


## RESEARCH ARTICLE

# Skewed morph ratios lead to lower genetic diversity of the heterostylous *Primula veris* in fragmented grasslands

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## Keywords

Distyly; floral morphs; genetic diversity; habitat fragmentation; heterostyly; semi-natural grasslands.

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## ABSTRACT

- Populations of heterostylous plant species are ideally composed of equal frequencies of two (distylous) or three (tristylous) morphologically different floral morphs. Intra-morph incompatibility helps to avoid inbreeding and to maintain genetic diversity, supporting plant fitness and long-term viability. Habitat fragmentation can lead to skewed morph ratios and thereby reduce the abundance of compatible mates. This, in turn, can result in a loss of genetic diversity. We tested whether the genetic diversity of heterostylous plants is affected by morph ratio bias using populations of the distylous grassland plant *Primula veris* in recently fragmented grasslands.
- We recorded morph frequencies and population sizes in 30 study populations of *P. veris* on two Estonian islands characterised by different degrees of habitat fragmentation. Examining variation of thousands of single nucleotide polymorphisms (SNPs) and heterostyly-specific genetic markers, we quantified overall and morph-specific genetic diversity and differentiation in these populations.
- Morph frequencies deviated more in smaller populations. Skewed morph ratios had a negative effect on the genetic diversity of *P. veris* in more fragmented grasslands. In the populations of better-connected grassland systems, genetic differentiation among S-morphs was higher than among L-morphs.
- Our study shows that deviations from morph balance are stronger in small populations and have a negative impact on the genetic diversity of the distylous plant *P. veris*. Together with the direct negative effects of habitat loss and decreased population size on the genetic diversity of plants, morph ratio bias may intensify the process of genetic erosion, thus exacerbating the local extinction of heterostylous species.

## INTRODUCTION

Loss and fragmentation of natural and semi-natural habitats have negative effects on biodiversity and ecosystem functioning (Hanski 2005; Haddad *et al.* 2015). Particularly affected are semi-natural grasslands, which have lost a significant proportion of their area throughout Europe during the last century (Laasimer 1965; Jiang *et al.* 2013; Cousins *et al.* 2015). Abandoning traditional management, such as grazing and mowing, leads to the overgrowth of semi-natural grasslands by shrubs and trees, which results in landscape changes and related possible decline in vascular plant species richness (Luoto *et al.* 2003). In addition, habitat loss and fragmentation lower genetic diversity within plant populations through genetic drift and inbreeding, and increase genetic differentiation among populations due to lower gene flow (Leimu *et al.* 2006; Honnay & Jacquemyn 2007; Aguilar *et al.* 2008; Angeloni *et al.* 2011).

Erosion of genetic diversity because of the loss and fragmentation of natural and semi-natural habitats might be particularly severe in heterostylous species, as their specialised mating system restricts the number of available mating partners. Heterostyly, known from at least 28 different plant families (Naiki 2012), is a

genetically controlled floral polymorphism expressed in different style length and anther position. It can occur with two- (distyly) or three- (tristyly) morphologically different floral forms (morphs) (Ganders 1979). In distylous species, short-styled individuals (S-morphs or thrums) exhibit lower positioning of the style compared to the stamens, which reach higher to the edge of petals. In contrast, in long-styled individuals (L-morphs or pins), the style is high, while stamens are low and hidden in the corolla. This spatial separation of reproductive organs within flowers restricts self-pollination, while the spatial matching of anthers and stigmas between S- and L-flowers favours cross-pollination (Darwin 1862; Keller *et al.* 2014). Reciprocal herkogamy is usually accompanied by a sporophytic incompatibility system that inhibits germination of pollen from the same individual and/or the same morph on the stigma (Ganders 1979). The morphological and physiological components thus jointly ensure fertilisation between the two floral morphs. In *Primula*, heterostyly is controlled by a heterostyly supergene comprised of several tightly linked genes that determine different traits characterising heterostyly, such as style length and the size of pollen grains. The supergene is hemizygous, being present in S-morphs and absent in L-morphs (Huu *et al.* 2016).

Negative frequency-dependent selection ensures that different morphs are usually represented in equal frequencies in sufficiently large populations of heterostylous plants (Boyd *et al.* 1990; Kery *et al.* 2003). However, decreasing size of populations caused by loss and fragmentation of habitats can lead to an imbalance of morphs (Endels *et al.* 2002; Kery *et al.* 2003). Previous studies demonstrated that individuals were less likely to find compatible mating partners in populations with skewed morph ratios than in populations with balanced morph ratios (Van Rossum *et al.* 2004; Meeus *et al.* 2012). Hence, unbalanced morph ratios may further amplify negative effects of habitat fragmentation on gene flow and genetic diversity, and can thereby lead to a population's reduced adaptability to changing environmental conditions. Consequently, populations with a biased morph ratio might have an increased risk of extinction (Leimu *et al.* 2010).

With legitimate fertilisation between two different morphs, offspring are expected to appear in equal amounts of S- and L-morphs. However, in the case of a cross between two L-morphs, the offspring are expected to be only L-morphs (Kery *et al.* 2003). Yet, although the heteromorphic incompatibility system should prevent intra-morph fertilisation, heterostylous plants hold a wide variation in the degree of intra-morph incompatibility, thus being strictly self-incompatible is not a rule in heterostylous plants (Barrett & Cruzan 1994). It has been found for *P. veris*, even though rarely, that long-styled morphs show moderate intra-morph compatibility, with some chance of the development of seed set through intra-morph pollination (14.7%). In contrast, the probability of successful reproduction as a result of crossing between short-styled morphs is negligible (0.6%; Wedderburn & Richards 1990; Brys & Jacquemyn 2015).

Because of the variation in self-compatibility between the two morph types (Wedderburn & Richards 1990), L-morphs can have a reproductive advantage in small and fragmented populations as they have more potential mates to reproduce with than S-morphs. This partial intra-morph incompatibility in L-morphs may be particularly important for ensuring reproduction in small and fragmented populations. It has been found that in fragmented populations with higher L-morph frequencies, inbreeding values are indeed lower than in populations with equal morph ratios (Van Rossum & Triest 2006). In conditions of pollination disruption, L-morph bias in small populations can thus help to buffer the negative effects of reduced abundance of individuals by increasing effective population size (Van Rossum & Triest 2006). However, recent studies have shown contrasting results in relation to the expected L-morph advantage. The less strict intra-morph compatibility in L-morphs can also lead to varying levels of genetic diversity within S- and L-morphs, as well as to differential patterns of gene flow (Meeus *et al.* 2012). For example, L-morphs may be more prone to higher (bi)parental inbreeding compared to S-morphs. A nationwide citizen science campaign in Estonia examining large-scale patterns of morph ratios of *P. veris* revealed a slightly higher frequency of S-morphs (Aavik *et al.* 2020). This result suggests that the stricter incompatibility and potentially higher genetic diversity in S-morphs can lead to higher survival and fitness of S-morphs.

*Primula veris* is a species characteristic of semi-natural grasslands, many of which have suffered a drastic decrease in area and connectivity over the past century. *P. veris* has been a model species in several previous studies on heterostyly and

habitat fragmentation (Van Rossum *et al.* 2004, 2006; Van Rossum & Triest 2006; Brys & Jacquemyn 2015; Aavik *et al.* 2020), as well as on the evolution of heterostyly (Huu *et al.* 2016, 2020; Potente *et al.* 2022). Thanks to its published high-quality genome (Nowak *et al.* 2015; Potente *et al.* 2022), this species serves as an ideal study system to explore the patterns of genetic diversity in response to fluctuations in morph ratios in recently fragmented grasslands. The present study will provide a novel insight into the morph-specific genetic differences between S- and L-morphs in response to landscape changes. For the first time in this species, genetic diversity indices and inbreeding coefficient are calculated separately for both morph types, with the expectation that morph-specific genetic patterns will elucidate the recently observed unexpected S-morph dominance (Aavik *et al.* 2020).

