Landscape genetic analysis suggests stronger effects of past than current landscape structure on genetic patterns of *Primula veris*

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**Abstract**

**Aim:** Recent changes in land use have led to substantial loss and fragmentation of semi-natural grasslands. We assessed the relative effect of current and historical landscape composition, and landscape change on the genetic diversity and gene flow of a characteristic grassland plant.

**Location:** Calcareous grasslands on Muhu and Saaremaa islands in Western Estonia, Europe.

**Methods:** We used landscape genetic methods to study the genetic patterns of the grassland plant *Primula veris*. We applied a high-throughput sequencing method (double-digest restriction site-associated DNA sequencing, ddRADseq) to obtain 2,619 putatively neutral single-nucleotide polymorphism (SNP) markers. We examined the impact of the historical (80 years ago) and current amount and edge density of 19 grasslands, the amount of woody elements in the surroundings of the study grasslands and the change in the area of these habitats on the current genetic diversity within populations and genetic differentiation between populations of *P. veris*.

**Results:** Genetic diversity within populations of *P. veris* was lower in landscapes with more pronounced grassland loss over the past 80 years. Higher historical grassland edge density in the surrounding landscape led to higher genetic diversity of *P. veris*. Higher historical proportion of grasslands between the study populations led to lower genetic differentiation, indicating higher (historical) gene flow between those populations.

**Main conclusions:** Although the study grasslands experienced a drastic loss in the area and connectivity over the past century, patterns of genetic diversity and gene flow of *P. veris* still largely mirror the effect of historical landscape, especially grassland edge density and at larger scales the historical proportion of grasslands between study populations. Thus, measures of genetic diversity and gene flow in *P. veris* may have a lagged response to landscape change, offering a window for preserving the still existing genetic diversity through immediate restoration activities.
INTRODUCTION

Recent changes in land use have led to drastic loss and fragmentation of natural and semi-natural habitats (Picó & Van Groenendael, 2007). In particular, land use has substantially changed towards more intensive agricultural practices, while traditional management has been abandoned (Picó & Van Groenendael, 2007; Prentice et al., 2006). During the last century, the area of semi-natural grasslands has drastically decreased in Europe (Cousins et al., 2015; Hooffman & Bullock, 2012) due to lack of traditional hay-making and extensive grazing. Decrease in the area and connectivity of habitats, and altered habitat conditions often lead to loss of biodiversity (Hahn et al., 2013; Honnay et al., 2007), including the decrease in genetic diversity of wild plant populations (Leimu et al., 2010). Genetic diversity provides adaptive potential to species for coping with environmental change and should therefore be one of the focal aims of conservation management (Aavik & Helm, 2018). Furthermore, inclusion of knowledge about genetic diversity in restoration activities has a high potential to increase restoration success (Mijangos et al., 2015) and is crucial for recovering structurally and functionally resilient ecosystems (Moreno-Mateos et al., 2020).

Fragmentation and loss of habitats often impose serious negative effects on genetic diversity because they potentially lead to reduced gene flow between populations and increased inbreeding and genetic drift (Honnay & Jacquemyn, 2007; Leigh et al., 2019; Leimu et al., 2006; Prentice et al., 2006; Young et al., 1996). Increasing application of landscape genetic tools has advanced knowledge about the influence of environmental heterogeneity on genetic diversity and demonstrated that, in addition to geographic isolation, also the characteristics of the landscape around and between plant populations can strongly shape genetic patterns of plants (Emel et al., 2020; Holdereregger et al., 2010; Lehmain et al., 2020; Manel et al., 2003). This knowledge is also crucial for the recovery of resilient populations during ecosystem restoration (Moreno-Mateos et al., 2020).

As plants mostly depend on various abiotic and biotic vectors for dispersal of pollen and seed (Holdereregger et al., 2010), landscape change affects plants mainly through influencing the movement of these vectors, either positively or negatively (Holdereregger et al., 2010). For insect-pollinated grassland species, for example, woody elements may act as a barrier for gene flow by inhibiting the movement of pollinating insects (Aavik et al., 2014; Aavik et al., 2017; Aguilar et al., 2006; Hahn et al., 2013; Schmitt et al., 2000). Corridors in barrier elements can facilitate pollen dispersal (Tewksbury et al., 2002), which is particularly critical for maintaining gene flow in insect-pollinated plant species (Aavik et al., 2014, 2017). On the other hand, edge habitats, for example on the borders of woody landscape elements (Söber et al., 2020) or between farmlands (Hass et al., 2018), can benefit pollinating insects by offering a more suitable microclimate (Bergman et al., 1996) and potential nesting and feeding areas (Neumüller et al., 2020) facilitating pollinator movement and thus gene flow across grasslands between edge habitats. Further, grazing cattle may facilitate the distribution of propagules of grassland plants, even of those not specifically adapted to animal seed dispersal (Holdereregger 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) as well as species diversity (Pärtel et al., 2007). However, nowadays, due to changes in land use, cattle is rarely being moved across larger spatial scales, which may have also contributed to reduced seed dispersal.

Plant populations, which have experienced severe fragmentation, may still maintain considerably high genetic diversity despite landscape changes (Reisch et al., 2017). This phenomenon may be caused by so-called genetic extinction debt, due to which genetic diversity has not yet reacted to the changes of landscape structure but might be expected to do so in the future (Aguilar et al., 2008). Indeed, several studies have found evidence of such time lags in the genetic patterns of plants (Aavik et al., 2019; Münzbergová et al., 2013; Reisch et al., 2017). Delayed responses to landscape change are more probable in long-lived plant species, while lagged responses in the genetic patterns of short-lived plants are rare (Aavik et al., 2017; Helm et al., 2009). Yet, there is still a gap in knowledge about the effects of change in landscape characteristics, such as the loss and transformation of semi-natural grasslands, on the gene flow between increasingly isolated grassland plant populations. A time lag in the response of genetic diversity and gene flow to habitat fragmentation may substantially influence conservation recommendations, but unfortunately, potentially delayed responses are often not accounted for despite the threat of further decay in genetic diversity of the species and eventually even extinction (Essl et al., 2015). However, delayed responses could also be seen as a possibility in conservation to restore the historical habitats of species before the negative effects of habitat loss manifest.

