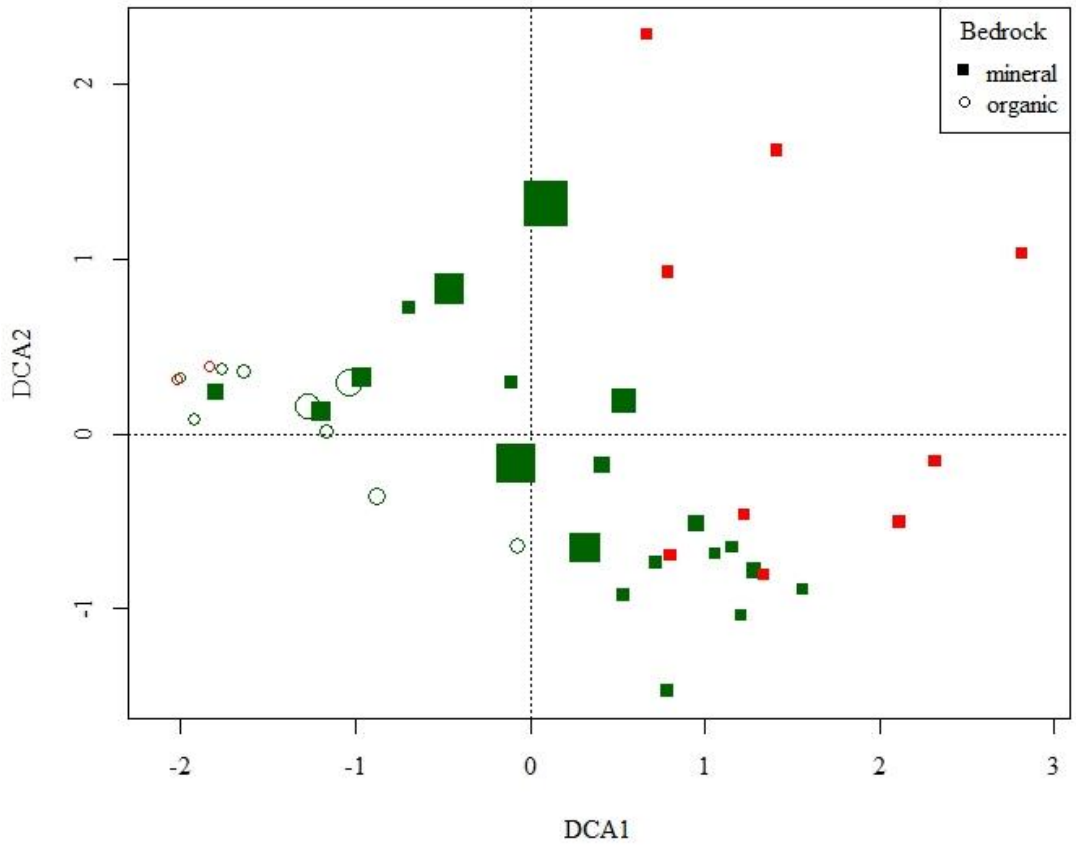


Figure 19: DCA of plant species composition of 40 sites on mineral (filled square) and organic (open circle) substrate with an actual (green) and extinct (red) population of *Arnica montana* in South-West Germany (length of gradient: 4.8361).

Table 22: Pairwise correlations coefficients between total number of rosettes (TR), percentage of flowering rosettes (PFR) and distribution (DIST.) of *Arnica montana* within a 25m²-square and local habitat property (vegetation cover, species number, weighted Ellenberg indicator value, soil chemistry/physic) on sites on mineral and organic substrate (significant levels: ***: p<0.001, **: p<0.005, *: p<0.05).

		Mineral Substrate (N=20)			Organic Substrate (N=9)		
		TR	PFR	DIST	TR	PFR	DIST
Altitude	[m]	0.01	**0.47** **	0.08	-0.13	**-0.68** **	-0.050
Vegetation Cover							
Gras	[%]	0.16	-0.12	0.10	-0.10	-0.25	-0.10
Herb	[%]	-0.13	0.10	-0.03	0.40	-0.39	0.38
Litter	[%]	-0.40	**-0.51** *	-0.44	-0.23	-0.10	-0.27
Moss	[%]	**-0.46** *	-0.06	**-0.49** *	-0.54	-0.06	-0.51
Bare soil	[%]	0.22	0.35	0.19	-0.09	-0.05	-0.09
Number of species							
Grases		0.34	-0.08	0.43	0.11	0.65	0.16
Herbs		-0.14	**0.60** **	-0.03	0.51	0.60	0.54
Weighted Ellenberg Indicator Value							
Light		0.14	0.24	0.18	0.32	-0.15	0.33
Temperature		-0.11	-0.23	-0.09	-0.67	-0.12	-0.63
Continentality		-0,04	-0.04	0.06	0.42	-0.24	0.40
Moisture		-0.07	-0.39	-0.09	**-0.72** *	-0.53	**-0.70** *
Reaction		-0.38	0.37	-0.32	0.25	0.43	0.32
Nutrient		-0.32	**0.58** **	-0.26	0.65	0.38	0.67
Mowing tolerance		**-0.55** *	**0.49** *	**-0.47** *	0.67	0.60	0.65
Grazing tolerance		-0.28	0.33	-0.14	0.43	0.37	0.37
Trampling tolerance		-0.20	0.15	-0.07	0.20	0.41	0.18
Forage value		**-0.56** **	0.36	**-0.51** *	0.33	**0.83** **	0.35
Soil chemistry/physic							
Soil depth	[cm]	-0.38	-0.25	-0.34	-0.14	-0.59	-0.14
WHC	[Gew.-%]	0.11	-0.13	0.12	-0.33	-0.18	-0.42
C	[%]	0.31	-0.30	0.23	-0.38	-0.46	-0.52
N	[%]	0.43	-0.08	0.42	-0.23	-0.39	-0.33
C/N		-0.05	**-0.50** *	-0.17	-0.22	-0.58	-0.35
P	[mg/kg]	0.39	0.03	0.34	0.03	-0.64	-0.03
K	[mg/kg]	0.15	0.07	0.30	-0.05	**-0.88** **	-0.12
Mg	[mg/kg]	0.03	**0.57** **	-0.05	-0.30	0.19	-0.37
Mn	[mg/kg]	**-0.52** *	0.16	**-0.49** *	-0.17	0.54	-0.18
Fe	[mg/kg]	-0.11	-0.10	-0.09	0.08	0.33	0.22
Al	[mg/kg]	**0.63** **	-0.05	**0.62** **	-0.18	-0.15	-0.12
pH (H_2O)		0.00	0.22	0.20	-0.38	0.03	-0.37
pH ($CaCl_2$)		-0.24	0.20	-0.05	-0.60	0.29	-0.59
Conductivity	[µS/cm]	0.26	-0.08	0.20	-0.30	-0.44	-0.40

Table 23: Habitat properties of sites with and actual (N=29) and an extinct (N=11) population of *A. montana* in South-West Germany (p: significance of pairwise Mann Whitney-U test) (continuation on next page).

