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IRIS REINULA

Genetic variation of grassland plants in changing landscapes

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Department of Botany, Institute of Ecology and Earth Sciences, Faculty of Science and Technology, University of Tartu, Estonia

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by Roman numerals:

I Aavik, T., Thetloff, M., Träger, S., Hernández-Agramonte, I. M., **Reinula, I.**, & Pärtel, M. (2019). Delayed and immediate effects of habitat loss on the genetic diversity of the grassland plant *Trifolium montanum*. *Biodiversity and Conservation*, 28, 3299–3319. https://doi.org/10.1007/s10531-019-01822-8

II **Reinula, I.**, Träger, S., Hernández-Agramonte, I. M., Helm, A., Aavik, T. (2021). Landscape genetic analysis suggests stronger effects of past than current landscape structure on genetic patterns of *Primula veris*. *Diversity and Distributions*, 27, 1648–1662. https://doi.org/10.1111/ddi.13357

III **Reinula, I.**, Träger, S., Järvine, H. T., Kuningas, V. M., Kaldra, M., & Aavik, T. (2024). Beware of the impact of land use legacy on genetic connectivity: A case study of the long-lived perennial *Primula veris*. *Biological Conservation*, 292, 110518. https://doi.org/10.1016/j.biocon.2024.110518

IV Träger, S., Rellstab, C., **Reinula, I.**; Zemp, N., Helm, A., Holderegger, R., Aavik, T. The effect of recent grassland overgrowth on the neutral and adaptive genetic variation of *Primula veris*. (manuscript)

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Author's contribution ('*' denotes a moderate contribution, '**' denotes a high contribution, '***' denotes a leading role).

	I	II	III	IV
Original idea		**	*	*
Study design		*	*	*
Data collection	*	**	*	**
Analysis and interpretation	*	***	***	**
Manuscript writing	*	***	***	*

LIST OF ABBREVIATIONS

AF	Allele frequency
AICc	Akaike information criterion corrected for small sample sizes
DAPC	Discriminant analysis of principal components
ddRAD	Double-digest restriction site-associated DNA sequencing
DIC	Deviance information criterion
EAA	Environmental association analysis
FDR	False discovery rate
F_{IS}	Inbreeding coefficient
F_{ST}	Genetic differentiation
H_E	Expected heterozygosity
H_O	Observed heterozygosity
IBD	Isolation by distance
LFMM	Latent factor mixed models
MAP	Pairwise mean assignment probability
PCR	Polymerase chain reactions
SNP	Single nucleotide polymorphism
π	Nucleotide diversity
%P	Percentage of polymorphic loci

1. INTRODUCTION

Natural ecosystems have been influenced by recent environmental and landscape changes in various ways (Picó & Van Groenendael, 2007). Land use has significantly changed towards more intensive agricultural practices and the prevalence of monocultures (Picó & Van Groenendael, 2007; Prentice et al., 2006). Additionally, the abandonment of traditional management practices has resulted in the overgrowth of semi-natural grassland habitats that depend on moderate grazing and mowing to persist. As a consequence, natural and semi-natural habitats, often characterised by exceptionally high species richness (Wilson et al., 2012), have become severely fragmented due to the loss in the area and connectivity of habitats (Fuchs et al., 2015; Lieskovský & Bürgi, 2018). Furthermore, the quality of habitats has shifted due to altered environmental conditions caused by climate change (Mantyka-pringle et al., 2012) and agricultural pollution (Carpenter et al., 1998). Not all organisms can adapt to these changes, leading to the loss of biodiversity (Díaz et al., 2019).

Biodiversity encompasses diversity at genetic, species and ecosystems level. However, studies about the impact of global change on biodiversity generally focus on examining the response of species richness (Fahrig, 2003). Yet, the genetic diversity of wild populations may also decline due to the factors mentioned above (Leimu et al., 2010). Loss of genetic variety, in turn, makes a species more vulnerable to environmental changes because it may limit populations' adaptive potential (Chung et al., 2023; Frankham et al., 2004) and result in lower fitness (Takkis et al., 2013). Consequently, as genetic diversity gives species the ability to adapt to future shifts in environmental conditions, sustaining and enhancing genetic diversity should be one of the focal aims of conservation actions. Furthermore, the inclusion of knowledge about genetic diversity in restoration activities has a high potential to increase long-term restoration success (Mijangos et al., 2015) and is crucial for recovering structurally and functionally resilient ecosystems (Moreno-Mateos et al., 2020). Encouragingly, maintaining genetic diversity is increasingly mentioned as one of the goals of international biodiversity strategies (Krug et al., 2022). Yet, an understanding of how environmental and landscape changes impact genetic diversity is far from being sufficient, limiting the successful implementation of theoretical knowledge in conservation practice.

Genetic diversity can be assessed at functionally neutral and adaptive parts of the genome. The diversity evaluated at putatively neutral genetic loci is primarily affected by neutral processes, such as gene flow and genetic drift, but not by local environmental factors, and is defined as neutral genetic diversity (Holderegger et al., 2006). Adaptive genetic diversity, in contrast, is the diversity assessed at putatively adaptive loci, i.e., at loci under natural selection and responding to environmental conditions (Holderegger et al., 2006). Traditionally, neutral genetic diversity has been the focus of conservation-oriented research due to methodological constraints. However, as a result of significant advances in molecular tools, the amount of studies exploring adaptive genetic variation not only in

model organisms but also in wild species has increased recently (Chung et al., 2023). In conservation, the main focus is still on neutral genetic diversity, but as environmental changes are getting more severe, it is increasingly important to include also knowledge of adaptive genetic diversity in conservation planning. Nevertheless, research on neutral genetic diversity should remain equally important since a distinct set of questions can be answered by studying these different aspects of genetic diversity (Chung et al., 2023; Holderegger et al., 2006). Neutral genetic diversity, for instance, can provide insights into the role of fragmentation and landscape structure on population structuring and movement of genes. On the other hand, adaptive genetic diversity can aid in revealing the impact of the environment on genetic diversity and assessing the adaptive potential. Because of different processes affecting diversity at adaptive and neutral loci, they are often not correlated with each other (Chung et al., 2023). Therefore, both components of genetic diversity should be considered when the aim is to obtain a comprehensive understanding of factors affecting biodiversity at the genetic level.

Genetic differentiation between populations generally increases with increasing geographical distance (Jenkins et al., 2010). This pattern is called isolation by distance (IBD; Wright, 1943). In population genetic research, the IBD assumption assumes that the landscape can be divided into two components: the suitable habitat and the unsuitable matrix surrounding it. Thus, classical population genetic approaches treat the matrix as uniform, even though it comprises diverse landscape elements with potentially distinct impacts on the gene flow of species (e.g., Aavik et al., 2014; Hahn et al., 2013). Landscape genetics is a relatively new research field that combines tools from population genetics and landscape ecology (Manel et al., 2003; Fig. 1). It stands separately from classical population genetic approaches as landscape genetic tools enable to assess how environmental heterogeneity, such as the configuration and characteristics of the landscape around and between populations, but also local environmental conditions, affect genetic diversity and gene flow in addition to the impact of geographic isolation and distance. However, the majority of landscape genetic studies are done with animals, while studies with plants are in the minority (Cruzan & Hendrickson, 2020; Storfer et al., 2010), indicating that more landscape genetic studies on plants are needed to increase understanding of the role of environmental heterogeneity on the genetic structuring of plant populations.

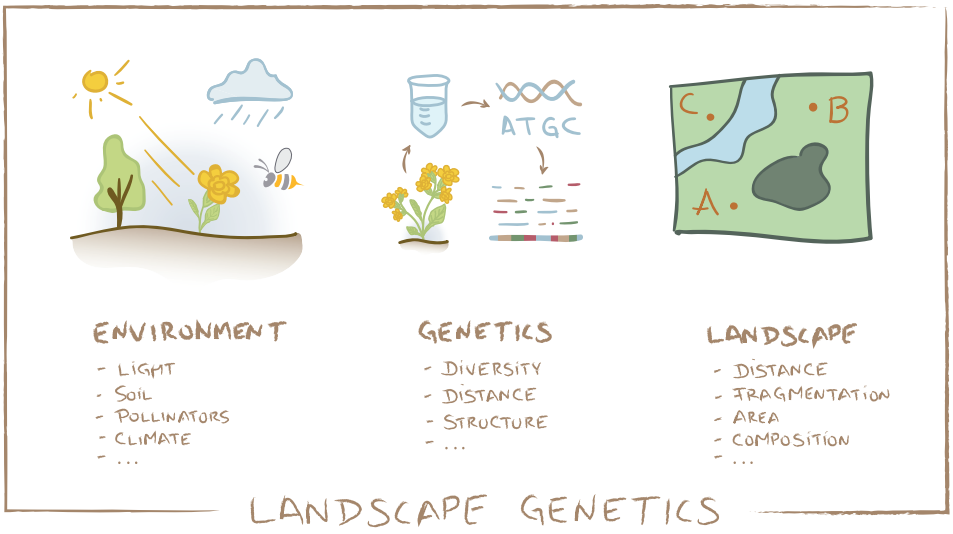
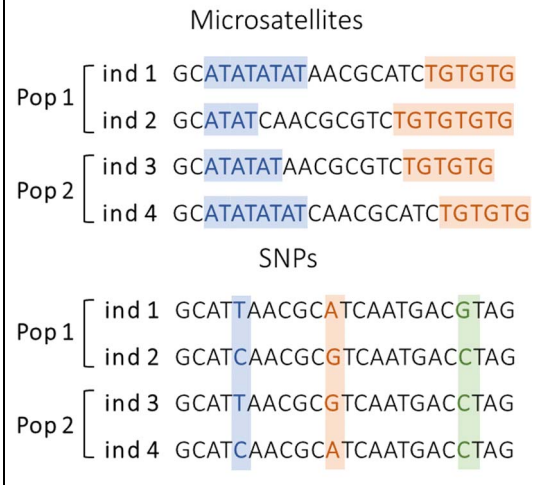


Figure 1. Components of landscape genetics. The field of landscape genetics studies how different components of landscape or environment affect the genetic variance of species or populations. Figure author Sabrina Träger.

Box 1. Measures of genetic diversity and variation based on various genetic markers can be used to quantify genetic diversity. For evaluating genetic diversity and gene flow (i.e., movement of genetic material from one population to another), the majority of landscape genetic studies have concentrated on neutral genetic markers, such as microsatellites. Genetic techniques have, however, greatly improved in recent years. The use of single-nucleotide polymorphism (SNP) molecular markers and next-generation sequencing techniques are just two examples of the genetic techniques available today that allow researchers to explore genetic diversity at a much higher number of loci, and to also concentrate on adaptive genetic diversity.



The most prevalent methods in landscape genetics are the 'link' and 'node' methods (Fig. 2; DiLeo & Wagner, 2016). The link method investigates paired relationships between populations, with the measure of gene flow, such as genetic differentiation between populations, used as the response variable, and various parameters of landscape characteristics between populations are used to explain patterns of gene flow (DiLeo & Wagner, 2016). The node method, on the contrary, focuses on within-population genetic diversity and examines the impact of landscape characteristics at and around the focal population on genetic diversity (DiLeo & Wagner, 2016). Given the stationary nature of plants, the node method is often preferred for studying plant populations. This method involves generating buffers of different radii around habitat patches, calculating, for example, the percentage of different land use types in these buffers, and relating the results with genetic data (Holderegger et al., 2010). The link method primarily assesses the effect of the characteristics and configuration of the landscape between populations and focuses on connectivity between populations, while the node method is more commonly applied to investigate the role of habitat area and the availability of suitable habitats in the surroundings of the focal population (DiLeo & Wagner, 2016).

