Genetic diversity at putatively adaptive but not neutral loci in *Primula veris* responds to recent habitat change in semi-natural grasslands

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Running head: Plant genetic response to habitat change

Note

The following manuscript is under consideration at *Molecular Ecology*.

Abstract

Recent habitat change in semi-natural grasslands due to a lack of management has been shown to affect the genetic diversity of grassland plants. However, it is unknown how such a change in local environment affects genetic diversity at adaptive loci. We applied RADseq (restriction-site associated DNA sequencing) to extract > 3,000 SNPs across 568 individuals from 32 Estonian populations of *Primula veris*, a plant species common to semi-natural grasslands. We evaluated the effect of recent grassland overgrowth due to management abandonment on the genetic diversity at both putatively neutral and adaptive loci, which we distinguished by applying three methods, i.e., linear and categorical environmental association analyses, and an F_{ST} outlier test. Effects of recent habitat change on genetic diversity differed between neutral and adaptive SNP sets. Genetic diversity assessed at putatively neutral loci was similar in open and overgrown habitats but showed a significant difference between these habitat types at putatively adaptive loci: overgrown (i.e. newly established) habitats exhibited higher genetic diversity at putatively adaptive loci compared to open (i.e. old) habitats, most likely due to the exertion of novel selection pressures imposed by new habitat conditions. This increase in genetic diversity at putatively adaptive loci in the new environment points to currently ongoing selection processes where genetic adaptation to the old habitat was lost through altered allele frequencies. Our study emphasises that a recent change in local habitat conditions may not be reflected in neutral loci whereas putatively adaptive loci can inform about potential ongoing selection in novel habitats.

Key words: environmental association analysis, genetic differentiation, habitat degradation, outlier test, semi-natural grasslands

Introduction

Habitat change is a consequence of ongoing anthropogenic landscape and climatic change (IPBES, 2018; IPCC, 2019). In particular, intensification of land use and abandonment of traditional management practices during the last century led to a dramatic degradation and isolation of habitats such as European semi-natural grasslands (Cousins, Auffret, Lindgren, & Tränk, 2015; Hooftman & Bullock, 2012). Historically, moderate and continuous human management (e.g. grazing by livestock) has led to open semi-natural grassland habitats with increased light availability and elevated niche partitioning, facilitating uniquely high levels of biodiversity (Habel et al., 2013; Wilson, Peet, Dengler, & Pärtel, 2012). Yet, abandonment of traditional management has gradually caused substantial loss in grassland area, and increased fragmentation and degradation due to overgrowth with dense woody vegetation or conversion to other land use types. This has resulted in negative effects on grassland biodiversity (Habel et al., 2013; Picó & Van Groenendael, 2007).

Likewise, the intra-specific genetic diversity of grassland plants can suffer from the degradation, fragmentation, and loss of semi-natural grasslands. Due to potentially reduced population sizes and increased landscape barriers, the genetic diversity of many insect-pollinated grassland plant species is compromised by interrupted pollen-mediated gene flow as a result of reduced pollinator movement in degraded and fragmented grasslands (e.g. DiLeo, Holderegger, & Wagner, 2018; Tewksbury et al., 2002). This likely aggravated gene flow contributes to potentially decreased genetic diversity and increased genetic differentiation in plant populations in affected grasslands (e.g. Honnay & Jacquemyn, 2007; Picó & Van Groenendael, 2007). However, plants might exhibit a delayed response to habitat changes (e.g. Aavik et al., 2019; Helm, Hanski, & Pärtel, 2006; Lehtilä et al., 2016).

For instance, life history traits, such as mating system and lifespan, can determine the speed and magnitude of a plant's response to a changed environment (Hamrick & Godt, 1996; Leimu, Mutikainen, Koricheva, & Fischer, 2006). With many grassland plants having a relatively long lifespan of up to several decades (Ehrlén & Lehtilä, 2002), such longevity might mask genetic effects induced by land use changes.

Genetic diversity is one of the central parameters in estimating a population's adaptive potential (Bilska & Szczecińska, 2016). However, because environmental change does not leave a signature in all parts of the genome (Nei, Suzuki, & Nozawa, 2010), such estimation requires differentiating between genetic diversity assessed at putatively neutral and adaptive loci (Bilska & Szczecińska, 2016). Here, we refer to putatively neutral loci when loci are affected by neutral processes such as gene flow but not by specific environmental factors. In contrast, putatively adaptive loci explicitly show a response to the tested environmental factors. Well-adapted populations might show a reduced diversity at adaptive loci due to beneficial mutations going towards fixation, and it is the adaptive potential at the population level that can be assessed with investigations of genetic diversity at adaptive loci (Milot et al., 2020). However, the difference in response of genetic diversity at putatively neutral and adaptive loci to habitat change has so far been mostly ignored, in particular in the context of recent land use change. The only existing studies that explicitly account for a difference in neutral and adaptive loci in plants concentrated on climatic factors (Dauphin et al., 2020; Sun et al., 2020). Moreover, in conservation genetics, most studies focussed on overall or genetic diversity at neutral loci so far, often using a set of neutral microsatellite markers, while ignoring adaptive regions of the genome (González et al., 2019; Wei & Jiang, 2020). However, it is exactly the adaptive regions that are important for the fate of a population.

With gradual grassland overgrowth, plant populations experience changed environmental conditions with novel selection pressures such as lower light availability, changes in soil chemical conditions, and altered and decreased pollinator communities (Helm, 2019), demanding for phenotypic plasticity of individuals or adaptation to the new habitat conditions. The adaptive potential of a population can be nourished from three sources: standing genetic variation (Barrett & Schluter, 2008; Radwan & Babik, 2012), gene flow (Slatkin, 1985) or, in the longer term, spontaneous mutations. Newly introduced barriers to gene flow should increase the importance of a population's standing genetic variation, and novel selection pressures induced by habitat change likely trigger a decrease in adaptation to the former grassland habitat. Thus, habitat change forces plants to either adapt to locally new habitat conditions predominantly based on their standing genetic variation in an aggravated gene flow scenario, to disperse to more favourable habitats, or to face local extinction (e.g. Cheptou, Hargreaves, Bonte, & Jacquemyn, 2017; Frankham, 2005).

In the present study, we were interested in the effect of recent overgrowth of Estonian semi-natural grasslands with woody vegetation over the past century on the genetic diversity at putatively neutral and adaptive loci in *Primula veris* populations, a long-lived grassland specialist plant. Our study is one of the first to test for a land use change effect on both putatively neutral and adaptive loci in *in-situ* wild plant populations. We applied double-digest restriction-site associated DNA sequencing (ddRADseq) in 32 populations of *P. veris* from open and recently overgrown grasslands. We distinguished between putatively neutral and adaptive loci by performing a combination of environmental association analyses and F_{ST} outlier tests. We specifically asked whether (1) genetic diversity at neutral loci of *P. veris* populations is negatively affected by overgrowth of grasslands due to

potentially reduced population size and/or aggravated gene flow; (2) genetic diversity at adaptive loci exhibits a different response to habitat change than genetic diversity at neutral loci; and (3) genetic diversity at adaptive loci is actually increased due to ongoing selection processes where genetic adaptations to the old, open habitat are slowly lost through altered allele frequencies of the beneficial alleles for open and overgrown habitats.