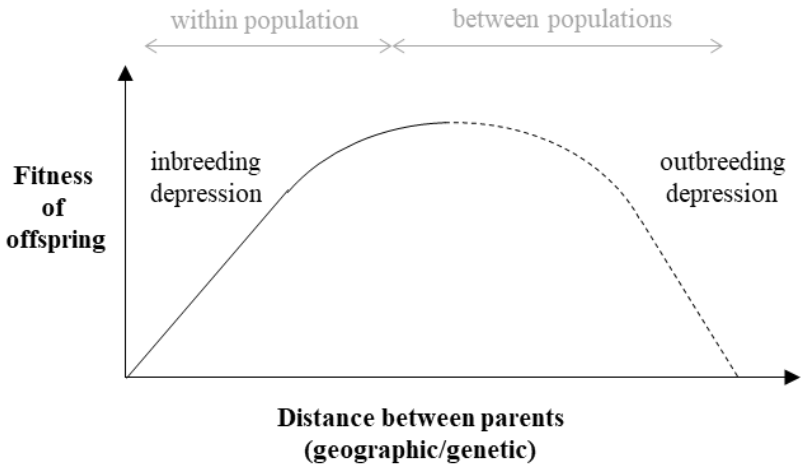
	Total					Mineral					Organic				
	yes		no		p	yes		no		p	yes		no		p
Number sites	29		11		-	20		9		-	9		2		
Altitude [m]	729.10	± 26.86	622.09	± 42.02	0.03	749.70	± 38	608.67	± 50.47	**0.03**	683.33	± 11.84	682.50	± 30.5	1.00
Vegetation Cover															
Gras [%]	57.24	± 3.97	68.18	± 5.41	0.15	50.25	± 4.85	64.44	± 5.86	0.11	72.78	± 3.13	85.00	± 5	0.12
Herb [%]	54.76	± 3.78	42.27	± 6.62	0.12	59.65	± 4.72	46.11	± 7.49	0.13	43.89	± 4.7	25.00	± 5	0.07
Moss [%]	48.45	± 6.12	58.73	± 8.08	0.35	40.25	± 6.76	58.44	± 9.99	0.16	66.67	± 10.99	75.00	± 15	1.00
Shrub [%]	1.88	± 1.07	2.27	± 2.27	0.12	2.33	± 1.54	0.00	± 0	0.07	0.78	± 0.43	12.50	± 12.5	0.62
Litter [%]	19.59	± 4.14	24.45	± 5.94	0.21	12.55	± 4.51	23.78	± 6.28	**0.04**	35.22	± 6.47	27.50	± 22.5	0.81
Bare soil [%]	5.59	± 1.22	8.05	± 2.65	0.34	6.85	± 1.5	5.94	± 1.83	0.83	2.78	± 1.88	17.50	± 12.5	0.06
Number of species															
Grases	7.97	± 0.37	8.18	± 0.82	0.59	7.60	± 0.38	7.67	± 0.91	0.90	8.78	± 0.8	10.50	± 0.5	0.22
Herbs	14.17	± 1	15.09	± 2.03	0.63	15.50	± 1.17	15.89	± 2.35	0.71	11.22	± 1.55	11.50	± 3.5	1.00
Shrub/Trees	0.97	± 0.22	0.73	± 0.27	0.66	0.80	± 0.24	0.56	± 0.29	0.56	1.33	± 0.47	1.50	± 0.5	0.63
Total	23.10	± 1.14	24.00	± 2.26	0.57	23.90	± 1.44	24.11	± 2.73	0.83	21.33	± 1.77	23.50	± 3.5	0.72
Vegetation Height															
Grasses [cm]	61.06	± 2.42	80.09	± 4.60	**0.00**	65.51	± 2.8	84.00	± 4.56	**0.00**	51.16	± 2.63	62.50	± 6.1	0.16
Herbs [cm]	36.04	± 2.96	40.98	± 6.45	0.54	42.27	± 2.94	45.44	± 7.05	0.60	22.20	± 4.28	20.90	± 2.9	1.00
Weighted Ellenberg Indicator Value															
Light	7.40	± 0.06	6.75	± 0.14	**0.00**	7.38	± 0.08	6.65	± 0.15	**0.00**	7.44	± 0.08	7.18	± 0.31	0.35
Temperature	4.52	± 0.08	4.99	± 0.19	**0.03**	4.52	± 0.09	4.87	± 0.2	0.18	4.50	± 0.14	5.57	± 0.24	**0.03**
Continentality	3.28	± 0.08	3.34	± 0.19	0.66	3.25	± 0.1	3.38	± 0.23	0.40	3.35	± 0.11	3.19	± 0.18	0.48
Moisture	5.82	± 0.22	5.85	± 0.33	0.66	5.17	± 0.16	5.45	± 0.23	0.20	7.26	± 0.18	7.66	± 0.25	0.16
Reaction	3.45	± 0.17	4.41	± 0.34	**0.01**	3.47	± 0.24	4.53	± 0.41	**0.02**	3.43	± 0.14	3.87	± 0.18	0.16
Nutrient	2.29	± 0.11	3.34	± 0.33	**0.00**	2.46	± 0.13	3.68	± 0.29	**0.00**	1.92	± 0.12	1.76	± 0.05	0.35
Mowing tolerance	4.31	± 0.17	5.13	± 0.42	0.06	4.53	± 0.21	5.57	± 0.38	**0.02**	3.82	± 0.23	3.18	± 0.01	0.24
Grazing tolerance	4.44	± 0.13	4.36	± 0.26	0.80	4.71	± 0.14	4.63	± 0.23	0.64	3.84	± 0.12	3.14	± 0.2	**0.03**
Trampling tolerance	4.25	± 0.1	4.36	± 0.18	0.60	4.46	± 0.12	4.48	± 0.17	0.96	3.80	± 0.12	3.83	± 0.57	0.81
Forage value	3.30	± 0.14	3.92	± 0.27	0.09	3.56	± 0.16	4.20	± 0.25	0.12	2.75	± 0.14	2.68	± 0.15	1.00

Table 23: Habitat properties of sites with and actual (N=29) and an extinct (N=11) population of A. montana in South-West Germany (p: significance of pairwise Mann Whitney-U test).