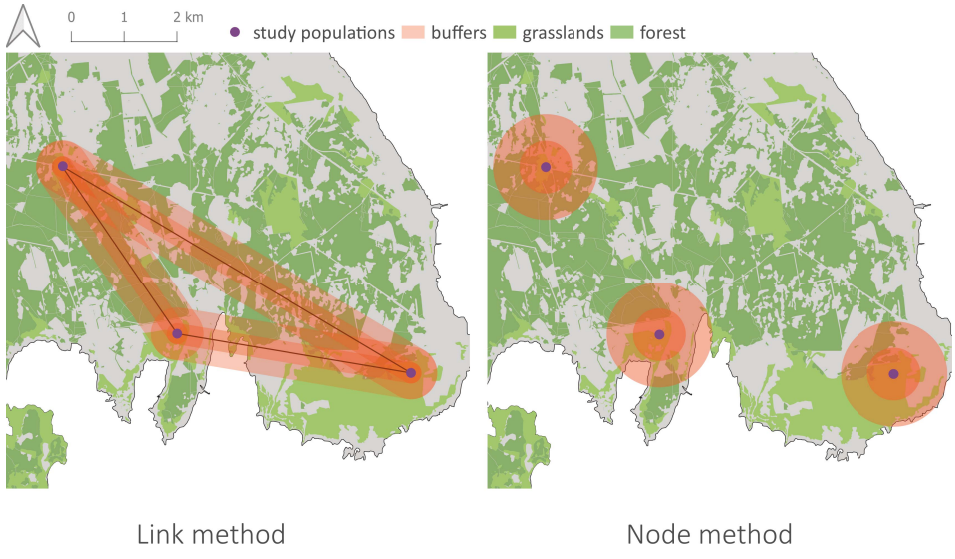


Figure 2. Two main methods for studying the influence of landscape structure on the distribution of genetic variation – the 'link' and 'node' methods. The link method focuses on relationships between populations and may use transects or corridors with different buffer radii between the populations to study the relationships between the genetic variation of these populations and the landscape between them. It is also possible to consider the whole landscape to study these paired relationships. The node method focuses on one population at a time, and assesses the role of the habitat conditions and the landscape surrounding that population on within-population genetic variation. Buffers with different radii are often used.

As indicated above, different landscape elements can influence gene flow between isolated plant populations differently, with some elements posing as complete barriers or facilitators of gene exchange, while other elements may allow gene flow from moderate to substantial levels (DiLeo & Wagner, 2016). Therefore, landscape features with distinct environmental characteristics should not be treated with equal effect when assessing the permeability of these elements on gene flow. With that in mind, landscape elements can be appointed different values for degrees of resistance (Balkenhol et al., 2015). For instance, a forest might be assigned a higher resistance value than a grain field, considering that the forest could pose a more formidable barrier for the gene flow between populations of grassland plants than an open field. These values could be added to a map, producing a resistance landscape. Subsequently, by marking a route (or several different ones) on this resistance landscape following the lowest resistance values, the accumulated resistance values yield what is termed landscape distance (e.g., least-cost paths). This may differ from geographical distance, meaning that the shortest landscape distance may not align with geographical distance. This data can be correlated with measures of gene flow between populations to examine how the structure of the landscape influences gene flow and genetic diversity (Holderegger et al., 2010). The main problem with this approach is the subjectivity of assigned resistance values, depending on the accuracy of expert evaluation (Peterman et al., 2019). Recent analysis methods, however, have found ways to surpass this, e.g., ResistanceGA (Peterman, 2018). Another option is the corridor- or transect-based approach that draws straight lines between population pairs and draws buffers with different radii around those lines to look at the influence of different landscape elements on the gene flow of study populations in those buffers (Fig. 2; Balkenhol et al., 2015; Spear et al., 2010). However, with this method, it may be possible that the most likely route between the populations will be outside the borders of the buffers (Balkenhol et al., 2015). Still, consensus about the best methodological approach to study the impact of landscape characteristics on the gene flow between plant populations is lacking (Balkenhol et al., 2009, 2015; Peterman & Pope, 2021; Shirk et al., 2017). In addition, it is also not clear how the revealing of impacts is affected by using different indices of genetic diversity and gene flow as response variables (Balkenhol et al., 2015; Epps & Keyghobadi, 2015).

In addition to landscape structure, environmental conditions at the study location may influence the genetic composition and diversity of plant populations. When the role of the environment on adaptive responses of plant populations is in focus, adaptive genetic variation needs to be addressed. Understanding such relationships is particularly relevant for assessing the adaptive potential to environmental change (Rellstab et al., 2015), but also for determining local adaptation to specific environmental conditions (Sork, 2018) as well as for exploring more complicated eco-evolutionary processes (Hand et al., 2015). As adaptive and neutral genetic diversity may not correlate with each other (Chung et al., 2023), it is important to first determine those loci which show signs of responding to selection imposed by environmental variables. This is most often

done by looking at the allele frequencies (frequencies of gene variants) of thousands of loci and comparing the frequencies to those assumed under neutral expectations. Allele frequencies of loci that exceed a certain threshold are considered outliers and are treated as potentially adaptive (Balkenhol et al., 2015). Genetic differentiation (F_{ST}) is commonly used as a genetic measure in the so-called outlier approach. Loci with F_{ST} values that are more or less diverged than expected from the distribution of genome-wide F_{ST} values are considered to be under selection. The second frequently used option is to relate allele frequencies of loci and environmental factors or gradients, and those allele frequencies showing a significant relationship to the studied environmental variables are considered putatively adaptive (Balkenhol et al., 2015). This is called environmental association analysis (EAA). The most commonly used statistical methods for EAA include parametric or nonparametric tests with categorical environmental factors, logistic regressions, matrix correlations, general linear models and mixed effects models to detect loci of adaptive relevance (Rellstab et al., 2015). Most studies using these approaches have focused on the role of climatic variables in determining variation at putatively adaptive loci, while other environmental variables crucial for plants, such as soil or light conditions, have remained neglected.

Biodiversity may respond to changes in landscape structure or environmental conditions with a lag (Chen et al., 2023). This phenomenon, coined as 'extinction debt', has been especially well-described in the species diversity patterns of plants (Chen et al., 2023; Helm et al., 2006; Lindborg & Eriksson, 2004). However, the genetic diversity of populations may exhibit similar delayed responses to alterations in land use or the environment, making it necessary to consider such lagged responses also in landscape genetic studies (Epps & Keyghobadi, 2015). For instance, plant populations subjected to significant fragmentation may still possess substantial genetic diversity despite changes in the landscape (Hahn et al., 2013). This phenomenon, where genetic diversity has not yet reacted to the changes in landscape structure, has also been referred to as 'genetic extinction debt' (Plue et al., 2017). The occurrence of time lags in response to landscape change might be influenced by various factors, such as the organism group (e.g., sessile plants may have a different response than mobile insects), species' life history traits, and the overall context of the landscape (Essl et al., 2015a). It is also possible that different indices of genetic diversity or gene flow react to landscape change at different speed and may thus show distinct results (Epps & Keyghobadi, 2015). One of the possibilities for detecting such time lags is to examine the relative impact of both historical and current landscape structure on current genetic variability (Epps & Keyghobadi, 2015). Historical landscape characteristics explaining more variation in the patterns of genetic diversity and gene flow than the characteristics of the current landscape would suggest a delayed response. Münzbergová and colleagues (2013), for example, showed that the genetic diversity of *Succisa pratensis* populations was connected to historical landscape connectivity, concluding a lagged response to reduced structural connectivity of habitats. Similarly, Reisch and colleagues (2017) found the genetic diversity of several calcareous grassland species to be related to the historical landscape

structure and not to the current one. However, plant species with short life span are less likely to exhibit signs of genetic extinction debt (Aavik et al., 2017; Helm et al., 2009). Yet, it can be expected that the genetic diversity of long-lived plants will also eventually respond to changes in landscape structure in the future when there is nothing to counteract habitat fragmentation. Although such lagged responses are increasingly addressed in conservation theory and practice, the length and existence of time lags in the reaction of genetic diversity to landscape change is still understudied and should be investigated in a more systematic way (Chen et al., 2023; Essl et al., 2015b).

During the last century, the area of semi-natural grasslands has drastically decreased throughout Europe (Cousins et al., 2015; Dengler et al., 2020; Haddad et al., 2015; Pazúr et al., 2024). Because of shifts in land management practices, many former pastures are undergoing transformations such as afforestation, overgrowth, or conversion into intensively managed agricultural fields. All of these processes have resulted in a decrease and fragmentation of grassland area as well as the deterioration of environmental conditions in the remaining grassland habitats. Consequently, many characteristic grassland species have either gone extinct locally or become rare (Hahn et al., 2013; Honnay et al., 2007). Given their substantial fragmentation, semi-natural grasslands serve as a suitable study system for examining the consequences of recent human-induced landscape changes for biodiversity. In Estonia, semi-natural grasslands have lost most of their historical area and have become more isolated during the last 100 years (Helm et al., 2006; Pärtel et al., 1999). The primary factor contributing to this decline and heightened isolation is the lack of traditional mowing and grazing practices because semi-natural grasslands require moderate management to persist. Analysing the effects of such a vast land use change on the genetic diversity of plant species in these grasslands is thus of great interest.

The main objective of the thesis was to predict the effect of recent land use change on the patterns of genetic diversity and gene flow of plants in spatially dynamic landscapes, using populations of grassland plants in a network of recently fragmented grasslands as a study system.

The specific aims of the thesis were:

(1) uncovering the effect of habitat area and connectivity, and other landscape characteristics on the neutral genetic diversity of plant populations **(I, II, III)**;

(2) examining the effect of landscape characteristics between populations on the contemporary and historical gene flow of plants in heterogeneous landscapes **(III)**;

(3) detection of possible time lags in the response of the genetic diversity and gene flow of plant populations to landscape change **(I, II)**;

(4) exploring the response of adaptive genetic diversity to landscape change **(IV)**;

(5) identifying if genetic diversity at adaptive loci exhibits a different response to habitat change than genetic diversity at neutral loci **(IV)**;

(6) exploring the effect of applying different landscape genetic methodological approaches on the results and conclusions (**II, III, IV**).

Finally, the thesis will provide recommendations about the use of genetic information in planning restoration and conservation activities.

2. METHODS

2.1. Study species

I used two study species to answer the questions of the thesis: *Trifolium montanum* (Fig. 3) and *Primula veris* (Fig. 3). These species were chosen because they are insect-pollinated outcrossing grassland plants and are thus especially vulnerable to landscape change (Clough et al., 2014). Insects' reaction to landscape change (Vasiliev & Greenwood, 2023) is most probably reflected also in the genetic variation of these plants.

Figure 3. Photos of the study species *Trifolium montanum* (left; photo author: Kalninis Dobilas) and *Primula veris* (right; author: Iris Reinula).

The focus of paper **I** was on the genetic diversity of *Trifolium montanum* (Fabaceae), a perennial plant characteristic of calcareous grasslands. *T. montanum* flowers in June and July and is a mostly outcrossing species depending mainly on pollination by bumblebees and bees (Pettersson & Sjödin, 2000). Vegetative reproduction in this species is limited (Klimeš & Klimešová, 1999). It has a relatively long life span (avg. four years, max 17 years; Tamm et al., 2002). *T. montanum* seeds lack a specific dispersal mechanism, leading to the majority of them staying in close proximity to the parent plant. The seed bank of *T. montanum* has a relatively short life span, with a significant portion of seeds losing their germination potential within a year (Thompson et al., 1997).

The focus of papers **II–IV** was on *Primula veris* (Primulaceae), a perennial plant common in calcareous grasslands, but occasionally, it can also occupy road

verges, forest edges and abandoned old fields on calcareous soils. *P. veris* is a heterostylous obligate outcrossing plant depending on insects for effective pollen flow. In Estonia, *P. veris* mostly flowers in May. The flowers are pollinated mainly by different species of Hymenoptera (e.g., bees) but also by some species of Coleoptera (beetles) and Lepidoptera (butterflies, moths). Pollen dispersal is limited to a few meters, and most of the seed dispersal to a few centimetres from the maternal plant (Brys & Jacquemyn, 2009). The clonal spread of the species is very limited (Rossum & Triest, 2007). The approximate average life span of *P. veris* is about 50 years (Ehrlén & Lehtilä, 2002).

2.2. Study design

All studies were done on the islands of Saaremaa and Muhu in Western Estonia (Fig. 4). As a study system, I used calcareous semi-natural grasslands, most of which fall into the category of alvars. Alvars are calcareous grasslands with a thin soil layer on limestone bedrock that are managed mainly by grazing. Lack of management leads to overgrowth of these grasslands by trees and bushes (mainly by *Juniperus communis*). Until the second half of the last century, alvars were quite widespread in Western Estonia (Laasimer, 1965). However, during the last century, most of these grasslands have been overgrown or turned into intensively managed fields due to land-use change and intensive agriculture (Helm & Tous-saint, 2020; Pärtel et al., 1999). The sites of papers **II** and **IV** were part of the EC LIFE+ programme restoration project "LIFE to Alvars" (LIFE13NAT/EE/ 000082; Helm, 2019). The aim of this project was to restore the most valuable but currently overgrown alvar grassland complexes in Western Estonia. The main technique for restoration was the removal of trees and bushes. Additionally, conditions were created for local farmers to manage these areas after restoration, and wide-based biodiversity monitoring was done (Prangel et al., 2023).

