Different molecular changes underlie the same phenotypic transition: origins and consequences of independent shifts to homostyly within species

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Abstract

The molecular basis of phenotypic convergence, a key topic in evolutionary biology and ecology, has been investigated especially between species. However, it remains unclear whether mutations in the same or different positions of the same gene, or in different genes underlie phenotypic convergence within species. A classic example of convergence is the transition from outcrossing to selfing in plants, illustrated by the repeated shift from heterostyly to homostyly. Heterostyly is characterized by the reciprocal position of male and female sexual organs in two (or three) distinct, incompatible floral morphs, while homostyly is characterized by a single, self-compatible floral morph. *Primula* has long served as the prime model for studies of heterostyly and homostyly. Here, we elucidate the phenotypic and molecular origins of homostyly in P. vulgaris and its microevolutionary consequences by integrating microsatellite analyses of both progeny arrays and natural populations characterized by varying frequencies of homostyles with DNA sequence analyses of the gene controlling the position of female sexual organs (*CYP?*). We found that: homostyles evolved repeatedly from short-styled individuals in association with different types of loss-of-function mutations in *CYP?* and, consequently, short-styled individuals occur at lower frequencies than long-styled individuals across populations with all three morphs; the shift to homostyly promotes a shift to selfing; and intra-population frequency of homostyles is positively correlated with selfing rate and inbreeding level, increasing genetic differentiation among populations. These results elucidate the connections between the genotypic and phenotypic levels of convergence and the effects of contrasting floral morphologies on reproductive strategies.

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ABSTRACT

The molecular basis of phenotypic convergence, a key topic in evolutionary biology and ecology, has been investigated especially between species. However, it remains unclear whether mutations in the same or different positions of the same gene, or in different genes underlie phenotypic convergence within species. A classic example of convergence is the transition from outcrossing to selfing in plants, illustrated by the repeated shift from heterostyly to homostyly. Heterostyly is characterized by the reciprocal position of male and female sexual organs in two (or three) distinct, incompatible floral morphs, while homostyly is characterized by a single, self-compatible floral morph. *Primula* has long served as the prime model for studies of heterostyly and homostyly. Here, we elucidate the phenotypic and molecular origins of homostyly in *P. vulgaris* and its microevolutionary consequences by integrating microsatellite analyses of both progeny arrays and natural populations characterized by varying frequencies of homostyles with DNA sequence analyses of the gene controlling the position of female sexual organs (*CYP?*). We found that: homostyles evolved repeatedly from short-styled individuals in association with different types of loss-of-function mutations in*CYP?* and, consequently, short-styled individuals occur at lower frequencies than long-styled individuals across populations with all three morphs; the shift to homostyly promotes a shift to selfing; and intra-population frequency of homostyles is positively correlated with selfing rate and inbreeding level, increasing genetic differentiation among populations. These results elucidate the connections between the genotypic and phenotypic levels of convergence and the effects of contrasting floral morphologies on reproductive strategies.

KEYWORDS

convergence, heterostyly, homostyly, loss-of-function mutations, mating system, *Primula*

1 INTRODUCTION

The independent evolution of similar phenotypes represents a central topic in evolutionary biology and ecology. Phenotypic convergence is hypothesized to occur as an adaptive response to similar selective pressures (Losos, 2011) and may or may not stem from the same molecular mechanism (Rosenblum, Parent, & Brandt, 2014; Storz, 2016). The genetic basis of phenotypic convergence has been elucidated in distantly related taxa, including the origin of carnivory (Fukushima et al., 2017; Palfalvi et al., 2020) and specialized photosynthetic pathways in plants (Christin, Weinreich, & Besnard, 2010; Goolsby, Moore, Hancock, de Vos, & Edwards, 2018), but remains unclear within species (Arendt & Reznick, 2007; Manceau, Domingues, Linnen, Rosenblum, & Hoekstra, 2010; Ralph & Coop, 2010).

Studies on the molecular basis of phenotypic convergence at the intraspecific level have provided contrasting results. For instance, the independent reduction of lateral plates in threespine sticklebacks (*Gasterosteus aculaetus*) is linked to the fixation of the same allele in different freshwater populations (Colosimo et al., 2005), providing an example of molecular convergence. In contrast, the loss of pigmentation in Gulf and Atlantic populations of beach mice (*Peromyscus polinotus*) in Florida appears to be caused by mutations in different genes (Hoekstra, Hirschmann, Bundey, Insel, & Crossland, 2006; Steiner, Römpler, Boettger, Schöneberg, & Hoekstra, 2009). This conflicting evidence underscores the importance of determining whether mutations occurring in the same or different positions of the same gene, or in different genes underlie phenotypic convergence. Such knowledge is crucial for a deeper understanding of convergence at both phenotypic and genotypic levels, yet studies that investigate such connections are scarce.