		Total				Mineral				Organic			
		yes		no		p	yes		no		p		
Soil chemistry/physic													
Soil depth	[cm]	45.10	± 7.71	61.90	± 14.42	0.23	23.57	± 3.46	48.99	± 14.24	0.07	92.96	± 13.85	120.00	± 0	0.37
WHC	[Gew.-%]	150.28	± 33.25	159.45	± 62.02	0.73	80.44	± 17.17	76.61	± 5.42	0.22	305.47	± 80.89	532.21	± 201.66	0.16
C	[%]	11.38	± 2.30	12.35	± 4.68	0.44	6.80	± 0.9	5.46	± 0.78	0.28	21.58	± 6.05	43.35	± 2.76	0.24
N	[%]	0.77	± 0.11	0.73	± 0.20	0.36	0.57	± 0.07	0.43	± 0.06	0.18	1.19	± 0.27	2.07	± 0.16	0.24
C/N		13.86	± 0.83	12.22	± 1.39	0.17	12.61	± 0.86	12.52	± 0.44	0.30	16.61	± 1.57	10.86	± 9.86	0.48
P	[mg/kg]	25.47	± 3.32	22.48	± 5.14	0.48	20.08	± 2.49	15.05	± 1.23	0.26	37.44	± 8.09	55.84	± 6.06	0.24
K	[mg/kg]	84.87	± 14.17	95.46	± 30.76	0.88	64.13	± 8.49	58.75	± 14.98	0.72	130.98	± 38.73	260.61	± 102.95	0.16
Mg	[mg/kg]	3.14	± 0.74	3.64	± 1.00	0.44	2.01	± 0.3	3.83	± 1.17	0.07	5.67	± 2.11	2.76	± 2.16	0.81
Mn	[mg/kg]	1.38	± 0.21	2.31	± 0.56	0.11	1.77	± 0.25	2.74	± 0.59	0.10	0.52	± 0.17	0.36	± 0.09	0.81
Fe	[mg/kg]	15.78	± 1.44	9.79	± 1.75	**0.02**	13.72	± 1.46	8.39	± 1.47	**0.02**	20.36	± 2.87	16.10	± 6.46	0.64
Al	[mg/kg]	44.92	± 6.08	33.27	± 9.55	0.23	52.27	± 7.69	34.44	± 11.69	0.12	28.57	± 7.57	28.00	± 8.03	0.81
pH (H_2O)		4.28	± 0.06	4.45	± 0.12	0.28	4.25	± 0.08	4.40	± 0.14	0.46	4.32	± 0.05	4.66	± 0.29	0.19
pH ($CaCl_2$)		3.92	± 0.05	4.29	± 0.15	**0.01**	3.87	± 0.06	4.20	± 0.15	**0.03**	3.98	± 0.05	4.66	± 0.46	0.10
Conductivity	[µS/cm]	98.66	± 9.77	113.00	± 29.08	0.81	80.65	± 8.02	71.33	± 5.26	0.74	138.67	± 21.13	300.50	± 50.5	**0.03**

Chapter 5

Crossing experiments provide strong evidence for outbreeding depression among populations of the rare plant species *Arnica montana*



Abstract

To counteract the ongoing loss of biodiversity, recent conservation activities often focus on restocking or reintroduction. The goal of both is to increase the population size and to minimize the risk of inbreeding within a local population and hence the loss of fitness. However, the introduction of foreign material can also lead to outbreeding depression when the receiver and donor populations are differentiated genetically. In the study presented here, the risk and extent of outbreeding was investigated for the strongly endangered plant species *Arnica montana*. Therefore, crossing experiments between plants from distinct populations were conducted under controlled conditions. From each crossing, the seed set respectively the filling rate was measured to estimate the fitness of the offspring. The results showed provided evidence for outbreeding depression if plants from different populations were crossed. This means that the filling rate was lower compared to crossings between plants from the same population. Based on that, restocking should only be conducted with plant material from the same population to avoid artificial mixture. When plant material from other populations is introduced for conservation reasons, the genetic distance between donor and receiver populations should be as small as possible to minimize the risk for outbreeding depression.

Introduction

In present times, loss of habitat, species and genetic diversity is an ongoing process worldwide and thus protection of biodiversity on the different levels is an urgent topic in nature conservation (CBD 1992). Due to human land-use changes and habitat destruction, formerly well distributed habitats and species became rare and populations are nowadays more strongly structural fragmented than in the past and hence isolated from each other today (Frankham et al. 2017; Baur 2021). The extent of isolation depends on the distance between remaining habitat patches and on life history traits of plants species, like dispersal ability of pollen and seeds (Kolb und Diekmann 2005; Ortego et al. 2015). For example, grassland species with a low dispersal ability are unable to conquer landscape barriers (e.g. forests) and hence populations are isolated functionally from each other. In contrast, wind-dispersed species can more easily overcome such barriers.

The fragmentation of populations leads to a loss of gene flow between populations when the exchange of genetic material is limited. Within isolated populations, natural mutations and selection as well as slight difference in ecological conditions leads to a shift in the frequency of alleles at certain loci (= genetic drift) (Holderegger und Segelbacher 2016). Due to the loss of gene flow between populations these differences can`t be compensated and the populations become genetically differentiated over time (Ellstrand und Elam 1993). This differentiation increases by long-lasting fragmentation of populations, especially if population size decreases (Frankham 2010).

If populations from species become isolated, gene flow only occurs between individuals within the population. Thus, it becomes more likely that mating between closely related individuals happens (Holderegger und Segelbacher 2016; Frankham et al. 2017). Closely related individuals have at least one same allele at a specific locus, which can then be given to the offspring from both parental individuals. Thus, the offspring is more likely homozygous at a certain locus. This is disadvantageous because homozygotes are less genetically diverse and thus less adaptable to changing environmental conditions which are more likely in present times of rapid climatic changes (Leimu et al. 2010). Furthermore, harmful alleles can be aggregated and thus become more prevalent. The loss of heterozygosity and genetic diversity results in a loss of fitness in the offspring which can be seen in e.g. reduced seed set, seedling survivability, flowering ability or increased mortality (Vrijenhoek 1994; Reed und Frankham 2003). This phenomenon is called inbreeding depression and leads to a higher risk of extinction of a local population (Brook et al. 2002). The presence of inbreeding depression in isolated populations is well proven for outcrossing plant species and more common in small populations (Waser 1993; Luijten et al. 2002; Paschke et al. 2002; Galloway et al. 2003; Grindeland 2008; Sletvold et al. 2012).

To counteract the effects of reduced genetic diversity and heterozygosity in small populations and increased inbreeding depression, nature conservation authorities apply the method of population enhancement. In this case, plant material from either the same or another population is introduced to an

existing population. Aim is to increase the genetic diversity and the amount of effective mating partners and thus the fitness of the population to counteract genetic loss (Whiteley et al. 2015; Hedrick und Garcia-Dorado 2016; Zippel und Lauterbach 2018; Bell et al. 2019). A second method is the introduction of species at a site without an actual occurrence. This aims to decrease the geographic fragmentation of populations and to enable the gene flow between remaining populations. However, the reintroduction of gene flow between long-time fragmented populations or the insertion of foreign plant material can be also disadvantageous, due to the potential effects of outbreeding. This means that the mating of genetically differentiated individuals may lead to a reduced fitness (Ottewell et al. 2016). This can be explained by e.g. chromosomal incompatibilities, which evolved, e.g. due to adaptation to different environmental conditions (Frankham et al. 2017). The success of both conservation methods can be rated by the amount of genetic rescue. It compares the offspring fitness of within-population crossings with crossings between populations (Frankham et al. 2017).