Materials and Methods

Study species

Primula veris L. (Primulaceae) is an herbaceous perennial rosette-forming hemicryptophyte most commonly occurring in calcareous grasslands. *Primula veris* prefers open habitats but can grow under shade with reduced reproduction (Brys & Jacquemyn, 2009). Its average life span reaches up to 50 years (Ehrlén & Lehtilä, 2002). In Estonia, the study region, *P. veris* generally flowers in May. The study species is an obligate outbreeder that depends on insect-pollination (mostly bees and bumblebees; Deschepper, Brys, & Jacquemyn, 2018). Pollen dispersal is spatially restricted to several meters (Brys & Jacquemyn, 2009). Self-pollination is prevented by heterostyly with two flower morphs, with low levels of successful intra-morph pollination (Wedderburn & Richards, 1990). Primary seed dispersal is limited to a few metres from the maternal plant (Brys & Jacquemyn, 2009).

Study sites and sampling

Study sites were located on dry calcareous grasslands, alvars, on the islands of Muhu and Saaremaa in Western Estonia (Figure 1). Alvars are semi-natural grasslands on Ordovician and Silurian bedrock with only a low soil depth (< 20 cm). Management, i.e. grazing livestock, in the area was abandoned 20 – 90 years ago. Our study sites were part of a large-

scale biodiversity inventory of an European Commission's LIFE+ Nature program restoration project ”LIFE to Alvars” (Helm, 2019), which included monitoring of genetic diversity of grassland plant species, including *P. veris*, in alvars at different successional stages of overgrowth (e.g. still open and recently overgrown). The mean temperature in the area is 17°C in summer and -3°C in winter, and the mean annual precipitation is about 680 mm (EWS, 2020).

We sampled 32 populations (i.e. spatially distinct patches) of *P. veris* distributed across two regions, Muhu and Saaremaa islands, in the summers of 2015 and 2016 (Figure 1; Table 1). Where possible, we chose pairs of closely located populations (i.e. within pollen- and seed-mediated gene flow distance) of contrasting habitat types (i.e. open and recently overgrown grasslands). Finally, 19 populations were located in open grasslands (i.e. old habitat; hereafter open habitats) and 13 populations were located in shrubby-overgrown grasslands (i.e. new habitat; hereafter overgrown habitats), comprising 10 population pairs with an average distance of 533 m and a minimum distance of 20 m between members of pairs. Such a paired sampling design has been shown to be efficient in detecting genomic signatures of local adaptation in environmental association analysis (EAA; Lotterhos & Whitlock, 2015) and allows the use of categorical EAA approaches (see below). Overgrown habitats represented mid-successional stages with at least 60% cover of shrubby vegetation, mostly *Juniperus communis*.

Within each population, we sampled three fresh leaves of 20 random flowering *P. veris* individuals (where possible) that were at least 50 cm apart. Leaves were stored in silica-gel until further processing. Approximate population census sizes of *P. veris* were estimated by assessing the number of both flowering and non-flowering individuals per population.

159 Environmental data

160 To characterize the environment of the study sites, we used data collected within the frame
161 of the "LIFE to alvars" project (Helm, 2019). We selected 16 *in-situ* measured environmental
162 variables regarding their potential to represent contrasting habitat types (open and
163 overgrown) from multiple environmental levels, i.e. "openness", "soil", and "biota". For
164 openness, we considered the total percentual shrub and tree coverage, respectively,
165 assessed within a radius of 10 m from the center of *P. veris* populations, and the light
166 availability above and below the herbal layer measured with Li-Cor LI-250 Light Meter and
167 LI-190SA Quantum Sensor (Lincoln, Nebraska, USA), in 1x1 m in the center of the
168 population. For soil, we considered average soil depth in cm based on ten random locations
169 taken within a 10 m radius around the central point of *P. veris* populations. In the same
170 radius, five soil samples were taken from random locations and pooled for chemical
171 analyses. From each sample, soil pH (KCl solution), available soil phosphorus (P; extraction
172 with acid ammonium lactate solution), pottasium (K), magnesium (Mg), calcium (Ca), and
173 soil organic content (OC, loss on ignition) were measured. For biota, we considered butterfly
174 and bumblebee abundance and richness and vascular plant richness within a 10 m radius
175 around the central points of *P. veris* populations. Butterflies and bumblebees were
176 monitored using standardised transect counts (Pollard, 1977). Each site was visited three
177 times for butterflies and two times for bumblebees over two years to cover phenological
178 aspects of different species (Helm, 2019).

179 In addition to the 16 *in-situ* measured environmental variables, we extracted climate
180 data for each population from CHELSA (Karger et al., 2017) with a resolution of 30 arc sec
181 from the reference period 1979-2013. To increase resolution, we applied a bi-linear
182 interpolation that accounts for the climate values in surrounding grid cells and the position

of the population within the grid cell. For our analyses, we used temperature (Bio1, annual mean temperature) and precipitation (Bio12, annual precipitation sum), because they represent the most comprehensive bioclimatic variables describing the climate in our study region.

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.

DNA extraction and ddRAD sequencing

Twenty-five mg of leaf material were pulverized with 2.3-mm chrome-steel beads (BioSpec Products, Bartlesville, USA) in a Mixer Mill 301 (Retsch, Haan, Germany). DNA was extracted using the LGC sbeadex plant kit (LGC, Berlin, Germany). 400 µl lysis buffer, consisting of 1% RNAse (100 mg/ml), 0.2% Proteinase K solution (20 mg/ml), and lysis buffer PN were added to pulverized samples, with an incubation time of 1 h at 65°C on a Thermomixer comfort (Eppendorf, Hamburg, Germany) at 300 rpm, and followed by a centrifugation at 2500 x g for 10 min. Lysates were transferred to binding solution, consisting of 420 µl binding buffer PN and 10 µl sbeadex particle solution. All following steps were conducted on a KingFisher Flex Purification System (Thermo Fisher Scientific, Waltham, USA), with the specification of using 400 µl of wash buffer PN1 twice, 400 µl of wash buffer PN2, and eluting purified DNA in 50 µl elution buffer AMP.

We applied a ddRADseq procedure by customizing an existing ddRADseq protocol (Westergaard et al., 2019). ddRADseq applies a double restriction enzyme digest followed by a size-selection of genomic fragments (Peterson, Weber, Kay, Fisher, & Hoekstra, 2012). RADseq provides a simple and cost-effective method to uncover thousands of polymorphic

markers, both neutral and adaptive, in model and non-model organisms (e.g. Davey et al., 2011). Because the aim of our study was to identify general patterns of genetic diversity assessed at neutral and adaptive loci across a high number of samples from many populations, rather than identifying specific genes involved in adaptation, we chose not to use whole genome or targeted sequencing. Such methods might be, however, worthwhile to consider in future analyses following the results of the present study.

For the detailed ddRADseq protocol see supplemental information (Supplemental Methods). Briefly, standardized DNAs of fully randomized samples were digested and purified before ligation to a combination of one of 48 EcoRI and 2 TaqI adapters, respectively, resulting in uniquely tagged barcoded DNA samples. DNA samples with the same TaqI adapter but different EcoRI adapters (48 samples) were pooled together and size selected for fragments of 450 bp length. The size-selected sample pools were selected for fragments containing biotin labelled TaqI adapters. Subsequently, polymerase chain reaction (PCR) was conducted, PCR products (ddRADseq libraries) were purified and their DNA concentration was measured to calculate molarity per ddRADseq library. Finally, samples with distinct TaqI multiplexing indices were combined to produce a final library of at least 5 nM consisting of 96 samples (2 x 48 uniquely barcoded samples from two multiplex indices). In addition, for sequencing, we used 15% of a standard Illumina library to increase index diversity.