To test the effect of morph ratio bias on the genetic patterns of the distylous *P. veris*, we examined morph frequencies, genetic diversity and gene flow of *P. veris* in a system of calcareous grasslands in western Estonia. First, we wanted to confirm whether morph frequencies in populations of *P. veris* in semi-natural grasslands are affected by plant population size. Following the unexpected findings on S-morph dominance from Aavik *et al.* (2020), we were also interested in exploring whether the frequency of one morph consistently dominates another morph in our study system, especially in smaller *P. veris* populations. Second, we hypothesised that morph ratio bias will have an overall negative impact on the genetic diversity of *P. veris*. Third, following the previous suggestions that there is no strict intra-morph incompatibility in L-morphs in *P. veris* (Wedderburn & Richards 1990; Brys & Jacquemyn 2015), we hypothesised that partial intra-morph compatibility of L-morphs would lead to higher inbreeding within L-morphs. We also hypothesised that stricter within-morph incompatibility between S-morphs would lead to higher genetic diversity within S-morphs. For the same reason, we also expected to see more gene flow and, thus, weaker genetic differentiation in L-morphs than in S-morphs among populations.

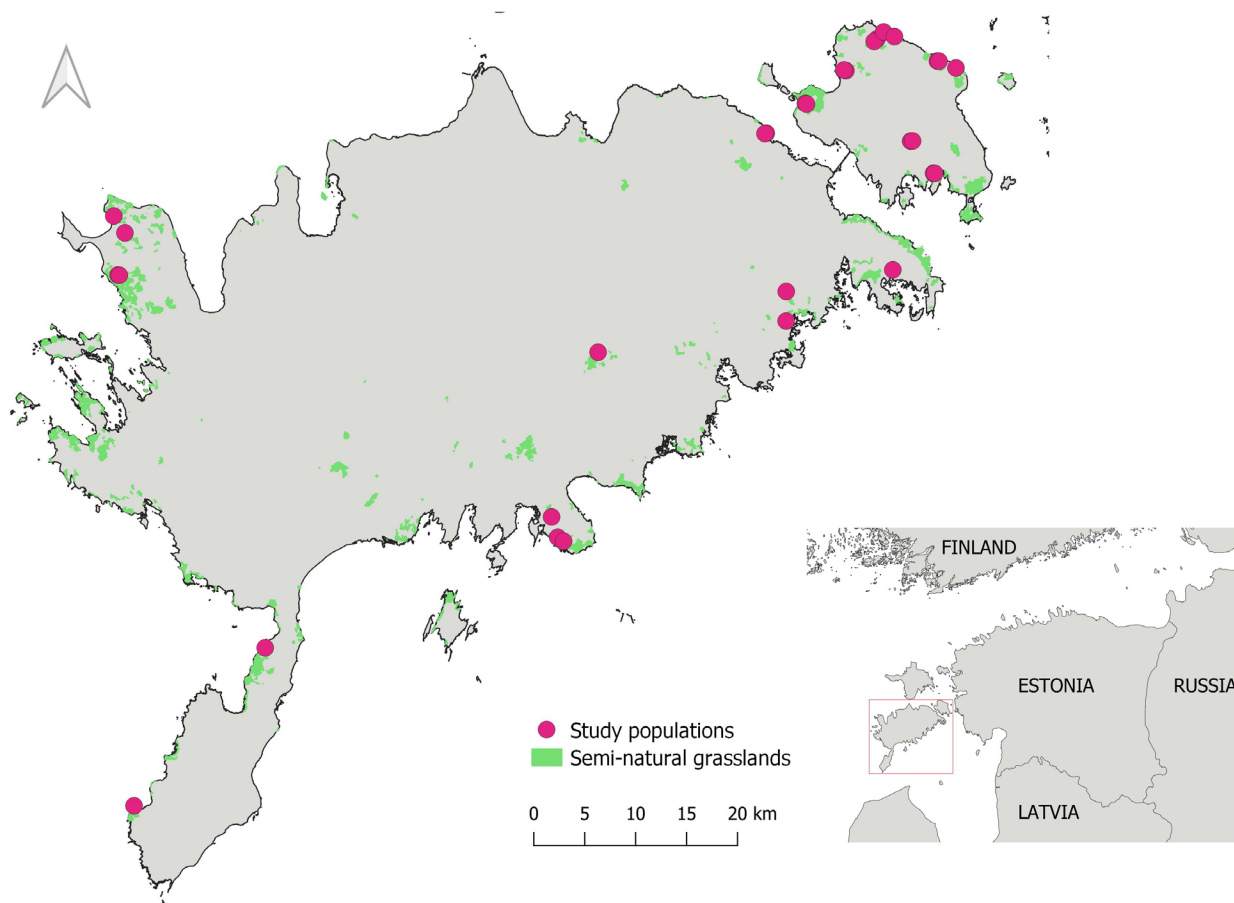
## MATERIAL AND METHODS

### Study species

*Primula veris* L. (Primulaceae) is a perennial rosette-forming hemicryptophyte, growing mainly in open habitats. In addition to semi-natural grasslands, *P. veris* can be found on road verges and forest edges (Brys & Jacquemyn 2009). *P. veris* is a distylous outcrossing species, whose reproduction highly depends on pollinator availability. *P. veris* is most commonly pollinated by Hymenoptera (bumblebees), but also species of Lepidoptera (butterflies) and Diptera (beetles) (Ornduff 1980; Deschepper *et al.* 2018). Average pollen dispersal has been shown to be ca 2 m, and seed dispersal is quite low, with an average of about 12 cm (Richards & Ibrahim 1978). *P. veris* mostly reproduces sexually, but has also been found to spread vegetatively to a certain extent (Tamm 1972). The lifespan for this perennial is considerable with an average longevity of 52.3 years (Ehrlén & Lehtilä 2002).

### Study sites

Study sites were located in Estonia on Saaremaa and Muhu islands (Fig. 1), where historically widespread semi-natural



**Fig. 1.** Locations of the 30 study populations of *Primula veris* on Saaremaa (larger island on the left) and Muhu (smaller island on the upper right), Estonia, together with the current distribution of semi-natural grasslands.

grasslands (alvars) have degraded extensively because of a decrease in traditional management through grazing. Formerly open alvars have become overgrown with shrubs and trees, mainly *Juniperus communis* and *Pinus sylvestris* (Pärtel *et al.* 1999a). Alvars have characteristically shallow calcareous soils, exhibit high plant species richness and are very rare on a global scale (Pärtel *et al.* 1999b). The study sites were part of a large-scale semi-natural grassland restoration project ‘LIFE to alvars’ (Helm 2019). In the framework of the project, >2500 ha of alvar grasslands were restored on the western Estonian mainland and islands between 2014–2019 by removing trees and shrubs, followed by management through grazing.

In statistical data analysis, the two islands, Muhu and Saaremaa, were considered as different regions because the genetic diversity of *P. veris* in Saaremaa and Muhu clearly differs (Reinula *et al.* 2021), with populations in Muhu being characterised by higher genetic diversity. The islands represent different extents of landscape change and land-use history, with populations on Saaremaa being affected more seriously by grassland loss and fragmentation. In Muhu, the dynamics of landscape change has been more subtle, and the remaining populations are spatially more connected. Besides the clear discrepancies between the genetic diversity of *P. veris* in Saaremaa and Muhu, the differential impact of land-use history on biodiversity in these islands is also reflected in lower richness of

vascular plants in the overgrown alvar grasslands of Saaremaa compared to similar overgrown grasslands in Muhu (Helm 2019).