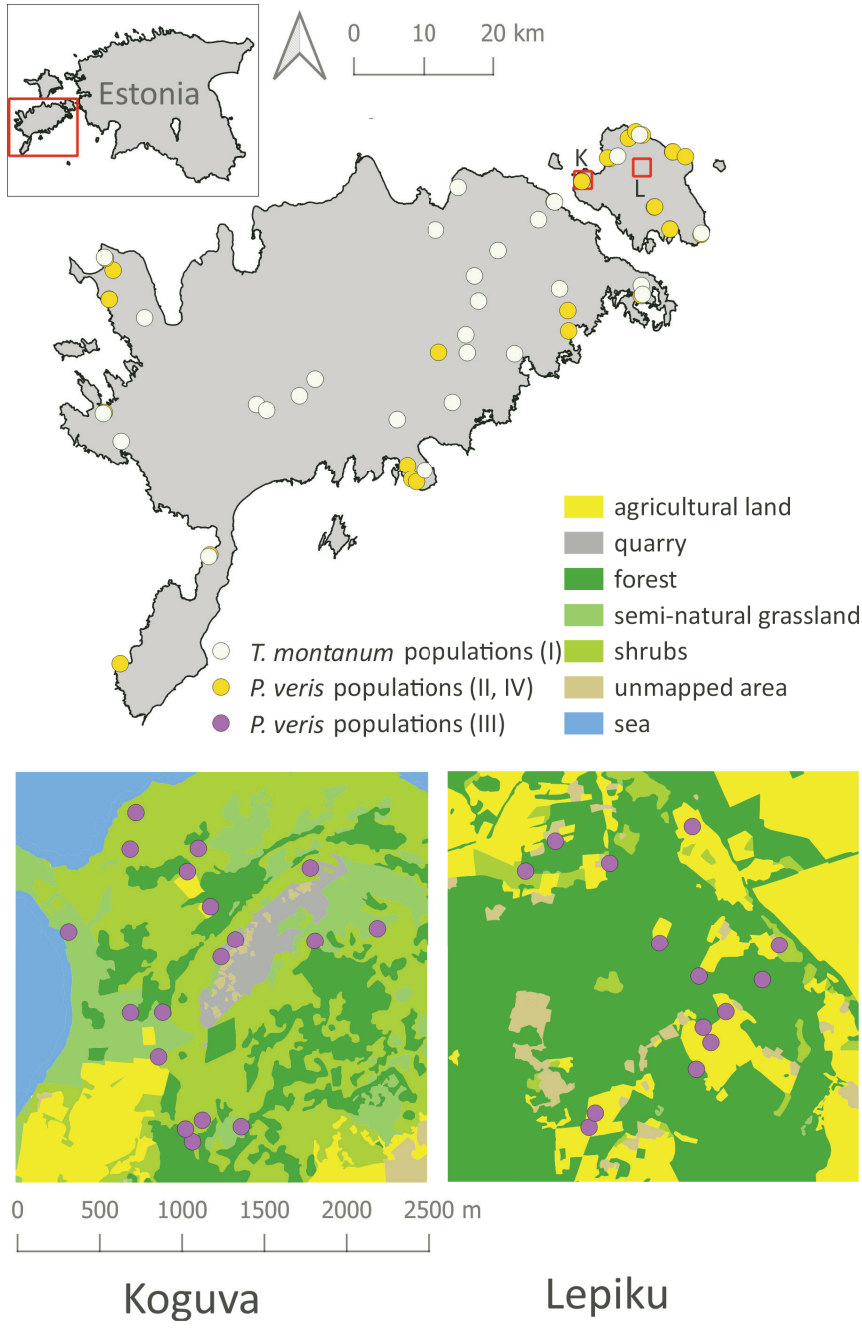


Figure 4. The map of the study populations of *Trifolium montanum* (**I**) and *Primula veris* (**II–IV**), and the study landscapes in Saaremaa and Muhu, Estonia. Papers **I, II,** and **IV** were done on a regional scale, and paper **III** was done on a landscape scale in two landscapes (Koguva and Lepiku, marked with red squares) in Muhu. The current land cover in the study landscapes examined in paper **III** is shown in the lower part of the figure.

For collecting samples in papers **I**, **II**, and **IV**, a node-specific study design (i.e., focal sites occurring throughout Saaremaa and Muhu were chosen) was used (Fig. 4). Samples were collected from 28 (**I**), 19 (**II**) or 32 (**IV**) populations. Populations from papers **II** and **IV** partly overlapped. In paper **II**, only populations in open habitats (Fig. 5) were sampled, whereas, in paper **IV**, populations from overgrown habitats (Fig. 5) neighbouring open grasslands were also examined (i.e., ten pairs of open and overgrown grasslands and 12 open and overgrown grasslands that were not paired). For paper **III**, a more detailed landscape-scale approach was used (Fig. 4). Two 2 × 2 km landscapes in Muhu island (Koguva and Lepiku) were selected as study landscapes. Both landscapes were historically dominated by semi-natural grasslands, but nowadays, grasslands in one of the landscapes (Lepiku) have mostly been overgrown or were turned into intensively managed fields, whereas the other study landscape (Koguva) still comprises vast areas of alvar grasslands. From these landscapes, all *P. veris* populations found in the area (14 and 18, respectively) were sampled. The leaves of 20 (**II–IV**) or 30 (**I**) randomly selected individuals (wherever possible) were collected from each study population. Leaf samples were stored on silica gel until further processing. Population sizes were also estimated. Populations were sampled before the active restoration of grassland complexes within the frames of the "LIFE to Alvars" project.

Figure 5. Open (left) and overgrown alvar grasslands (right) in the study populations of papers **II** and **IV** in Saaremaa and Muhu. Author: Iris Reinula.

2.3. Laboratory work

Leaf material was ground, and DNA was extracted using the Dneasy 96 Plant Kit (QIAGEN; **I**) or the LGC sbeadex plant maxi kit (LGC) with some modifications (**II–IV**). For genetic analysis of *T. montanum* (**I**), we used 11 following microsatellite markers: ats002, ats006, ats029, ats032, Tm10, Tm12, Tm13, Tm16, Tm17, Tm21 and Tm24. Microsatellite regions were amplified in polymerase chain reactions (PCR) following the protocol by Matter and colleagues (2012). For the genetic analysis of *P. veris* (**II–IV**), we used double-digest restriction site-associated DNA sequencing (ddRADseq; Peterson et al., 2012). Shortly, the ddRADseq method uses two restriction enzymes (in the present thesis, EcoRI and TaqI) to cut standardised DNA in a two-step process. Following a purification

step, DNA fragments were ligated to corresponding adapters (48 EcoRI adapters and 2 TaqI adapters). Samples containing different EcoRI adapters but the same TaqI adapter (48 samples) were pooled together. Pooled samples were size-selected for fragments with a length of 450 bp and biotin-labelled TaqI adapters. Then, a PCR was done, and PCR products (ddRAD libraries) were purified. For the final steps prior to sequencing, the molarity of the final ddRAD libraries was calculated according to their mean DNA fragment size. Sequencing libraries with distinct multiplex indices were combined, resulting in a final library of at least 5 nM consisting of 96 individuals. Pooled libraries were prepared according to the guidelines of the sequencing facility and sequenced on an Illumina HighSeq2500 (Illumina, Inc, San Diego, CA, USA) at the Functional Genomics Center Zurich (Switzerland), using one lane per library with 125 cycles in single-end read (125 bp), high-output mode.

2.4. Bioinformatics analysis and acquisition of genetic data

For paper **I**, we used FSTAT 2.9.3.2 (Goudet, 1995) to test for linkage disequilibrium between loci within populations and to assess deviations from Hardy–Weinberg equilibrium (HWE) expectations per locus in the populations. Reads (sequenced DNA fragments) from papers **II–IV** were bioinformatically analysed and filtered. Reads were demultiplexed, and PCR duplicates were removed using STACKS version 1.47 (Catchen et al., 2011, 2013). Sequences were filtered using TRIMMOMATIC v0.36 (Bolger, Lohse, & Usadel, 2014) and then aligned and mapped against a draft reference genome of *P. veris* (Nowak et al., 2015) using BURROWS-WHEELER ALIGNER v0.7.17 (BWA; Li, 2013). SNP detection was done using FREEBAYES v1.1.0-54-g49413aa (Garrison & Marth, 2012). To exclude SNPs with low quality and individuals with too much missing data, SNP filtering was done using VCFTOOLS v0.1.12b (Danecek et al., 2011) following DDOCENT SNP Filtering Tutorial (Puritz, Hollenbeck, et al., 2014; Puritz, Matz, et al., 2014) with some adjustments. Indels (i.e., insertions or deletions of bases in the genome) and loci potentially in linkage disequilibrium were removed using VCFTOOLS. Loci showing excess heterozygotes were filtered in R v3.4.2 (R Development Core Team, 2017). The genotyping error of filtered SNPs was calculated by the weighted mean of error rates using replica samples (i.e., positive controls) with TIGER v1.0 (Bresadola et al., 2020).

Putatively adaptive SNPs were identified through linear (mixed effect models) and categorical environmental association analysis (EAA) to detect SNPs associated with environmental factors related to habitat overgrowth (**II–IV**)**,** and F_{ST} outlier test using BAYESCAN v2.1 (Foll & Gaggiotti, 2008) to detect SNPs under potential diversifying or balancing selection (**II–IV**). For the linear EAA analysis, we used latent factor mixed models (lfmm_ridge and lfmm_test functions in LFMM v2.0 in R; Caye et al., 2019), examining linear relationships of the allele frequency (AF) at each SNP with each environmental variable (see below), while accounting for population structure with random latent factors.

False discovery control was applied using the inflation factor λ and the χ2 distribution (Caye et al., 2019; François et al., 2016) to adjust p-values and the Benjamini-Hochberg algorithm (Benjamini & Hochberg, 1995) with a false discovery rate (FDR) of 0.05. Categorical EAA comprised paired t-tests and Wilcoxon signed-rank tests on population AFs of geographically close but environmentally diverged population pairs. The putatively beneficial allele for open habitat was determined for each SNP, and the average AF change from open to overgrown habitat was calculated within pairs. Deviations from random ratios were tested using an exact two-sided binomial test in R. Using linear and categorical EAAs to identify potentially adaptive genetic loci in relation to specific environmental factors and to assess the impact of habitat variation (which stem from these same environmental factors) on genetic diversity indices could lead to circularity issues. To see if this might be a problem, 100 SNP datasets with randomised genotype assignments were created and subjected to the same EAA and F_{ST} outlier analyses. Additionally, we did all the same steps (for both original and shuffle data), but without FDR in linear EAA and with FDR in both linear and categorical EAA, to further confirm the validity of our approach and results. Putatively adaptive SNPs were either excluded to separate neutral SNPs (**II**, **III**) or used for further analysis (only EAA; **IV**).

We calculated population-based genetic diversity indices: allelic richness (A_R; calculations were based on a minimum sample size of 13 individuals; using FSTAT; **I**), gene diversity (H_E; Nei, 1973; using FSTAT; **I**) and inbreeding coefficient (F_{IS}; Wright, 1965); using FSTAT for **I**, using the package "genepop" (Rousset, 2008) in R (R Core Team, 2024) for **II**, **III**), unbiased expected heterozygosity (uH_E; using GenAlex version 6.503, Peakall & Smouse, 2005, 2012; **II**, **III**), observed heterozygosity (H_O; using GenAlex; **II**), percentage of polymorphic loci (%P; using GenAlex; **II**) and mean nucleotide diversity (π) using VCFTOOLS within a window of 125 bp over all loci for each population (**III–IV**). We calculated population-based pairwise genetic distance indices: genetic differentiation (F_{ST}; **II–IV**), using the package "genepop" (Rousset, 2008) in R (R Core Team, 2024) as a measure of "historical" gene flow and pairwise mean assignment probability (MAP; **III**) in R (R Core Team, 2024) using *AssignPop* (Chen et al., 2018) as a measure of "recent" gene flow (i.e., in about 1–2 generations).

