The effect of recent habitat change on genetic diversity at putatively adaptive and neutral loci in *Primula veris* in semi-natural grasslands

S. Träger*[1,2], C. Rellstab[3], I. Reinula[2], N. Zemp[4], A. Helm[2], R. Holderegger[3,5], T. Aavik[2]

[1]Institute of Biology/Geobotany and Botanical Garden, Martin Luther University Halle-Wittenberg, Große Steinstr. 79/80, 06108 Halle (Saale), Germany

[2]Department of Botany, Institute of Ecology and Earth Sciences, University of Tartu, Lai 40, 51005 Tartu, Estonia

[3]WSL Swiss Federal Research Institute, Zürcherstrasse 111, 8903 Birmensdorf, Switzerland

[4]Genetic Diversity Centre (GDC), ETH Zurich, Universitätstrasse 16, 8092 Zürich, Switzerland

[5]Institute of Integrative Biology (IBZ), ETH Zurich, Universitätstrasse 16, 8092 Zürich, Switzerland

*Corresponding author. E-mail: sabrina.traeger@botanik.uni-halle.de

Running head: Plant genetic response to habitat change

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 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 following management abandonment on the genetic diversity at 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. For validation, we randomised the genotype to sample assignments. 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 than open (i.e. old) habitats, 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 is potentially lost through altered allele frequencies. Our study suggests that a recent change in local habitat conditions may not be reflected in neutral loci whereas putatively adaptive loci can inform about potential selection processes.

Key words: environmental association analysis, genetic differentiation, habitat degradation, permutation, 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 supported development of 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, conversion to other land use types or abandonment of traditional management have gradually caused substantial loss in grassland area and increased fragmentation, i.e. due to overgrowth with dense woody vegetation. 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 suffers 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 (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 change (e.g. Aavik et al., 2019; Helm, Hanski, & Pärtel, 2006; Lehtilä et

62 al., 2016). For instance, life history traits, such as mating system and lifespan, determine the
63 speed and magnitude of a plant's response to a changed environment (Hamrick & Godt,
64 1996; Leimu, Mutikainen, Koricheva, & Fischer, 2006). With many grassland plants having a
65 relatively long lifespan of up to several decades (Ehrlén & Lehtilä, 2002), such longevity
66 might mask genetic effects induced by habitat change.

67 Genetic diversity is one of the central parameters in estimating a population's adaptive
68 potential (Bilska & Szczecińska, 2016). However, because environmental change does not
69 leave a signature in all parts of the genome (Nei, Suzuki, & Nozawa, 2010), such estimation
70 requires differentiating between genetic diversity assessed at putatively neutral and
71 adaptive loci (Bilska & Szczecińska, 2016). Here, we refer to putatively neutral loci when loci
72 are affected by neutral processes such as gene flow but not by specific environmental
73 factors. In contrast, putatively adaptive loci explicitly show a response to tested
74 environmental factors. Well-adapted populations might show a reduced diversity at
75 adaptive loci due to beneficial mutations going towards fixation, and it is the adaptive
76 potential at the population level that can be assessed with investigations of genetic diversity
77 at adaptive loci (Milot et al., 2020). However, the difference in response of genetic diversity
78 at putatively neutral and adaptive loci to habitat change has been mostly ignored so far, in
79 particular in the context of recent land use change. The only existing studies that explicitly
80 account for differences in neutral and adaptive loci in plants concentrated on climatic
81 factors (Dauphin et al., 2020; Sun et al., 2020). Moreover, in conservation genetics, most
82 studies focussed on overall or genetic diversity at neutral loci, often using a set of neutral
83 microsatellite markers, while ignoring adaptive regions of the genome (González et al.,
84 2019; Wei & Jiang, 2020). However, it is exactly the adaptive regions that are important for
85 the fate of a population.

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

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

133 project ”LIFE to Alvars” (Helm, 2019), which included monitoring of genetic diversity of
134 grassland plant species, including *P. veris*, in alvars at different successional stages of
135 overgrowth (e.g. still open and recently overgrown). The mean temperature in the area is
136 17°C in summer and -3°C in winter, and the mean annual precipitation is about 680 mm
137 (EWS, 2020).

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

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

155 Environmental data

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

174 In addition to the 16 *in-situ* measured environmental variables, we extracted climate
175 data for each population from CHELSA (Karger et al., 2017) with a resolution of 30 arc sec
176 from the reference period 1979-2013. To increase resolution, we applied a bi-linear
177 interpolation that accounts for the climate values in surrounding grid cells and the position
178 of the population within the grid cell. For our analyses, we used temperature (Bio1, annual

179 mean temperature) and precipitation (Bio12, annual precipitation sum), because they
180 represent the most comprehensive bioclimatic variables describing the climate in our study
181 region.

182 To test which environmental factors significantly differed between the two habitat
183 types, we performed a (non-paired) two-sample t-test for each of the 18 environmental
184 variables.