### Sampling

#### *Collection of samples for genetic analyses*

Extensive biodiversity monitoring was carried out on the study sites before and after restoration. The data used in this study were collected before restoration. Samples from the start of genetic monitoring of *P. veris*, a characteristic species in these grasslands, were collected in the summers of 2015 and 2016. Leaf samples of *P. veris* were collected from 30 populations on Saaremaa and Muhu islands in grasslands at two different successional stages – open (18) and overgrown (12) grasslands (Table 1). The average shrub cover was  $32 \pm 6.5\%$  in open grasslands and  $71 \pm 9.6\%$  in overgrown grasslands. From each population, one to three leaves from up to 20 individuals, if possible, were collected and stored on silica gel until further processing. At least 1 m was left between sampled individuals to avoid collecting samples of potentially genetically identical individuals. A total of 571 samples from open and overgrown populations from Saaremaa and Muhu were used for further analysis. Later, the morph type of each genetically analysed individual was identified in the lab *sensu* Huu *et al.* (2016) (see

**Table 1.** Genetic diversity measures.  $H_0$  – observed heterozygosity;  $uH_E$  – unbiased expected heterozygosity accounting for sample size;  $F_{IS}$  – inbreeding coefficient; %P – percentage of polymorphic loci of the study populations of *Primula veris* in Muhu and Saaremaa, Estonia. Habitat – habitat successional stage. Individuals observed – number of morph types identified per population, from which the population-level morph bias was calculated. Genetic samples – number of samples used in genetic analyses. Respective values for S- and L-morphs determined in the lab are given in brackets. Populations removed from analyses due to low number of individuals per morph type are marked with an asterisk. Pop – population ID.

pop	region	habitat	population size	individuals observed (S:L)	morph bias	genetic samples (S:L)	$H_0$ (S:L)	$uH_E$ (S:L)	$F_{IS}$ (S:L)	%P (S:L)
1	Saaremaa	Open	100	26 (15;11)	-0.154	6 (1*;5)	0.22 (0.24;0.22)	0.21 (0.24;0.21)	-0.07 (NA;-0.05)	55.17 (23.82;53.28)
2	Saaremaa	Overgrown	100	43 (26;17)	-0.209	20 (11;9)	0.23 (0.23;0.23)	0.23 (0.22;0.23)	-0.02 (-0.02;-0.01)	74.61 (66.15;68.17)
3	Saaremaa	Open	100	58 (33;25)	-0.138	20 (10;10)	0.25 (0.25;0.24)	0.24 (0.24;0.24)	-0.03 (-0.06;-0.01)	75.14 (68.55;67.98)
4	Muhu	Overgrown	1000	101 (46;55)	0.089	20 (11;9)	0.26 (0.25;0.27)	0.26 (0.25;0.28)	0.01 (0.01;0.02)	87.67 (73.89;82.98)
5	Muhu	Open	100	60 (35;25)	-0.167	19 (8;11)	0.27 (0.27;0.27)	0.26 (0.27;0.26)	-0.02 (0.01;-0.04)	84.5 (76.72;80.27)
6	Saaremaa	Overgrown	800	66 (38;28)	-0.152	20 (9;11)	0.27 (0.28;0.27)	0.28 (0.28;0.27)	0.01 (-0.01;0.03)	84.99 (80.11;79.47)
7	Saaremaa	Open	400	93 (42;51)	0.097	19 (12;6)	0.26 (0.25;0.28)	0.26 (0.26;0.26)	0 (0.02;-0.06)	84.73 (80.57;69.77)
8	Muhu	Overgrown	1000	108 (57;51)	-0.056	20 (8;12)	0.28 (0.28;0.28)	0.28 (0.28;0.28)	0 (-0.02;0.01)	87.9 (76.68;84.5)
9	Muhu	Open	500	80 (40;40)	0.000	19 (7;7)	0.27 (0.25;0.28)	0.28 (0.28;0.28)	0.03 (0.11;-0.01)	87.13 (74.66;77.02)
10	Muhu	Overgrown	20	14 (10;4)	-0.429	20 (10;9)	0.28 (0.26;0.29)	0.27 (0.26;0.28)	-0.03 (0.01;-0.05)	84.15 (76.98;78.59)
11	Saaremaa	Open	5000	111 (63;48)	-0.135	20 (9;11)	0.26 (0.26;0.26)	0.27 (0.27;0.27)	0.04 (0.03;0.04)	78.24 (72.21;74.66)
12	Muhu	Overgrown	500	50 (24;26)	0.040	20 (11;9)	0.24 (0.23;0.25)	0.26 (0.25;0.26)	0.07 (0.07;0.03)	80.15 (75.61;72.48)
13	Muhu	Open	100	49 (32;17)	-0.306	15 (7;7)	0.26 (0.26;0.25)	0.26 (0.26;0.26)	0.02 (0.0;0.05)	79.27 (72.18;70.99)
14	Saaremaa	Open	2000	147 (87;60)	-0.184	20 (12;7)	0.27 (0.29;0.25)	0.28 (0.28;0.28)	0.03 (-0.01;0.11)	88.74 (85.11;76.22)
15	Saaremaa	Open	2000	131 (75;56)	-0.145	18 (13;4*)	0.28 (0.29;0.26)	0.28 (0.28;0.28)	0.01 (-0.02;0.08)	87.71 (84.47;68.51)
16	Muhu	Open	1500	58 (28;30)	0.034	19 (6;12)	0.27 (0.26;0.26)	0.28 (0.28;0.28)	0.06 (0.06;0.07)	89.5 (74.35;86.64)
17	Muhu	Open	100	30 (21;9)	-0.400	20 (7;10)	0.26 (0.23;0.27)	0.28 (0.25;0.28)	0.06 (0.09;0.04)	89.84 (70.99;84.05)
18	Saaremaa	Open	2000	127 (69;58)	-0.087	20 (10;9)	0.27 (0.27;0.26)	0.27 (0.26;0.27)	-0.01 (-0.04;0.02)	83.96 (77.21;77.25)
19	Saaremaa	Overgrown	5000	116 (61;55)	-0.052	20 (9;11)	0.26 (0.27;0.26)	0.27 (0.29;0.26)	0.05 (0.09;0.03)	91.1 (86.37;80.19)
20	Muhu	Overgrown	800	116 (74;42)	-0.276	20 (8;12)	0.29 (0.29;0.29)	0.28 (0.28;0.29)	-0.02 (-0.02;-0.01)	90.53 (78.74;85.46)
21	Muhu	Open	500	100 (68;32)	-0.360	19 (13;5)	0.28 (0.29;0.27)	0.28 (0.28;0.28)	-0.02 (-0.04;0.03)	89.35 (84.16;77.22)
22	Saaremaa	Overgrown	100	36 (22;14)	-0.222	17 (9;8)	0.26 (0.25;0.27)	0.27 (0.26;0.27)	0.02 (0.04;0)	86.33 (77.14;77.86)
23	Saaremaa	Open	1000	102 (62;40)	-0.216	19 (9;9)	0.21 (0.21;0.22)	0.21 (0.21;0.22)	0 (-0.01;0)	78.69 (68.66;68.24)
24	Saaremaa	Open	300	57 (37;20)	-0.298	15 (7;8)	0.21 (0.22;0.2)	0.22 (0.24;0.2)	0.05 (0.08;0.01)	78.39 (71.49;63.02)
25	Saaremaa	Overgrown	100	39 (31;8)	-0.590	7 (3*;4*)	0.22 (0.23;0.22)	0.24 (0.22;0.25)	0.09 (-0.05;0.15)	69.68 (49.27;62.1)
26	Muhu	Overgrown	50	30 (18;12)	-0.200	20 (9;11)	0.27 (0.27;0.26)	0.27 (0.26;0.27)	0.01 (-0.07;0.03)	80.95 (69.31;77.4)
27	Muhu	Open	20	14 (5;9)	0.286	19 (4*;15)	0.27 (0.26;0.27)	0.26 (0.26;0.26)	-0.02 (0.03;-0.03)	82.89 (62.94;81.3)
28	Saaremaa	Open	100	27 (18;9)	-0.333	11 (6;4*)	0.22 (0.21;0.23)	0.25 (0.22;0.27)	0.11 (0.06;0.17)	81.14 (63.44;69.08)
29	Muhu	Overgrown	150	40 (20;20)	0.000	17 (11;6)	0.28 (0.28;0.28)	0.29 (0.29;0.28)	0.04 (0.05;0.01)	91.22 (87.14;76.91)
30	Muhu	Open	3000	122 (53;69)	0.131	19 (9;9)	0.27 (0.27;0.27)	0.28 (0.28;0.29)	0.04 (0.03;0.08)	89.73 (80.34;83.4)

the method below) because this information was unfortunately not recorded during collection of samples for genetic analysis.