Whether the response of genetic patterns of plants to current and historic landscape structure is detected may also depend on the applied method as well as the metrics of genetic diversity (Epps & Keyghobadi, 2015). In node-based analyses, the effect of landscape features (i.e. amount of different habitats) in a circular area at and around a study population on population-based genetic diversity is tested, using, for example, linear model or regression analyses (DiLeo & Wagner, 2016). With such on-site analyses, effects on genetic connectivity via potential immigration and establishment of pollen, seeds or juvenile plants can be tested. Link-based approaches, in contrast, focus on examining the effect of landscape features in a direct or indirect path between pairs of populations on
their genetic differentiation ($F_{ST}$), using Mantel test or (multivariate) generalized linear model approaches with distance matrices (DiLeo & Wagner, 2016). Such between-site analyses provide the possibility to test for facilitating or inhibiting effects of the intervening landscape on gene flow and its vectors (e.g. pollinators) via pollen and seeds. The two approaches complement each other and should ideally be used in concert (DiLeo & Wagner, 2016). Yet, this has been rarely done so far (DiLeo & Wagner, 2016).

In the current study, we examined the genetic diversity of *Primula veris* populations occurring in semi-natural calcareous grasslands in Western Estonia that have experienced a dramatic loss of their area during the last century (Helm et al., 2006). In addition, we assessed genetic differentiation between the study populations as an indirect measure of gene flow. We examined the response of these node- and link-based genetic metrics to current and historical (pre-fragmentation; from the 1930s) landscape configuration and to landscape change. We proposed the following questions: (a) Does the amount of grassland in the landscape affect the genetic diversity of populations and gene flow between populations? (b) Does the amount of woody elements (forests and shrubs) in the landscape influence the genetic diversity of populations and gene flow between populations? (c) What is the role of grassland edge density on the genetic diversity of *P. veris*? (d) Do historical landscape characteristics have a stronger effect on current genetic diversity than current landscape features due to relatively long life span of *P. veris*, that is do patterns of genetic diversity exhibit a lagged response to landscape change? The knowledge obtained in the present study is crucial for interpreting measures of biodiversity in spatio-temporally dynamic landscapes and for planning conservation activities of threatened habitats.

## 2 | METHODS

### 2.1 | Study species

*Primula veris* L. (*Primulaceae*) is an herbaceous perennial plant with an approximate life span of up to 50 years (Ehrln & Lehtilä, 2002). *Primula veris* flowers in May in Estonia and is mostly found in dry, species-rich grasslands, in shrub or woodland ridges and edges and on calcareous cliffs. It is a light-demanding and drought-tolerant species (Brys & Jacquemyn, 2009). *Primula veris* is an obligate outcrossing plant depending on insects for effective pollen flow. The flowers are mostly pollinated by different species of Hymenoptera (e.g. bees), but also some species of Coleoptera (beetles) and Lepidoptera (butterflies). Pollen dispersal of *P. veris* is generally limited to a few metres from parental plants and seed dispersal is restricted to a few centimetres from maternal plants (Brys & Jacquemyn, 2009) but occasional longer dispersal distances might be reached by domestic and wild animals (Auffret & Plue, 2014; Plue et al., 2019; Rico et al., 2014). The study species is heterostylos, that is it has two morphologically different flower types. Mostly, only inter-morph pollen exchange results in successful fertilization (Ganders, 1979).

### 2.2 | Study area

Study sites were located in semi-natural calcareous grasslands—alvars—in Western Estonia on the islands of Muhu and Saaremaa (58.6°, 23.25°; 58.42°, 22.5°; Figure 1a). Annual mean temperature in the area is 6.9°C, and precipitation is 600 mm (Estonian Weather Service, 2020). Alvars have characteristic shallow calcareous soils occurring on limestone bedrock. Estonian alvars are typically located near coastal areas in Northern and Western Estonia, making the climate of these grasslands more humid and with a smaller temperature range. Alvars have lost more than two thirds of their area during the last century because traditional grazing needed to sustain the grasslands has been mostly abandoned. As a consequence, most of the alvars have overgrown with trees and shrubs (Helm et al., 2006; Pärtel et al., 1999).

Samples were collected from 19 populations of *P. veris*, occurring on currently managed open alvars on Muhu and Saaremaa (Figure 1a). The sites were part of the EC LIFE+programme restoration project “LIFE to Alvars” (LIFE13NAT/EE/000082; Helm, 2019). Population sizes ranged from c. 20 to 5,000 individuals. In each population, 1–3 leaves from 20 individuals, where possible (range 6–20), of *P. veris* were randomly sampled in the summers of 2015 and 2016. In total, samples were collected from 350 individuals (Table 1). The distance between sampled individuals within a population was at least one metre. Samples were stored on silica gel until DNA extraction.

### 2.3 | Genetic analyses

Samples were weighed to 25 mg of leaf material and ground for 2 min using two 2.3-mm metal beads in a Mixer Mill 301 (Retsch GmbH). DNA was extracted using the LGC sbeadex plant maxi kit (LGC) with some modifications. Extraction steps of binding, washing and elution were done on a KingFisher Flex Purification System (Thermo Fisher Scientific). For a more detailed description of DNA extraction, see Träger et al. (2021).

Extracted DNA was prepared for sequencing using double-digest restriction site-associated DNA sequencing (ddRADseq; Peterson et al., 2012). An extended description of sequencing library preparation is provided in Träger et al. (2021). Briefly, the ddRADseq method uses two restriction enzymes (in the present study, EcoRI and TaqI) to cut standardized 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 the length of 450 bp and biotin-labelled TaqI adapters. Then, a polymerase chain reaction (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. In order to exclude the possibility of contamination and to calculate the genotyping error of SNPs, the sample set per library included negative (no sample DNA) and positive (sample replica) controls.