185 DNA extraction and ddRAD sequencing

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

197 We applied a ddRADseq procedure by customizing an existing ddRADseq protocol
198 (Westergaard et al., 2019). ddRADseq applies a double restriction enzyme digest followed
199 by a size-selection of genomic fragments (Peterson, Weber, Kay, Fisher, & Hoekstra, 2012).
200 RADseq provides a simple and cost-effective method to uncover thousands of polymorphic
201 markers, both neutral and adaptive, in model and non-model organisms (e.g. Davey et al.,

202 2011). Because the aim of our study was to identify general patterns of genetic diversity
203 assessed at neutral and adaptive loci across a high number of samples from many
204 populations, rather than identifying specific genes involved in adaptation, we chose not to
205 use whole genome or targeted sequencing. Such methods might be, however, worthwhile
206 to consider in future analyses following the results of the present study.

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

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

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

226 Bioinformatic analysis

227 Sequence data were demultiplexed, and PCR duplicates were filtered using the functions
228 "process_radtags" and "clone_filter" of STACKS v1.47, respectively (Catchen et al., 2011;
229 Catchen, Hohenlohe, Bassham, Amores, & Cresko, 2013). We used trimmed sequencing
230 reads (generated sequences), applying TRIMMOMATIC v0.36 (Bolger, Lohse, & Usadel, 2014)
231 with the following conditions: (1) removing Illumina adapter matches allowing a maximum
232 of two mismatches, (2) removing leading and trailing low quality or N bases below a quality
233 score of 5, (3) performing a 5 bp sliding window quality check and trimming sequence ends
234 if quality dropped below 15, and (4) dropping sequences, which were < 50 bp after previous
235 quality checks. Pre-filtered sequence reads were aligned and mapped against the reference
236 genome of *P. veris* (Nowak et al., 2015) using BURROWS-WHEELER ALIGNER v0.7.17 (BWA; Li,
237 2013). SNPs were called using FREEBAYES v1.1.0-54-g49413aa (Garrison & Marth, 2012)
238 applying default values except a minimum-mapping-quality of 5, a minimum-base-quality of
239 5, and evaluating the 10 best SNP alleles. We only used SNPs which met quality criteria of
240 the DDOCENT SNP filtering pipeline (Puritz, Hollenbeck, & Gold, 2014; Puritz, Matz, et al.,
241 2014) with customizing the following parameters: minimum quality score of 20, minor allele
242 count of 3, and maximum missing value proportion of 20% across all individuals. Loci
243 potentially in linkage disequilibrium were filtered using VCFTOOLS 0.1.15 (*geno-r2* function;
244 Danecek et al., 2011) keeping one random SNP of potentially linked SNP pairs with a
245 threshold of 0.8. Loci showing an excess of heterozygotes (> 60% of the samples identified
246 as heterozygotes) were filtered in R v3.4.2 (R Development Core Team, 2017). The

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

249 Compilation of adaptive and neutral SNP sets

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

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

265 Environmental association analyses

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

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

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

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

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

308 The use of linear and categorical EAAs for extracting putatively adaptive loci in respect
309 to specific environmental factors to test the effect of habitat differences (which are caused
310 by the same environmental factors) on genetic diversity indices might result in a circularity
311 problem. This is because major AF and heterozygosity are not completely independent
312 (Rosenberg & Jakobsson, 2008) and thus, differences in genetic diversity between habitat
313 types might reflect the results of EAAs. To address this potential problem, we created 100
314 SNP_overall data sets with fully randomised genotype to sample assignments in order to
315 create an empirical null distribution of the calculated parameters and statistics. Each of

316 those 100 SNP_overall data sets (hereafter randomised data sets) went through the same
317 process of EAAs and F_{ST} outlier analysis as the original data set to extract putatively neutral
318 and adaptive loci.

319 Population genetic diversity

320 For measurements of genetic diversity at putatively neutral and adaptive loci, we calculated,
321 for all SNP sets of the original and for SNP_neutral and SNP_adapt of each randomised data
322 set, observed heterozygosity (H_o) using GENALEX 6.503 (Peakall & Smouse, 2012), and mean
323 nucleotide diversity (π) using VCFTOOLS within a window of 125 bp over all loci for each
324 population.

325 The effect of habitat type (open or overgrown) and SNP set (SNP_neutral or
326 SNP_adapt), and their interaction, on genetic indices (H_o, π) was tested using linear mixed
327 effect models with population (1 – 32) and region (Muhu or Saaremaa) as random effects
328 (lmerTest v3.1-0, *lmer* function; Kuznetsova, Brockhoff, & Christensen, 2017), for the
329 original and each randomised data set. The latter random effect accounted for potential
330 differences in landscape history among regions. To quantify the importance of fixed factors
331 and their interaction we used the Likelihood Ratio Test to obtain *p* values, analysing the
332 variance between the full and reduced models with Satterthwaite approximation (χ^2 and
333 associated p values). Here, we were particularly interested in the interaction of habitat type
334 and SNP set. A significant interaction implies that genetic diversity indices assessed at
335 putatively neutral and adaptive loci behave differently in open and recently overgrown
336 habitats. If the interaction term was found significant, we tested for differences of genetic
337 diversity indices in the different habitats for each SNP set using post-hoc tests with least-
338 square means (lsmeans v2.30-0, *lsmeans* function; Lenth, 2016). We also tested for an effect

339 of population size on genetic diversity indices by including it as a fixed effect in the above
340 mixed effect models. Due to non-significant effects of population size and its interaction, we
341 only present the results of mixed effect models without population size.