#### Characterisation of morph ratios of the study populations

To obtain population-level information about the morph ratios within each study grassland, the frequencies of S- and L-morphs were recorded in the summer of 2017, before grassland restoration. In the same populations where the genetic samples had been collected, at least 100 random individuals of *P. veris*, if possible, were visually inspected for their floral morph type, with a distance between sampled individuals similar to when collecting genetic samples, *i.e.* observed individuals were at least 1 m apart (Table 1). Therefore, in some populations, morph identity could be assessed for a lower number of individuals, even when the population was >100 individuals. The size of *P. veris* populations was assessed both when collecting samples for genetic analysis (in 2015 and 2016) as well as in 2017 by estimating the amount of flowering *P. veris* individuals. In the case of very large and/or dense populations, flowering cowslip individuals were counted in a 10 m × 10 m area, and this estimate was multiplied by the rest of the area covered by cowslips with similar densities to obtain an approximate estimate for the whole population. A *t* test was used to check whether there were any significant differences between the size estimates from different time points.

#### Data generation

##### Quantification of genetic diversity and genetic identification of morph types

We used 2619 neutral single-nucleotide polymorphisms (SNPs) from previously generated RAD-seq data in the same study populations from Saaremaa and Muhu (for more detail see Träger *et al.* 2021) to estimate observed and unbiased expected heterozygosity ( $H_O$  and  $uH_E$ , respectively; Table 1) and the percentage of polymorphic loci (%P). These measures of genetic diversity were calculated using GenAlex 6.503 (Peakall & Smouse 2012) for each study population of *P. veris*. Inbreeding coefficient ( $F_{IS}$ ) and pairwise genetic differentiation ( $F_{ST}$ ) were calculated using genepop version 1.0.5. (Rousset *et al.* 2017) in R 3.6.1 (R Core Team 2019). The morph types of plants sampled for genetic analysis were unfortunately not determined in the field. For morph identification of genetically analysed samples, we took advantage of the hemizygous nature of the heterostyly locus (present only in S-morph; Huu *et al.* 2016; Potente *et al.* 2022) to assign morph type to each sample. For a more detailed description of lab analyses, see Supporting Information. The indices of genetic diversity were also calculated separately for S-morphs and L-morphs in order to compare the genetic patterns of different morphs (Table 1).

##### Quantification of morph ratios

Population-level morph ratio bias was calculated from at least 100 individuals (where possible) by subtracting the number of L-morphs from S-morphs and dividing this value by the total number of individuals  $(L\text{-morph} - S\text{-morph}) / (L\text{-morph} + S\text{-morph})$ . This measure of directional morph ratio bias (from -1 to +1) indicates towards which morph type the deviation is skewed. Negative values indicate an excess of S-morphs (with a value -1 suggesting the presence of only

S-morphs), and positive values indicate an excess of L-morphs (with a value of 1 indicating to the presence of only L-morphs). In the case of 0, S- and L-morphs have an equal frequency, *i.e.* a balanced morph ratio. We also calculated the absolute morph bias as a separate explanatory variable to measure the extent, rather than the direction, of morph bias by taking the absolute value from the above-described indicator of morph ratio bias.

#### Statistical analyses

To test whether morph ratios were more skewed in smaller populations, we fitted a linear regression model. The population size was log-transformed to obtain a normal distribution. To take into account the potential effect of land-use history on morph ratios, region was considered as one of the explanatory factors in the subsequent analysis. Next, we tested the factors influencing genetic diversity of populations using linear regression models, including morph ratio bias, population size, habitat succession (open or overgrown) and region (Saaremaa or Muhu) as explanatory variables, and genetic diversity indices ( $H_O$ ,  $uH_E$ , %P) and inbreeding coefficient ( $F_{IS}$ ) as response variables. All possible double interactions of the explanatory variables were also included in the models. Linear models were used to analyse the effects of explanatory variables on  $H_O$ ,  $uH_E$  and  $F_{IS}$ . For %P, generalised linear models were applied because polymorphic loci are expressed in percentages and have a binomial distribution.

To analyse the effects of population-level morph ratio bias, population size, habitat succession and region on the genetic diversity of S- and L-morphs separately, linear mixed-effects models were applied, with the population as a random variable to take into account the fact that the genetic indices for S-morphs and L-morphs were calculated from the same population. Populations with <5 samples per morph were removed from all genetic analyses. The morph identity (S or L) was considered as a fixed variable in these models. Estimated marginal means *post-hoc* test from R package emmeans (Searle *et al.* 1980) was used to compare group means for interacting variables.

For linear and linear mixed effect models, we used the stepAIC function (both backward and forward selection) from the R package MASS (Venables & Ripley 2002) to select models according to the lowest AIC. Since stepAIC function cannot be used for generalised linear models, we chose the best models for the %P by starting with models including all uncorrelated explanatory variables, and continued by removing non-significant variables in a stepwise manner until the model with fewer variables explained the variation significantly better than more complex models, using likelihood ratio tests.

A paired-samples *t* test was used to examine whether the genetic differentiation ( $F_{ST}$ ) between the two morphs was significantly different. Multivariate generalised linear mixed models (R package MCMCglmm; Hadfield 2010) were used to test the effect of morph type, region and geographical distance on genetic differentiation of populations. In this model, genetic differentiation ( $F_{ST}$ ) was used as a dependent variable and the morph type (S or L), geographical distance (isolation by distance or IBD pattern), region, and their interactions as explanatory variables. All statistical analyses were performed in R 3.6.1 (R Core Team 2019).