Reads (sequenced DNA fragments) were bioinformatically analysed and filtered (see Appendix S1, for more info). Genotype information was extracted from the resulting VCF file using PGDSPIDER v2.1.1.3 (Lischer & Excoffier, 2012). Putatively adaptive SNPs were excluded using environmental association analysis (EAA) to detect SNPs associated to environmental factors related to habitat overgrowth and fragmentation and outlier tests to detect SNPs under potential diversifying or balancing selection (see Träger et al. (2021), for more information) to ensure that only putatively neutral SNPs were used in the further analyses. Population-based genetic diversity indices (unbiased expected and observed heterozygosity, \( \text{uH} \), and \( \text{H}_e \), respectively, and percentage of polymorphic loci, %P) were calculated using GenAlEx version 6.503 (Peakall & Smouse, 2005, 2012). Unbiased expected heterozygosity accounts for differences in population sizes and is shortened as expected heterozygosity (\( \text{H}_e \)) below. Inbreeding coefficients (\( F_{IS} \)) and genetic differentiation (\( F_{ST} \)) were calculated using the package "genepop" (Rousset, 2008) in R version 3.4.2 (R Core Team, 2017).

2.4 Landscape data

For assessing the role of landscape characteristics on the genetic patterns of \( P. \) veris, we used historical and current data about the distribution of calcareous grasslands and woody landscape elements because overgrowth of grasslands with trees and shrubs has been the most profound change in these landscapes. We obtained historical landscape data (woody elements and grasslands) from digitalized maps of 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). Current grasslands were assessed using the map layers of Estonian Seminatural Community Conservation Association and Estonian Environmental System (EELIS). Current woody elements (forests and shrubs) were obtained from Estonian Basic Map (1:10,000; Estonian Land Board). ArcGIS version 10.4 (ESRI, 2016) was used to merge initial map layers and digitalize the historical maps. Map analyses were done in QGIS version 2.18.14 (QGIS Development Team, 2017).
We used both node- and link-based approaches for assessing the effect of landscape characteristics on genetic diversity and gene flow (DiLeo & Wagner, 2016; Figure 1). For the node-based approach, we calculated the current and historical amount (i.e. area within the buffer) and grassland edge density (i.e. the length of grassland habitat edge in the buffer; m/ha) of alvar grasslands and woody elements (i.e. combined area of forests and shrubs) on-site surrounding the study populations in circular buffers with radii of 500 m, 1,000 m and 2,000 m (Figure 1b). Different buffer radii were used to determine the scale, at which the role of landscape composition on genetic diversity in *P. veris* is the strongest and to take into account the possible different dispersal ranges of the species and its pollinators. For extracting and calculating the amounts of landscape elements in buffers, we used SQL queries in SpatiaLite database in QGIS with native QGIS functions buffer, calculate area, clip and field calculator. For calculating edge densities, we used the LecoS plugin (Jung, 2016) in QGIS. In addition, and similarly as for landscape elements, we assessed the area covered by water in the buffers because several populations are located close to the sea. We also calculated the change in the amount of grassland and woody elements since the 1930s in the same buffers. For the link-based approach, we calculated the proportion of current and historical amount of alvar grassland and woody elements, area covered by waterbodies and change in the amount of grassland and woody elements in straight corridors of different widths (200 m, 500 m and 1,000 m radius) between population pairs (Figure 1c). Again, different buffer radii were used to take into account the possible different dispersal ranges of the species and its pollinators. Here, the proportion of areas was used, as the corridors were of different length, and thus, the actual areas were not comparable. For constructing the corridors, we created a polyline layer of all possible direct linear connections between populations with the LineString function and calculated the proportions of landscape elements in buffers around the corridors using the Python console in QGIS with the native QGIS functions buffer, calculate area, clip and field calculator. In addition, we calculated geographic distance starting from the centre of the population in a straight line between population pairs using again the Python console (function measureLength(line)) in QGIS.

### 2.5 Data analysis

Because predictor variables from different buffer radii used for the node-based approach were correlated with each other (Appendix S2), we made full models for each buffer radius separately (Appendix S3), using only uncorrelated variables in one model. We excluded woody elements change ($r = 500$ m, $1,000$ m, $2,000$ m), water amount ($r = 1,000$ m, $2,000$ m) and historical grassland amount ($r = 2,000$ m) in models due to many strong correlations with other variables.

### Table 1

| Population ID | Region | Site   | Longitude  | Latitude   | Samples before filtering | Samples after filtering | $H_e$ | $H_o$ | $F_{IS}$ | %P |
|---------------|--------|--------|------------|------------|--------------------------|-------------------------|------|------|---------|----|
| 1             | Saaremaa | Asva1  | 23.061226  | 58.45345   | 6                        | 6                        | 0.21 | 0.22 | -0.07   | 0.55|
| 2             | Saaremaa | Kahtla1| 23.240038  | 58.465636  | 20                       | 20                       | 0.24 | 0.25 | -0.03   | 0.75|
| 3             | Muhu    | Koguva | 23.091042  | 58.610773  | 20                       | 20                       | 0.26 | 0.27 | -0.02   | 0.85|
| 4             | Saaremaa | Köruse | 21.93925   | 58.446525  | 20                       | 20                       | 0.26 | 0.26 | 0.00    | 0.85|
| 5             | Muhu    | Lõetsa1| 23.314138  | 58.649971  | 20                       | 20                       | 0.28 | 0.27 | 0.03    | 0.87|
| 6             | Saaremaa | Lõu    | 22.201352  | 58.122065  | 20                       | 20                       | 0.27 | 0.26 | 0.04    | 0.78|
| 7             | Muhu    | Mäla   | 23.27088   | 58.579382  | 16                       | 15                       | 0.26 | 0.26 | 0.02    | 0.79|
| 8             | Saaremaa | Neeme  | 21.946529  | 58.483978  | 20                       | 20                       | 0.28 | 0.27 | 0.03    | 0.89|
| 9             | Saaremaa | Neeme  | 21.926973  | 58.49856   | 20                       | 18                       | 0.28 | 0.28 | 0.01    | 0.88|
| 10            | Muhu    | Nõmmküla| 23.208551  | 58.686868  | 20                       | 19                       | 0.28 | 0.27 | 0.06    | 0.90|
| 11            | Muhu    | Nõmmküla| 23.204176  | 58.666753  | 20                       | 20                       | 0.28 | 0.26 | 0.06    | 0.90|
| 12            | Saaremaa | Orrinömme| 23.023776  | 58.584617  | 20                       | 20                       | 0.27 | 0.27 | -0.01   | 0.84|
| 13            | Muhu    | Paenase| 23.153634  | 58.641189  | 20                       | 19                       | 0.28 | 0.28 | -0.02   | 0.89|
| 14            | Saaremaa | Vanamõisa| 22.674132  | 58.243252  | 20                       | 20                       | 0.21 | 0.21 | 0.00    | 0.79|
| 15            | Saaremaa | Vanamõisa| 22.685017  | 58.225494  | 17                       | 15                       | 0.22 | 0.21 | 0.05    | 0.78|
| 16            | Muhu    | Võiküla1| 23.384982  | 58.544683  | 20                       | 20                       | 0.28 | 0.28 | -0.03   | 0.89|
| 17            | Muhu    | Võiküla2| 23.308743  | 58.551047  | 20                       | 20                       | 0.26 | 0.27 | -0.02   | 0.83|
| 18            | Saaremaa | Võrsna | 23.746727  | 58.389119  | 11                       | 11                       | 0.25 | 0.22 | 0.11    | 0.81|
| 19            | Muhu    | Üügu   | 23.238277  | 58.67114   | 20                       | 19                       | 0.28 | 0.27 | 0.04    | 0.90|