Pooled libraries were prepared according to guidelines of the sequencing facility and sequenced on an Illumina HiSeq2500 at the Functional Genomics Centre Zurich (FGCZ, Switzerland), using one lane per library with 125 cycles in single-end read (125 bp), high-output mode. The sample set per library included a negative (no sample DNA) and a positive

(sample replica; different positive controls in different libraries) control to exclude the possibility of contamination and to calculate the genotyping error of SNPs.

Bioinformatic analysis

Sequence data were demultiplexed, and PCR duplicates were filtered using the functions "process_radtags" and "clone_filter" of STACKS v1.47, respectively (Catchen et al., 2011; Catchen, Hohenlohe, Bassham, Amores, & Cresko, 2013). We used trimmed sequencing reads (generated sequences), applying TRIMMOMATIC v0.36 (Bolger, Lohse, & Usadel, 2014) with the following conditions: (1) removing Illumina adapter matches allowing a maximum of two mismatches, (2) removing leading and trailing low quality or N bases below a quality score of 5, (3) performing a 5 bp sliding window quality check and trimming sequence ends if quality dropped below 15, and (4) dropping sequences, which were < 50 bp after previous quality checks. Pre-filtered sequence reads were aligned and mapped against the reference genome of *P. veris* (Nowak et al., 2015) using BURROWS-WHEELER ALIGNER v0.7.17 (BWA; Li, 2013). SNPs were called using FREEBAYES v1.1.0-54-g49413aa (Garrison & Marth, 2012) applying default values except a minimum-mapping-quality of 5, a minimum-base-quality of 5, and evaluating the 10 best SNP alleles. We only used SNPs which met quality criteria of the DDOCENT SNP filtering pipeline (Puritz, Hollenbeck, & Gold, 2014; Puritz, Matz, et al., 2014) with customizing the following parameters: minimum quality score of 20, minor allele count of 3, and maximum missing value proportion of 20% across all individuals. Loci potentially in linkage disequilibrium were filtered using VCFTOOLS 0.1.15 (*geno-r2* function; Danecek et al., 2011) keeping one random SNP of potentially linked SNP pairs with a threshold of 0.8. Loci showing an excess of heterozygotes (> 60% of the samples identified as heterozygotes) were filtered in R v3.4.2 (R Development Core Team, 2017). The

252 genotyping error of filtered SNPs was calculated by the weighted mean of error rates using
253 replica samples (i.e. positive controls) with TIGER v1.0 (Wegmann Lab, 2019).

254 ## Compilation of adaptive and neutral SNP sets

255 From the total SNP data set (SNP_overall), we identified putatively adaptive SNPs
256 (SNP_adapt) that were either (a) linearly associated to environmental factors or (b)
257 categorically associated to habitat types (see below). An additional putatively adaptive SNP
258 set (SNP_BayeScan) was compiled by identifying SNPs under potential diversifying or
259 balancing selection applying an F_{ST} outlier test using BAYESCAN v2.1 (Foll & Gaggiotti, 2008).
260 The models for BAYESCAN ran with default parameters (10 prior odds, 5,000 iterations with a
261 thinning interval of 10, a burn in of 50,000 and 20 pilot runs of 5,000 iterations). Potential
262 F_{ST} outlier loci were extracted for q-values 0.05.

263 The putatively neutral SNP set (SNP_neutral) was gained by excluding both putatively
264 adaptive SNP sets (SNP_adapt and SNP_BayeScan) from SNP_overall. Subsequently, we
265 calculated population genetic diversity parameters for both SNP_neutral and SNP_adapt.
266 We did not use the SNP_BayeScan set for the estimation of genetic diversity indices at
267 adaptive loci, because the SNPs identified by BAYESCAN might represent loci putatively
268 involved in adaptation to other environmental factors than investigated here and were only
269 used to better define SNP_neutral.

270 ## Environmental association analyses

271 To detect putative signatures of natural selection in open and overgrown habitats, we used
272 EAA that correlates environmental variation (describing the local habitat) with genetic
273 variation of a population (Rellstab, Gugerli, Eckert, Hancock & Holderegger, 2015). Here, we
274 performed EAAs with two types of relationships: linear and categorical.

For the linear analysis, we used latent factor mixed models (*lfmm_ridge* and *lfmm_test* functions in LFMM v2.0 in R; Caye, Jumentier, Lepeule, & François, 2019), which test for a linear relationship of the allele frequency (AF) at each SNP with each environmental variable while accounting for population structure with random latent factors. All 32 populations were considered, and all 18 environmental variables were used in EAA. We did not remove correlated variables, because our aim was to identify all SNPs with any sign of environmental adaptation for the compilation of the different SNP sets. For all subsequent analyses, however, we concentrated on those SNPs that were associated with environmental variables that significantly differed among the two habitat types. In LFMM, the number of latent factors has to be set by the user and is recommended to be based on the number of genetic clusters in the study systems (see below) and the inflation factor λ (François, Martins, Caye, & Schoville, 2016). To control for false discoveries, we adjusted the *p* values per environmental variable using λ and the χ^2 distribution (Caye et al., 2019; François et al., 2016) and applied the Benjamini-Hochberg algorithm (Benjamini & Hochberg, 1995) with a false discovery rate (FDR) of 0.05.

For the categorical analysis, we performed three different pairwise analyses on population AFs of the 20 populations that were sampled in pairs, i.e., geographically close, but environmentally diverged (open-overgrown; Table 1): a paired t-test, a paired Wilcoxon signed-rank test, and a sign test. To reduce false positive findings, only SNPs whose population AFs were significantly different between habitat types in all three categorical tests were considered for further analyses. For the t- and Wilcoxon tests, we used an α value of 0.05. In the sign test, we checked whether AF differences between the two populations of all pairs were consistent (i.e. had the same sign). We considered SNPs

298 significant if they were consistent in a minimum of eight out of ten comparisons (pairwise
299 AF differences of 0 were treated as consistent). In these categorical analyses, population
300 structure is not directly incorporated, but since they test for differences within pairs,
301 population structure can be ignored. It is unlikely that population structure would lead to
302 different signs of AF differences in different pairs. For all further analyses on putatively
303 adaptive SNPs (SNP_adapt), we concentrated on the SNPs that were (a) associated in LFMM
304 to those environmental variables that significantly differed among the two habitat types
305 and/or (b) were significant in all three categorical tests.

306 For the SNP_adapt set, we also wanted to know whether we find a non-random pattern
307 of AF change ("direction") between the two populations of habitat pairs. For each SNP, we
308 identified the putatively beneficial allele (i.e. major allele; AF > 0.5) for the open habitat and
309 then calculated the average AF change of this beneficial allele from open to overgrown
310 habitat (the historical habitat change) within pairs. We then counted how many SNPs
311 exhibited a beneficial AF decrease or increase from open to overgrown habitats and used an
312 exact two-sided binomial test in R to check if this pattern deviated from a random 1:1 ratio.

313 Population genetic diversity

314 For measurements of genetic diversity at putatively neutral and adaptive loci, we calculated,
315 for all SNP sets, observed heterozygosity (H_o) using GenAlEx 6.503 (Peakall & Smouse, 2012),
316 and mean nucleotide diversity (π) using VCFtools within a window of 125 bp over all loci for
317 each population. Note that both parameters do not rely on allele frequencies and are thus
318 not confounded with the identification of putatively adaptive loci based on allele
319 frequencies (EAA) described above.