2.5. Environmental data

For paper **IV**, we selected 16 in-situ measured environmental variables with the potential to represent contrasting habitat types (open and overgrown) falling into the following categories: "openness", "soil", "biota", and "climate". For openness, we assessed the total percentage of shrub and tree cover, and the light availability above and below the herbal layer measured with a light meter. For soil, we measured average soil depth, soil pH (KCl solution), available soil phosphorus (P; extraction with acid ammonium lactate solution), potassium (K), magnesium

(Mg), calcium (Ca), and soil organic content (OC, loss on ignition). As variables characterising biotic environment, we used butterfly and bumblebee abundance and richness, and vascular plant richness. In addition, we extracted climate data for each population from CHELSA (Karger et al., 2017): temperature (Bio1, annual mean temperature) and precipitation (Bio12, annual precipitation sum), totalling 18 environmental variables. To test which environmental factors significantly differed between the two habitat types, we performed a (non-paired) two-sample t-test for each of the 18 environmental variables.

2.6. Landscape data

To assess the role of landscape characteristics on the genetic patterns of *T. montanum* (**I**) and *P. veris* (**II–III**), we used historical and current landscape data. We obtained historical landscape data (woody elements and grasslands) from digitalised maps of a historical vegetation survey, which was carried out in the 1930s, that is when the distribution of Estonian alvar grasslands was at its maximum (Laasimer, 1965; **II**, **III**). The area of current grasslands was assessed by combining the most recent information on the current distribution of Estonian semi-natural grasslands originating from the database of grassland inventory by the Estonian Semi-natural Grassland Conservation Association and from the Estonian Nature Information System (Estonian Environmental Agency; **I–II**). Other current landscape data was obtained from the Estonian Topographic Database (1:10,000; Estonian Land Board; **I–III**) and aerial photos of the study area from 2015 (Estonian Land Board; **III**). Spatial analyses were done in QGIS version 2.18.14 (QGIS Development Team, 2017) and ArcGIS 10.2 software (ESRI 2012).

More precisely, we estimated the current and historical patch area of study grasslands (**I**), the area of current and historical cover of alvar grasslands and woody vegetation in circular buffers with radii of 500, 1000 and 2000 m (**I**, **II**), their change (**II**), and the grassland edge density (i.e., the length of grassland habitat edge in the buffer; m/ha; **II**). We also calculated the proportion of the current and historical areas of alvar grassland and woody elements in straight corridors of different widths (200 m, 500 m and 1,000 m radius) between population pairs and the change in the relative proportion of these elements (**II**). In the landscape-scale study, we estimated the proportion of current semi-natural grassland, agricultural land, shrubs, forest and quarry in straight corridors with a radius of 50 m (**III**). Additionally, for the same study, we assessed the current land use for the whole study landscapes (2.5 x 2.5 km) and divided the landscapes into the same five land use categories (**III**).

2.7. Data analysis

The genetic structure of populations was analysed with discriminant analysis of principal components (DAPC) in "adegenet" v2.1.1 (Jombart, 2008) package (**III**, **IV**). DAPC uses uncorrelated principal component analysis (PCA) variables for discriminant analysis (Jombart et al., 2010). The optimal number of principal components was found using cross-validation, and either populations were used as clusters (**III**) or the function *find.clusters* was used to determine the optimal number of clusters within the data sets (**IV**).

For the node-based analyses, after excluding some strongly correlated landscape variables with different radii, we conducted linear models to examine the effect of previously mentioned landscape variables and population size (**I, II**) on different genetic diversity indices (A_R, **I**; H_E, **I**; F_{IS}, **I**, **II**; uH_E, **II**; H_O, **I, II**). We carried out generalized linear models to examine the response of %P applying a quasibinomial error distribution (package "lmerTest", Kuznetsova et al., 2017; **II**). We also included region (Muhu/Saaremaa) as a co-variable in the analysis to account for potential differences in the land use trajectories between regions and included the interactions between region and other landscape variables in the models (**II**). We started with full model(s) and, based on Akaike information criterion corrected for small sample sizes (AICc; Burnham & Anderson, 2002) and used either model averaging with a multi-model inference analysis to determine the most important predictor variables ("MuMIn"; Barton, 2018; **I**) or stepwise selection with the function *stepAICc* (http://www.chris toph-scherber.de/stepAICc.txt; **II**) to find the models with the best fit. We considered models with $\Delta AICc < 2$ as equally good. Because AICc is not calculated for generalized linear models with quasibinomial distribution used for %P as a response variable, we began by building the full models and manually excluded the least important variables (according to p-value), starting with interactions. We compared the models with likelihood ratio tests until the model with fewer variables explained the variation in data significantly ($p < 0.05$) better than the respective more complex model (**II**). Additionally, we analysed the effect of landscape change (proportional change in the patch area of the study grasslands, and the area of grasslands and woody habitats in the surroundings of the study populations) on the probability of the occurrence of recent bottleneck in the study populations (**I**). For this, we used generalized linear models applying a binomial error distribution.

For the link-based analyses, after excluding some strongly correlated landscape variables with different radii, we conducted multivariate generalized mixed effect models (package "MCMCglmm"; Hadfield, 2010) to examine the effect of previously mentioned landscape variables on pairwise genetic differentiation (F_{ST}; **II**, **III**) and pairwise mean assignment probability (MAP; **III**). Population identity (**II**; **III**) and region (**II**) of both populations in a pair were used as random variables in covariance matrices to account for potential non-independence of data points in distance matrices (maximum likelihood population effect (MLPE) models; Clarke et al., 2002; van Strien et al., 2012). We made all possible model

combinations with geographic distance forced in the models and strongly correlated variables in different initial models. We also made null models with only random variables. We then assessed all models according to the deviance information criterion (DIC), and models with $\Delta DIC < 2$ were treated as equally good. We also assessed the influence of geographic distance on F_{ST} with multivariate generalized mixed effect models (package "MCMCglmm"; Hadfield, 2010) and characterised the scale of it with multiple simple linear functions (package "stats" 3.4.2, *lm* function; Chambers, 1992; **IV**).

In paper **III**, we created resistance surfaces using "ResitanceGA" (Peterman, 2018) to assess the influence of landscape elements on gene flow. We evaluated the influence of geographic distance between *P. veris* populations and land cover raster data of five land use categories (except quarry in Lepiku) on the two tested gene flow indices (F_{ST} and MAP) per landscape, resulting in two different resistance surfaces for each landscape. In addition to the default models resulting from the analysis (null model, model with geographical distance, model with resistance distance), we made an additional model with both geographical distance and resistance distance. For each landscape and genetic distance metric, we evaluated these four models with the Akaike information criterion corrected for small sample sizes (AICc; Burnham & Anderson, 2002). We treated models with $\Delta AICc < 2$ as equally good.

In paper **IV**, we examined the influence of habitat type (open or overgrown) and SNP set (neutral or adaptive), and their interaction, on genetic indices (Ho, π) using linear mixed effect models ("lmerTest" v3.1-0, lmer function; Kuznetsova et al., 2017), for the original and each randomised data set. The significance of fixed factors and their interaction was assessed via the likelihood ratio test, analysing the variance between the full and reduced models with Satterthwaite approximation (c2 and associated p-values). Particularly, we focused on the interaction between habitat type and SNP set, which, if significant, indicates differing behaviours of genetic diversity indices at putatively neutral and adaptive loci in open and overgrown habitats. Post-hoc tests with least square means ("lsmeans" v2.30-0, Lenth, 2016) were conducted to examine differences in genetic diversity indices between habitats for each SNP set in case of a significant interaction. To compare results between the original and randomised data sets, we calculated permutation p-values for each factor, assessed the distribution of putatively adaptive SNPs with increasing and decreasing beneficial allele frequencies (AF) using a t-test, and analysed the mean Ho and π per population across all 100 randomised data sets using a mixed-effect model analysis as done for the original data set.

3. RESULTS

In all study systems, the area of grasslands has significantly decreased since the 1930s. In the study system explored in paper **I**, most grassland patches had lost more than 90% of their original area. The area of alvars surrounding the study populations in a circular buffer (r = 1,000 m) explored in paper **II** had decreased on average by about 53 % since the 1930s. Among the two landscapes examined in paper **III**, Koguva, the less fragmented landscape, had 88 % of the area historically covered by calcareous grasslands. In Lepiku, the more fragmented landscape, semi-natural grasslands occupied 82 % of the landscape. Nowadays, 16 % of the study area of Koguva is covered by semi-natural grasslands, while in Lepiku, only 0.1 % of the area is covered by semi-natural grasslands.

3.1. Within-population neutral genetic diversity

Different measures of genetic diversity of *P. veris* were, on average, higher in Muhu than in Saaremaa (**II**). At the landscape scale, the genetic diversity was significantly ($p < 0.05$) higher in Lepiku, the more fragmented landscape, than in Koguva (**III**). Allelic richness (A_R) of *T. montanum* populations increased with the current population size (**I**), whereas population size did not affect the genetic diversity of *P. veris* populations (**II**).

Different measures of genetic diversity for both study species, *T. montanum* and *P. veris*, had varying responses to past and current landscape variables (Table 1). Observed heterozygosity (H_O) and inbreeding coefficients (F_{IS}) of the *T. montanum* study populations (**I**) responded significantly to historical landscape variables (Fig. 6). H_O increased with higher historical grassland area in the surroundings of the study populations. Correspondingly, F_{IS} decreased with higher historical landscape-scale habitat area. By contrast, allelic richness (A_R), as well as expected heterozygosity (H_E) of *T. montanum*, responded to current landscape variables. H_E increased significantly with both the current patch area and current landscape-scale area of grasslands in the surroundings of the study populations (Fig. 6). Genetic diversity (H_O, H_E and %P) of *P. veris* populations (**II**) increased with higher historical edge density (Fig. 7). The change in the area of grasslands had a negative effect on the proportion of polymorphic loci (%P) at smaller spatial scales (r = 500 m and r = 1,000 m) but a positive effect in models, which included landscape variables calculated within the buffer with the largest radius (r = 2,000 m). At a larger spatial scale (buffer radius of 2,000 m), H_O decreased in landscapes with a more substantial loss of grassland area. Historical grassland area had a significant negative effect on H_E at the buffer radius of 1,000 metres but a positive effect on %P (r = 500 m). Current grassland area had a significant positive effect on %P at a smaller scale (r = 500 m), but at larger scales, a negative effect on H_E (r = 2,000 m) and H_O (r = 1,000 m, Saaremaa; r = 2,000 m). Current grassland edge density positively affected %P (r = 500 m) but, surprisingly, had a negative effect at a larger spatial scale (r = 2,000 m). In one model (r = 1,000 m), current grassland edge density had a negative effect for

%P in Muhu but a positive effect in Saaremaa. The historical area of woody elements in the surroundings of the study populations had a positive effect on %P. The current area of woody elements in the surroundings of the study populations had a negative effect on %P in Muhu.

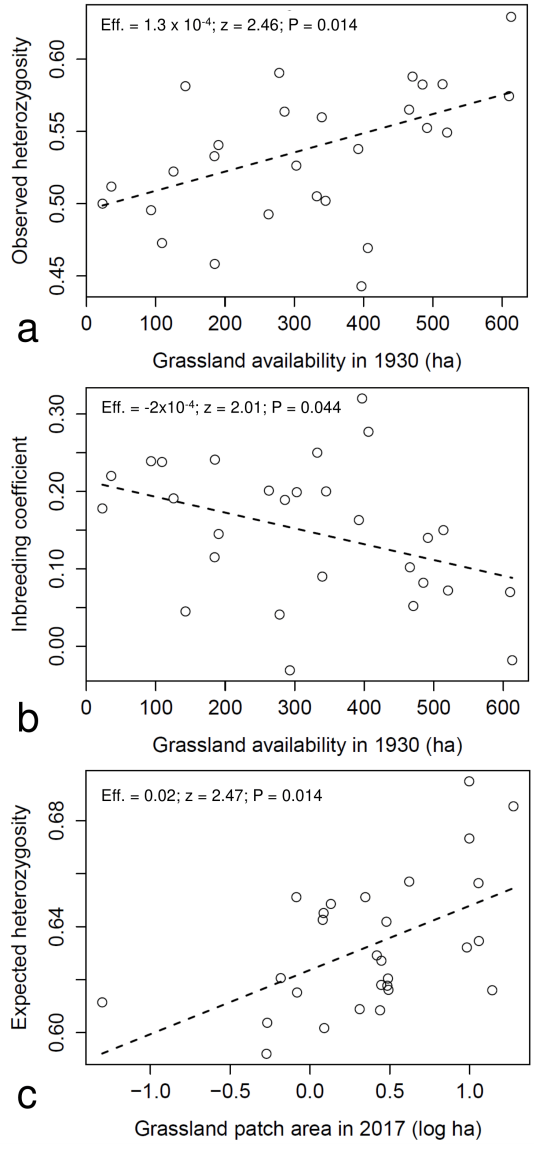


Figure 6. The effect of past and current landscape variables on the genetic diversity of 28 populations of *Trifolium montanum* on the islands of Muhu and Saaremaa, Estonia (**I**). The effect of historical landscape-scale grassland availability in the surroundings of the study populations (r = 2000 m) on observed heterozygosity (H_O; a) and inbreeding coefficient (F_{IS}; b), and the effect of current grassland patch area (log-transformed) on expected heterozygosity (H_E). Effect sizes, z- and p-values are based on model averaging using multi-model inference analysis.