342 We compared results of the original data set with those of the randomised data sets by
343 (a) calculating a "permutation *p*-value" for each factor (habitat type, SNP set and their
344 interaction), i.e. the number of *p*-values of the randomised data set model outputs which
345 were smaller than the *p*-value of the original data set model output divided by the total
346 amount of randomisations (100); (b) inspecting whether the proportion of putatively
347 adaptive SNPs with decreasing and increasing beneficial AF, respectively, of the original data
348 set is significantly different than the distribution of the proportion of SNP_adapt with
349 decreasing and increasing beneficial AF of the randomised data sets, using a t-test; and (c)
350 calculating the mean H_o and π per population over all 100 randomised data sets and
351 conducting a mixed-effect model analysis as done for the original data set.

352 Population genetic structure and potential gene flow

353 Population genetic structure using SNP_neutral and SNP_overall was analysed using
354 discriminant analysis of principle components (DAPC) in adegenet v2.1.1 in R (Jombart,
355 2008). DAPC uses uncorrelated principal component analysis (PCA) variables for
356 discriminant analysis, producing synthetic discriminant functions that maximize between-
357 group variation while minimizing within-group variation (Jombart, Devillard, & Balloux,
358 2010). We used cross-validation with 50 replicates to determine the number of principal
359 components (PCs) to be retained to avoid overfitting. The function *find.clusters* was used to
360 determine the optimal number of clusters within the data sets. For validation, we also
361 applied a hierarchical clustering tree analysis on a Nei's genetic distance matrix using mmod

362 v1.3.3 and the *aboot* function from poppr v2.8.4 in R (Kamvar et al., 2019), with a cut-off of
363 50 and a bootstrap sample of 1,000.

364 Pairwise genetic differentiation (F_{ST}) among populations for SNP_neutral and
365 SNP_overall was calculated using genepop v1.0.5 (Rousset et al., 2017) in R. Potential effects
366 of geographic distance and habitat type "distance" (open-open, overgrown-overgrown,
367 open-overgrown/overgrown-open) on genetic differentiation (F_{ST}) were tested for both SNP
368 sets using multivariate generalized linear mixed models fitted with Markov chain Monte
369 Carlo techniques (MCMCglmm) with 2,000,000 iterations and 500,000 burnins (MCMCglmm
370 2.29, *MCMCglmm* function; Hadfield, 2010) to account for non-independence of pairwise
371 distance data. We present results of the best model according to DIC for each SNP set.

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

383

Results

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

Putatively adaptive loci

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

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

406 SNPs from linear EAAs were used for further analyses (SNP_adapt = 77 SNPs). Using
407 randomised data sets, the mean number of SNP_adapt was 82 (± 20 SD) SNPs.

408 In the 77 SNPs that were putatively involved in adaptation to habitat type, 53 SNPs
409 showed a decrease of the average beneficial AF (for the old, open habitat) in the new,
410 overgrown compared to the open habitat (Figure 2). The binomial test revealed that this
411 pattern was significantly different from a random expectation ($p < 0.01$). However, AF
412 differences between habitat types were small; average AF change was 0.09 (range 0.04-
413 0.16) in the 53 SNPs with decreasing, and 0.08 (range 0.03-0.15) in the 24 SNPs with
414 increasing beneficial AF. Yet, the maximum AF difference found between two populations of
415 a pair in any SNP was 0.56 (Figure S 3). Using randomised data sets, the mean proportion of
416 SNP_adapt loci with decreasing beneficial AF was 52 (± 9 SD) %, and the mean proportion of
417 SNP_adapt loci with increasing beneficial AF was 48 (± 9 SD) % (Figure S 4).

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

422 Population genetic diversity

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

429 $p < 0.05$), with populations in overgrown habitats exhibiting increased H_o compared to
430 populations in open habitats (Figure 3a). The random factors region and population
431 accounted for 45.1% and 32.8% of variation in the data for H_o. For the mean of randomised
432 data sets, there was no significant interaction nor habitat effect on H_o ($p = 0.58$ and $p = 0.24$,
433 respectively) but a significant SNP set effect ($p < 0.001$; Figure S 5a and Figure S 6a). The
434 "permutation *p*-value" for H_o for the factor habitat type was $p = 0.05$, for SNP set it was
435 $p = 0.7$, and for the interaction it was $p = 0.04$.

436 For nucleotide diversity (π), there was a marginally (non-)significant interaction effect of
437 habitat type and SNP set ($p = 0.056$; Table 2). For SNP_neutral, π ranged from 0.0024 and
438 0.0033 across all study populations (Table 1). There was no significant difference of π
439 between populations in open and overgrown habitats (Figure 3b; post-hoc test: $p = 0.97$).
440 For SNP_adapt, π ranged from 0.0022 and 0.0032 across all study populations (Table 1). π of
441 populations in overgrown habitats was higher than π of populations in open habitats, but
442 this difference was not significant (Figure 3b; post-hoc test: $p = 0.21$). The random factors
443 region and population accounted for 0% and 68.4% of variation in the data for π. For the
444 mean of randomised data sets, there was no significant interaction nor habitat effect on π
445 ($p = 0.36$ and $p = 0.73$, respectively) but a significant SNP set effect ($p < 0.001$; Figure S 5b
446 and Figure S 6b). The "permutation *p*-value" for π for the factor habitat type was $p = 0.14$,
447 for SNP set it was $p = 0$, and for the interaction it was $p = 0.32$.