The effect of habitat type (open or overgrown) and SNP set (SNP_neutral or SNP_adapt), and their interaction, on genetic indices (H_o, π) was tested using linear mixed effect models with population (1 – 32) and region (Muhu or Saaremaa) as random effects (lmerTest v3.1-0, *lmer* function; Kuznetsova, Brockhoff, & Christensen, 2017). The latter random effect accounted for potential differences in landscape history among regions. To quantify the importance of fixed factors and their interaction we used the Likelihood Ratio Test to obtain *p* values, analysing the variance between the full and reduced models with Satterthwaite approximation (χ^2 and associated p values). Here, we were particularly interested in the interaction of habitat type and SNP set. A significant interaction implies that genetic diversity indices assessed at putatively neutral and adaptive loci behave differently in open and recently overgrown habitats. If the interaction term was found significant, we tested for differences of genetic diversity indices in the different habitats for each SNP set using post-hoc tests with least-square means (lsmeans v2.30-0, *lsmeans* function; Lenth, 2016). We also tested for an effect of population size on genetic diversity indices by including it as a fixed effect in the above mixed effect models. Due to non-significant effects of population size and its interaction, we present the results of mixed effect models without population size, only.

Population genetic structure and potential gene flow

Population genetic structure using SNP_neutral and SNP_overall was analysed using discriminant analysis of principle components (DAPC) in adegenet v2.1.1 in R (Jombart, 2008). DAPC uses uncorrelated principal component analysis (PCA) variables for discriminant analysis, producing synthetic discriminant functions that maximize between-group variation while minimizing within-group variation (Jombart, Devillard, & Balloux, 2010). We used cross-validation with 50 replicates to determine the number of principal

components (PCs) to be retained to avoid overfitting. The function *find.clusters* was used to determine the optimal number of clusters within the data sets. For validation, we also applied a hierarchical clustering tree analysis on a Nei's genetic distance matrix using mmod v1.3.3 and the *aboot* function from poppr v2.8.4 in R (Kamvar et al., 2019), with a cut-off of 50 and a bootstrap sample of 1,000.

Pairwise genetic differentiation (F_{ST}) among populations for SNP_neutral and SNP_overall was calculated using genepop v1.0.5 (Rousset et al., 2017) in R. Potential effects of geographic distance and habitat type "distance" (open-open, overgrown-overgrown, open-overgrown/overgrown-open) on genetic differentiation (F_{ST}) were tested for both SNP sets using multivariate generalized linear mixed models fitted with Markov chain Monte Carlo techniques (MCMCglmm) with 2,000,000 iterations and 500,000 burnins (MCMCglmm 2.29, *MCMCglmm* function; Hadfield, 2010) to account for non-independence of pairwise distance data. We present results of the best model according to DIC for each SNP set.

After a first visual inspection of the genetic and geographic distance relationship, we fitted multiple simple linear functions (package stats 3.4.2, *lm* function; Chambers, 1992) to further characterize potential isolation by distance (IBD; Van Strien, Holderegger, & Van Heck, 2015) and to estimate the maximum geographic distance up to which gene flow as indicated by genetic differentiation might be prevalent compared to other genetic processes, such as genetic drift. One set of linear models included pairwise F_{ST} values as response variable and increasing geographic distance as explanatory variable, the other complementary set of linear models fitted F_{ST} against a constant for the difference in geographic distance to 100 km (i.e. the maximum distance between study populations). The

366 threshold for potential gene flow was estimated as the point where the sum of the residual
367 standard errors of sets of complementary models stayed constant.

368

369 ## Results

370 Sequencing of ddRADseq fragments yielded on average about 150 M raw sequences per
371 library, with on average about 1.2 M sequences per sample. SNP calling and quality filtering
372 resulted in 4,588 SNPs. From those, 3,084 SNPs remained after excluding loci potentially in
373 linkage equilibrium and with an excess of heterozygotes, in a total of 568 individuals from
374 32 populations. The genotyping error of quality-filtered SNPs was 0.004. Negative controls
375 did not result in sequences.

376 ### Putatively adaptive loci

377 From the 18 environmental variables describing the habitat of populations, six significantly
378 (t-test, $p \leq 0.05$) differed among the two habitat types (open and overgrown; Figure S 1):
379 shrub coverage, light above and below the herbal layer, butterfly species richness and
380 abundance, and plant species richness.

381 In the linear EAA, based on the number of clusters in the DAPC (K = 6, see below) and
382 based on the fact that the inflation factor λ in un-adjusted p values did not vary
383 considerably from K = 3-10 in all environmental variables, we chose K = 6 latent factors for
384 the LFMM analysis. With an FDR of 0.05, we identified eight SNPs being associated to an
385 environmental variable (Table S 1). Only three of them were associated to one of the six
386 variables that significantly differed between the habitat types (butterfly abundance).

In the categorical EAA comparing the two habitat types, we identified 99 SNPs with the paired t-test ($p \leq 0.05$), 95 with the Wilcoxon test ($p \leq 0.05$), and 557 SNPs with the sign test. Seventy-four SNPs were identified in all three pairwise tests, but none of them overlapped with the three LFMM SNPs (Figure S 2). The 74 SNPs from categorical EAAs and the three SNPs from linear EAAs were used for further analyses (SNP_adapt = 77 SNPs).

In the 77 SNPs that were putatively involved in adaptation to habitat type, 53 SNPs showed a decrease of the average beneficial AF (for the old, open habitat) in the new, overgrown compared to the open habitat (Figure 2). The binomial test revealed that this pattern was significantly different from a random expectation ($p < 0.01$). However, AF differences between habitat types were small; average AF change was 0.09 (range 0.04-0.16) in the 53 SNPs with decreasing, and 0.08 (range 0.03-0.15) in the 24 SNPs with increasing beneficial AF. Yet, the maximum AF difference found between two populations of a pair in any SNP was 0.56 (Figure S 3).

The BayeScan analysis resulted in 391 potential F_{ST} outlier loci (SNP_BayeScan). These SNPs, together with those from SNP_adapt, were removed from the SNP_overall to create SNP_neutral (2619 loci). There was almost no overlap between SNP_adapt and SNP_BayeScan (Figure S 2).

Population genetic diversity

There was a significant interaction effect of habitat type and SNP set on observed heterozygosity (H_o; Table 2). For SNP_neutral, H_o ranged from 0.21 to 0.30 across all study populations (Table 1). There was no significant difference of H_o between populations in open and overgrown habitats (Figure 3a; post-hoc test: $p = 0.91$). For SNP_adapt, H_o ranged from 0.18 to 0.31 across all study populations (Table 1). Importantly, there was a significant

410 difference of H_o between populations in open and overgrown habitats (post-hoc test:
411 $p < 0.05$), with populations in overgrown habitats exhibiting increased H_o compared to
412 populations in open habitats (Figure 3a). The random factors region and population
413 accounted for 45.1% and 32.8% of variation in the data for H_o.

414 For nucleotide diversity (π), there was a marginally significant interaction effect of
415 habitat type and SNP set ($p = 0.056$; Table 2). For SNP_neutral, π ranged from 0.0024 and
416 0.0033 across all study populations (Table 1). There was no significant difference of π
417 between populations in open and overgrown habitats (Figure 3b; post-hoc test: $p = 0.97$).
418 For SNP_adapt, π ranged from 0.0022 and 0.0032 across all study populations (Table 1). π of
419 populations in overgrown habitats was higher than π of populations in open habitats, but
420 this difference was not significant (Figure 3b; post-hoc test: $p = 0.21$). The random factors
421 region and population accounted for 0% and 68.4% of variation in the data for π.

422 Population genetic structure and potential gene flow

423 DAPC of SNP_neutral identified six genetic clusters across the 32 populations with 200 PCs
424 retained (Figure 4a). The first two discriminant functions from DAPC analysis and the
425 hierarchical clustering tree analysis highlighted a differentiation by geographic regions,
426 mainly by the islands Muhu and Saaremaa (Figure 4b,d). Importantly, the separation of
427 genetic clusters was not based on habitat types (Figure 4c,d).