## RESULTS

### Population-level morph ratios

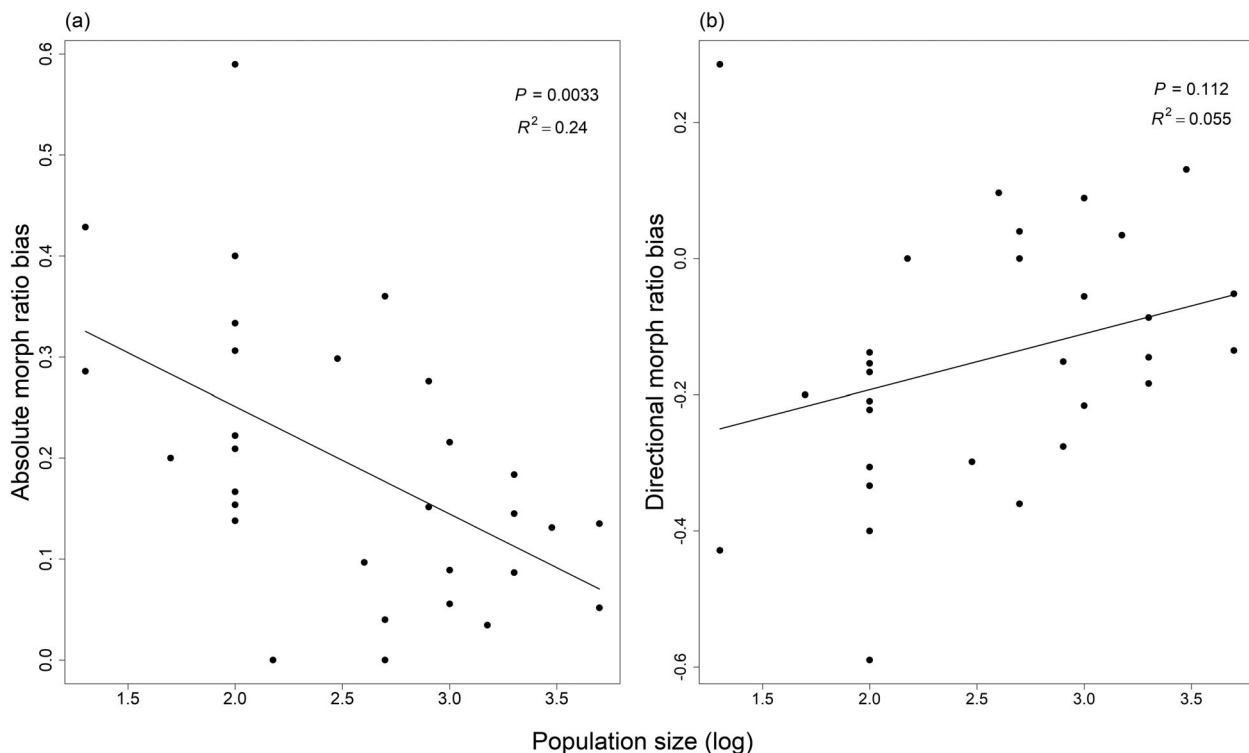
In total, we recorded 1210 S-morphs and 941 L-morphs across the 30 study populations of *P. veris*. Two populations had equal numbers of S- and L-morphs, six populations had more L-morphs and 22 populations had more S-morphs. The absolute morph ratio bias (0 – equal balance, 1 – only one morph present) ranged from 0 to 0.59, and directional bias (–1 – only S-morphs, 1 – only L-morphs) ranged from –0.59 to 0.29. The average absolute morph ratio bias was 0.16, and average directional morph ratio bias was –0.15 (*i.e.* skewed towards the prevalence of S-morphs). There were no significant differences between population size estimates from different time points, thus, the estimates from 2017 were used in subsequent analyses. The linear model on the effect of population size on morph bias showed that the absolute morph bias was negatively correlated with population size ( $P = 0.003$ ; Fig. 2a), whereas the directional bias was not significantly affected by population size ( $P = 0.104$ ; Fig. 2b).

### Genetic diversity of *P. veris* populations in response to morph bias

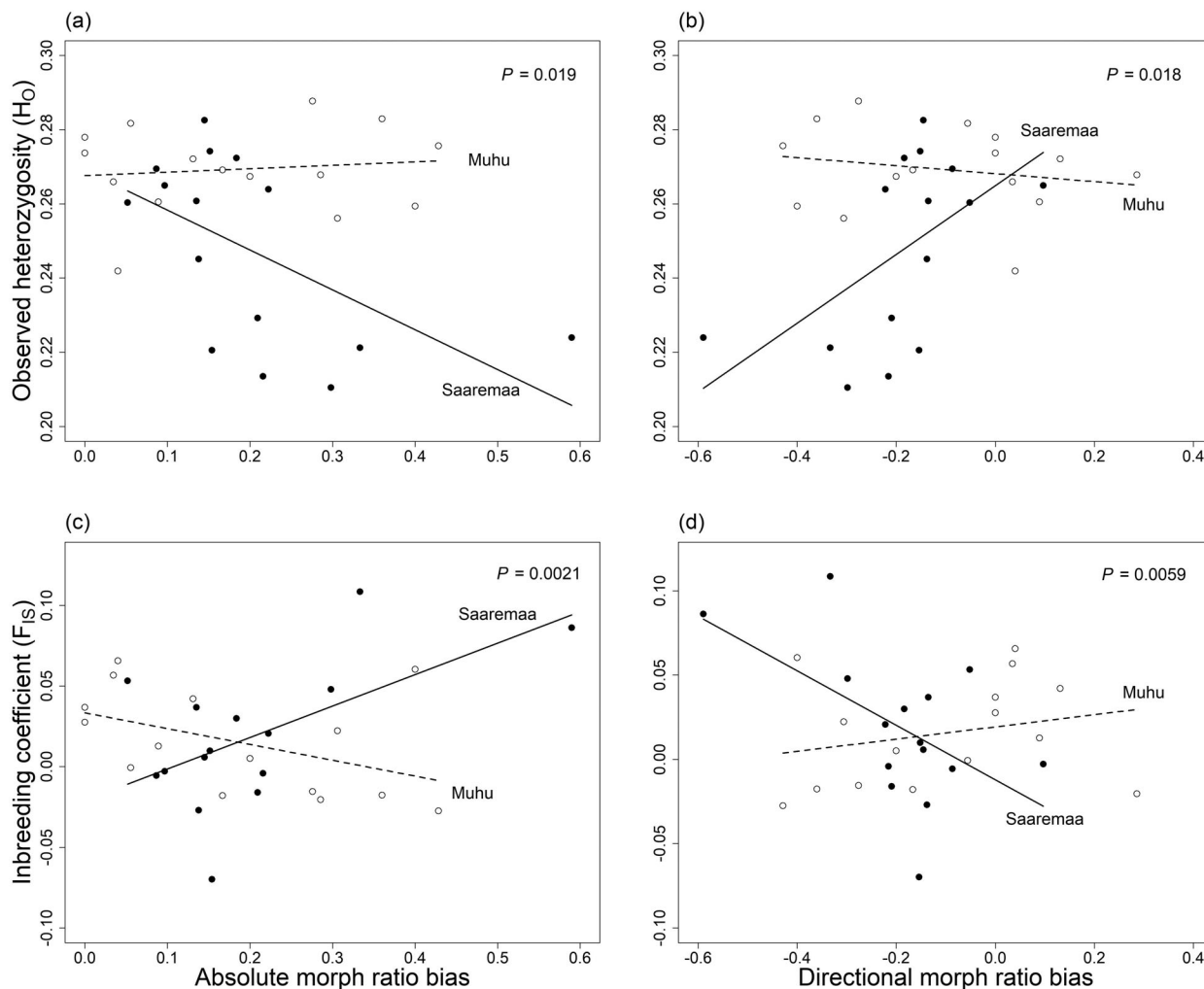
The observed heterozygosity ( $H_O$ ) in the study populations of *P. veris* ranged from 0.210 to 0.288 (Fig. 3a, Table 1). The explanatory variables in the best model for  $H_O$  included morph bias, region, population size and the interaction between morph bias and region (Table 2). The model showed that on Saaremaa, which has been more affected by fragmentation,  $H_O$  was positively correlated with absolute morph bias ( $P = 0.019$ ),

but not correlated with morph bias in Muhu's populations (Fig. 3a, Table 2). The model including directional bias, instead of absolute bias, also showed that  $H_O$  was positively correlated with directional morph bias ( $P = 0.018$ ) in Saaremaa only (Fig. 3b, Table 2).  $H_O$  was the lowest in Saaremaa's populations skewed towards the dominance of S-morphs and highest in populations skewed towards the dominance of L-morphs (Fig. 3b). However, because population size had no effect on  $H_O$  ( $P = 0.112$ ; Table 2), this is indicating a significant effect of morph deviation on genetic diversity, regardless of population size.