448 Population genetic structure and potential gene flow

449 DAPC of SNP_neutral identified six genetic clusters across the 32 populations with 200 PCs
450 retained (Figure 4a). The first two discriminant functions from DAPC analysis and the
451 hierarchical clustering tree analysis highlighted a differentiation by geographic regions,

452 mainly by the islands Muhu and Saaremaa (Figure 4b,d). Importantly, the separation of
453 genetic clusters was not based on habitat types (Figure 4c,d).

454 Pairwise F_{ST} values assessed using SNP_neutral were mostly moderate with an average
455 of 0.10 (± 0.05 SD) ranging from 0.01 to 0.25. We found a positive significant relationship
456 between pairwise F_{ST} and geographic distances among all populations ($p_{MCMCglmm} < 0.001$;
457 Table S 2), indicating isolation by distance (IBD; Figure 5). Model fitting showed that values
458 of residual standard error (RSE) of the models roughly reached a plateau between 15 to
459 30 km in geographic distance between populations (Figure S 7), indicating a potential
460 threshold up to where genetic differentiation is driven by geographic distance and gene flow
461 rather than random (genetic) processes. The habitat type did not explain patterns in genetic
462 differentiation (Table S 2).

463 Results of all analyses using SNP_overall were highly similar to when using SNP_neutral.
464 For completeness, these results are presented as supplemental information (Table S 2 – S 4,
465 Figure S 7 – S 10).

466

467 ## Discussion

468 Habitat degradation due to abandoned management and related loss and isolation of
469 European semi-natural grasslands (Auffret et al., 2018; Habel et al., 2013) during the last
470 century has been shown to negatively impact the biodiversity of these grasslands, both at
471 the species and the genetic level (e.g. Helm et al., 2006; Picó & Van Groenendael, 2007). Yet,
472 the effect of abandoned management, which can result in gradual overgrowth of grasslands
473 with woody vegetation, on the genetic diversity at adaptive loci of grassland plants has
474 remained unknown. Here, we examined the effect of recent overgrowth of abandoned

semi-natural calcareous grasslands (Estonian alvars) on the genetic diversity at putatively neutral and adaptive loci of the perennial herb *Primula veris*. Our study revealed that *P. veris* populations in the new, overgrown habitats likely had a similar level of genetic diversity at putatively neutral loci as in the old, open habitats, despite substantial change in environmental conditions. Genetic diversity at putatively adaptive loci, however, was higher in the new, overgrown compared to the old, open habitats. Neutral genetic structure and gene flow as indicated by neutral genetic differentiation was not (yet) affected by grassland overgrowth. We are among the first to demonstrate how recently changed non-climatic selection pressures are potentially in the process of changing adaptive genetic patterns of wild populations. Most other studies concentrated on the effects of climate on plants (e.g. Dauphin et al., 2020; Sun et al., 2020) or used manipulative experiments in otherwise natural habitats (Laurentino et al., 2020). Hence, our study is an example of potential *in-situ* "adaptation in action", where genetic diversity at adaptive loci is increasing due to a slow loss of previous genetic adaptations.

Overgrowth of semi-natural grasslands has often a negative impact on genetic diversity of grassland-specialist plants due to a decrease in habitat quality and potential development of barriers for gene flow (Aavik & Helm, 2018; Picó & Van Groenendael, 2007). However, in our study, neutral genetic diversity of *P. veris* was similar in open and overgrown habitats, indicating that habitat and landscape change do not necessarily restrict gene flow among populations of *P. veris*. Still, an environmental impact on the neutral part of a genome might only show after several generations (e.g. Landguth et al., 2010). *Primula veris* is a perennial plant with an average lifespan of up to 50 years and can persist with reduced reproduction even when the environment has changed due to overgrowth (Ehrlén & Lehtilä, 2002). Hence, genetic diversity of such populations potentially reflects the state before their

499 habitats started to change (Reinula, 2018). In addition, heterozygosity indices, as used in our
500 study, have been suggested to respond more slowly to environmental change as compared
501 to, for example, measures of inbreeding (Lloyd, Campbell, & Neel, 2013; Lowe, Boshier,
502 Ward, Bacles, & Navarro, 2005). Deschepper et al. (2017), who examined patterns of neutral
503 genetic diversity of *P. veris* in grassland and forest populations in Belgium, also reported no
504 significant difference in H_o between habitat types. Yet, their study system, i.e. forests,
505 represents a late-successional stage, whereas ours represents a mid-successional stage
506 (shrubby overgrowth). Consequently, neutral genetic patterns of *P. veris* might need a very
507 long time (up to centuries) to reach an equilibrium with the new overgrown environment.