428 Pairwise F_{ST} values assessed using SNP_neutral were mostly moderate with an average
429 of 0.10 (± 0.05 SD) ranging from 0.01 to 0.25. We found a positive significant relationship
430 between pairwise F_{ST} and geographic distances among all populations ($p_{MCMCglmm} < 0.001$;
431 Table S 2), indicating isolation by distance (IBD; Figure 5). Model fitting showed that values
432 of residual standard error (RSE) of the models roughly reached a plateau between 15 to

433 30 km in geographic distance between populations (Figure S 4), indicating a potential
434 threshold up to where genetic differentiation is driven by geographic distance and gene flow
435 rather than random (genetic) processes. The habitat type did not explain patterns in genetic
436 differentiation (Table S 2).

437 Results of all analyses using SNP_overall were highly similar to when using SNP_neutral.
438 For completeness, these results are presented as supplemental information (Table S 2 – S 4,
439 Figure S 4 – S 7).

440

441 ## Discussion

442 Habitat degradation due to abandoned management and related loss and isolation of
443 European semi-natural grasslands (Auffret et al., 2018; Habel et al., 2013) during the last
444 century has been shown to negatively impact the biodiversity of these grasslands, both at
445 the species and the genetic level (e.g. Helm et al., 2006; Picó & Van Groenendael, 2007). Yet,
446 the effect of abandoned management, which can result in gradual overgrowth of grasslands
447 with woody vegetation, on the genetic diversity at adaptive loci of grassland plants has
448 remained unknown. Here, we examined the effect of recent overgrowth of abandoned
449 semi-natural calcareous grasslands (Estonian alvars) on the genetic diversity at putatively
450 neutral and adaptive loci of the perennial herb *Primula veris*. Our study revealed that *P.*
451 *veris* populations in the new, overgrown habitats had a similar level of genetic diversity at
452 putatively neutral loci as in the old, open habitats, despite substantial change in
453 environmental conditions. Genetic diversity at putatively adaptive loci, however, was higher
454 in the new, overgrown compared to the old, open habitats. Neutral genetic structure and
455 gene flow as indicated by neutral genetic differentiation was not (yet) affected by grassland

456 overgrowth. We are among the first to demonstrate how recently changed non-climatic
457 selection pressures are in the process of changing adaptive genetic patterns of wild
458 populations. Most other studies concentrated on the effects of climate on plants (e.g.
459 Dauphin et al., 2020; Sun et al., 2020) or used manipulative experiments in otherwise
460 natural habitats (Laurentino et al., 2020). Hence, our study is an example of *in-situ*
461 "adaptation in action", where genetic diversity at adaptive loci is increasing due to a slow
462 loss of previous genetic adaptations.

463 Overgrowth of semi-natural grasslands has often a negative impact on genetic diversity
464 of grassland-specialist plants due to lower habitat quality and potential creation of barriers
465 for gene flow (Aavik & Helm, 2018; Picó & Van Groenendael, 2007). However, in our study,
466 neutral genetic diversity of *P. veris* was similar in the open and the overgrown habitats,
467 indicating that habitat and landscape change do not necessarily restrict gene flow among
468 populations of *P. veris*. On the other hand, an environmental impact on the neutral part of a
469 genome might only show after several generations (e.g. Landguth et al., 2010). *Primula veris*
470 is a perennial plant with an average lifespan of up to 50 years and can persist with reduced
471 reproduction even when the environment has changed as a consequence of overgrowth
472 (Ehrlén & Lehtilä, 2002). Hence, genetic diversity of such populations potentially reflects the
473 state before their habitats started to change (Reinula, 2018). In addition, heterozygosity
474 indices, as used in our study, have been suggested to respond more slowly to environmental
475 change as compared to, for example, measures of inbreeding (Lloyd, Campbell, & Neel,
476 2013; Lowe, Boshier, Ward, Bacles, & Navarro, 2005). Deschepper et al. (2017), who
477 examined patterns of neutral genetic diversity of *P. veris* in grassland and forest populations
478 in Belgium, also reported no significant difference in H_o between habitat types. Yet, their
479 study system, i.e. forests, represents a late-successional stage, whereas ours represents a

480 mid-successional stage (shrubby overgrowth). Consequently, neutral genetic patterns of *P.*
481 *veris* might need a very long time (up to centuries) to reach an equilibrium with the new
482 overgrown environment.

483 In contrast to genetic diversity at putatively neutral loci, genetic diversity assessed at
484 putatively adaptive loci differed between habitat types in our study. Populations in recently
485 overgrown grasslands showed higher genetic diversity at putatively adaptive loci compared
486 to populations in open grasslands (Figure 3a,b). This is most likely caused by new selection
487 pressures in the new, overgrown habitats compared to the old, open habitats. The open
488 grasslands used in our study have been in an open state for several hundreds of years,
489 whereas ongoing overgrowth started only about 90 years ago (Helm et al., 2006). Hence,
490 populations of *P. veris* in open grasslands experienced homogenous selection pressures for
491 a very long time, which increased the frequencies of alleles beneficial for an open habitat
492 and led to reduced heterozygosity and genetic diversity at adaptive loci. The majority of the
493 putatively adaptive SNPs (53 out of 77) exhibited a smaller average beneficial (i.e. major) AF
494 for open habitat conditions in populations from the new, overgrown compared to those
495 from the old habitat. This led to an increase in heterozygosity and genetic diversity in
496 populations of the new, overgrown habitats (Figure 2). Considering *P. veris'* potential
497 longevity, this indicates that populations in overgrown habitats are still adapting to their
498 new selection pressures (e.g. lower light availability, reduced or altered pollinator
499 community), i.e. many alleles potentially beneficial for the new, overgrown habitat are still
500 far from fixation (i.e. homozygosity). Consequently, the genetic diversity at putatively
501 adaptive loci of *P. veris* populations in overgrown grasslands has not yet been reduced. A
502 similar pattern was found in the conifer tree *Pinus cembra*, where populations in the core of
503 the current niche exhibited a decreased genetic diversity at adaptive loci, but no difference

504 in the one at neutral loci, compared to populations at the niche margin, which most likely
505 present unstable or novel habitats (Dauphin et al., 2020).

506 An increase of non-beneficial alleles for the open habitat in the overgrown populations,
507 and thus an increase in genetic diversity and heterozygosity at putatively adaptive loci in
508 overgrown populations, can be achieved in two ways: (1) the alternative alleles at SNP loci
509 were either already present in lower frequencies in populations of open habitats (i.e.
510 standing genetic variation), or (2) arrived to overgrowing grasslands by gene flow, before
511 they were subject to positive selection in the overgrown habitat. As shown in Figure S 3,
512 beneficial alleles in populations of the open habitat were rarely fixed, strongly pointing
513 towards the importance of standing genetic variation. This implies that even populations
514 with reduced genetic diversity at putatively adaptive loci (e.g. in open grasslands) may
515 possess the ability to react to habitat changes due to the low, but crucial amount of
516 standing genetic variation (e.g. Morris, Bowles, Allen, Jamniczky, & Rogers, 2018). Still, we
517 found gene flow as indicated by genetic differentiation potentially spreading alleles
518 between habitats which, in theory, can contribute to the increased genetic diversity at
519 adaptive loci in overgrown populations. The potential gene flow distances found in our
520 study (up to 30 km; Figure 4, Figure 5) stand in marked contrast to the potential dispersal
521 ranges of pollen and seeds of *P. veris* for which very short distances up to 12 m and 0.5 m,
522 respectively, have been found (Antrobus & Lack, 1993; Richards & Ibrahim, 1978). Yet,
523 pollinating insects of *P. veris* have been shown to occasionally travel up to 2 km (Kreyer,
524 Oed, Walther-Hellwig, & Frankl, 2004; Zurbuchen, Bachofen, Müller, Hein, & Dorn, 2010)
525 and dispersal distances are often underestimated in ecological studies (e.g. Bullock, Shea, &
526 Skarpaas, 2006). Historical rotational grazing of domestic animals and movement of wild
527 animals (e.g. deer, moose, wild boar) might also have facilitated seed dispersal for longer

528 distances even for *P. veris* which is not adapted to zoochory (Plue, Aavik, & Cousins, 2019).
529 Overall, especially standing genetic variation but also gene flow are capable of supplying *P.*
530 *veris* populations undergoing habitat changes with new or alternative alleles fostering
531 adaptation to new habitat conditions in our study region.