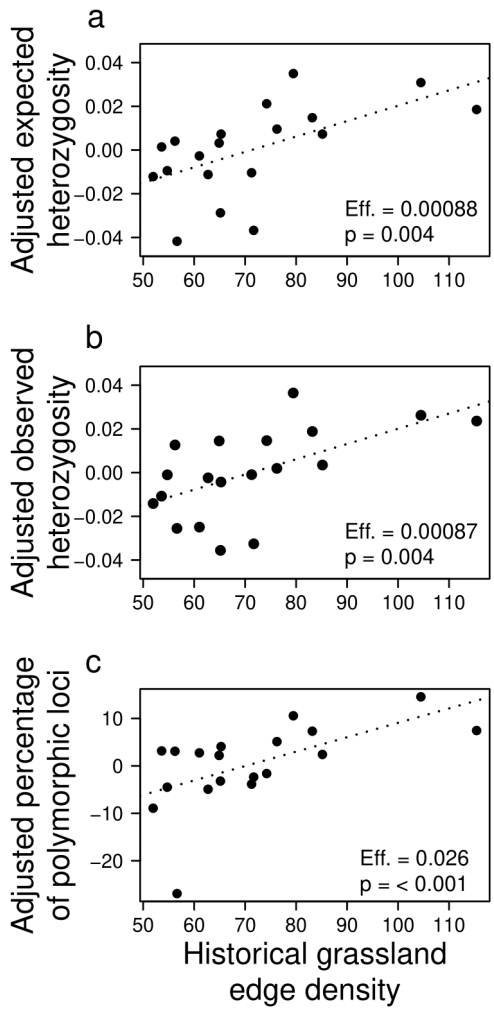


Figure 7. Relationship between genetic diversity indices (a – expected heterozygosity H_E; b – observed heterozygosity H_O; c – proportion of polymorphic loci P%) of *Primula veris* study populations on Muhu and Saaremaa in Estonia and the historical grassland edge density (**II**). Genetic diversity indices are plotted as residuals (adjusted) after removing the effect of the influence of the current grassland area (only for %P) in a circular buffer with a radius of 500 m from the study populations and region. 'Eff.' indicates the effect size from the respective best model according to AICc, and the p-value represents the corresponding values from the best model.

Table 1. Direction of significant effects of current and historical landscape elements on the genetic diversity and gene flow of *Trifolium montanum* (**I**) and *Primula veris* (**II–III**) populations in Saaremaa and Muhu islands in Estonia. Overall results per species (only for genetic diversity indices, observed heterozygosity H_O, inbreeding coefficient F_{IS}, allelic richness A_R, expected heterozygosity H_E and percentage of polymorphic loci %P) and more specific results per index are shown. Interaction effects with region ('reg') are shown with Saaremaa ('Saare') or Muhu added in the parentheses. The radius of different circular buffers is shown in meters for paper **II**. 'geo. dist.' stands for geographical distance between pairs of populations, 'pop. size' for population size, 'F_{ST}' for genetic differentiation, and 'MAP' for pairwise mean assignment probability. Note that higher F_{ST} means less gene flow and higher MAP means more gene flow.

				current landscape variables					historical landscape variables				
	reg	geo. dist.	pop. size	grass-land patch area	grassland area in buffers	forest and shrubs	grassland edge density	agri-cultural land	grass-land patch area	grassland area in buffers	forest and shrubs	grassland edge density	grassland area change in buffers
T. montanum			+	+	+					+			
H_O										+			
F_{IS}										–			
A_R			+										
H_E				+	+								
P. veris	–				+/–	–	+/–			+/–	+	+	+/–
H_E	–				– (2000 Saare)					– (1000)		+ (500, 1000)	
H_O	–				– (1000 Saare, 2000)					– (1000)		+ (500)	– (2000)
F_{IS}													
%P					+ (500)	– (1000 Muhu, 2000 Muhu)	+ (500); – (2000)			+ (500)	+ (500, 1000, 2000 Saare)	+ (500, 2000)	– (500, 1000, 1000 Muhu); + (2000, 1000 Saare)
F_{ST} (**II**)		+								–			
F_{ST} (**III**)		+				+ (shrubs)							
MAP		–				+ (forest)		–					

3.2. Within-population adaptive genetic diversity

In paper **IV**, we identified 78 SNPs from categorical EAAs, which overlapped between the two tests (paired t-test and Wilcoxon test) and three SNPs from linear EAAs. These SNPs were used for further analyses. In the 81 SNPs that were putatively involved in adaptation to habitat type, 55 SNPs showed a decrease in the average beneficial AF (for the old, open habitat) in the new, overgrown habitat compared to the open habitat. The binomial test revealed that this pattern significantly differed from a random expectation ($p < 0.01$). There was no significant difference in H_O or nucleotide diversity (π) in the neutral SNP dataset between populations in open and overgrown habitats (post-hoc test for H_O: $p = 0.91$; for π: $p = 0.97$; Fig. 8). For the adaptive SNP dataset, there was a significant difference of H_O between populations in open and overgrown habitats (post-hoc test: $p < 0.05$), with populations in overgrown habitats exhibiting increased H_O compared to populations in open habitats (Fig. 8). For π, there was a marginally (non-)significant interaction effect of habitat type and SNP set ($p = 0.056$; Fig. 8).

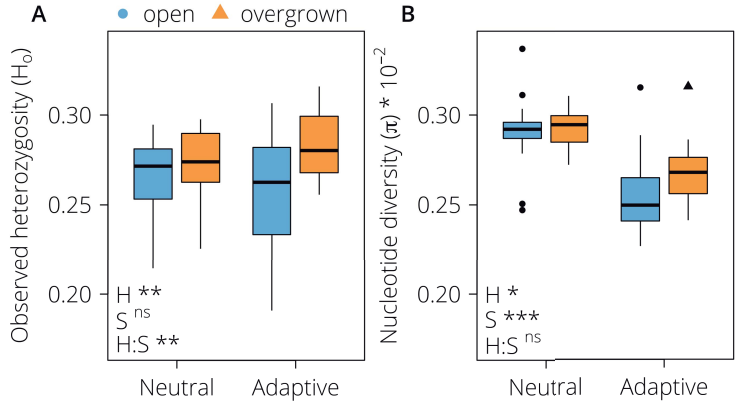


Figure 8. Boxplots of observed heterozygosity (H_O; A) and nucleotide diversity (π; B) at putatively neutral and adaptive loci in open (blue, circles) and overgrown (orange, triangles) grasslands from paper **IV**. Factors: habitat (H): open and overgrown; SNP_set (S): SNP_neutral and SNP_adaptive. Significance values: $^{ns}\ p > 0.05$; $^{*}\ p \leq 0.05$; $^{**}\ p < 0.01$; $^{***}\ p < 0.001$.

3.3. Indicators of gene flow between populations

The average genetic differentiation (F_{ST}) among populations was significantly lower in Koguva than in Lepiku. The average pairwise mean assignment probabilities (MAP) in Koguva and Lepiku were not significantly different. DAPC showed that Lepiku and Koguva populations separate into two genetically distinct clusters, with Lepiku populations showing greater variance among populations compared to Koguva populations (**III**). The isolation by distance (IBD) for *P. veris* reached a plateau in about 15–30 km (**IV**).

In the link-based approach of paper **II**, the best generalized mixed effect models about the role of different landscape elements between pairs of populations on pairwise F_{ST} according to DIC had a buffer radius of 1,000 m around a straight line between populations. In the best model, geographic distance had a significant positive effect on F_{ST}, while a higher historical proportion of grasslands led to lower genetic differentiation (i.e., indicating more gene flow; Fig. 9).

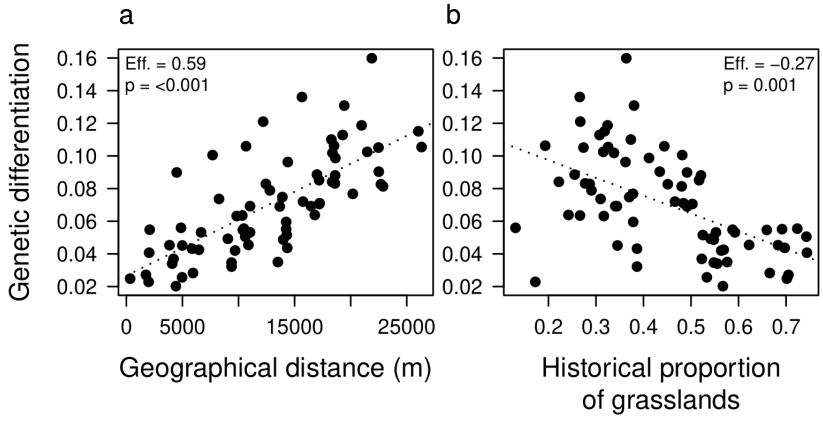


Figure 9. The relationship between pairwise genetic differentiation (F_{ST}) of *Primula veris* study populations (**II**) in the node-based analysis and geographical distance (a) and historical proportion of grasslands (b). Eff. indicates the effect sizes of the best generalized mixed effect model and the p-values of the best model according to DIC.

The resistance surface analysis (ResistanceGA) applied in paper **III** revealed that according to AICc (ΔAICc < 2), the null models with only random variables were the best-supported models when using F_{ST} as a measure of gene flow in both landscapes, and also when pairwise mean assignment probability (MAP) was used as a measure of gene flow in Lepiku. For MAP in Koguva, there were two models with equal support: the null model and the one with geographical distance. In contrast, the corridor-based analysis of paper **III** revealed the significant influence of some landscape variables. The best-supported models in the less fragmented site (Koguva) included a significant positive effect of geographical distance and shrubs on F_{ST}. In Lepiku, the more fragmented site, the best-supported models demonstrated a surprising significant negative effect of geographical distance on F_{ST}. Among the best-supported models in Koguva, a significant negative effect of geographical distance on MAP was found (note that genetic distance grows with higher F_{ST} values and declines with higher MAP). In Lepiku, the best models featured the significant positive effect of forest and the significant negative effect of agricultural land on MAP.

4. DISCUSSION

4.1 The effect of landscape characteristics on the neutral genetic diversity

In this thesis, I studied the patterns of neutral genetic diversity and gene flow of grassland plants at both regional and landscape scales, using semi-natural grasslands as a study system and applying different landscape genetic methods. In addition, I examined the adaptive genetic variation in populations of a grassland plant*, Primula veris,* in response to the overgrowth of grasslands. I found that a larger grassland area and a higher proportion of grasslands in the surroundings of the study populations generally had a positive effect on the genetic diversity of grassland plants (**I–II**). Additionally, in paper **I**, I found that population size positively affects the genetic diversity of *Trifolium montanum*. The positive relationship between population size and/or patch area and plant genetic diversity is a commonly observed finding (Balkenhol et al., 2015; Honnay & Jacquemyn, 2007), that demonstrates the importance of preserving large populations for maintaining biodiversity at the genetic level (Hahn et al., 2013; Toma et al., 2015) even in case genetic measurements are not applied. The finding also adds validity to the recently proposed recommendation to implement effective population size as one of the proxies for genetic diversity in conservation applications (Hoban et al., 2024). However, the finding of "the larger – the better" is not so straightforward. In paper **II**, I found that an increasing grassland edge density results in higher genetic diversity of *P. veris*. Several possibilities may explain this finding. First, heterogeneity of environmental conditions caused by more edge habitats may support a higher variation of plant genotypes adapted to different environmental conditions (Karbstein et al., 2020). Second, such heterogeneous grasslands may be more attractive to pollinators who are more likely to find shelter and habitats, but possibly also more resources for feeding in grasslands intertwined with a moderate area of woody elements compared to more monotonous large grasslands (Bergman et al., 1996; Neumüller et al., 2020). Thus, in addition to the characteristics of the habitat patch itself, the broader availability of grasslands, as well as grassland heterogeneity, are significant for maintaining the genetic diversity of grassland plants.