Unbiased expected heterozygosity ( $uH_E$ ) ranged from 0.208 to 0.288 (Table 1). The explanatory variables in the best model for  $uH_E$  included region, population size and their interaction (Table 2). The linear model showed that  $uH_E$  was significantly higher on the better-connected Muhu than on Saaremaa ( $P = 0.013$ ), but did not respond to morph bias. The inbreeding coefficient ( $F_{IS}$ ) ranged from –0.069 to 0.109 (Table 1) and the explanatory variables in the best model for  $F_{IS}$  included morph bias, region, population size and the interaction between morph bias and region (Table 2). The model showed that  $F_{IS}$  was affected by population size and region, with higher values in Muhu ( $P = 0.006$ ; Table 2) and as population size increased ( $P = 0.041$ ; Table 2).  $F_{IS}$  also increased with higher deviation from absolute morph balance ( $P = 0.002$ ; Fig. 3c), but only in Saaremaa's populations. The model including the effect of directional morph bias on  $F_{IS}$  also revealed higher inbreeding in Muhu ( $P = 0.024$ ; Table 2) and higher  $F_{IS}$  values with increasing population size ( $P = 0.042$ ; Table 2), as well as showing an increase in  $F_{IS}$  with higher deviation from morph balance in Saaremaa's populations ( $P = 0.006$ ; Fig. 3d, Table 2).



**Fig. 2.** Effect of population size on (a) absolute and (b) directional morph ratio bias in *Primula veris* populations on the islands of Muhu and Saaremaa, Estonia.  $P$ -value from the respective best model and adjusted  $R^2$  from the best model.



**Fig. 3.** Effects of absolute and directional morph ratio bias in the study populations of *Primula veris* on Muhu and Saaremaa, Estonia on (a, b) observed heterozygosity and on (c, d) inbreeding coefficient. Negative deviation values express dominance of S-morphs, positive values indicate dominance of L-morphs. Empty dots are populations from Muhu, filled dots from Saaremaa. Dashed and solid lines represent linear regression lines between observed heterozygosity or inbreeding coefficient and morph ratio bias for Muhu and Saaremaa, respectively.  $P$ -value is significant relationship between morph ratio bias and region from the respective best model.

The highest  $F_{IS}$  values occurred in populations dominated by S-morphs (Fig. 3d). The percentage of polymorphic loci (%P) ranged from 55.17% to 91.22% (Table 1). The explanatory variables in the best model for %P included region and population size, with lower %P values in Saaremaa ( $P < 0.001$ ) and higher values with an increase in population size ( $P = 0.002$ ; Table 2).

### Genetic diversity of morph types

For S-morphs,  $H_O$  ranged from 0.211 to 0.290,  $uH_E$  ranged from 0.211 to 0.292,  $F_{IS}$  ranged from -0.074 to 0.113, and %P ranged from 62.96% to 87.13% (Table 1). For L-morphs,  $H_O$  ranged from 0.201 to 0.295,  $uH_E$  ranged from 0.203 to 0.289,  $F_{IS}$  ranged from -0.062 to 0.107, and %P ranged from 53.30% to 86.64% (Table 1). The linear mixed-effects models revealed that  $H_O$ ,  $uH_E$  and %P were affected by the interaction of morph type and region (Table S1). The *post-hoc* test (Table S2) revealed that L-morphs in Muhu (more stable, well-connected habitats) populations had significantly higher  $H_O$  than

L-morphs in Saaremaa, which has been more affected by loss and fragmentation of grasslands (Fig. 4a). S- and L-morphs in Muhu were characterised by higher  $uH_E$  than S- and L-morphs in Saaremaa (Fig. 4b, Table S2). %P of L-morphs in Muhu was higher than that of S-morphs in Muhu and L-morphs in Saaremaa, and L-morphs in Saaremaa had lower %P values than S-morphs in Saaremaa (Fig. 4c, Table S2).

### Genetic differentiation of populations by morph

Pairwise genetic differentiation ( $F_{ST}$ ) values of S-morphs ranged from 0 to 0.278 ( $0.108 \pm 0.048$ ) and for L-morphs from 0.008 to 0.243 ( $0.100 \pm 0.048$ ). Pairwise genetic differentiation was significantly higher in S- than L-morphs ( $t$  test:  $t = -4.57$ ,  $df = 495$ ,  $P < 0.001$ ). Multivariate generalised linear mixed models showed that  $F_{ST}$  values were overall positively correlated with geographical distance ( $P < 0.001$ ; Fig. 5, Table S3), as expected.  $F_{ST}$  was also affected by the interaction between region and morph type ( $P = 0.001$ ; Table S3), with significantly

**Table 2.** Results of the models on the effects of absolute and directional morph bias, region (Muhu and Saaremaa), population size (log) and their interactions on observed heterozygosity ( $H_O$ ), unbiased expected heterozygosity ( $uH_E$ ), inbreeding coefficient ( $F_{IS}$ ) and polymorphic loci (%P) of the study populations of *Primula veris* in Muhu and Saaremaa, Estonia. For  $H_O$  and  $F_{IS}$ , models for both absolute morph bias and directional morph bias are presented.

response variable	explanatory variable	estimate	SE	t-value	$Pr(> t )$
observed heterozygosity ( $H_O$ )	(Intercept)	0.241	0.018	13.546	<b>&lt;0.001</b>
	absolute morph bias	0.033	0.034	0.978	0.337
	region Saaremaa	-0.002	0.011	-0.167	0.868
	population size	0.009	0.006	1.649	0.112
	absolute morph bias: region Saaremaa	-0.115	0.046	-2.508	<b>0.019</b>
	(Intercept)	0.246	0.014	17.846	<b>&lt;0.001</b>
	directional morph bias	-0.019	0.021	-0.882	0.386
	region Saaremaa	-0.008	0.009	-0.887	0.384
	population size	0.009	0.005	1.690	0.104
	directional morph bias: region Saaremaa	0.094	0.037	2.543	<b>0.018</b>
unbiased expected heterozygosity ( $uH_E$ )	(Intercept)	0.256	0.016	15.644	<b>&lt;0.001</b>
	region Saaremaa	-0.066	0.025	-2.672	<b>0.013</b>
	population size	0.007	0.007	1.064	0.297
	region Saaremaa: population size	0.016	0.009	1.703	0.101
	(Intercept)	-0.033	0.034	-0.977	0.338
inbreeding coefficient ( $F_{IS}$ )	absolute morph bias	-0.04	0.063	-0.632	0.533
	region Saaremaa	-0.063	0.021	-3.010	<b>0.006</b>
	population size	0.023	0.011	2.154	<b>0.041</b>
	absolute morph bias: region Saaremaa	0.297	0.087	3.436	<b>0.002</b>
	(Intercept)	-0.036	0.027	-1.299	0.206
	directional morph bias	0.016	0.043	0.366	0.717
	region Saaremaa	-0.043	0.018	-2.402	<b>0.024</b>
	population size	0.022	0.01	2.139	<b>0.042</b>
	directional morph bias: region Saaremaa	-0.221	0.073	-3.011	<b>0.006</b>
	(Intercept)	0.912	0.286	3.190	<b>0.004</b>
polymorphic loci (P%)	region Saaremaa	-0.576	0.153	-3.769	<b>&lt;0.001</b>
	population size	0.397	0.115	3.452	<b>0.002</b>

Bold indicates significant ( $<0.05$ )  $P$ -values.

higher  $F_{ST}$  between S- than L-morphs in Muhu, but not in Saaremaa (Fig. 5), which has experienced more drastic landscape changes.