508 In contrast to genetic diversity at putatively neutral loci, genetic diversity assessed at
509 putatively adaptive loci differed between habitat types in our study. Populations in recently
510 overgrown grasslands showed higher genetic diversity at putatively adaptive loci compared
511 to populations in open grasslands (Figure 3a,b). This is most likely caused by changed
512 selection pressures in the new, overgrown habitats compared to the old, open habitats. The
513 open grasslands used in our study have been in an open state for several hundreds of years,
514 whereas ongoing overgrowth started only about 90 years ago (Helm et al., 2006). Hence,
515 populations of *P. veris* in open grasslands have experienced homogenous selection
516 pressures for a very long time, which increased the frequencies of alleles beneficial for an
517 open habitat and led to reduced heterozygosity and genetic diversity at adaptive loci. The
518 majority of putatively adaptive SNPs (53 out of 77) exhibited a smaller average beneficial
519 (i.e. major) AF for open habitat conditions in populations from the new, overgrown
520 compared to those from the old habitat. This led to an increase in heterozygosity and
521 genetic diversity in populations of the new, overgrown habitats (Figure 2). Considering *P.*
522 *veris'* potential longevity, this indicates that populations in overgrown habitats are still

523 adapting to their new selection pressures (e.g. lower light availability, reduced or altered
524 pollinator community), i.e. many alleles potentially beneficial for the new, overgrown
525 habitat are still far from fixation (i.e. homozygosity). Consequently, the genetic diversity at
526 putatively adaptive loci of *P. veris* populations in overgrown grasslands has not yet been
527 reduced. A similar pattern was found in the conifer tree *Pinus cembra*, where populations in
528 the core of the current niche exhibited a decreased genetic diversity at adaptive loci, but no
529 difference in the one at neutral loci, compared to populations at the niche margin, which
530 most likely present unstable or novel habitats (Dauphin et al., 2020).

531 An increase of non-beneficial alleles for the open habitat in overgrown populations, and
532 thus an increase in heterozygosity at putatively adaptive loci in overgrown populations, can
533 be achieved in two ways: (1) the alternative alleles at SNP loci were either already present in
534 lower frequencies in populations of open habitats (i.e. standing genetic variation), or (2)
535 arrived to overgrowing grasslands by gene flow, before they were subject to positive
536 selection in the overgrown habitat. As shown in Figure S 3, beneficial alleles in populations
537 of the open habitat were rarely fixed, strongly pointing towards the importance of standing
538 genetic variation. This implies that even populations with reduced genetic diversity at
539 putatively adaptive loci (e.g. in open grasslands) may possess the ability to react to habitat
540 changes due to the low, but crucial amount of standing genetic variation (e.g. Morris,
541 Bowles, Allen, Jamniczky, & Rogers, 2018). Still, we found gene flow as indicated by genetic
542 differentiation potentially spreading alleles between habitats which, in theory, can
543 contribute to the increased genetic diversity at adaptive loci in overgrown populations. The
544 potential gene flow distances found in our study (up to 30 km; Figure 4, Figure 5) stand in
545 marked contrast to the potential dispersal ranges of pollen and seeds of *P. veris* for which
546 very short distances up to 12 m and 0.5 m, respectively, have been reported (Antrobus &

547 Lack, 1993; Richards & Ibrahim, 1978). Yet, pollinating insects of *P. veris* have been shown to
548 occasionally travel up to 2 km (Kreyer, Oed, Walther-Hellwig, & Frankl, 2004; Zurbuchen,
549 Bachofen, Müller, Hein, & Dorn, 2010) and dispersal distances are often underestimated in
550 ecological studies (e.g. Bullock, Shea, & Skarpaas, 2006). Historical rotational grazing of
551 domestic animals and movement of wild animals (e.g. deer, moose, wild boar) might also
552 have facilitated seed dispersal for longer distances in *P. veris* which is not adapted to
553 zoochory (Plue, Aavik, & Cousins, 2019). Overall, standing genetic variation but also gene
554 flow are capable of supplying *P. veris* populations undergoing habitat changes with new or
555 alternative alleles fostering adaptation to new habitat conditions in our study region.

556 Importantly, the fact that we detected a significant effect of habitat type on genetic
557 diversity (H_o) when using the putatively adaptive SNP set but not with the neutral SNP set
558 suggests the need to examine genetic diversity at neutral and adaptive loci separately when
559 studying the genetic response of plant species to environmental changes. Besides, in the
560 overall SNP set (Figure S 10), there was no significant effect of habitat type on genetic
561 diversity, which indicates that genetic diversity of an overall SNP set represents rather
562 neutral genetic patterns (Dauphin et al., 2020). In conservation genetics and restoration,
563 most assessments have been based on overall or neutral genetic diversity so far (González
564 et al., 2019; Wei & Jiang, 2020), even though its stand-alone importance is questionable
565 (Teixeira & Huber, 2021) and it is likely the putatively adaptive regions of a genome that are
566 important for the fate of a population in a changed environment.

567 The different effects of habitat type on genetic diversity at putatively neutral and
568 adaptive loci could partly be due to the unevenness in the number of loci at neutral and
569 putatively adaptive regions (2,619 versus 77, respectively). However, the ddRADseq

570 procedure used in our study should result in a “random” and representative subset of both,
571 neutral and adaptive SNPs. Here, we were not particularly interested in the actual molecular
572 mechanisms underlying adaptation, but in general patterns at putatively adaptive loci,
573 which should also become visible with 77 SNPs if these patterns are of substantial nature.
574 Additionally, the reliability of the detected SNPs can be assumed high, because the
575 genotyping error for our SNPs was low (0.004) and the SNP sets were identified using a draft
576 genome (Hoban et al., 2016), which covers about 63% of the whole 479.22 Mb genome of
577 the study species *P. veris* (Nowak et al., 2015).