Other landscape elements in the surroundings of habitats may also play a role in determining the genetic diversity of grassland plants. In paper **II**, I found that an increasing present-day proportion of woody elements had a negative effect on the genetic diversity of *P. veris.* It is in line with the previous observations that a higher area of forests and shrubs in the surrounding landscape can have a negative effect on the genetic diversity of grassland plants (Aavik et al., 2014, 2017; Aguilar et al., 2006; DiLeo et al., 2018; Hahn et al., 2013; Schmitt et al., 2000). However, I also found that the higher proportion of historical woody elements in the surroundings of the study populations had a positive effect on the genetic diversity of *P. veris*. This controversial result is most probably explained by the

specific relative proportion of woody elements. Historically, the landscapes in the study region of Western Estonia were very open, with vast areas covered by alvar grasslands. In such landscapes, a moderate area of forest and shrubs may have increased the heterogeneity of the landscapes and hence improved the suitability of these areas for pollinators, as demonstrated by the positive effect of edge density. Nowadays, we witness the other end of the gradient, i.e., the majority of formerly open grassland landscapes are overgrown by forests and shrubs. The domination of woody elements may act rather as barriers than facilitators for feeding, movement and shelter for pollinators. Nevertheless, in paper **I**, I did not detect the impact of woody elements on the genetic diversity of *T. montanum*. These differences between species could partially be attributed to different characteristics of species such as dispersal distance, life span, etc. (Honnay et al., 2006, 2007). It is thus important to avoid broad generalisations about the influence of different landscape elements.

4.2 The effect of landscape characteristics between populations on gene flow

It is generally assumed that populations further apart are genetically more different. Such a relationship is called isolation by distance (IBD). In paper **II**, I found that the populations of *P. veris* at a regional scale indeed are isolated by distance. However, at landscape scale (**III**), the pattern only occurred in a more stable landscape (Koguva), showing that IBD should not always be assumed, especially in dynamic landscapes, which have experienced rapid turnover of landscape elements in the recent past. Additionally, the study scale may influence whether IBD is observed, as distinct patterns may emerge at different scales (Twyford et al., 2020). I found that the genetic differentiation of *P. veris* reaches a plateau with a distance of about 15–30 km between populations **(IV)**. From there on, populations further apart were not significantly more different. Thus, it may show that even if the IBD pattern is not detectable at smaller scales in more disturbed landscapes, it may still emerge at larger scales. Depending on the context, the lack of IBD may also refer to the stronger importance of some other factor determining patterns of genetic structuring, such as the significant impact of some particular landscape elements or strong human intervention (e.g., sowing of non-local seed mixes).

The gene flow between the spatially separated populations of insect-pollinated grassland plants, such as *P. veris*, in my study system can mainly take place (1) via the movement of pollinators, (2) through seed dispersal, which can also be mediated by grazing animals (Kiviniemi & Eriksson, 1999; Plue et al., 2019; Rico et al., 2014) and wild animals (Auffret & Plue, 2014; Iravani et al., 2011), but also (3) as a result of the movement of agricultural and other machinery (Auffret, 2011). It is thus very plausible that different landscape elements between populations influence the gene flow of such plants because of the distinct effect of these elements on the movement of dispersal vectors (Bolliger et al., 2014;

Prevedello & Vieira, 2010). The results of papers **II–III** confirm this assumption. In paper **II**, I found that a higher historical proportion of grasslands between the populations of *P. veris* resulted in lower genetic differentiation, i.e., supported gene flow. In paper **III**, I found that a higher proportion of shrubs and fields led to a lower level of gene flow, whereas a higher proportion of forest acted as a facilitator of gene flow. It is expected that a higher amount of suitable habitat between study patches would result in higher gene flow between them (Balkenhol et al., 2015). Shrubs, as woody elements inhibiting gene flow, may pose a barrier to some pollinators (DiLeo et al., 2018). Surprisingly, forest had the opposite effect by supporting gene flow between the study populations. It is possible that when there are more grasslands, for example, pollinators may tend to avoid entering woody elements, whereas, in landscapes with a low area of grassland, pollinators may opt to choose the next best movement option, e.g., through forest (Zurbuchen et al., 2010), being the case in the fragmented study landscape of Lepiku, where this result was found. Alternatively, the supporting effect of forest on gene flow could, in fact, reflect the negative role of agricultural fields as the percentage of forest in the landscape surrounding the study populations was highly negatively correlated to the proportion of fields. Intensively managed agricultural land may pose a barrier to pollinators due to unsuitable microclimate (Bergman et al., 1996). However, the influence of landscape elements was only revealed using the corridor-based approach and not the resistance-based approach, suggesting that results based on only one approach may not be comprehensive.

Generally, it can be concluded from the results of the thesis that higher spatial connectivity, i.e., a larger proportion of semi-natural grasslands between grassland habitats, supports the gene flow between spatially isolated grassland plant populations. Nevertheless, "the larger the better" notion is not as straightforward as hypothesised initially because the genetic diversity also benefits from higher heterogeneity of microhabitats in the studied grassland complexes, which is mirrored by the positive effect of grassland edge density. Further, grazing cattle may facilitate the distribution of propagules of grassland plants, even of those not specifically adapted to animal seed dispersal (Holderegger et al., 2010). Therefore, grazing, especially rotational grazing, can be an important mechanism for maintaining genetic diversity via increased gene flow (DiLeo et al., 2017; Honnay et al., 2006; Jacquemyn et al., 2010; Plue et al., 2019; Rico et al., 2014). Because most of the study grasslands should be grazed, following the restoration activities and the creation of grazing infrastructure in the frames of the LIFE project "LIFE to Alvars", the genetic diversity and gene flow of grassland plants in our study system will be maintained and enhanced.

4.3 Time lags in the response of the genetic diversity and gene flow to landscape change

The landscapes studied in this thesis have gone through a significant change during the last 100 years. Both study species are quite long-lived (*T. montanum* avg. four years, max 17 years, Tamm et al., 2002; *P. veris* avg. 52 years, Ehrlén & Lehtilä, 2002). It could be expected that their genetic diversity has not reacted to the landscape change as only a few generations may have passed since grasslands were large and well-connected. It is, therefore, important to consider not only the current but also the historical landscape structure when exploring the impact of landscape characteristics on the genetic patterns of such long-lived plant species. In papers **I–II,** I included the variables of historical landscape characteristics in the analyses. In paper **I**, I found that while some indicators of genetic diversity of *T. montanum* populations respond to contemporary landscape (H_E), some still reflect the impact of historical landscape conditions (H_O, F_{IS}). In paper **II**, I found that the genetic diversity of *P. veris* populations responded more strongly to historical landscape structure than to the current one. Similarly, the gene flow measured by genetic differentiation F_{ST} between the study populations of *P. veris* was positively affected by the historical and not the present-day proportion of grassland between the populations (**II**). In paper **III**, where the two study landscapes had been covered by grasslands a century ago but had followed distinct trajectories of land use change after that, I found that the effect of different landscape elements on the gene flow of *P. veris* was not similar in these landscapes, i.e., depended on landscape context. This finding suggests that different land use trajectories may also strongly determine the observed patterns of gene flow. Schmidt and colleagues (2009) also found a differential effect of hedges in different landscapes on the genetic diversity of *Geum urbanum* populations, indicating the dependency of the genetic structuring of plants on landscape context. Similarly to our findings, several previous studies have found a lag in the reaction of genetic diversity to landscape change (Münzbergová et al., 2013; Plue et al., 2017; Reisch et al., 2017). However, working in the same region, Aavik and colleagues (2017) found that the genetic diversity of an annual plant *Rhinanthus osiliensis* reacts to contemporary landscape and not to the current one, supporting the idea that long-lived plants are more likely to react to landscape change with a time lag. Notably, the life span of species may change with overgrowth (Schleuning & Matthies, 2009). While both study species of this thesis are perennial, the life span of *T. montanum* is still significantly shorter than that of *P. veris*, and thus, lagged responses in *P. veris* are more common in our study system. Conclusively, potential time lags should be considered when planning conservation activities based on the observed genetic structure and diversity (Essl et al., 2015a). Such a lagged response could also be seen as an opportunity for conservation, i.e., enabling the restoration of the habitats before genetic diversity has had a chance to react, and, this way, preserving the still-existing genetic diversity of grassland plant populations.

4.4 The impact of different methods

It is clear from the thesis results that landscape elements may have a different effect on genetic diversity and differentiation depending on the studied scale. In a regional-scale study (**II**), I found an effect of grassland on the gene flow of *P. veris*, but at landscape scale (**III**), this effect was not observed. Additionally, the IBD was partially observed at the smaller scale (up to 2 km, **III**), while a clear IBD pattern was present at the regional scale, i.e., up to 30 km, (**II**) but was not detectable from there on. The importance of scale was also revealed in papers **I–II**, with some effects being present with one buffer radius but not the others. Ecological processes, such as seed dispersal and pollen flow, can occur at different spatial scales and may be influenced by different factors (DiLeo et al., 2018). Twyford and colleagues (2020) also suggested that multiple scales should be studied to understand the genetic structure of a species as a whole. Thus, results from one scale should not be extrapolated automatically to another, and the study scale should be carefully considered when constructing the study design (Anderson et al., 2010) and planning conservation activities.

In paper **I**, I found that some genetic diversity indices responded to the historical amount of landscape features (observed heterozygosity, H_O; inbreeding coefficient, F_{IS}), whereas some to current ones (allelic richness, A_R; expected heterozygosity, H_E; correlated with each other). It has been previously suggested that allelic richness reveals the influence of landscape changes quicker than heterozygosity (Epps & Keyghobadi, 2015), and our results seem to partly confirm that. Similarly, Aavik and colleagues (2017) found that allelic richness, but not heterozygosity, reacted to the area of forest surrounding the populations of *Rhinanthus osiliensis* in wet grasslands, which had experienced drastic loss in the area over the past century. Additionally, in paper **II**, the influence of forest was only revealed with the percentage of polymorphic loci (%P), and none of the landscape elements had an influence on the inbreeding coefficient (F_{IS}). In paper **III**, I observed discrepancies in the response of two different gene flow indices of *P. veris* to landscape characteristics between study populations. F_{ST}, which is known to reflect more historical gene flow (Epps & Keyghobadi, 2015), responded to landscape elements in the more stable landscape. MAP, which should capture recent gene flow, reacted to landscape elements in the landscape that has gone through more drastic changes. Thus, when studying the influence of more recent landscape change on the gene flow of perennial plants, it would be advisable to use assignment-based measures, such as MAP. The results of this thesis show that the relative response to current and historical landscape variables revealed may depend on the measure of genetic diversity or variation used. It is, therefore, important to use various indices to ensure that the different responses between landscape characteristics and genetic variation will be revealed.

Recent advances in molecular tools have raised questions whether switching from traditional microsatellite markers, usually encompassing up to a couple of tens of loci, to thousands of SNPs will substantially increase knowledge while covering the accumulating costs related to improved infrastructure and demands

for higher and more specialised capacity to analyse sequence data (Puckett, 2017). In paper **I**, I used microsatellites, and in papers **II–IV**, single nucleotide polymorphisms (SNPs) as molecular markers. It is not possible to compare the quality of results based on these two different sets of markers in this thesis because different markers were used for different species. Fischer and colleagues (2017) found that genetic diversity indices of *Arabidopsis halleri* based on microsatellites and SNPs differed and suggested using SNPs in future studies. However, the total cost and work effort for obtaining SNP markers in the current thesis were substantially higher than the ones related to microsatellites for analysing the comparable number of populations and individuals, which is why it is always necessary to evaluate the costs and benefits before choosing the type of markers. Moreover, the speed of evolution of these markers may differ, with microsatellite markers typically exhibiting a higher evolution rate per locus per generation than SNPs (Selkoe & Toonen, 2006). Consequently, shifts in genetic diversity in response to landscape alterations might be detectable using microsatellites but not SNPs, potentially impacting the outcomes of studies based on the chosen genetic marker (Epps & Keyghobadi, 2015). Yet, the number of SNP markers is usually several magnitudes higher than that of microsatellites, which might, to some extent, compensate for the slower rate of evolution in this type of markers. In addition, exploring thousands of SNPs throughout the genome enables to search for loci of putative adaptive relevance (**IV**), which may be a desirable aim in conservation activities targeting the recovery of adaptive potential. Thus, the choice of markers adds another important layer to consider when designing a landscape genetic study.