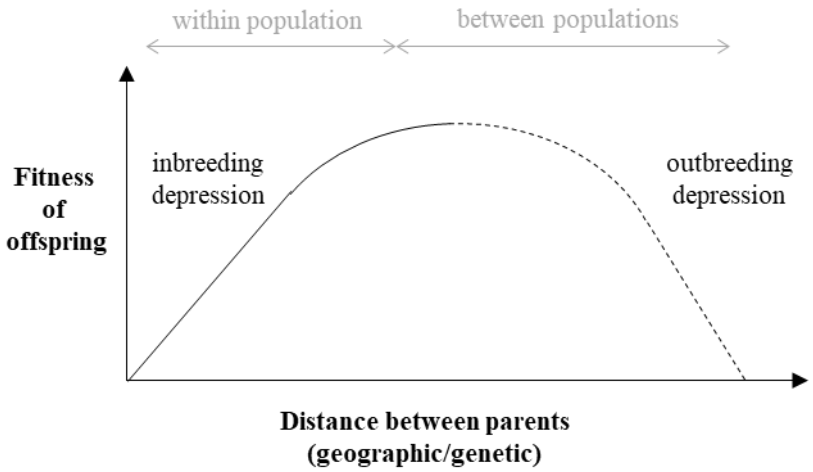


Figure 20: Proven effects of inbreeding depression (solid line) and theoretical effects of outbreeding depression (dashed line) on the fitness of offspring (Kaye 2001, adapted).

By applying reintroduction or re-stocking, the extent of inbreeding and potential outbreeding depression needs to be estimated and balanced in advance to improve the offspring fitness. In theory, the highest offspring fitness can be reached by intermediate values for genetic or geographic distance between mating partners (Figure 20) (Kaye 2001; Frankham et al. 2017; Hoffmann et al. 2021). Until know, the disadvantage of inbreeding on plant fitness was proven as well as the advantage of interpopulation crossing on small populations suffering from inbreeding (Luijten et al. 2002; Paschke et al. 2002; Bossuyt 2007; Grindeland 2008; Sletvold et al. 2012). However, most studies conducted the interpopulation crossing only between populations from sites with a low geographic distance and also didn`t investigate the influence of genetic distances (e.g. Raabová et al. 2009). Thus until today, the general recommendation for re-establishing and re-stocking is to use plant material from a population

with low geographic distances (Ottewell et al. 2016; Zippel und Lauterbach 2018). This approach is easily applicable in nature conservation. However, especially for species with a low dispersal ability and a fragmented distribution, geographical and genetic distances between populations are not necessarily correlated (Phillipsen et al. 2015). The present study aims to analyse this problem for the endangered and rare plant species *Arnica montana*. Artificial crossings between geographical distinct populations have been conducted in a greenhouse experiment to investigate the offspring fitness. The data were linked with genetic data to answer the following questions:

- **Does geographic distance between source and receiver population effect the offspring fitness?**
- **Is offspring fitness correlated with genetic distance between the two parental populations?**

Material and methods

Study species and plant material

Arnica montana is a perennial species from the *Asteraceae* family that grows in nutrient-poor grasslands and dry heathlands on acidic soil in Central Europe. The species can either propagate vegetatively or reproduce generatively, for which the pollination by insects (e.g. syphids, bees, butterflies) is mandatory (Luijten 2001). Since decades, the species is threatened by extinction and an ongoing loss of populations is detected in all European countries (IUCN 2021). In South-West Germany, remnant populations occur in separate geographic regions in the Black Forest, Swabian Alb, and Upper Swabia. Between and within the regions the populations are isolated from each other, which is increased further by an ongoing loss of suitable habitats. The natural dispersal ability of the species is low which enhances the functional isolation of populations (Strykstra et al. 1998; Trapp et al. 2018).

For the study, in June/July 2018, ripe seeds from 22 different wild populations of *A. montana* in South-West Germany were collected. Within each population seeds from ten different flower heads were collected, whereby a minimum distance of 5 m between two flower heads was mandatory to avoid obvious multiple sampling of the same individual. The seeds from different populations were stored in separate paper bags and kept dry and at room temperature until further proceeding.

After sampling, all collected seeds were x-rayed in the lab with a Faxitron X-Ray MX-20 (Faxitron Bioptics, LLC, USA) with 18kV for 10 seconds. This gentle method enables an easy selection of seeds with a well-developed embryo (Tausch et al. 2012). Empty or unripe seeds were discarded. Filled seeds from each population were placed in separate petri dishes on water saturated filter paper. For seed germination, the petri dishes were placed in a climate chamber (Rumed, type 1301, Rubarth Apperate GmbH, Germany) with a temperature of 22°C at day (14 hours) and of 14°C at night (10 hours). After three weeks, the seedlings were transplanted into 4x6 cell trays with a mixture of acidic cultivation soil (pH 3.8, Alpenflor) and acidic sand (Silex) and were cultivated outside in the botanical garden of the

University of Regensburg. All plants were individually marked to keep track of the origin wild population. After further three months of cultivation, each plantlet was potted into a single plant pot (diameter 13cm) with the same mixture of acidic cultivation soil and acidic sand used before. During the cultivation, the plants were watered regularly with deionised or rainwater and never fertilized. During the winter period from November to March, the pots were placed in concrete enclosures with removable glass cover to preserve the plants from cold snaps. The rest of the year, the plants were cultivated in an unheated, open caged greenhouse. The first plants started to flower in May 2019.

Crossing design and experimental set up

For the crossing experiment, cultivated plants from four different populations were used as receiver populations (R1-4, Table 24). These geographically separate receiver populations were located within different natural regions of the distribution range in South-West Germany (Figure 23 in appendix). For each receiver population a crossing within the population was conducted by crossing two different plants of the same population (DX.0, intrapopulation). Hence, the geographic distance of this crossing was 0.0 km. In addition, further plants from the receiver populations were pollinated with pollen from 4 to 6 different donor populations (interpopulation crossing). The geographic distance (GGD) between receiver and donor population ranged from 0.6 to 156.0 km (Table 24). Each receiver plant was pollinated with the pollen from only one other donor plant. Each pair of receiver and donor population was replicated independently between 3 to 5 times with different plants. The number of replications depended on the number of available, flowering plants. In total, 105 receiver plants were pollinated with pollen from different donor sites.

Table 24: Design of crossing between pollen receiver (R1-4) and pollen donor (DX.X) sites with geographic distance (GGD) and number of replicates per crossing.

Receiver	Donor	GGD [km]	Number of replications
R1	D1.0	0.0	3
	D1.1	1.9	3
	D1.2	11.0	3
	D1.3	11.2	5
	D1.4	17.2	5
	D1.5	72.5	4
	D1.6	105.5	5
R2	D2.0	0.0	3
	D2.1	0.6	5
	D2.2	8.6	5
	D2.3	20.2	5
	D2.4	22.0	5
	D2.5	96.2	4
R3	D3.0	0.0	4
	D3.1	0.7	4
	D3.2	4.8	4
	D3.3	7.4	4
	D3.4	52.9	4
R4	D4.0	0.0	5
	D4.1	0.9	5
	D4.2	2.1	3
	D4.3	6.2	5
	D4.4	6.7	5
	D4.5	10.0	5
	D4.6	156.0	3

The crossing experiment took place in early summer 2019 and 2020 and depended on the individual flowering time of the plants in the greenhouse. The pollination of a receiver plant started when the first flower within a flowering head opened. Then a plant from a donor population was selected for pollination. The pollen transfer from donor and receiver plant was done by gently rubbing them together. This was done for each crossing pair twice a day during the whole flowering time until all flowers of the receiver flower head had flowered. Unless the short time needed for the pollen transfer, each flower head was bagged individually with nylon fabric with small mesh size to avoid uncontrolled pollination (Figure 21).