## DISCUSSION

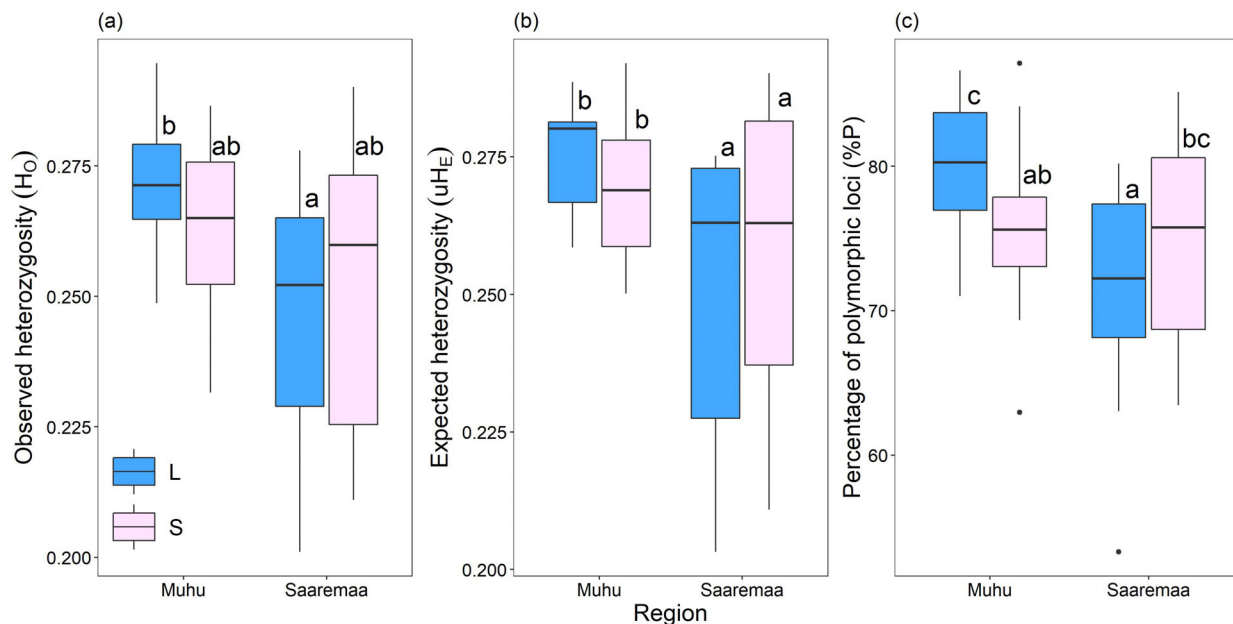
Habitat loss and fragmentation have a negative impact on biodiversity, including plant and pollinator species diversity and abundance, as well as the diversity at genetic level (Aguilar *et al.* 2008). Heterostylous species may be especially affected because they depend on pollen transfer between two or three reciprocal floral morphs for effective reproduction and maintaining genetic diversity because of intra-morph self-incompatibility (Jacquemyn *et al.* 2012). With habitat loss and fragmentation, plant populations tend to become small and spatially isolated from each other, which may result in deviations from the equal balance of morph types through stochastic effects (Kery *et al.* 2003). The negative effect of decreasing population size of the grassland plant *P. veris* on morph balance was also confirmed by the current study. In addition, we found that the genetic diversity of *P. veris* is negatively affected by morph ratio bias, but the effect differs depending on land-use context. More importantly, we found that for some indices of genetic diversity, this response partially depended on morph

identity (S or L), as L-morphs showed larger differences in genetic diversity between regions.

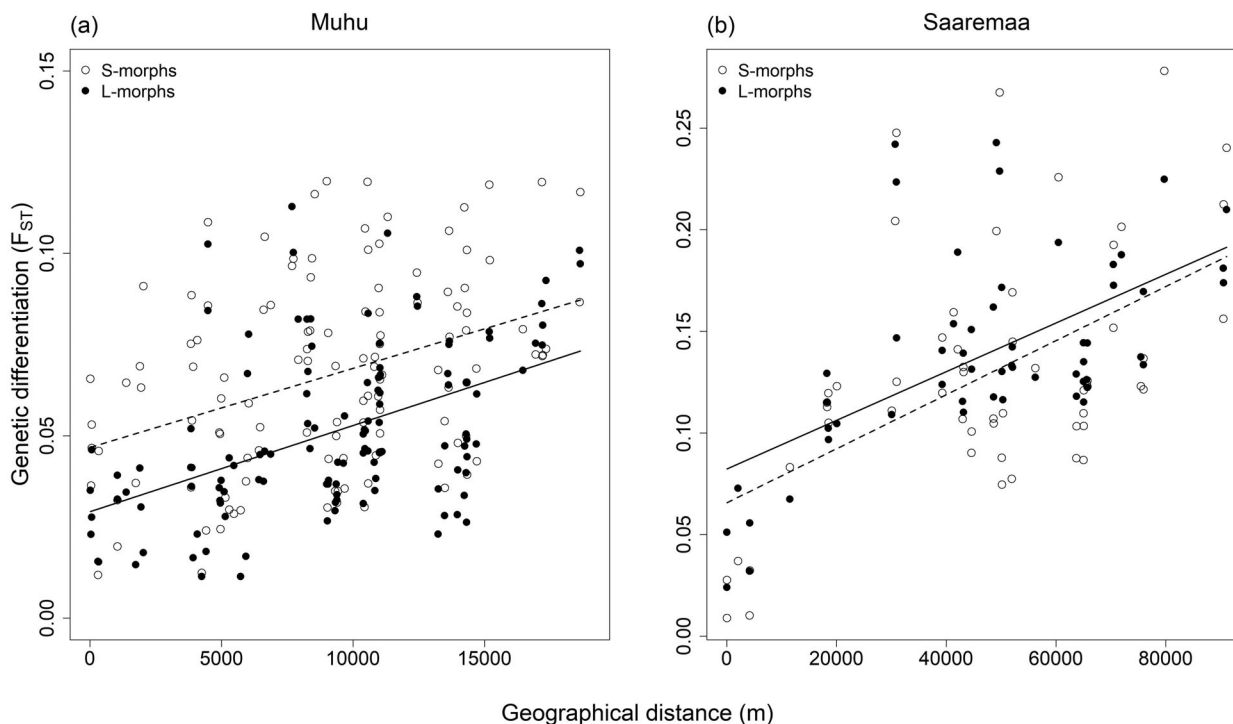
### Morph ratio bias affects the genetic diversity of *Primula veris*

Population size and deviation from equal morph balance are the most important factors that negatively affect the genetic diversity of heterostylous plants (Van Rossum *et al.* 2004; Meeus *et al.* 2012). Our results corroborate these general findings, as genetic diversity was negatively correlated with both population size and morph ratio bias (Figs 2 and 3). Because morph bias appears to often be strongly related to a reduction in population size (Endels *et al.* 2002; Kery *et al.* 2003), heterostylous species can be under dual negative pressure of both decreasing population size and deviations from equal morph frequencies. Indeed, in Saaremaa, which experienced stronger grassland loss and fragmentation, populations with more skewed morph ratios had lower  $H_O$  and higher  $F_{IS}$ , while in Muhu, which was less affected by anthropogenic landscape disturbance, this correlation was not detected (Fig. 3). Hence, our study suggests that negative pressures of decreasing population size, deviations from equal morph frequencies, or both, may put heterostylous species under increased risk of extinction.





**Fig. 4.** Morph effects on (a) observed heterozygosity, (b) unbiased expected heterozygosity, and (c) percentage of polymorphic loci in the study populations of *Primula veris* on Muhu and Saaremaa, Estonia. Letters a, b, and c represent significance of differences between groups from *post-hoc* tests. S, short-styled plants; L, long-styled plants.