4.5 The response of adaptive genetic diversity to landscape change

Most assessments of genetic diversity in conservation and restoration planning and evaluation have been based on overall or neutral genetic diversity (Gonzalez-Robles et al., 2020; Wei & Jiang, 2021). However, the stand-alone importance of neutral variation in light of environmental change is questionable because the putatively adaptive regions of a genome are vital for the fate of a population in a changed environment (Teixeira & Huber, 2021). The majority of studies that have looked at the influence of environment on adaptive genetic diversity have focused on detecting loci relevant for responding to climatic variables, whereas I focused on the influence of the overgrowth of grasslands, which poses a major environmental change for plants inhabiting these valuable habitats (**IV**). Genomic approaches (i.e., analysis involving thousands of SNPs from all over the genome) are increasingly suggested as a tool for conserving and restoring populations with high adaptive potential (Theissinger et al., 2023). Ideally, whole genome sequencing (WGS) would be used to capture most of the genetic diversity, but this is often not feasible (especially in conservation) due to the complexity and the cost of the analysis. Thus, in paper **IV**, I explored the impact of grassland overgrowth

on the putatively adaptive *vs* neutral genetic diversity in the populations of *P. veris* using a reduced representation of the genome (ddRAD). The overgrowing of these habitats started about 90 years ago, and before that, these habitats had remained open for centuries (Helm et al., 2006; Pärtel et al., 1999). Contrary to our initial expectations (that an overgrowth would lead to reduced adaptive genetic diversity), I found that the adaptive genetic diversity is higher in overgrown grassland habitats, while neutral genetic diversity did not respond to overgrowth. Overgrowth may cause a change in several environmental conditions, such as light availability and other microclimatic variables, but may also initiate shifts in the biotic environment, such as pollinator abundance and composition (Prangel et al., 2023). This may indicate that in overgrown habitats, we observe evolution in process, i.e., a selection pressure imposed by novel environmental conditions temporally increases the genetic diversity in adaptive regions but not in neutral regions. This selection pressure is expressed in the change of allele frequencies: alleles (gene variants) that may have been beneficial in open conditions may now not be optimal in an overgrown environment, leading to a drop in the frequency of these alleles. At the same time, the frequency of other alleles beneficial in the new conditions is increasing. There were more alleles beneficial (i.e., closer to the fixed state) in open habitats that showed decreasing frequencies in overgrown habitats, indicating the reduced level of fixation and supporting the idea of allele frequencies changing due to changed habitat conditions. Considering the longevity of *P. veris*, it may mean that populations in overgrown habitats are still adapting to the new changed conditions. Similarly, the adaptive genetic diversity of *Pinus cembra* was higher in the periphery of the species niche, suggesting that in the peripheral, less suitable conditions, there is a selection pressure to adapt to the new conditions (Dauphin et al., 2020). These studies confirm the value of populations inhabiting marginal or sub-optimal environmental niches as they act as sources of genetic variation for responding to further environmental changes (Pearman et al., 2024).

5. CONCLUSIONS

Genetic diversity is an essential part of biodiversity that should not be neglected when planning and conducting conservation and restoration activities because it provides populations with the ability to adapt to future environmental changes. Recent land use shifts have resulted in reduced area and connectivity of habitats, and lower suitability of landscapes between populations (Cousins et al., 2015; Haddad et al., 2015; Wilson et al., 2016) with detrimental consequences for population sizes of many plant populations relying on natural and semi-natural habitats. Studying the impact of these patterns of landscape change on genetic diversity – the component of biodiversity often neglected in conservation practice (Laikre et al., 2020) – is thus vital to preserve and enhance the adaptive potential of fragmented plant populations in an era of ongoing environmental change.

The aim of this thesis was to assess the impact of historical and current landscape structure on the genetic diversity and gene flow patterns of plants in landscapes characterised by substantial recent loss in the area and connectivity of habitats to improve the understanding of the effects of land use change on genetic diversity and related long-term evolutionary potential. For this, I utilised various landscape genetic methods to investigate neutral genetic diversity and gene flow among populations of grassland plants, both regionally (**I**, **II**) and at landscape level (**III**), using semi-natural grasslands as a study system. Understanding these relations in different temporal and spatial scales, and testing the applicability of different methods is necessary to make broader and well-informed conclusions for further steps of conservation and restoration. Additionally, I sought to enhance understanding of how changes in land use affect the genetic diversity relevant to adaptation. Although adaptive genetic diversity is essential for the survival of species in shifting environmental conditions, the effects of the loss of grassland habitats on adaptive genetic variation have been hardly studied before. Paper **IV** in this thesis is among the first to capture the possible ongoing evolution due to changing landscape and local environmental conditions following land use change.

Overall, the results of this thesis show that the overgrowing of grassland habitats at landscape scale has a negative influence on the genetic diversity of grassland plants, but these influences may manifest with a time lag. Yet, completely homogenous open grasslands do not offer the best setting for maintaining genetic diversity either. Both studies on neutral as well as adaptive genetic variation show that moderate heterogeneity provided by occasional woody elements and increased edge density support higher genetic diversity. In addition, I show that grassland habitats have to be spatially connected to facilitate the gene flow between populations.

It is important to choose appropriate indices when measuring genetic diversity and differentiation of plants, particularly when the focus is on long-lived plant species inhabiting spatially dynamic habitats, to ensure that the impact of landscape change will be revealed. Ideally, several different indices should be used in combination, but it is not done very often. Additionally, the scale of the study can

influence the results, and findings from one scale may not be transferrable to the other. Even more, results may depend on the trajectory of land use change and thus, landscape genetic studies would benefit from the replication of study landscapes. Finally, different analytical approaches may show varying results, and thus, it should be considered whether several methods could be simultaneously applied.

From a conservational point of view, the existence of lagged responses in the genetic diversity of grassland plants to landscape change may pose an opportunity to restore their habitats and connectivity before the negative effects in the patterns of genetic variation emerge. When restoring grassland habitats, overall environmental heterogeneity within habitats should be maintained, e.g., by preserving some woody elements, as this promotes genetic diversity due to environmental variation and increased opportunities for mutualistic partners, such as pollinators and seed vectors.

Future studies should put more focus on the adaptive part of genetic diversity and set a particular emphasis on the influence of landscape changes, as this is a highly understudied avenue. Rapidly developing genetic methods help to increase the feasibility of such studies. However, genetic diversity in neutral regions should not be neglected as it helps to understand the role of gene flow and drift in shaping the genetic structure of populations. Additionally, as plants mostly depend on pollinators, wind, and seed vectors for dispersal, incorporating the studies on the role of these organism groups and factors in future landscape genetic research more directly is advisable.

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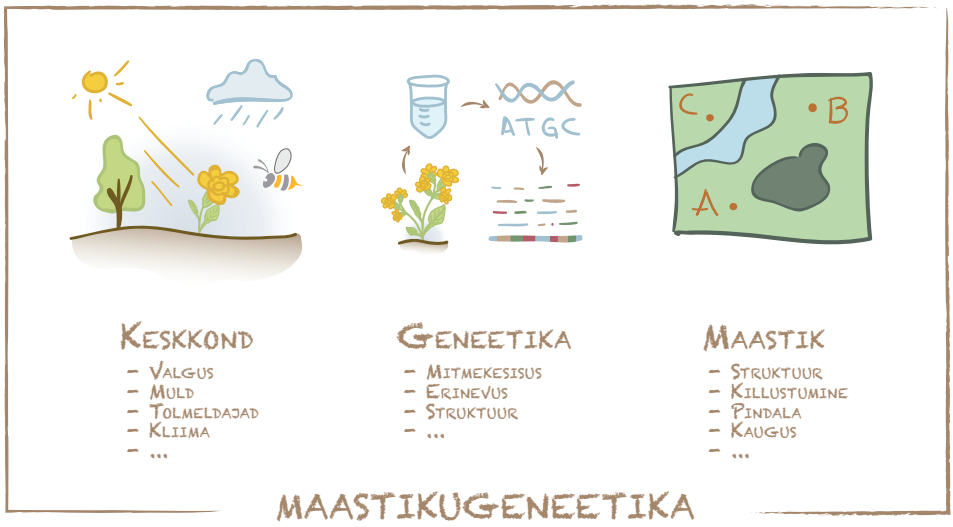
SUMMARY IN ESTONIAN

Niidutaimede geneetiline varieeruvus muutuvas maastikus

Sajandi jooksul toimunud maakasutuse ja keskkonnamuutused on looduslikke ökosüsteeme mitmel moel mõjutanud. Suurt mõju on avaldanud nii põllumajanduse intensiivistumine kui ka traditsiooniliste maakasutusvõtete kadu. Eriti silmatorkavad on selle tagajärjed poollooduslikel rohumaadel ehk pärandniitudel, mida varasemalt traditsiooniliselt karjatati või niideti. Tänaseks on suurem osa pärandniitudest kas põllumaaks muudetud või majandamise lakkamisel puude ja põõsastega kinni kasvanud. Nii on kunagised elurikkad rohumaad killustunud ja allesjäänud aladel muutunud keskkonnatingimused niidutaimede jaoks ebasobivaks.

Elurikkuse võib tinglikult jagada kolmeks komponendiks: liigiline, geneetiline ja ökosüsteemide elurikkus. Kuigi liigilist mitmekesisust uuritakse kõige rohkem, on oluline tähelepanu all hoida ja looduskaitses arvesse võtta ka geneetilist mitmekesisust – just viimane tagab liikide ja populatsioonide hakkamasaamise muutuvate keskkonnatingimustega. Tasub tähele panna, et geneetilise mitmekesisuse võib jagada omakorda kaheks: neutraalseks ja adaptiivseks geneetiliseks mitmekesisuseks. Viimane peegeldab mitmekesisust, mida hinnatakse looduslikule valikule reageerivast genoomi osast ning mis seetõttu otseselt mõjutab populatsioonide ja liikide toimetulekut muutuvates keskkonnatingimustes. Siiski on teadmised ka neutraalsest geneetilisest mitmekesisusest olulised, sest annavad teavet elupaikade kao ja maastikumuutuste rollist populatsioonidevahelises geenivoolus ja geenitriivis ning nendega seotud populatsioonide dünaamika mustrite kujunemises.

Taimepopulatsioonide geneetilist mitmekesisust võivad mõjutada mitmed tegurid, sealhulgas populatsiooni suurus, ümbritseva maastiku ja kasvukoha omadused ning õietolmu ja seemnete vektorid (näiteks mesilased, liblikad ja kariloomad). Nende tegurite mõju võib avalduda ajalise viibega, mille tuvastamine tagab looduskaitse- ja taastamistegevuste edukuse. Maastikugeneetika on teadusharu, mis kombineerib maastikuökoloogia ja populatsioonigeneetika meetodeid, hindamaks ülalmainitud tegurite mõju populatsioonide geneetilisele mitmekesisusele ja populatsioonide vahelisele geenivoolule (joonis 10).



Joonis 10. Maastikugeneetika komponendid. Maastikugeneetika uurib, kuidas maastiku struktuur või keskkonna omadused mõjutavad populatsioonide geneetilist varieeruvust. Autor: Sabrina Träger.

Doktoritöös uuriti maastikumuutuste mõju niidutaimede geneetilise mitmekesisuse ja geenivoolu mustritele Lääne-Eesti maastikel, mis on viimase saja aasta jooksul kaotanud suurema osa ajalooliselt laialt levinud pärandniitude pindalast. Doktoritöö eesmärk oli teada saada, kuidas mõjutavad taimede neutraalset geneetilist mitmekesisust ja geenivoolu elupaiga pindala, sidusus ja teised maastikuelemendid. Neile küsimustele vastuste leidmiseks kasutasin erinevaid maastikugeneetilisi uurimisvõtteid. Tuginedes maastike iseloomustamisel nii kaasaegsetele kui ka ajaloolistele kaartidele uuriti, kas mainitud tegurite mõjud võivad avalduda ajalise viibega. Selleks eraldati võimalikud adaptiivsed lookused neutraalsetest ning uuriti maastikumuutuste mõju erinevusi neutraalsete ja adaptiivsete lookuste vahel. Doktoritöö peamiste tulemuste põhjal antakse soovitusi, kuidas geneetilist teavet looduskaitselistesse tegevustesse rakendada.