532 Importantly, the fact that we detected a significant effect of habitat type on genetic
533 diversity (H_o) when using the putatively adaptive SNP set but not with the neutral SNP set
534 emphasizes the need to examine genetic diversity at neutral and adaptive loci separately
535 when studying the genetic response of plant species to environmental changes. Besides, in
536 the overall SNP set (Figure S 7), there was no significant effect of habitat type on genetic
537 diversity, which indicates that genetic diversity of an overall SNP set represents rather
538 neutral genetic patterns (Dauphin et al., 2020). In conservation genetics and restoration,
539 most assessments have been based on overall or neutral genetic diversity so far (González
540 et al., 2019; Wei & Jiang, 2020), even though it is most likely the putatively adaptive regions
541 of a genome that are important for the fate of a population in a changed environment.

542 The different effects of habitat type on genetic diversity at putatively neutral and
543 adaptive loci could partly be due to the unevenness in the number of loci at neutral and
544 putatively adaptive regions (2,619 versus 77, respectively). However, the ddRADseq
545 procedure used in our study should result in a "random" and representative subset of both,
546 neutral and adaptive SNPs. Here, we were not particularly interested in the actual molecular
547 mechanisms underlying adaptation, but in general patterns at putatively adaptive loci,
548 which should also become visible with 77 SNPs if these patterns are of substantial nature.
549 Additionally, the reliability of the detected SNPs can be assumed high, because the
550 genotyping error for our SNPs was low (0.004) and the SNP sets were identified using a draft

551 genome (Hoban et al., 2016), which covers about 63% of the whole 479.22 Mb genome of
552 the study species *P. veris* (Nowak et al., 2015).

553 In addition to genetic diversity, population size is another factor that is affected by
554 habitat change, and which also directly influences genetic diversity (Leimu et al., 2006). The
555 census sizes of our *P. veris* populations were similar in open and overgrown habitats and
556 were not associated with genetic diversity at neutral and adaptive loci. In contrast to our
557 results, a meta-analysis showed a clear relationship between population size and (neutral)
558 genetic diversity, including long-lived and self-incompatible plant species, such as *P. veris*
559 (Leimu et al., 2006). Populations in our study exhibited a minimum of about 100 individuals,
560 which is larger than the overall census for small populations in the study by Leimu et al.
561 (2006). Consequently, our *P. veris* populations might be still sufficiently large to counteract
562 population size driven effects on genetic diversity.

563 ## Conclusions

564 Our landscape genomic investigation of *Primula veris* in Estonian semi-natural grasslands is
565 one of the first to demonstrate the effects of land use change on the genetic diversity at
566 putatively adaptive loci of *in-situ* wild plant species. We show that the effect of recent
567 overgrowth of grasslands is not genetically manifested when considering neutral SNPs, even
568 after almost a century of ongoing environmental changes. Yet, genetic diversity assessed at
569 putatively adaptive loci was higher in populations in overgrown compared to open habitats,
570 most probably due to allele frequency changes of standing genetic variation. Thus, even
571 populations in degraded and fragmented habitats may possess the ability to adapt to
572 habitat changes due to their important standing genetic variation in addition to potential
573 allele immigration due to gene flow.

574 For perennial long-lived plant species, such as *P. veris*, long time spans might pass
575 before habitat change can be detected at neutral regions of the genome whereas habitat
576 effects at adaptive loci could be noticeable much faster. Consequently, a repeated
577 monitoring of genetic diversity at both neutral and adaptive loci and further investigations
578 of contemporary gene flow at different spatial scales would be highly valuable to identify
579 potential genetic consequences of recent and ongoing environmental change in natural and
580 semi-natural habitats. In addition, extending our results to whole-genome and targeted
581 sequencing approaches would be vital to reliably identify genes and gene networks
582 putatively involved in the adaptation of *P. veris* to habitat change, and to assess the relative
583 importance of loss of previous and gain of new genetic adaptations to the altered
584 environment.

585

586 ## Acknowledgements

587 We thank the Genetic Diversity Centre Zurich (GDC) for laboratory support, the Functional
588 Genomic Centre Zurich (FGCZ) for Illumina sequencing, A. Rogivue for introducing the lead
589 author to EAA, and B. Dauphin for extracting climate data and support in EAAs. We are
590 grateful for financial support from the Estonian Research Council (MOBJD427, PUT589 and
591 PRG874), the COST "G-BIKE" action (CA18134), the European Regional Development Fund
592 (Centre of Excellence EcolChange), and European Commission LIFE+ Nature program
593 (LIFE13NAT/EE/000082). We also thank three anonymous reviewers for their valuable
594 comments and suggestions on previous versions of this manuscript.

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856

857 ## Data Accessibility

858 Sequence data used in this study will be made available at the European Nucleotide Archive

859 (ENA) upon acceptance (ERS5253979 – ERS5254546). R-scripts, genotypic and

860 environmental data will be provided at the Dryad Digital Repository upon acceptance (xxx).

861 ## Author Contributions

862 T.A., S.T. and A.H designed the conceptual approach and carried out field work. S.T. and I.R.

863 conducted laboratory work. S.T. and N.Z. performed bioinformatic analyses. S.T. and C.R.

864 analysed the data. R.H. contributed with discussing the results in a broader ecological

865 context. S.T. wrote the manuscript with major contributions from C.R. All authors read,

866 commented and approved the final version of the manuscript.

867 ## Competing interests

868 The authors declare no competing interests.

Supplemental Information

Supplemental Methods

Figure S 1 Environmental variables and their response to habitat type (open – overgrown).

Figure S 2 Venn diagram of shared putatively adaptive loci by different methods.

Figure S 3 SNP allele frequencies and their behaviour in open and overgrown habitats.

Figure S 4 Residual standard error results for IBD analyses, measured using the overall (3,084 loci) and neutral (2,619 loci) SNP sets.

Figure S 5 Genetic structure of *Primula veris* populations in the study area measured using the overall set of loci (3,084 loci).

Figure S 6 Isolation by distance pattern measured at the overall (3,084 SNPs) set of loci for *Primula veris* populations.

Figure S 7 Genetic and nucleotide diversity (H_o and π) at the overall set of SNPs (3,084 loci).

Table S 1 Number of associations between SNPs and environmental variables.

Table S 2 Results of generalized mixed effect models for the effect of geographic distance and habitat type distance on pairwise genetic differentiation (F_{ST}) for the neutral (2,619 loci) and overall (3,084 loci) SNP sets.

Table S 3 Genetic diversity measures using the overall set of SNPs (3,084 loci) for the studied populations of *Primula veris*.