Figure 21: Bagged flower heads with nylon fabric with small mesh size to avoid uncontrolled pollination.

As control, some randomly selected flower heads were bagged during the whole experiment without artificial pollination. For these flower heads, no seeds filled with an embryo were detected at the end of the experiment (data not shown). Thus, uncontrolled selfing within the bags didn`t happen. Due to this self-incompatibility of *A. montana*, artificial emasculation by removing anthers from the receiver flowers was not necessary.

Fitness measurement

The flower heads of the receiver plants were kept bagged at the flowering stems until the seeds were ripe. This was due when the achenes fell off from the flower heads and were collected within the nylon bags. The seeds within each bag were transferred to separate paper bags and stored dry and at room temperature. For each flower head, the total number of built seeds was counted. Furthermore, the number of seeds filled with an embryo were determined by x-raying the seeds with the Faxitron X-Ray MX-20 (Faxitron Bioptics, LLC, USA) with 18kV for 10 seconds. Following, for each flower head of the receiver plants the percentage of filled seeds was calculated as the ratio of filled to total seeds. For *A. montana*, the filling rate is a good and easy measurable proxy for the fitness of the offspring (see chapter 3).

Genetic distance (F_{ST})

Genetic distance between all populations used for the crossing experiment was determined via microsatellites using fresh leaf tissue from all populations used as receiver or donor population. In maximum, 16 rosettes per population were sampled across the populations whereas a minimum distance

of 1 m was mandatory. The leaf tissue was stored in filter bags and dried in silica gel. Following, the DNA was extracted from each sample by applying the CTAB method (Rogers und Bendich 1994) adapted by Reisch (2007). The DNA content was measured with a microvolume spectrophotometer (NanoDrop One, ThermoFischer, Scientific, Germany) and diluted with water to 7.8ng/µl. Following, the genotypes of all samples were determined by investigating the alleles at nine different microsatellite loci. Van Rossum and Raspé (2018) developed the required arnica-specific primers for the polymerase chain reaction (PCR). The forward and reverse primers were combined in two separate multiplexes to reduce the number of single PCRs (Multiplex A: AG-10, AG-4B, AG-1, AG-11, CT-2 / Multiplex B: CT-5, ATC-3, AG-2B, ATC-2). Thus, for each sample only two separate multiplexed PCRs were necessary. Each multiplex reaction contained following ingredients: 3.2µl DNA (7.8 ng/µl), 5.0µl 2xMastermix S (VWR), 0.8µl H_2O, 0.5µl multiplexed forward primers (10 µM), 0.5µl multiplexed reverse primers (10µM). The applied thermocylcer program followed Van Rossum and Raspé (2018). The size of the fragments was analysed with an automated sequencer (GenomeLab GeXP from Beckman Coultier, method Frag-4). Therefore, 1µl of each PCR product was mixed with 24.8µl Sampling Loading Solution (SLS, Beckman Coulter) and 0.2µl Size Standard 400 (CEQ, Beckman Coultier, Germany). The size of the detected fragments was determined with BioNumerics (Applied Maths, Version 7.6.3). Based on that, GenAlEx (Version 6.51b2) was used to calculate the pairwise genetic distance F_{ST} between each receiver and donor population.

Data analysis

Data analysis was conducted in R (Version 4. 0.1). The mean filling rate (FR) for each crossing pair of a receiver and donor site was calculated as the average of the conducted replications. Based on this rate, the genetic rescue (GR) for all pairs of crossing was calculated, as follow (Frankham et al. 2017):

$$GR = {}^{(\text{FR between populations} - \text{FR within population})}/_{\text{FR within population}}$$

Values of genetic rescue larger than 0 represent a beneficial influence of the interpopulation crossing on the fitness. In contrast, negative values indicate a loss of fitness in case of interpopulation crossings and thus mean the evidence of outbreeding depression. The correlation between the height of genetic rescue and geographic respectively genetic distance was tested with non-parametric correlations (Spearmann rank).

Results

In total, the flower heads built 11,921 seeds with an overall filling rate of 63.33 % (Table 25). The filling rates of crossings within the four receiver populations ranged from intermediate values (R2: 64%, R4: 58%) to high filling rates (R1: 90%, R3: 89%). The filling rates resulting from the interpopulation crossings ranged in general from 5 to 86%.

The genetic distance (F_{ST}) between the donor and receiver sites ranged from 0.01 to 0.26. For each of the four receiver populations, the values of genetic distance between receiver and donor population didn`t correlate with the geographic distance ($p > 0.05$). For plants from receiver population R1 and R4 the filling rate of the seeds was negatively correlated with the genetic distance (Table 26 in appendix). Furthermore, the filling rate decreased with increasing geographic distance.

Table 25: Geographic (GGD) and Genetic (GD) distance between receiver and donor sites of *A. montana* with resulting mean filling rate and genetic rescue of crossing.

Receiver	Donor	GGD [km]	GD (FST)	Filling rate MW	±SE	Genetic Rescue
R1	D1.0	0.0	0.00	0.90	0.02	0.000
	D1.1	1.9	0.07	0.86	0.06	-0.043
	D1.2	11.0	0.13	0.81	0.09	-0.094
	D1.3	11.2	0.08	0.61	0.14	-0.323
	D1.4	17.2	0.06	0.78	0.04	-0.126
	D1.5	72.5	0.22	0.38	0.16	-0.581
	D1.6	105.5	0.09	0.82	0.05	-0.081
R2	D2.0	0.0	0.00	0.64	0.14	0.000
	D2.1	0.6	0.04	0.65	0.11	0.008
	D2.2	8.6	0.20	0.75	0.05	0.162
	D2.3	20.2	0.06	0.75	0.08	0.172
	D2.4	22.0	0.11	0.45	0.15	-0.298
	D2.5	96.2	0.16	0.61	0.16	-0.053
R3	D3.0	0.0	0.00	0.89	0.02	0.000
	D3.1	0.7	0.01	0.65	0.19	-0.274
	D3.2	4.8	0.12	0.86	0.06	-0.035
	D3.3	7.4	0.03	0.54	0.02	-0.394
	D3.4	52.9	0.04	0.50	0.09	-0.434
R4	D4.0	0.0	0.00	0.62	0.16	0.000
	D4.1	0.9	0.10	0.39	0.15	-0.390
	D4.2	2.1	0.18	0.60	0.04	-0.033
	D4.3	6.2	0.22	0.58	0.13	-0.069
	D4.4	6.7	0.26	0.19	0.07	-0.691
	D4.5	10.0	0.16	0.42	0.16	-0.325
	D4.6	156.0	0.25	0.05	0.04	-0.920

The values for genetic rescue of the interpopulation crossings ranged from – 92.0 % to 17.2 % and were in average -19.3 % (Table 25). The positive values for genetic rescue were only recognized for two crossings of receiver population R2. All other crossings showed negative values, which means that the filling rate of the interpopulation crossing was lower than of the associated crossing within the receiver population. In general and separate for the four receiver sites, the genetic rescue of the interpopulation crossings decreased with geographic and genetic distance (Table 27 in appendix). For genetic distances large than 0.22, the offspring fitness clearly decreased at least 58.1% and more (Figure 22).