| Geographic coordinates, the number of sampled individuals before and after bioinformatic filtering, expected heterozygosity ($H_e$), observed heterozygosity ($H_o$), inbreeding coefficient ($F_{IS}$) and the percentage of polymorphic loci (%P) in 19 study populations of *Primula veris* on the islands of Muhu and Saaremaa, Estonia.
All statistical analyses were done in R version 3.4.2 (R Core Team, 2017). We conducted linear models to examine the effect of landscape variables on expected and observed heterozygosity and inbreeding coefficient of *P. veris*. We carried out generalized linear models to examine the response of the percentage of polymorphic loci applying a quasibinomial error distribution (package “lme4Test” (Kuznetsova et al., 2017)). We also included region (Muhu/Saaremaa) as a co-variable in the analysis to take into account potential landscape historical differences between regions (Appendix S4). In addition, we included the interactions between region and other landscape variables in the models. We also added population size in the models, but because it had no significant effect in any of the models, we present results without population size. We made several full models (Appendix S3) because of the correlations between landscape variables (Appendix S2). For linear models, we used Akaike information criterion corrected for small sample sizes (AICc; Burnham & Anderson, 2002) with the function stepAICc (http://www.chris.toph-scherber.de/stepAICc.txt) to find the models with the best fit. stepAICc function excludes and adds variables one at a time from the full models to find the model with the lowest AICc. After finding the best model, it further tries to exclude or add variables to find the best model until adding or excluding variables no longer reduces the AICc. We considered models with ΔAICc < 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 with building the full models and manually excluded the least important variables (according to p-value) starting with interactions and compared the models with likelihood ratio tests until the model with fewer variables was explaining the variation in data significantly (p < .05) better than the respective more complex model.

To examine the effect of landscape composition between study populations on the gene flow in *P. veris* (i.e., link-based analysis), we conducted multivariate generalized mixed effect models (package “MCMCglmm”; Hadfield, 2010) with genetic differentiation FST as predictor variables. We made separate models for landscape variables calculated within buffers with different radii (200, 500, and 1,000 m). Some of the initially calculated landscape variables (change in the proportion of grasslands and woody elements, proportion of water bodies) were excluded because of the strong correlation with other variables (r > .6; Appendix S5). The change in the proportion of grasslands was not included in the further analysis because it had stronger correlations with other variables than historical proportion of grasslands. Population identity and region 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. We only used the data from population pairs that were not further than 27 km from each other, because isolation by distance (IBD) analysis reached a plateau at about 27 km (Träger et al. 2021) indicating that at larger distances other (random) factors than geographic distance and/or landscape characteristics might be more important drivers of genetic differentiation. We chose the best models according to deviance information criterion (DIC). We used model selection based on DIC to compare different models to a null model, which included only geographic distance as an independent variable and population identity and region of both populations in a pair as random variables.

3 | RESULTS

3.1 | Landscape analysis

Landscape variables calculated within different buffers (r = 500, 1,000, 2,000 m) showed very similar trends regarding the loss of alvar grasslands and increase in the area of forests and shrubs (Appendix S4). For example, the current average amount of alvar grasslands surrounding the study population in a circular buffer (r = 1,000 m) was 86.04 ± 56.34 ha (mean ± SD), while historical amount of alvar grasslands was 184.29 ha ± 51.20 ha (Appendix S4). On average, the amount of alvars decreased by 98.25 ha (% ± 47.65) since the 1930s. The amount of woody elements, on the contrary, has increased: woody elements cover currently 174.43 ± 44.49 ha, while the amount covered by forests and shrubs historically was on average 18.87 ± 32.66 ha. As a result, the amount of woody elements has increased by 155.56 ± 41.23 ha since the 1930s. The average percentage of current grassland amount in a corridor with a radius of 1,000 m around a straight line between two populations was 14% ± 11% (Appendix S6). The average proportion of historical alvar grasslands in the corridor was 45% ± 15%, mirroring the loss of semi-natural grasslands from the landscape (31% ± 15%). The proportion of woody elements cover currently nearly half (47% ± 13%) of the landscape between the study populations, while historically it was very low, covering on average 3% of the corridor area. Hence, the increase of the proportion covered by woody elements has been on average 44% ± 12%.