Table S 4 Results of linear mixed effect models for H_o, π for the overall SNP set (3,084 loci) of the studied *Primula veris* populations.

Tables

Table 1 Location information and genetic diversity measurements for the studied populations of *Primula veris* in Muhu and Saaremaa, Estonia. Habitat – affiliation to open or overgrown grassland; pair – affiliation to a pair of closely situated populations (0 indicates affiliation to no pair); N – sample size after SNP filtering; population size – estimated number of individuals per population; H_o – observed heterozygosity; π – nucleotide diversity.

									Neutral SNP set		Adaptive SNP set	
Pop ID	**Region**	**Site**	**Habitat**	**Pair**	**Latitude [°N]**	**Longitude [°E]**	**N**	**Population size**	H_o	π	H_o	π
1	Saaremaa	Asva1	open	1	58.445	23.061	6	100	0.22	0.0033	0.23	0.0032
2	Saaremaa	Asva2	overgrown	1	58.419	23.064	20	200	0.23	0.0027	0.25	0.0026
3	Saaremaa	Atla	overgrown	0	58.302	21.936	10	100	0.30	0.0031	0.30	0.0026
4	Saaremaa	Kahtla1	open	0	58.466	23.240	20	200	0.25	0.0028	0.23	0.0023
5	Muhu	Koguva	overgrown	2	58.611	23.091	20	2000	0.26	0.0027	0.27	0.0024
6	Muhu	Koguva	open	2	58.611	23.091	19	500	0.27	0.0028	0.26	0.0024
7	Saaremaa	Kõruse	overgrown	3	58.447	21.939	20	1000	0.27	0.0029	0.25	0.0025
8	Saaremaa	Kõruse	open	3	58.447	21.939	19	1000	0.26	0.0028	0.23	0.0023
9	Muhu	Lõetsa1	overgrown	4	58.650	23.313	20	1000	0.28	0.0029	0.31	0.0027
10	Muhu	Lõetsa1	open	4	58.650	23.314	19	1000	0.27	0.0029	0.25	0.0025
11	Muhu	Lõetsa2	overgrown	0	58.644	23.345	20	100	0.28	0.0029	0.31	0.0027
12	Saaremaa	Lõu	open	0	58.122	22.201	20	5000	0.26	0.0031	0.22	0.0026
13	Muhu	Mäla	overgrown	5	58.579	23.270	20	800	0.24	0.0029	0.27	0.0028
14	Muhu	Mäla	open	5	58.579	23.271	15	500	0.26	0.0030	0.24	0.0026
15	Saaremaa	Neeme	open	0	58.484	21.947	20	1500	0.27	0.0029	0.22	0.0024
16	Saaremaa	Neeme	open	0	58.499	21.927	18	3000	0.28	0.0029	0.28	0.0028
17	Muhu	Nõmmküla	open	0	58.669	23.209	19	1000	0.27	0.0029	0.30	0.0026
18	Muhu	Nõmmküla	open	0	58.667	23.204	20	500	0.26	0.0028	0.28	0.0025
19	Saaremaa	Orinõmme	open	6	58.585	23.024	20	3500	0.27	0.0028	0.26	0.0024
20	Saaremaa	Orinõmme	overgrown	6	58.584	23.024	20	5000	0.26	0.0028	0.27	0.0024
21	Muhu	Paenase	overgrown	7	58.641	23.159	20	1000	0.29	0.0029	0.30	0.0025

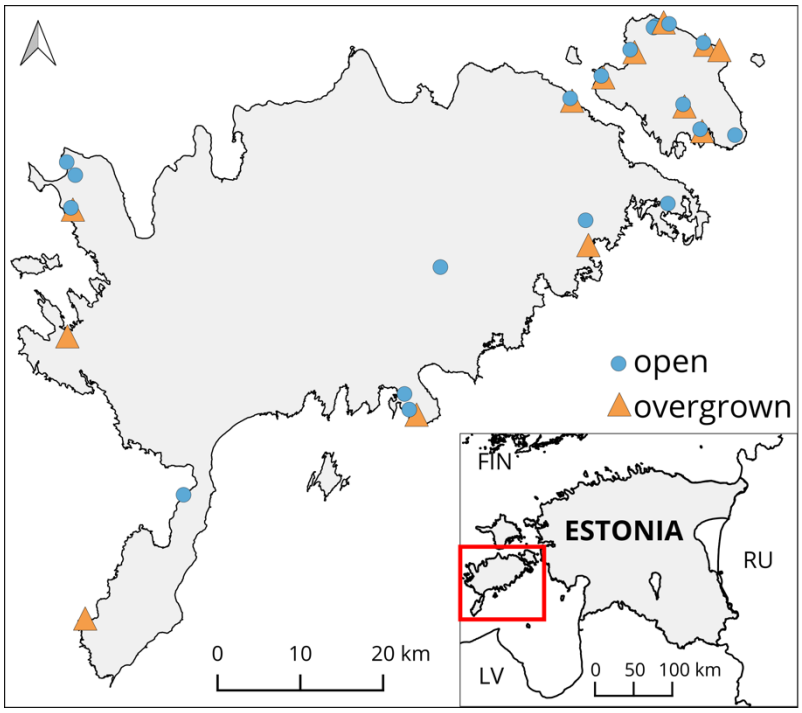
22	Muhu	Paenase	open	7	58.641	23.154	19	1000	0.28	0.0028	0.30	0.0024
23	Saaremaa	Türju	overgrown	0	57.980	21.993	17	100	0.26	0.0028	0.25	0.0025
24	Saaremaa	Vanamõisa	open	0	58.243	22.674	19	1000	0.21	0.0024	0.20	0.0023
25	Saaremaa	Vanamõisa	open	8	58.225	22.685	15	500	0.21	0.0025	0.18	0.0022
26	Saaremaa	Vanamõisa	overgrown	8	58.222	22.697	7	100	0.22	0.0031	0.25	0.0032
27	Muhu	Võiküla1	open	0	58.545	23.385	20	500	0.28	0.0028	0.28	0.0024
28	Muhu	Võiküla2	overgrown	9	58.551	23.309	20	10000	0.27	0.0030	0.29	0.0027
29	Muhu	Võiküla2	open	9	58.551	23.309	19	10000	0.27	0.0029	0.27	0.0026
30	Saaremaa	Võrsna	open	0	58.389	22.747	11	100	0.22	0.0027	0.23	0.0027
31	Muhu	Üügu	overgrown	10	58.675	23.222	17	500	0.28	0.0029	0.29	0.0028
32	Muhu	Üügu	open	10	58.671	23.238	19	4000	0.27	0.0029	0.27	0.0025
Open mean								1800	0.26	0.0029	0.25	0.0025
Overgrown mean								1700	0.27	0.0029	0.28	0.0027

894

Table 2 Results of linear mixed effect models for observed heterozygosity (H_o), and nucleotide diversity (π) of *Primula veris* populations. Fitted parameters (± SE), t-values and significance (p_{Model}) is given for the full model of each genetic index, H_o and π. Factors: habitat (H): open and overgrown; SNP_set (S): SNP_neutral and SNP_adaptive. RF_{region} and RF_{pop} denote the use of region (Muhu and Saaremaa) and population (1–32) as random factors (RF). The importance of the fixed factor interaction is given as χ^2 and *p*-values for each genetic measurement. For H_o and π, the importance of the fixed factor interaction is given for the comparison of the full model (with H * S) with the next simplest model (H + S).