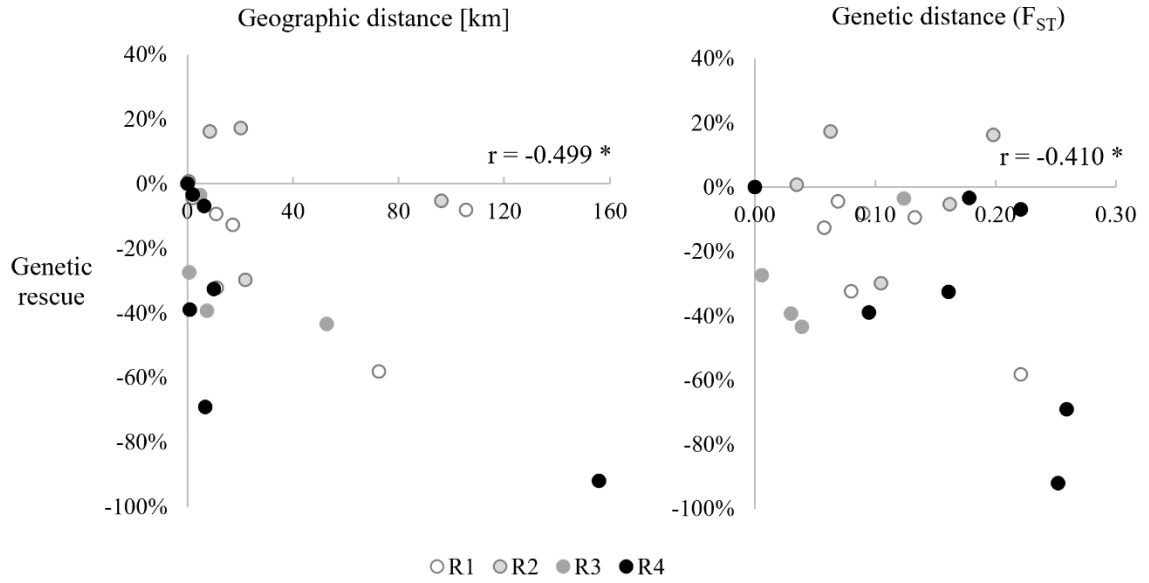


Figure 22: Genetic rescue in relation to geographic (left) and genetic (right) distance of 25 pairs of crossings of four different receiver sites (R1-R4) (r = spearman rank correlation coefficient, * = p<0.05).

Discussion

The present study showed that interpopulation crossings have a negative impact on genetic rescue in the offspring of *A. montana*. Hence, the occurrence of outbreeding depression could be proven for this species. Due to the reduced filling rate, it is likely that the overall survivability of the offspring will be reduced (see chapter 3). This shows that artificial mixture of distinct populations should be avoided.

Furthermore, the study showed a negative impact of geographic distance on genetic rescue. A similar pattern was shown before e.g. for the outbreeding species *Gentianella germanica* (Fischer und Matthies 1997) and underlines the occurrence of outbreeding depression for *A. montana* if populations are far distinct. But in contrast, in several other studies no (clear) negative impact of geographic distance on the offspring fitness was detected (Luijten et al. 2002; Bailey et al. 2006; Billingham et al. 2007; Raabová et al. 2009; Palacio-Lopez et al. 2018). All these studies have in common that the range of geographic distance was rather low (< 50km), and it is very likely that the critical geographic distance was not yet reached. In the present study, especially the crossing between populations with a distance of approximately 160km resulted in a high decline of offspring fitness. The corresponding populations of this crossing originated from different regions that are distinct from each other since long time and following the isolation between the populations exists since long time (see chapter4). Within small geographic distances no clear impact of the distance on the offspring fitness was shown and the result fits to the findings of previously mentioned studies (Luijten et al. 2002; Billingham et al. 2007; Raabová et al. 2009; Palacio-Lopez et al. 2018). Small geographic distances showed either positive effects on the offspring fitness (D2.2: GGD=8.6km, GR=16.2%) or negative (D4.1: GGD=0.9km, GR=-39.0%). Thus,

based on the results of the study no geographic distance could be specified from which outbreeding depression is clearly visible.

Increasing genetic distance led to a decrease in the offspring fitness of the conducted interpopulation crossings. Up to a genetic distance of F_{ST} = 0.22, the fitness declined up to -43.4%. For larger genetic distances, the offspring fitness decreased between -58.1 to -92.0%. The findings are a prove for outbreeding depression in *A. montana* and support the recommendations of Ottewell et al. (2014) who defined a genetic distance larger than 0.15 as high and artificial mixture shouldn`t take place. For the species *Lotus scoparius* the pattern of outbreeding depression was already given for lower values of genetic distance (Montalvo und Ellstrand 2001). In contrast, a study on *Aster amellus* didn`t reveal outbreeding depression up to a genetic distance of 0.19 (Raabová et al. 2009). Hence, the critical genetic distance where outbreeding depression clearly occurs seems to be species-specific.

As described before, the offspring fitness of interpopulation crossings up to a genetic distance of F_{ST} < 0.22 declined compared to the intrapopulation crossing. However, the height of genetic rescue wasn`t clearly effected by the height of genetic distance. Other studies on the effect of genetic distance on offspring fitness revealed that also the environmental distance and home site advantages influences the success of crossing (Montalvo und Ellstrand 2001; Frankham et al. 2011; Del Vecchio et al. 2019). *A. montana* occurs on a wide range of soil-acidic, nutrient-poor habitats with different hydrological conditions (Blachnik und Zehm 2017; LUBW 2020a). Environmental differences can lead to a genetic differentiation between population due to the need of adaptation to different local habitat conditions (Pagel et al. 2020). Unfortunately, those adaptive genetic differences can barely be detected by neutral genetic markers like the applied microsatellite (SSR) markers. Hence, to calculate the overall effect of interpopulation crossings on the offspring fitness also environmental distances should be considered in future studies.

Conclusions

The artificial crossing experiment between geographical and genetic distinct populations of *A. montana* showed a clear decline in fitness and genetic rescue when the interpopulation distance increased. Hence, the occurrence of outbreeding depression for this species could be proven and the transfer of seed or plant material should only be conducted if necessary (e.g. full clonality within receiver population). In this case, the investigation of genetic distance between the two populations should be mandatory in advance because offspring fitness clearly drops if genetic distance is too large (F_{ST} > 0.22). Geographic distance shouldn`t be used alone for the determination of receiver and source populations because crossings of near-by populations can also be disadvantage if genetic distance is large. This is further promoted by the proven lack of correlation between genetic and geographic distance of *A. montana* within distinct regions.