3.2 | Landscape genetic analysis

Sequencing of ddRAD fragments yielded about 150 million raw sequences per library, with about 1.2 million sequences per sample. SNP calling resulted in 411,426 raw SNPs, which were reduced to 2,619 putatively neutral SNPs after quality filtering and exclusion of putatively adaptive loci in a total of 338 individuals across 19 populations. Mean expected heterozygosity (H_E) was 0.26 ± 0.02, observed heterozygosity (H_O) was 0.26 ± 0.02, and percentage of polymorphic loci (%P) was 53% ± 8%. Mean inbreeding coefficient (Fis) was 0.01 ± 0.04 (Table 1). Mean FST between pairs of *P. veris* populations was 0.07 ± 0.03.
Table 2. Directions of effects, Akaike information criterion corrected for small sample sizes (AICc; current table includes models with AICc < 2) and adjusted R² (adj. R²) values of the best fit linear models of the effect of current and historical grassland amount, change in grassland amount, historical amount of woody elements, current and historical grassland edge density, region (Muhu/Saaremaa) and interactions of region with current and historical grassland amount, change in the grassland amount, the historical amount of woody elements amount, and current and historical grassland edge density on genetic diversity of *Primula veris* populations in the node-based analysis.

| Gen div ind | Current grassland amount | Historical grassland amount | Grassland amount change | Historical woody elements amount | Current grassland edge density | Historical grassland edge density | Water | Region | Region: current grassland amount | Region: historical grassland amount | Region: historical woody elements amount | Region: current grassland edge density | Region: historical grassland edge density | AICc | Adj. R² |
|-------------|--------------------------|-----------------------------|-------------------------|---------------------------------|-------------------------------|----------------------------------|-------|--------|---------------------------------|---------------------------------|--------------------------------------------|-------------------------------|---------------------------------|------|--------|
| r = 500 m   |                          |                             |                         |                                 |                               |                                  |       |        |                                 |                                 |                                            |                                |                                 |      |        |
| Hₑ         | 8.8 x 10⁻⁴               | 1.2 x 10⁻³                  | 8.7 x 10⁻⁴             | 1.3 x 10⁻³                     | -3.8 x 10⁻²                   | -4.3 x 10⁻²                     | -93.45 | .52   | -91.73                         | -94.61                         | -94.14                                 | -93.06                         | -92.84                         |      |        |
| Hₑ         | 2.6 x 10⁻⁴               | 4.4 x 10⁻²                  | 1.3 x 10⁻³             | -1.1 x 10⁻⁰                    | -8.7 x 10⁻²                   | -1.3 x 10⁻⁰                    | -7.5 x 10⁻⁴ | 7.5 x 10⁻⁴ | -92.49                         | -92.00                         | -92.93                                 | -91.93                         | -91.72                         |      |        |
| r = 1,000 m |                          |                             |                         |                                 |                               |                                  |       |        |                                 |                                 |                                            |                                |                                 |      |        |
| Hₑ         | -2.7 x 10⁻⁴             | -7.9 x 10⁻⁵                 | -3.0 x 10⁻⁴            | 7.0 x 10⁻⁴                     | -3.5 x 10⁻²                   | -4.6 x 10⁻⁴                    | -92.00 | .48   | -90.97                         | -92.93                         | -92.49                                 | -91.93                         | -91.72                         |      |        |
| Hₑ         | 6.5 x 10⁻⁵              | 9.4 x 10⁻⁵                 | -3.0 x 10⁻⁴            | 7.5 x 10⁻⁴                     | 1.6 x 10⁻²                    | 3.4 x 10⁻⁴                     | -9.2 x 10⁻⁵ | 9.4 x 10⁻⁵ | -86.18                         | -86.18                         | -85.80                                 | -85.43                         | -85.43                         |      |        |
| r = 2,000 m |                          |                             |                         |                                 |                               |                                  |       |        |                                 |                                 |                                            |                                |                                 |      |        |
| Hₑ         | -4.8 x 10⁻³             | 4.8 x 10⁻⁴                  | 6.2 x 10⁻³             | 4.9 x 10⁻⁴                     | -6.6 x 10⁻³                   | -5.4 x 10⁻³                    | 1.6 x 10⁻⁴ | 1.6 x 10⁻⁴ | -87.30                         | -87.07                         | -85.80                                 | -85.43                         | -85.43                         |      |        |
| Hₑ         | 1.9 x 10⁻⁵              | -5.3 x 10⁻⁵                 | -3.8 x 10⁻⁵            | 5.5 x 10⁻⁴                     | -2.5 x 10⁻²                   | -1.3 x 10⁻⁴                    | 88.88  | .55   | 89.87                          | -89.87                         | -89.87                                 | -88.87                         | -88.87                         |      |        |
| Hₑ         | 3.6 x 10⁻³              | -4.7 x 10⁻³                 | -3.8 x 10⁻⁵            | -2.0 x 10⁻³                    | -2.6 x 10⁻²                   | -1.7 x 10⁻⁴                    | 1.6 x 10⁻⁴ | 1.6 x 10⁻⁴ | -88.97                         | -88.97                         | -88.97                                 | -88.97                         | -88.97                         |      |        |
| Hₑ         | -9.2 x 10⁻⁵             | -6.5 x 10⁻⁵                 | -9.2 x 10⁻⁵            | -6.6 x 10⁻²                    | -2.7 x 10⁻²                   | -9.2 x 10⁻⁵                    | -88.97 | .44   | -88.97                         | -88.97                         | -88.97                                 | -88.97                         | -88.97                         |      |        |