578 When using linear and categorical EAA to test effects of habitat type on genetic
579 diversity, we must consider a potential circularity issue. Nevertheless, the results of the
580 randomised data sets indicate that, for H_o, the habitat type related results (including the
581 interaction with SNP set) of the original data are likely not concerned by circularity. Yet, this
582 is the case for π. Potentially, since our study sites are just in the beginning of a habitat
583 change process (i.e. in terms of perennial plants like *P. veris*), more time might be needed
584 until a stronger habitat change effect can be detected by π. Importantly, even though the
585 number of putatively adaptive loci using randomised data sets is in the range of putatively
586 adaptive loci found using the original data set, the putatively adaptive loci from the original
587 data set do not show a random pattern with respect to their change in beneficial AF (Figure
588 S 4). In addition, applying the same mixed-effect model statistic to the mean randomised
589 data set, for both H_o and π, there is no habitat type or interaction effect anymore (Figure S
590 5), as can be expected when breaking the apparent link between genotype and habitat type
591 sample assignment of the original data set. Therefore, caution is needed when interpreting
592 the results, especially those using π, but the overall conclusion of potential early stages of
593 “adaption in action” processes remains. To our knowledge, there is no previous study

594 applying EAA to examine putatively neutral and adaptive genetic diversity that used
595 permutations for validation. In contrast, such validations are common practice in genome
596 wide association studies (e.g. Kreiner, Tranel, Weigel, Stinchcombe, & Wright, 2021; Tibbs
597 Cortes, Zhang, & Yu, 2021) which, together with our results, might call for their wider
598 application in landscape genomic analyses.

599 In addition to genetic diversity, population size is another factor that is affected by
600 habitat change, and which also directly influences genetic diversity (Leimu et al., 2006). The
601 census sizes of our *P. veris* populations were similar in open and overgrown habitats and
602 were not associated with genetic diversity at neutral and adaptive loci. In contrast to our
603 results, a meta-analysis showed a clear relationship between population size and (neutral)
604 genetic diversity, including long-lived and self-incompatible plant species, such as *P. veris*
605 (Leimu et al., 2006). Populations in our study exhibited a minimum of about 100 individuals,
606 which is larger than the overall census for small populations in the study by Leimu et al.
607 (2006). Consequently, our *P. veris* populations might be still sufficiently large to counteract
608 population size driven effects on genetic diversity.

609 Conclusions

610 Our landscape genomic investigation of *Primula veris* in Estonian semi-natural grasslands is
611 one of the first to demonstrate the potential effects of land use change on the genetic
612 diversity at putatively adaptive loci of *in-situ* wild plant species. We show that the effect of
613 recent overgrowth of grasslands might not be genetically manifested when considering
614 neutral SNPs, even after almost a century of ongoing environmental changes. Yet, genetic
615 diversity assessed at putatively adaptive loci was higher in populations in overgrown
616 compared to open habitats, most probably due to allele frequency changes of standing

617 genetic variation. Thus, even populations in degraded and fragmented habitats may possess
618 the ability to adapt to habitat changes due to their important standing genetic variation in
619 addition to potential allele immigration due to gene flow. However, permutation analyses
620 indicated that caution is needed when interpreting results based on linear and categorical
621 EAA, especially when working with nucleotide diversity.

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

633

634 ## Acknowledgements

635 We thank the Genetic Diversity Centre Zurich (GDC) for laboratory support, the Functional
636 Genomic Centre Zurich (FGCZ) for Illumina sequencing, A. Rogivue for introducing the lead
637 author to EAA, and B. Dauphin for extracting climate data and support in EAAs. We are
638 grateful for financial support from the Estonian Research Council (MOBJD427, PUT589 and
639 PRG874), the COST “G-BIKE” action (CA18134), the European Regional Development Fund

(Centre of Excellence EcolChange), and European Commission LIFE+ Nature program (LIFE13NAT/EE/000082). We also thank three anonymous reviewers for their valuable comments and suggestions on previous versions of this manuscript.

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Data Accessibility

Sequence data used in this study will be made available at the European Nucleotide Archive (ENA) upon acceptance (ERS5253979 – ERS5254546). R-scripts, genotypic and environmental data will be provided at the Dryad Digital Repository upon acceptance (xxx).

Author Contributions

T.A., S.T. and A.H designed the conceptual approach and carried out field work. S.T. and I.R. conducted laboratory work. S.T. and N.Z. performed bioinformatic analyses. S.T. and C.R.

analysed the data. R.H. contributed with discussing the results in a broader ecological context. S.T. wrote the manuscript with major contributions from C.R. All authors read, commented and approved the final version of the manuscript.

Competing interests

The authors declare no competing interests.

928 Supplemental Information

929 Supplemental Methods

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

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

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

933 Figure S 4 Proportion of putatively adaptive SNPs with de- and increasing beneficial AF of
934 the original and randomised data sets.