Artiklites **I–III** keskenduti neutraalse geneetilise mitmekesisuse hindamisele Muhus ja Saaremaal olevatel poollooduslikel rohumaadel ning neid ümbritsevatel maastikel. Käsitleti nii populatsioonisisest geneetilist mitmekesisust ja ümbritseva maastiku mõju sellele (**I–II**) kui ka populatsioonidevahelist geneetilist erinevust ning nende vahel oleva maastiku mõju (**II–III**). Artiklites **I** ja **II** vaadeldi neid seoseid regionaalsel skaalal (Saaremaal ja Muhus). Artiklis **III** keskenduti aga maastiku skaalale (proovialad suurusega 2 × 2 km) ning kõrvutati geenivoolu mustreid kahel erineva sidususastmega maastikul, Koguva sidusamal ja Lepiku killustunud maastikul. Artiklis **IV** tuvastati lookused, mis reageerisid pärandniitude keskkonnatingimuste muutustele ning on seega eeldatavalt adaptiivsed. Lisaks hinnati, kuidas mõjutab niidukoosluste kinnikasvamine taimepopulatsioonides adaptiivset ja geneetilist mitmekesisust. Artiklis **I** uuriti mägi-

ristikut (*Trifolium montanum*), mis on pikaealine putuktolmlev ning poolloouslikele rohumaadele iseloomulik taim. Artiklites **II–IV** oli uurimisliigiks harilik nurmenukk (*Primula veris*), mis on samuti pikaealine, poolloouslikel rohumaadel tavapärane ja sõltub tolmeldamisel putukatest.

Artiklis **I** kasutati geneetiliste markeritena mikrosatelliite, mis võimaldavad uurida ainult neutraalset geneetilist mitmekesisust. Artiklites **II–IV** kasutati aga ühenukleotiidseid polümorfisme (SNP), mis annavad võimaluse uurida nii neutraalset kui ka adaptiivset geneetilist mitmekesisust. Adaptiivsete ja neutraalsete lookuste eraldamiseks kasutasin erinditel põhinevat analüüsi (*outlier analysis*), kus teatud piirväärtusest kõrgemal olevate alleelisagedustega lookused loetakse adaptiivseteks. Teise meetodina kasutati keskkonnaassotsiatsiooni uuringut (*environmental association study*, EAA), mille puhul kõrvutatakse keskkonnagradiente või faktoreid alleelisagedustega.

Artiklis **I–II** leiti, et elupaigaks olev rohumaa suurus ja uurimispopulatsioone ümbritseva rohumaa osakaal mõjutab niidutaimede geneetilist mitmekesisust üldiselt positiivselt. Artiklis **II** tuvastati, et kui nurmenukupopulatsioone ümbritsesid kõrgema servaalade tihedusega rohumaad, oli populatsioonide geneetiline mitmekesisus kõrgem. Seega, kuigi võimalike elupaikade pindala on geneetilise mitmekesisuse toetamisel oluline, on tähtis ka elupaikade heterogeensus, mis võib olla kasulik nii niidutaimedele kui ka nende tolmeldajatele. Geneetilisele mitmekesisusele võivad mõju avaldada ka muud maastikuelemendid. Artiklis **II** leiti, et metsa tänapäevasel osakaalul oli nurmenuku geneetilisele mitmekesisusele negatiivne mõju, samas kui metsa ajaloolise osakaalu kasvuga kaasnes kõrgem geneetiline mitmekesisus. Sellise vastuolulise tulemuse üheks võimalikuks seletuseks on metsa erinev osakaal tänapäeval ja ajalooliselt. Ajalooliselt oli uuritud maastikes metsa pigem vähem või üldse mitte ja iga puittaimedega maastikuelemendi olemasolu võis mõjuda keskkonna heterogeensust suurendava positiivse tegurina. Tänapäeval on metsa pindala nendel maastikel märkimisväärselt suurem ja sellisel puhul võib mets olla pigem levikubarjääriks.

Eeldatavasti on üksteisest ruumiliselt kaugemalolevad populatsioonid geneetiliselt erinevamad (*isolation by distance*, IBD). Doktoritöö artiklis **II** leiti sellele eeldusele suuremal skaalal kinnitust, kuid maastikuskaalal ilmnes see muster vaid sidusamas Koguva maastikus (**III**). Lisaks geograafilisele kaugusele võivad ka erinevad maastikuelemendid taimede geenivoolu mõjutada. Jõuti teadmiseni, et rohumaade suurem osakaal populatsioonide vahel soodustas geenivoolu (**II**). Maastikuskaalal läbi viidud uuringu tulemuste kohaselt takistas põõsastiku ja haritava maa suurem osakaal populatsioonidevahelist geenivoolu, metsa osakaalu tõus aga soodustas seda (**III**). Kuna metsa osakaal oli haritava maa osakaaluga vähemsidusas niidukoosluste võrgustikus negatiivselt korreleeritud, siis ei saa kindlalt vastata, kas geenivoolu mustritele on olulisem metsa positiivne või haritava maa negatiivne mõju. Põõsastiku positiivne mõju ilmnes aga sidusamal maastikul. Võib öelda, et sidusamad ja heterogeensemad maastikud soodustavad niidutaimede geenivoolu.

Eelnevad mõjud võivad avalduda ajalise viibega ehk vaadeldud geneetilised mustrid ei peegelda praegu veel maastikumuutuste mõju. Eriti tähelepanelik tasub

selles osas olla uuringutes, kus keskendutakse hiljuti muutunud või veel muutuva struktuuriga maastikele, nagu siin doktoritöös vaadeldud uurimissüsteem, kus niiduelupaigad on maastikumuutuste tõttu kaotanud viimase saja aasta jooksul peaaegu 90% oma kunagisest pindalast. Ajaloolise ning kaasaegse maastiku struktuuri mõju võrdlev uurimine on üks viise, kuidas selliseid võimalikke viibeid tuvastada. Kui ajalooline maastikustruktuur selgitab geneetilise mitmekesisuse ja geenivoolu mustreid paremini kui kaasaegne, on põhjust eeldada viibe ehk n-ö geneetilise väljasuremisvõla olemasolu. Artiklites **I–II** vaadeldi nii kaasaegse kui ka ajaloolise maastiku mõju geneetilisele mitmekesisusele. Artiklis **I** leiti, et mõned geneetilise mitmekesisuse indeksid reageerisid ajaloolisele, teised aga tänapäevasele maastikule. Artiklis **II** tuvastati peamiselt ajaloolise maastikustruktuuri mõju. Artiklis **III** uuriti hariliku nurmenuku populatsioonide vahelist geenivoolu kahel maastikul, mis olid ajalooliselt kaetud suure ja sidusa pärandniitude võrgustikuga, kuid mis erinevate maakasutusvõtete tõttu on tänaseks kujunenud üsna erinevaks. Leidsin, et sama maastikuelemendi mõju võib erinevates maastikes olla erinev või suisa vastandlik, mis viitab, et geenivoolu ja geneetilise mitmekesisuse mustrite tõlgendamisel tuleb arvestada ka maakasutuse muutuse iseloomu ning kiirusega. Lisaks uuriti artiklis **III** erinevate geenivoolu indeksite kasutamise mõju vaadeldavatele geenivoolu ja maastiku struktuuri vahelistele seostele. Doktoritöö tulemused kinnitavad, et erinevad geneetilise mitmekesisuse ja geenivoolu indeksid võivad maastikumuutuste mõju kohta anda erinevaid vastuseid. Doktoritöö artiklite võrdlus näitab, et ka uuritav skaala võib tulemusi mõjutada. Näiteks avaldus artiklis **II** kirjeldatud regionaalsel skaalal (u 70 × 50 km) tehtud uuringus rohumaa osakaalu positiivne mõju geenivoolule, kuid maastikuskaalal (2 × 2 km) see mõju ei tuvastunud (**III**). Ka eeldus geograafilise kauguse ja geneetilise erinevuse vahelisest seosest (IBD) avaldus selgemalt keskmisel ja suuremal uuritud ruumiskaalal (kuni 30 km), kuid oli väga nõrk või kadus maastikuskaalal, aga ka suurimal uuritud skaalal (> 30 km).

Suur osa geneetilise mitmekesisuse hinnangutest põhineb looduskaitses neutraalsel geneetilisel mitmekesisusel, kuigi otseselt vajalik populatsioonide ellujäämiseks muutuvates keskkonnatingimustes on just adaptiivne geneetiline mitmekesisus. Adaptiivset mitmekesisust on seni vaadeldud peamiselt kliimamuutuste kontekstis. Käesolev doktoritöö on üks väheseid, mis uurib maakasutuse muutuse (niidukoosluse kinnikasvamine majandamise lakkamisel) mõju adaptiivsele mitmekesisusele (**IV**). Üllatuslikult leiti doktoritöös, et kinnikasvanud niiduelupaikades olevate populatsioonide adaptiivne geneetiline mitmekesisus on kõrgem kui veel avatud elupaikades olevatel populatsioonidel. Neutraalne geneetiline mitmekesisus kinnikasvamisele ei reageerinud. Selline tulemus võib viidata, et kinnikasvavates elupaikades määrab geneetilist varieeruvust muutuvate keskkonnatingimuste põhjustatud loodusliku valiku surve, mis ajutiselt suurendab adaptiivset geneetilist mitmekesisust.

Doktoritöö tulemused näitavad, et elupaikade kinnikasvamine mõjutab niidutaimede geneetilist mitmekesisust negatiivselt, kuid need mõjud võivad avalduda ajalise viibega. Geneetilist mitmekesisust aitab alal hoida ka maastiku hetero-

geensus. Töö tulemused näitavad, et geneetiline mitmekeisus on kõrgem rohumaal, kus leidub mõõdukalt põõsaid ja puid või puuderühmi ning rohkelt servaalasid. Looduskaitselisest vaatenurgast hinnatest tuleks rohumaid taastades alles jätta mõned puud ja põõsad ning tagada, et maastik oleks sobilik tolmeldajatele ja seemnete levitajatele, kellest sõltub paljude taimeliikide geenivariantide levik, millest mõned võivad osutuda keskkonnamuutustega toimetulekul väga oluliseks. Hoida ja taastada tuleb rohumaade ruumilist sidusust, mis toetab populatsioonidevahelist geenivoolu. Ajalisi viibeid võib aga käsitelda kui varuvõimalust looduskaitsele – veel on võimalik õigeaegse taastamisega peatada edasist elurikkuse kadu.

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PUBLICATIONS

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List of publications

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Reinula, I., Träger, S., Järvine, H. T., Kuningas, V. M., Kaldra, M., & Aavik, T. (2024). Beware of the impact of land use legacy on genetic connectivity: A case study of the long-lived perennial *Primula veris*. *Biological Conservation*, 292, 110518. https://doi.org/10.1016/j.biocon.2024.110518

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Uurimistoetused ja stipendiumid

2020 ITC konverentsigrant COSTi tegevuselt CA18201 ConservePlants
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2017 Kristjan Jaagu vahetusõpingute stipendium
2016 Üliõpilaste teadustööde riikliku konkursi Eesti Teaduste Akadeemia presidendi lootustandvate sähvatuste diplom bakalaureusetöö "Taimede geneetilise ja liigilise mitmekesisuse vahelised seosed" eest.
2015 Tartu Ülikooli tulemusstipendium

Organisatsiooniline ja erialane tegevus

2023 Bakalaureusetöö juhendamine: Vete-Mari Kuningas. 2023, juh. Tsipe Aavik, Iris Reinula. Maastiku struktuuri mõju hariliku nurmenuku (*Primula veris*) populatsioonide geneetilisele mitmekesisusele ja geenivoolule. Tartu Ülikool, Ökoloogia ja maateaduste instituut, Botaanika osakond.
2021–... Retsensent ajakirjades Global Ecology and Conservation, Biological Conservation
2019–… Harrastusteaduskampaania "Eesti otsib nurmenukke" ja rahvusvahelise kampaania "Looking for Cowslips" korraldamine, https://nurmenukk.ee
2014–… Tartu üliõpilaste looduskaitseringi liige

Muud publikatsioonid

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Erialane enesetäiendus

2021 kursuse "Adaptation genomics" läbimine (Physalia courses)

2021 kursuse "RAD-seq data analysis" läbimine (Physalia courses)

2019 ALTER-Net-i (A Long-Term Biodiversity, Ecosystem and Awareness Research Network) suvekool Peyresqis, Prantsusmaal

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