**Fig. 5.** Relationship between geographical distance (m) and pairwise genetic differentiation ( $F_{ST}$ ) in S- and L-morphs in the study populations of *Primula veris* on (a) Muhu and (b) Saaremaa, Estonia. Dashed and solid lines represent linear regression lines between pairwise genetic differentiation and geographical distance for S- and L-morphs, respectively.

Genetic diversity can react to landscape changes with a time lag, and the current genetic patterns may therefore reflect historical landscape structure (Epps & Keyghobadi 2015). As the

average life span of the study species *P. veris* is around 50 years (Ehrlén & Lehtilä 2002), and the species can regenerate to a limited extent vegetatively (Tamm 1972), it is uncertain when

the genetic consequences of deviating morph frequencies might be manifested. Furthermore, the plant populations should experience fragmentation for several generations for genetic consequences to become detectable (Aguilar *et al.* 2008). Such a lag in the response of species to habitat loss may be one of the reasons for the negative effects of morph deviations on within-population genetic diversity in the populations of Saaremaa, where landscape changes have been more severe and took place earlier than in Muhu. Compared to the rest of Estonia, semi-natural grasslands in western Estonia are still in relatively good condition, despite considerable overgrowth of grasslands. The grasslands in both Saaremaa and Muhu used to be part of extensive and connected areas (Laasimer 1965). It is likely that this landscape change is not yet reflected in biodiversity (Helm *et al.* 2006; Reinula *et al.* 2021). Thus, the overall high homogeneity in genetic diversity (Table 1) could still reflect the historically high area and connectivity of these grasslands. A study on *P. vulgaris* in Flanders demonstrated that the negative genetic effects of long-term habitat fragmentation were apparent in seedling plants but not in adult plants (Van Geert *et al.* 2008). In our study, however, we sampled only adult plants. Consequently, there could be a time lag before habitat change-induced effects on morph deviation and genetic diversity of long-lived grassland plant species can be detected.

It has been observed that the genetic diversity could be higher in populations dominated by L-morphs, which may be due to their partial intra-morph compatibility leading to a higher number of potential mating partners. For example, in a study on *Pulmonaria officinalis*, the highest genetic diversity appeared in populations skewed towards L-morphs (Meeus *et al.* 2012), and a study on *P. veris* found that inbreeding values were the lowest in populations dominated by L-morphs (Van Rossum & Triest 2006). Similarly, our results show that genetic diversity was the lowest in populations with S-morph dominance (Fig. 4). In addition,  $F_{IS}$  was the highest in populations skewed towards S-morphs. Populations with S-morph dominance could thus be more prone to inbreeding and loss of genetic diversity. With fewer L-morphs supposedly characterised by higher intra-morph compatibility, individuals in these S-dominated populations might suffer from reduced availability of suitable mates, *i.e.* smaller effective population size, leading to lower within-population genetic diversity. The effects appearing with directional morph ratio bias suggest that changes in genetic diversity did not happen only because of stochastic events, but rather were controlled by morph type abundance.

#### Genetic diversity and genetic differentiation of S- and L-morphs

Variation in the strength of the intra-morph incompatibility reaction between S- and L-morphs affects outcrossing opportunities within populations, as the ovules of the morph with the weaker incompatibility reaction can be fertilised by pollen of both S- and L-flowers (Wedderburn & Richards 1990). Because of potential intra-morph compatibility in L-morphs and consequent (biparental) inbreeding, we expected to see a higher inbreeding value in L-morphs compared to S-morphs and lower genetic diversity values in L-morphs compared to S-morphs. Our results did not confirm this hypothesis: genetic diversity and inbreeding did not differ between the morph

types. However, we again observed regional effects on the genetic diversity within S- and L-morphs. For  $H_O$ ,  $uH_E$  and % P, L-morphs in Muhu had higher genetic diversity than L-morphs in Saaremaa. Populations in Muhu also had generally higher diversity than populations in Saaremaa. This finding suggests that L-morphs are more prone to the effects of landscape change, being more pronounced in Saaremaa, while S-morphs appear more stable and do not show changes in genetic diversity as swiftly.

In addition to differences in genetic diversity between morph types, we were interested in the patterns of genetic differentiation ( $F_{ST}$ ) between S- and L-morphs. We expected to see higher  $F_{ST}$  for S-morphs than for L-morphs because of more gene flow between L-morphs caused by weaker self-incompatibility. Indeed, the results showed that S-morphs had a significantly higher overall  $F_{ST}$  than L-morphs (Fig. 5). We also explored the response of  $F_{ST}$  within separate morphs to geographical distance (IBD pattern). We found that increasing spatial distance between populations of *P. veris* had a significant positive effect on  $F_{ST}$  for both S- and L-morphs. S-morphs had consistently higher  $F_{ST}$  values than L-morphs. The higher  $F_{ST}$  in S-morphs was statistically significant only in Muhu. Together with previous suggestions that S-morphs are more strictly self-incompatible (Wedderburn & Richards 1990; Van Rossum & Triest 2007), these findings suggest that partial intra-morph compatibility in L-morphs may also lead to higher levels of gene flow between populations, as reflected in lower genetic distances between L-morphs.

#### CONCLUSIONS

The results of our study indicate that deviations in morph frequencies have a negative impact on genetic diversity of the distylous grassland plant *P. veris*. However, the effects were partially determined by the study region and morph identity. The effects were stronger in a region where grassland loss and isolation have been more severe (Saaremaa). When no conservation efforts are made, populations in Muhu will probably follow the same trend in the future because the genetic diversity in Muhu populations might still show a lagged response to morph frequency deviations from the optimal 1:1 ratio. Also, the lower genetic diversity in L-morphs in Saaremaa compared to that in Muhu suggests that landscape change in Saaremaa may already be manifested in the differential patterns of genetic diversity of S- and L-morphs not detectable in the populations of Muhu. In semi-natural grasslands, management *via* grazing or mowing should be considered as an important conservation measure because this prevents these habitats from overgrowth and supports biodiversity. In order to maintain the genetic diversity of insect-pollinated grassland species in general, and heterostylous plants in particular, it is important to restore and maintain the populations to support effective gene flow *via* the movement of pollinating insects, but also by grazing animals.

Previous studies suggest that S- and L-morphs might not be equal in their mating patterns (Keller *et al.* 2014; Deschepper *et al.* 2018). However, knowledge on this topic is insufficient, and more studies on the differences between morphs are needed. Future studies should also consider landscape history and temporal dynamics of landscape change, *e.g.* how long plant populations have been present in habitats with changing

area and contiguity. This knowledge may help to indicate how long these populations can survive under disrupted conditions and when the negative consequences of skewed morph ratios on genetic diversity can be expected. Such information will also help in decision-making for which populations are in more immediate need of conservation and protection.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table S1.** Results of models on the effects of morph type (S and L), absolute and directional morph bias, region (Muhu and Saaremaa), population size (log) and their interactions on observed heterozygosity ( $H_O$ ), unbiased expected heterozygosity ( $uH_E$ ), inbreeding coefficient ( $F_{IS}$ ) and polymorphic loci (%P) of the study populations of *Primula veris* in Muhu and Saaremaa, Estonia.

**Table S2.** Results of testing differences among group means for significance (Tukey's HSD test) in Saaremaa and Muhu on observed heterozygosity ( $H_O$ ), unbiased expected heterozygosity ( $uH_E$ ) and percentage of polymorphic loci (%P) of study populations of *Primula veris* in Muhu and Saaremaa, Estonia. S, short-styled plants (S-morphs); L, long-styled plants (L-morphs).

**Table S3.** Results of the model on effects of morph type (S and L), region (Muhu and Saaremaa), geographic distance and their interactions on genetic differentiation ( $F_{ST}$ ) of the study populations of *Primula veris* in Muhu and Saaremaa, Estonia.

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