935 Figure S 5 Mean of genetic and nucleotide diversity (H_o and π) at putatively neutral and
936 adaptive SNPs of 100 randomised data sets.

937 Figure S 6 Genetic and nucleotide diversity (H_o and π) at putatively neutral and adaptive
938 SNPs per population of 100 randomised data sets.

939 Figure S 7 Residual standard error results for IBD analyses, measured using the overall
940 (3,084 loci) and neutral (2,619 loci) SNP sets.

941 Figure S 8 Genetic structure of *Primula veris* populations in the study area measured using
942 the overall set of loci (3,084 loci).

943 Figure S 9 Isolation by distance pattern measured at the overall (3,084 SNPs) set of loci for
944 *Primula veris* populations.

945 Figure S 10 Genetic and nucleotide diversity (H_o and π) at the overall set of SNPs (3,084 loci).

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

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

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

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

954 Tables

955 Table 1 Location information and genetic diversity measurements for the studied populations of *Primula veris* in Muhu and Saaremaa, Estonia.
956 Habitat – affiliation to open or overgrown grassland; pair – affiliation to a pair of closely situated populations (0 indicates affiliation to no pair);
957 N – sample size after SNP filtering; population size – estimated number of individuals per population; H_o – observed heterozygosity; π –
958 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

960 Table 2 Results of linear mixed effect models for observed heterozygosity (H_o), and
961 nucleotide diversity (π) of *Primula veris* populations of the original data set. Fitted
962 parameters (± SE), t-values and significance (p_{Model}) is given for the full model of each
963 genetic index, H_o and π. Factors: habitat (H): open and overgrown; SNP_set (S): SNP_neutral
964 and SNP_adaptive. RF_{region} and RF_{pop} denote the use of region (Muhu and Saaremaa) and
965 population (1–32) as random factors (RF). The importance of the fixed factor interaction is
966 given as χ^2 and *p*-values for each genetic measurement. For H_o and π, the importance of the
967 fixed factor interaction is given for the comparison of the full model (with H * S) with the
968 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

969

Figures

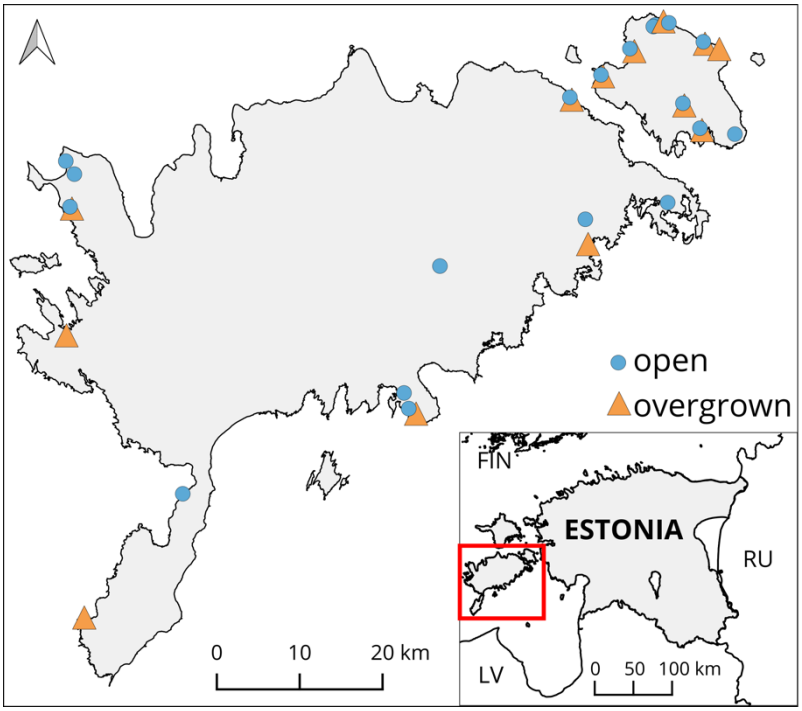


Figure 1 Map of the study area on Muhu and Saaremaa islands in Western Estonia. *Primula veris* populations are depicted with blue filled circles when occurring in open semi-natural grassland and with orange filled triangles when occurring in recently overgrown historical semi-natural grasslands.