Note: Some landscape variables were correlated, so several initial models were made (Appendix S3). Genetic diversity indices (Gen div ind) used were expected heterozygosity (Hₑ) and observed heterozygosity (Hₑ). Landscape variables were calculated as the areas in buffers (r = 500 m, 1,000 m, 2000 m) around the study populations (Figure 1). Significant values (p < .05) are highlighted in bold.
Different measures of genetic diversity were on average higher in Muhu than in Saaremaa (Table 2). The response of genetic diversity to landscape variables in the node-based approach depended on the scale \((r = 500, 1,000 \text{ and } 2,000 \text{ m})\), as well as the measure of genetic diversity. In the best models with the lowest AICc, historical grassland edge density exhibited a most constant and significant positive effect on different variables of genetic diversity. In particular, both \(H_o\) and \(H_e\) increased with historical edge density within a 500-metre buffer radius around populations (Table 2; Figure 2). Furthermore, \(H_o\) showed a significant positive response to historical edge density also in the models with landscape variables calculated within the buffers of 1,000 and 2,000 metres (Table 2). At larger spatial scale (buffer radius of 2,000 m), \(H_e\) decreased in landscapes with stronger loss of grassland area. Historical grassland amount had a significant negative effect on \(H_e\) at the buffer radius of 1,000 metres. However, as the historical edge density was strongly negatively correlated with historical habitat amount \((r = -0.88 \text{ Appendix S2})\) at this spatial scale, it is not possible to discriminate the effect of grassland amount from the effect of edge density. Historical grassland edge density also had a positive effect on \%P (500 m, Figure 2; 2,000 m) as did historical grassland amount (500 m; Appendix S7). Current grassland amount had a significant positive effect on \%P at smaller scale \((r = 500 \text{ m})\), but at larger scales a negative effect on \(H_o\) (2,000 m) and \(H_e\) (1,000 m, Saaremaa; 2,000 m). The change in the area of grasslands had a negative effect on \%P at smaller spatial scales \((r = 500 \text{ m} \text{ and } r = 1,000 \text{ m})\), but a positive effect in models, which included landscape variables calculated within the buffer with the largest radius (2,000 m). Current grassland edge density had a positive effect on \%P \((r = 500 \text{ m})\), but surprisingly, a negative effect at larger spatial scale \((r = 2,000 \text{ m})\). In one model \((r = 1,000 \text{ m})\), current grassland edge density had a negative effect for \%P in Muhu, but a positive effect in Saaremaa. Historical woody elements amount had a positive effect on \%P (500 m; 1,000 m; 2,000 m, Saaremaa). Current amount of woody elements had a negative effect on \%P (1,000 m, 2,000 m) in Muhu. None of the variables had a significant effect on \(F_{IS}\).

In the link-based approach, the best generalized mixed effect models about the role of different landscape elements between pairs of populations on pairwise \(F_{ST}\) according to DIC were with a buffer radius of 1,000 m around a straight line between populations (Appendix S8; Tables 3 and 4). The best model included the effect of geographic distance, historical proportion of grasslands and the current proportion of woody elements in a buffer. In this model, geographic distance had a significant positive effect on \(F_{ST}\) (Figure 3a), while higher historical proportion of grasslands led to lower genetic differentiation (Figure 3b). As the DIC of the best model differed less than 2 from four other models, a single best model may not be determined. However, the significant variables in these models still stayed the same, so we report the results of only the best model.

4 | DISCUSSION

In the current study, we found that depending on the spatial scale and the measure of genetic diversity, both historical and current landscape variables drive the genetic patterns of the perennial grassland plant Primula veris. In general, the results indicate that the
landscape composition at the scale of 500 and 1,000 metres in the surroundings of the populations of insect-pollinated grassland plants is more relevant in shaping the within-population genetic diversity, while landscape parameters at larger scale are of lesser importance. These distances also reflect the spatial scales, where most of the pollinating insects, such as bumblebees (Redhead et al., 2016) and other wild bees (Zurbruchen et al., 2010) forage, indicating the potential role of pollinators as mediators of gene flow in *P. veris*, a self-incompatible insect-pollinated plant.

We observed a significant positive effect of historical grassland edge density on the genetic diversity of *P. veris*. We also found that the replacement of open grassland habitats by woody elements as a result of abandoning traditional grassland management affects the genetic diversity and differentiation of populations of the grassland plant *P. veris*. In particular, we detected that an overall decline of grassland can lead to decreased genetic diversity of *P. veris*. In addition, the historical proportion of grasslands showed a significant negative effect on genetic differentiation as a measure of gene flow between populations. This as well as the significant effect of past edge density suggests lagged responses in the genetic patterns to landscape change.

### 4.1 The role of landscape composition on genetic diversity and gene flow

We found that higher (historical) proportion of alvar grasslands between the study populations has a negative effect on the genetic differentiation ($F_{ST}$) of *P. veris* populations, indicating more gene flow between populations with higher proportion of historical grasslands between them. Thus, high proportion of suitable habitats between grassland plant populations supports intra-specific gene flow by ensuring functional connectivity, that is the movement of seed and pollen (Auffret et al., 2017). In the study area, pollinator observations on “LIFE to alvars” (Helm, 2019) project sites revealed that the overgrowth of alvars with shrubs and trees was accompanied by a significant loss in the diversity and abundance of important pollinator groups—butterflies and bumblebees (Prangel, 2017). It is thus very likely that

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TABLE 3 Deviance information criterion (DIC) values of models from the link-based analysis for three buffer zones ($r = 200, 500$ and $1,000$ m) for the direct path between population pairs of *Primula veris* on the islands of Muhu and Saaremaa