Effect	Value	SE	t	p_{Model}	χ^2	*p*
H_o ~ H * S + RF_{region} + RF_{pop}					**Importance of H * S**	
Habitat	0.027	0.008	3.304	0.002		
SNP_set	0.008	0.005	1.793	0.083		
habitat:SNP_set	-0.021	0.007	-2.942	0.006	8.110	0.004
π ~ H * S + RF_{region} + RF_{pop}					**Importance of H * S**	
Habitat	$1.4e^{-4}$	$7.0e^{-5}$	2.009	0.051		
SNP_set	$3.4e^{-4}$	$3.5e^{-5}$	9.573	<0.001		
habitat:SNP_set	$-1.1e^{-4}$	$5.6e^{-5}$	-1.985	0.056	3.949	0.047

904 Figures



905

906 Figure 1 Map of the study area on Muhu and Saaremaa islands in Western Estonia. *Primula*
907 *veris* populations are depicted with blue filled circles when occurring in open semi-natural
908 grassland and with orange filled triangles when occurring in recently overgrown historical
909 semi-natural grasslands.

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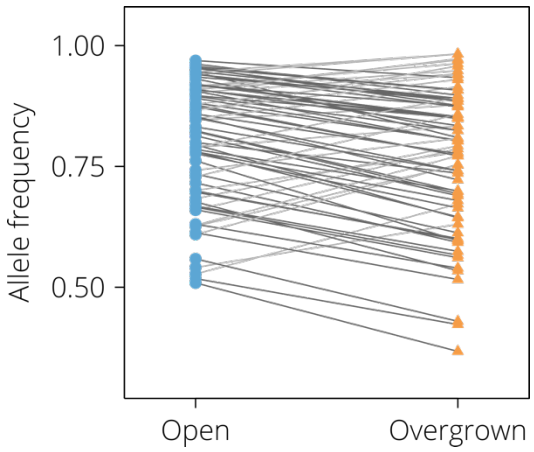
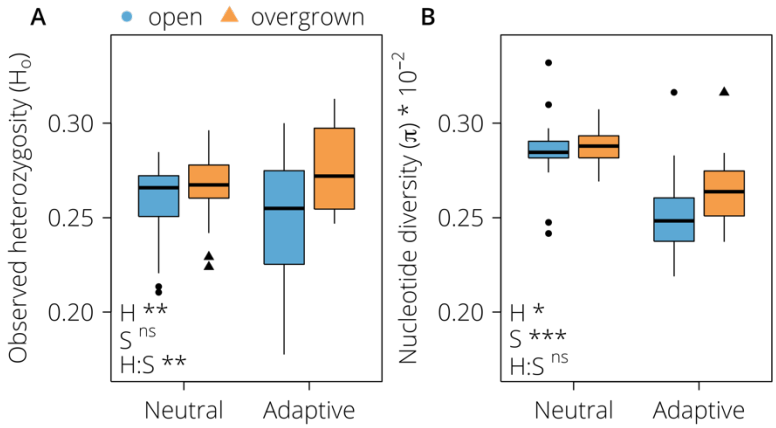


911

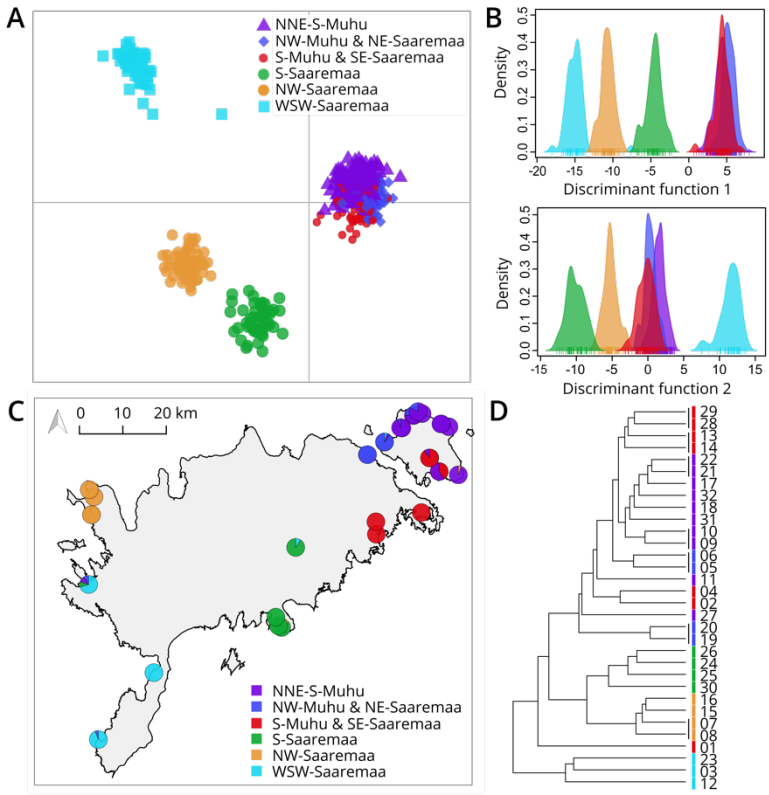
912 Figure 2 Patterns of allele frequency (AF) change of the 77 putatively adaptive SNPs
913 (SNP_adapt) averaged across pairs of populations for each SNP. For each SNP, the putatively
914 beneficial allele frequency for the open habitat (AF > 0.5) is shown. SNPs were derived from
915 linear and categorical environmental association analyses. SNPs with decreasing/increasing
916 AF in overgrown habitats (orange triangles) compared to open habitats (blue circles) are
917 indicated with dark grey/light grey lines, respectively. For detailed results, see Figure S 3.

918



919

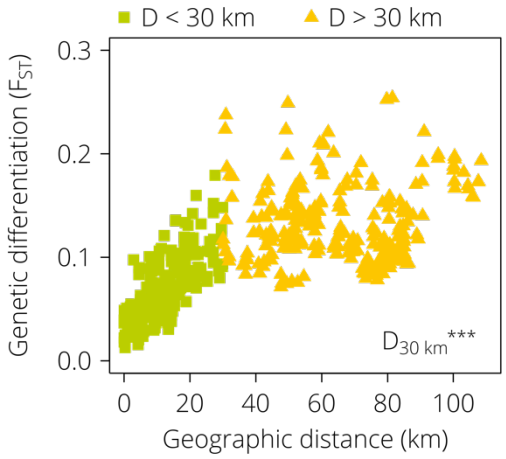
920 Figure 3 Boxplots of observed heterozygosity (H_o; A) and nucleotide diversity (π; B) at
921 putatively neutral and adaptive loci in open (blue, circles) and overgrown (orange, triangles)
922 grasslands. Factors: habitat (H): open and overgrown; SNP_set (S): SNP_neutral and
923 SNP_adaptive. Significance values: ^{ns} $p > 0.05$; * $p \leq 0.05$; ** $p < 0.01$; *** $p < 0.001$.



924

925 Figure 4 Genetic structure of *Primula veris* populations in the study region. (A) Result of the
926 discriminant analysis of principle components on the putatively neutral SNP set resulting in
927 six geographical clusters (C; named by cardinal directions). The distribution of the six
928 clusters per discriminant function is shown in panel B. (D) Result of the hierarchical
929 clustering tree analysis with population codes (see Table 1) and colour coding by cluster.

930



931

932 Figure 5 Relationship between genetic differentiation (F_{ST}) and geographic distance for all
933 possible pairs of 32 *Primula veris* populations, measured at putatively neutral loci.
934 Population pairs with a geographic distance (D) less or equal to 30 km are visualized as
935 green squares. Population pairs with a geographic distance greater than 30 km are given as
936 yellow triangles. The effect of geographic distance on F_{ST} is highly significant ($p < 0.001$) up
937 to a threshold of 30 km.