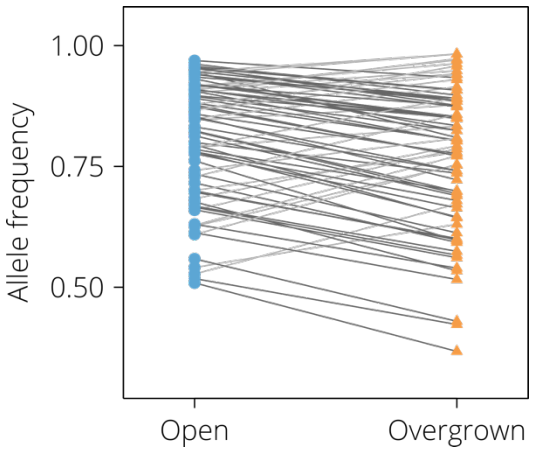
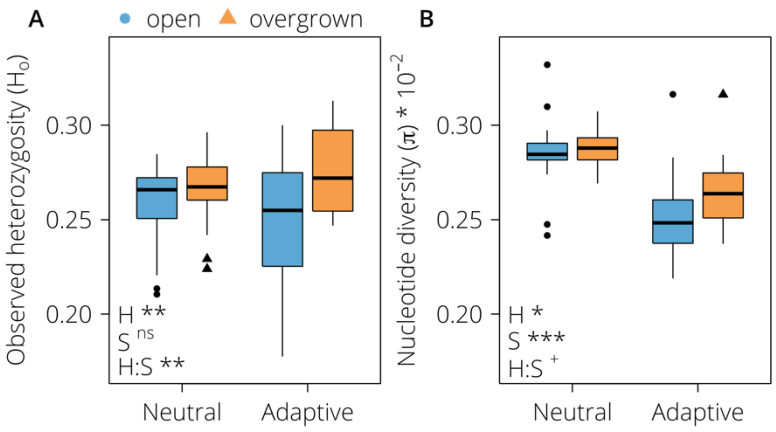


Figure 2 Patterns of allele frequency (AF) change of the 77 putatively adaptive SNPs (SNP_adapt) averaged across pairs of populations for each SNP. For each SNP, the putatively beneficial allele frequency for the open habitat (AF > 0.5) is shown. SNPs were derived from linear and categorical environmental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



985

986 Figure 3 Boxplots of observed heterozygosity (H_o; A) and nucleotide diversity (π; B) at
987 putatively neutral and adaptive loci in open (blue, circles) and overgrown (orange, triangles)
988 grasslands. Factors: habitat (H): open and overgrown; SNP_set (S): SNP_neutral and
989 SNP_adaptive. Significance values: ^{ns} $p > 0.05$; $^{+}$ $p = 0.056$; * $p \leq 0.05$; ** $p < 0.01$;
990 *** $p < 0.001$.

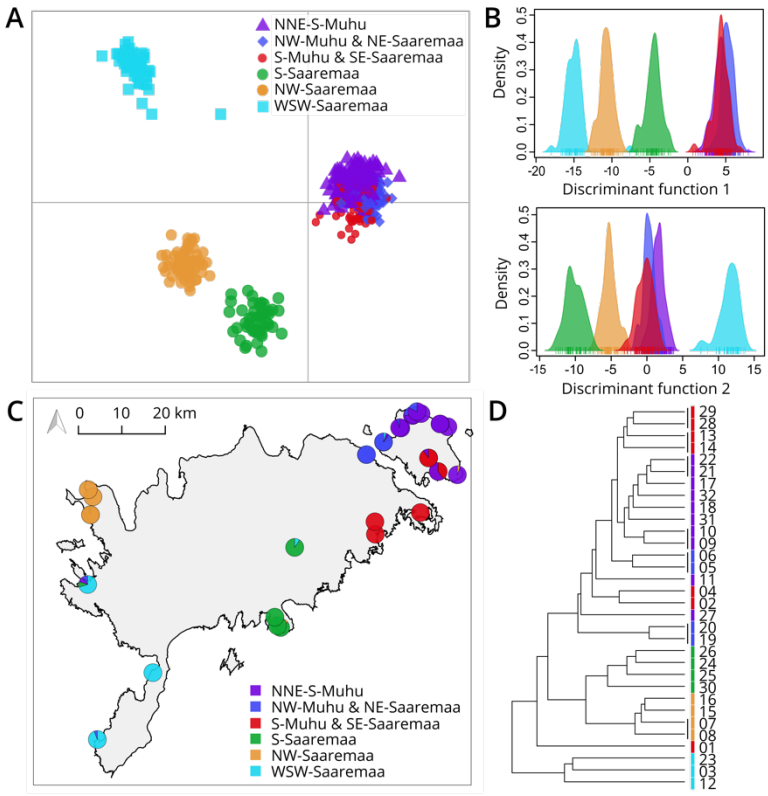


Figure 4 Genetic structure of *Primula veris* populations in the study region. (A) Result of the discriminant analysis of principle components on the putatively neutral SNP set resulting in six geographical clusters (C; named by cardinal directions). The distribution of the six clusters per discriminant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

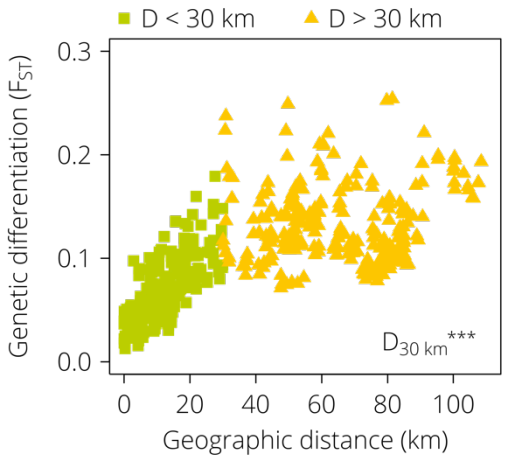


Figure 5 Relationship between genetic differentiation (F_{ST}) and geographic distance for all possible pairs of 32 *Primula veris* populations, measured at putatively neutral loci. Population pairs with a geographic distance (D) less or equal to 30 km are visualized as green squares. Population pairs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