| Nr  | Model | $r = 200$ m | $r = 500$ m | $r = 1,000$ m |
|-----|-------|-------------|-------------|---------------|
| 1   | $F_{ST}$ - dist | 573.00 | 573.00 | 573.00 |
| 2   | $F_{ST}$ - dist + curr. grass | 574.00 | 573.58 | 574.50 |
| 3   | $F_{ST}$ - dist + hist. grass | 569.07 | 568.18 | 561.36 |
| 4   | $F_{ST}$ - dist + curr. woody | 571.72 | 570.19 | 566.59 |
| 5   | $F_{ST}$ - dist + hist. woody | 574.87 | 574.19 | 573.52 |
| 6   | $F_{ST}$ - dist + curr. grass + hist. grass | 570.98 | 569.93 | 563.33 |
| 7   | $F_{ST}$ - dist + curr. grass + curr. woody | 571.90 | 569.48 | 567.09 |
| 8   | $F_{ST}$ - dist + curr. grass + hist. woody | 575.04 | 572.53 | 572.53 |
| 9   | $F_{ST}$ - dist + hist. grass + curr. woody | 570.21 | 568.13 | 560.58* |
| 10  | $F_{ST}$ - dist + hist. grass + hist. woody | 570.85 | 569.10 | 561.55 |
| 11  | $F_{ST}$ - dist + curr. woody + hist. woody | 573.68 | 571.94 | 567.52 |
| 12  | $F_{ST}$ - dist + curr. grass + hist. grass + curr. woody | 571.82 | 569.15 | 562.23 |
| 13  | $F_{ST}$ - dist + curr. grass + hist. grass + hist. woody | 572.65 | 570.17 | 562.60 |
| 14  | $F_{ST}$ - dist + curr. grass + curr. woody + hist. woody | 573.64 | 570.93 | 568.74 |
| 15  | $F_{ST}$ - dist + hist. grass + curr. woody + hist. woody | 572.22 | 570.00 | 562.45 |
| 16  | $F_{ST}$ - dist + curr. grass + hist. grass + curr. woody + hist. woody | 573.73 | 570.65 | 563.69 |

Note: Variables in the model: $F_{ST}$—genetic differentiation, dist—geographic distance, curr. grass—current grassland proportion, hist. grass—historical grassland proportion, curr. woody—current woody elements proportion, hist. woody—historical woody elements (forests and shrubs) proportion. All variables were calculated as proportional amounts in the buffers and rank transformed. DIC values for models containing only geographic distance as explanatory variable are independent of the buffer zone and have thus the same value. Model DIC values with $\Delta$DIC < 2 within the buffer radius are highlighted in bold. The best model for each radius according to DIC is underlined.

*The overall best model according to DIC.
TABLE 4 Results of the best fit generalized mixed effect model of the effect of current grassland amount, historical grassland amount, the current proportion of woody elements, historical proportion of woody elements and geographic distance on pairwise genetic differentiation (\(F_{ST}\)) of Primula veris populations

| Effect size          | I-95% | U-95% | \(P_{\text{MCMC}}\) |
|----------------------|-------|-------|----------------------|
| Historical proportion of grasslands | −0.27 | −0.44 | −0.091 | 0.001*** |
| Current proportion of woody elements | −0.13 | −0.30 | 0.033 | 0.11 |
| Geographic distance | 0.59  | 0.44  | 0.74  | <0.001*** |

Note: We present the results of the best model (radius = 1,000 m) according to deviance information criterion (DIC). I-95% and U-95% – lower and upper limit of 95% confidence interval, respectively. Significance value: ***\(p<.001\). Statistically significant results are highlighted in bold.

changes in landscape structure, such as the overgrowth of alvars and consequent loss of suitable habitats, can lead to deficiency of pollen vectors, which, in turn, is likely to have an overall negative effect on genetic diversity and gene flow. However, in semi-natural grasslands such as alvars, achieving functional connectivity may be also ensured by the movement of cattle or sheep as plant seed vectors between grassland complexes. With the recovery of cattle-grazing, semi-natural habitats might be preserved by preventing overgrowth of grasslands with shrubs on the one hand and by spreading plant seeds within and among grassland patches on the other (Rico, Holderegger, et al., 2014). At the study sites, sheep and cattle were introduced (after sample collection) as the main management tools for ensuring the maintenance of “LIFE to alvars” project sites.

We found that the proportion of polymorphic loci (%P) and observed heterozygosity (\(H_o\)) of \(P. veris\) populations were generally lower in landscapes, which had experienced more drastic grassland loss. In addition, %P decreased with decreasing habitat amount (\(r = 500\) m), whereas \(H_o\), surprisingly, increased with decreasing habitat amount (\(2,000\) m). The area of grasslands has been found to have a positive effect on the genetic diversity in grassland plants (e.g. Aavik et al., 2019; Hahn et al., 2013; Toma et al., 2015). This is partly mediated by the positive effect of habitat amount on population size and thus the potential gene pool (DiLeo & Wagner, 2016; Leimu et al., 2006; Young et al., 1996). However, in our study, population sizes of \(P. veris\) might be still sufficiently large to not affect genetic diversity because we did not find a significant relationship between these two factors. The longevity of the study species should also be considered, because it might additionally buffer potential habitat and population size effects on the measures of genetic diversity (see section “Delayed response of genetic patterns to landscape change” below). Yet, in the long term, it can be expected that loss of habitat amount may lead to decreased population size that in turn could increase the negative influence of genetic drift (Balkenhol et al., 2015), which causes the random loss of alleles, as well as inbreeding (Young et al., 1996).

Surprisingly, we observed a relatively constant positive effect of (historical) grassland edge density on the genetic diversity (\(H_e\), \(H_o\), %P) of \(P. veris\). It has been demonstrated that edge density (Sowińska Świękosz, 2020), particularly the density of those edges which occur between different habitat types, may be very good predictors of plant species richness (Ma et al., 2013). However, we are not aware of any previous studies, which would have detected such strong effects of landscape-scale edge density on plant genetic diversity. There are several possibilities how edge density can affect within-population genetic diversity of plants. First, edge habitats usually represent environmentally more heterogeneous habitats, which, in turn, can select for a broader range of genotypes adapted to different environmental conditions (Vellend & Geber, 2005) compared to homogeneous conditions. However, as the present study relies on neutral genetic markers, which should not respond to selection, this might not be the most applicable explanation. Second, edges of grasslands may be important habitats for pollinators, whose contribution to the gene exchange in insect-pollinated plants may be substantial (e.g. Jabis et al., 2011). Indeed, pollinators have been shown to prefer edge habitats due to more feeding and nesting opportunities (Díaz-Forero et al., 2011, 2013; Söber et al., 2020) as well as more suitable microclimate (Bergman et al., 1996) and possibly to shrubs due to being potential landmarks (Cranmer et al., 2012). Additionally, in our study, current and historical grassland edge densities were strongly negatively correlated with current and historical amount of grasslands, respectively, meaning sites with more grasslands have less grassland edges per area unit. Thus, the negative influence of the amount of grasslands on genetic diversity observed in some of the models could likely mirror the preference of pollinators towards landscapes with more edge habitats. Therefore, alvar grasslands should still harbour some degree of shrub coverage (Helim, 2019), while the connectivity between sites should be maintained (Bergman et al., 2018; Steffan-Dewenter & Tscharntke, 1999) and overgrowth avoided.