Appendix

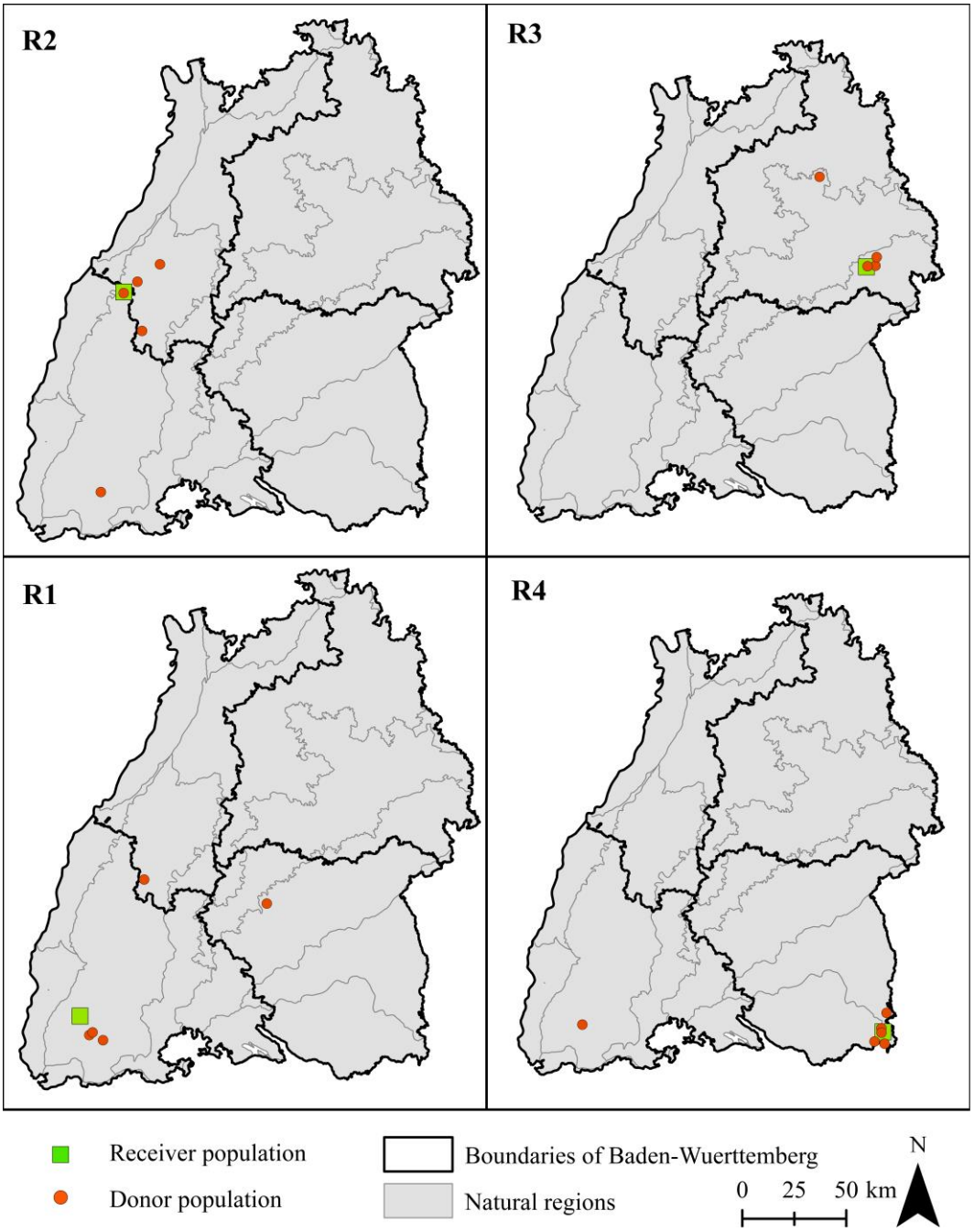


Figure 23: Geographic location of four receiver populations of Arnica *montana* (R1-4, green square) and corresponding pollen donor sites in South-West-Germany.

Table 26: Pairwise correlation coefficient between seed filling rate of *A. montana* and geographic (GGD) / genetic (F_{ST}) distance for four different receiver populations (R1-R4) (levels of significance: p<0.01 = **, p<0.05 = *).

Receiver	GGD		F_{ST}	
R1	-0.238	ns	-0.397	*
R2	-0.108	ns	0.018	ns
R3	-0.576	**	-0.190	ns
R4	-0.376	*	-0.358	*

Table 27: Pairwise correlation coefficient between genetic rescue of *A. montana* and geographic (GGD) / genetic (F_{ST}) distance for four different receiver populations (R1-R4) (levels of significance: p<0.05 = *).

Receiver	GGD		Fst	
R1	-0.607	ns	-0.607	ns
R2	-0.371	ns	-0.029	ns
R3	-0.900	*	-0.400	*
R4	-0.714	*	-0.643	*
Total	-0.499	*	-0.410	*

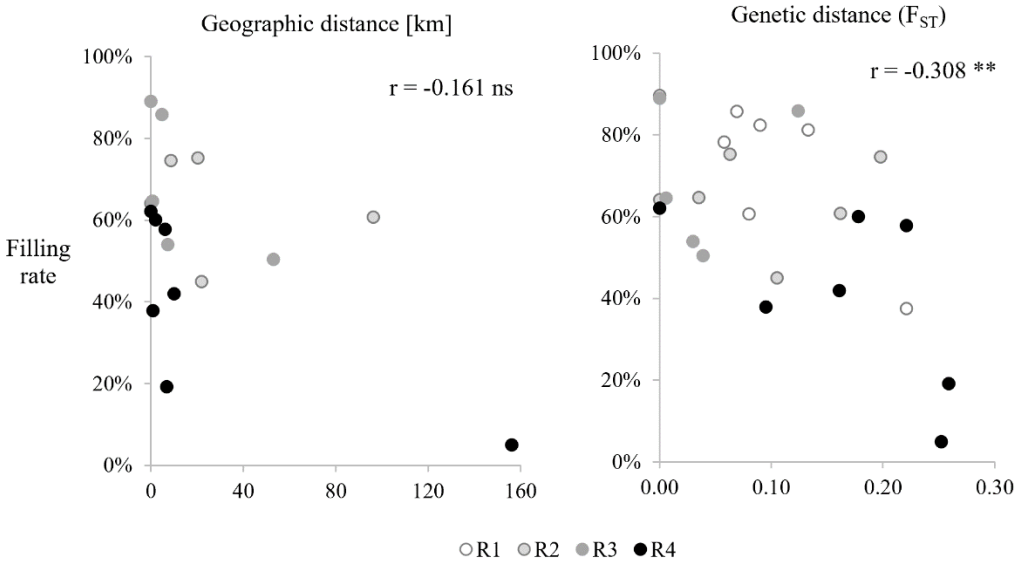


Figure 24: Pairwise correlation between seed filling rate and geographic (left) and genetic (right) distance for four different receiver populations of *A. montana*.

Chapter 6

General conclusions and perspectives for nature conservation

The global loss of biodiversity is recognizable since decades. To counteract this development, conservation biology aims to improve the assessment and the management of threatened species (Gerber et al 2010). Therefore, P*opulation Viability Analysis* (PVA) can be conducted to evaluate risk factors for the extinction of a species. Originally, such analyses focused only on the calculation of extinction probabilities and minimum viable population size without including environmental or demographic factors (Boyce 1992). Later on, genetic stochasticity was used to predict the extinction risk of a certain population and hence a species (Thompson 1991). In the meantime, it became more common to include several biotic and abiotic parameters as well as species-specific life history traits to evaluate the risk of extinction of a species (Coulson et al. 2001). Based on this process, recommendations for nature conservation can be given. The aim of the presented thesis was first to investigate the constitution of the threatened plant species *Arnica montana* in South-West Germany and following to find parameters that influence its viability and to give recommendations for future conservation efforts. In summary, influencing factors on the landscape, the habitat and the population level were found:

- **Landscape level: Regions as conservation units**

  The landscape analysis in chapter 4 revealed that the landscape in the last 150 years became more forest dominated and proved that remaining nutrient-poor grasslands habitats became isolated from each other. Furthermore, distinct regions in South-West Germany have slightly different landscape histories, topography and climatic conditions. Hence, the present isolation of *Arnica* populations within and between regions in South-West Germany persists already since more than a century. It is likely that populations are adapted to such local conditions. These findings are promoted by the high genetic differentiation between the populations, regions and genetic clusters, described in chapter 2. For perennial, clonal growing plants like *A. montana* it takes a while until structural fragmentation is visible on the genetic level (Nybom 2004; Ottewell et al. 2016). This underlines the long-lasting isolation of *Arnica* populations within the study area. Based on these findings, each distinct region should be seen as single conservation respectively management units (Vogler und Desalle 1994; Heywood und Iriondo 2003; Frankham 2010, 2015; Ottewell et al. 2016). Furthermore, landscape history should be considered in conservation activities as it can explain e.g. large-scale genetic pattern.

  In addition, the loss of nutrient-poor habitats increased in last decades (BfN 2020). For South-West Germany, this development was also shown in detail by the monitoring reports of the federal species protection program. Species like *A. montana* that are adapted to conditions of nutrient-poor habitats suffer from this habitat loss the most, because the availability of alternative habitats is limited. Hence, the constitution of *A. montana* can be improved in general by the avoidance of pure habitat destruction by e.g. afforestation, abandonment or intensification of land usage.

- **Habitat level: Maladapted management and nutrient supply promotes species extinction**

  The survey of local habitat qualities on the occurrence of *A. montana* (chapter 4) showed, that the species got locally extinct due to changing habitat qualities. The results revealed that the vegetation structure and composition differed at sites with respectively without *A. montana*. The differences were related to a local increase in nutrient supply and maladapted management. Hence, the vegetation became dominated by grass species and its growth height increased. Following, the light availability near the soil surface decreased and hence unsuitable conditions for *A. montana* occurred (Vermeer und Berendse 1983). Increased nutrient supply further led to an reduction of census population size and seedling survivability decreased (Hollmann et al. 2020). For the future preservation of the species, habitat conditions need to suit to the specific requirements of *A. montana*.

  Next to that, *A. montana* evolved originally as pasture weed and hence its life history traits are adapted to this type of management. By cattle grazing during the flowering time of *A. montana*, the biomass of other species can be reduced and at the same time, open soil as germination niche appears. If mowing is the only applicable type of local management, it is mandatory recommended to remove the biomass and litter manually out of the flowering period.

- **Population level: Filling rate as proxy for generative fitness and extent of clonality**

  The results of chapter 4 showed that higher nutrients supply was harmful for the number of rosettes but promoted at the same time the building of flowering stems. By that, the possibility of pollination of flowers of the same genet become more likely which harms the effective seed production. Furthermore, the correlation analysis in chapter 3 showed that the flowering ability of a population can`t give a full insight to the generative fitness or genetic constitution of *A. montana*. Hence, the census vegetative and generative population size is rather an uninformative proxy for the evaluation of the constitution of populations of *A. montana*.

  Chapter 2 could show that the majority of the actual populations don`t suffer from a reduced genetic diversity or inbreeding. The latter is avoided in *A. montana* due its fully self-incompatibility. Due to the vegetative propagation of the species, the genetic diversity could be preserved until now while effective generative reproduction seems strongly reduced. Predominantly small populations showed a reduced genetic diversity and a higher extent of clonality. Following, conservation efforts should focus on increasing local population sizes to enable the continuance of populations.

  At the same time, small populations showed a lower generative fitness which can be estimated by the filling rate of the seed set (chapter 3). This in turn is negatively correlated with the extent of clonality. Hence, by measuring the filling rate within flower heads, information on the genetic constitution and generative reproduction ability are assessable at the same time. This survey can be

done directly in the field because filled and viable seeds are black and hard, whereas empty seeds are whitish and compressible. Thus, it is cost-effective and fast measurement and can be easily applied during the monitoring of populations.

- **Reintroduction: Keep it local**

  The conducted crossing experiment (chapter 5) revealed the presents of outbreeding depression for *A. montana* in case of interpopulation crossings. This is a unique evidence of the negative impact of the introduction of foreign plant material on a local population. In detail, a negative impact of geographic and genetic distance between pollen donor and receiver populations was shown. This led to the recommendation that seed exchange between populations and random seed propagation should be avoided. This is also promoted by the high genetic differentiation between natural populations, shown in chapter 2. In case of reintroduction, the determination of genetic distance between two populations can help to calculate the extent of outbreeding depression in advance. Furthermore, population enhancement should only be conducted with local plant material and artificial mixture of different origins shouldn`t take place.

Future perspectives

The conducted studies give an entire insight on the constitution of *A. montana* in South-West Germany and how it is influenced. Based on the findings, it was possible to give recommendations for applied conservation activities on the landscape, habitat and population level as well as for reintroductions. However, it needs to be mentioned, that this was only possible because investigations on the species, population, habitat and landscape level were combined, set into context and viewed under the involvement of species-specific life history traits and requirements. In recent times, *A. montana* was investigated in several scientific research projects but rarely an insight on several risk factors was given and the conservation effort couldn`t be fully improved. Hence, all the above-mentioned approaches are necessary for conducting efficacious and sustainable population viability analysis and hence species protection. Furthermore, the inclusion of historic and especially genetic data is helpful for species protection because they can give a detailed insight on the time when the species had a favourable status. Especially for the protection of endangered and rare species, which are more likely specialists, this holistic approach is necessary to understand the current constitution in detail and how it is influenced respectively can be improved. In future, the results of detailed (and preferable standardised) population viability analysis of several different (endangered) plant species could be then combined to find unified pattern to protect several species.

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