The results revealed a positive effect of historical (but not current) amount of woody elements in the proximity of studied alvar grasslands on the percentage of polymorphic loci (%P) of \(P. veris\). The effect of forest on the genetic diversity of insect-pollinated grassland plants is usually assumed to be negative as it can act as a barrier for pollinators and thus diminish gene flow (Aavik et al., 2014, 2017; DiLeo et al., 2018). However, Hahn et al. (2013) did not detect the negative influence of forest on the genetic diversity and gene flow of \(Trifolium montanum\). It has been hypothesized that forest would reduce the movement of pollinators and thereby hamper gene flow in insect-pollinated grassland plants when these woody landscape elements cover more than half of the landscape (Aavik et al., 2019). Yet, several pollinators are able to cross forests and shrubs up to certain distances (e.g. Zuruchen et al., 2010) and some forest cover may be even beneficial to, for example, butterflies (Bergman et al., 2018). In addition, the relatively low historical amount of forest and shrubs may have acted similarly to grassland edges for pollinators providing feeding and nesting sites as well as better microclimate.
4.2 | Delayed response of genetic patterns to landscape change

We found support for patterns of genetic diversity of *P. veris* to exhibit a lagged response to landscape change, although some of the genetic variables responded to current landscape parameters. Notably, historical grassland edge density had a significant effect on genetic diversity (*H_e, H_o, %P*) of *P. veris* as did historical grassland amount. Historical amount of woody elements surrounding the study populations had a significant positive effect on percentage of polymorphic loci of *P. veris* populations (see previous paragraph). In addition, historical but not current proportion of grasslands between study populations led to lower genetic differentiation (*F_{ST}*) suggesting higher levels of gene flow between those plant populations.

Working in the same region as the study area, Aavik et al. (2019) found some evidence of lagged responses (at larger scale, r = 2,000 m) of the genetic diversity of *T. montanum* to landscape variables in alvar grasslands, but Helm et al. (2009) found that the genetic diversity of *Briza media* is influenced mostly by contemporary landscape parameters. These somewhat contrasting results could partially be explained by different dispersal characteristics of the species as *B. media* is a wind-dispersed plant whereas *T. montanum* and *P. veris* are insect-pollinated. Another characteristic influencing the existence of a lagged response may be the life span of the species. Long-lived species are more likely to exhibit a lagged response to landscape change than short-lived species (Epps & Keyghobadi, 2015). As *P. veris* is a long-lived herbaceous species (i.e. life span up to 50 years; Brys & Jacquemyn, 2009), observing delayed responses can indeed be expected. In contrast, a study in the same region (Aavik et al., 2017) found that the genetic diversity of an annual plant *Rhinanthus osiliensis* reacts to contemporary landscape. However, a growing number of studies on grassland plants have demonstrated so-called genetic extinction debt (Münzbergová et al., 2013; Plue et al., 2017; Reisch et al., 2017), showing these lagged responses should be studied more in depth (e.g. Cousins et al., 2007). On the other hand, if the genetic diversity of grassland plants such as *P. veris* has not yet reacted to changes in landscape structure, there are still good opportunities for restoration activities to maintain high levels of standing genetic diversity crucial in an era of global change.

5 | CONCLUSIONS

The present study is one of the few to use simultaneously both node- and link-based approaches for analysing the effects of landscape change on genetic patterns of plants. We demonstrate that despite the vast landscape change in Estonian alvars, genetic diversity as well as patterns of gene flow of *Primula veris* still reflects past landscape context. Therefore, relationships between current habitat amount and genetic patterns should be interpreted with caution. Clarifying the impact of landscape change on the genetic diversity and differentiation of plants is thus crucial for guiding decision-making in nature conservation policy. Where possible, maps about historical distribution of habitats may help to determine delays in biodiversity patterns. Such delays may provide opportunities for successful restoration of semi-natural grasslands before they suffer from the consequences of substantially decreased genetic diversity.

Most of the effects of landscape variables on the measures of within-population genetic diversity of *P. veris* were apparent at the scales of 500 and 1,000 m, while the role of landscape was less evident at the scale of 2,000 m. Because these distances correspond
to the foraging distances of several important pollinator groups (e.g., bumblebees and bees), it is very probable that pollinating insects have an important role in supporting the gene flow of insect-pollinated grasslands plants compared to, for example, cattle-mediated seed dispersal, which has been lacking from these grasslands for a long period of time. The significant contribution of pollinators may also be reflected by the substantial positive effect of landscape-scale density of grassland edges as suitable feeding habitats for pollinators on the within-population genetic diversity of *P. veris*. Thus, we advocate that in future landscape genetic studies of insect-pollinated plants, the response of plant genetic patterns to landscape factors should be examined in the framework of plant-pollinator interactions. When pollinators play a major role in the gene exchange of grassland plants, the analyses should consider the perception of the landscape composition and scales from the viewpoint of pollinators.

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**CONFLICT OF INTEREST**

The authors declare no conflicts of interest.

**PEER REVIEW**

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**DATA AVAILABILITY STATEMENT**

Sequence data used in this study will be made available at the European Nucleotide Archive (ENA) upon acceptance of Träger et al. (2021; study accession no. PRJE840977). Genetic diversity and differentiation, and landscape data are available at the Dryad Digital Repository (DOI https://doi.org/10.5061/dryad.h70rxwdj).

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Author contributions: T.A., I.R. and A.H designed the conceptual approach and carried out field work. S.T. and I.R. conducted laboratory work. S.T. performed bioinformatic analyses. A.H. and I.H-A. provided maps for landscape data. I.R. and S.T. analysed the data. I.R. wrote the first draft of the manuscript. All authors read, commented and approved the final version of the manuscript.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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