A striking example of convergence in plants is the transition from outcrossing to selfing (Stebbins, 1957). An important advantage driving this repeated evolutionary shift is the ability of selfers to reproduce when pollinators and/or mates are scarce (i.e., reproductive assurance; Darwin, 1876; Eckert, Samis, & Dart, 2006). Additionally, selfers have a genetic advantage over outcrossers due to an increase in the number of gene copies transmitted to the next generation (i.e., automatic advantage; Busch & Delph, 2012; Fisher, 1941). Transitions to selfing are enabled by the loss of self-incompatibility (SI). The shift from SI to self-compatibility (SC) is usually driven by mutations inactivating the genes responsible for self-pollen recognition (e.g., *SCR* or *SRK* in Brassicaceae, and *SLF* in Solanaceae; Shimizu & Tsuchimatsu, 2015) and has been investigated both between and within species (Igic, Lande, & Kohn, 2008). Furthermore, the transition

to selfing is frequently accompanied by a reduced spatial separation between male and female reproductive organs (i.e., herkogamy), which facilitates autonomous pollination (Sicard & Lenhard, 2011). However, the genetic basis of herkogamy has only recently been discovered (Huu et al., 2016; Huu, Keller, Conti, Kappel, & Lenhard, 2020; Luo & Widmer, 2013), making it now possible to elucidate the genotypic changes underlying the reduction of herkogamy and associated transition to selfing.

A classic model to study the relationship between herkogamy and mating system is the shift from heterostyly to homostyly (Barrett, 1992, 2010a). Heterostyly is a complex floral polymorphism whereby two (distyly) or three (tristyly) floral morphs differing in the position of reproductive organs co-occur (Darwin, 1877). In distylous species, flowers of the long-styled morph (LS-morph, also known as *pin*) exhibit the stigma above the anthers, whereas flowers of the short-styled morph (SS-morph, also known as *thrum*) show the reciprocal arrangement (Ganders, 1979; Figure 1). The reciprocal herkogamy observed in heterostylous taxa simultaneously reduces the frequency of self-pollination and promotes pollen export to individuals of the opposite floral morph (Keller, Thomson, & Conti, 2014; Lloyd & Webb, 1992). Moreover, heterostyly is often associated with a polymorphism in pollen type and stigmatic papillae, as well as a heteromorphic SI that reduces self- and intra-morph fertilization (Dulberger, 1992). Therefore, heterostyly, reported in at least 28 angiosperm families, is considered a typical example of convergent adaptation for outcrossing (Barrett, 2019).

Considering that complex phenotypes can be lost in multiple ways (Roscito et al., 2018), repeated losses of heterostyly to homostyly are expected. Homostylous flowers are characterized by a reduction of herkogamy and, usually, loss of SI (de Vos, Wuest, & Conti, 2014). Both long- and short-homostylous morphs are known, but the latter have been reported only rarely (Dowrick, 1956; Lewis & Jones, 1992; Ray & Chisaki, 1957). In long-homostyles, both anthers and stigmas are placed high in the corolla-tube (HO-morph from here on; Figure 1), facilitating self-pollination. Hence, homostyles can produce seeds in the absence of pollinators, enabling reproductive assurance (Carlson, Gisler, & Kelso, 2008; de Vos, Keller, Isham, Kelso, & Conti, 2012; Jia, Hu, Liu, & Jiang, 2017; Piper et al., 1984; Yuan et al., 2017), and have higher selfing rates than heterostyles (Belaoussoff & Shore, 1995; Husband & Barrett, 1993; Piper et al., 1986; Schoen, Johnston, L'Heureux, & Marsolais, 1997; Yuan et al., 2017; Zhou et al., 2017; Zhong et al., 2019). Consequently, the transition from heterostyly to homostyly provides an ideal model to study shifts of mating systems.

The phenotypic origins of homostyly have been investigated at the inter- and intraspecific levels. Phylogenetic analyses inferred convergent shifts to homostyly in ancestrally heterostylous lineages of Primulaceae (de Vos, Hughes, Schneeweiss, Moore, & Conti, 2014; Mast et al., 2006; Zhong et al., 2019), Pontederiaceae (Kohn, Graham, Morton, Doyle, & Barrett, 1996), Rubiaceae (Ferrero, Rojas, Vale, & Navarro, 2012), Gentianaceae (Kissling & Barrett, 2013), and Plumbaginaceae (Costa, Torices, & Barrett, 2019). At the intraspecific level, population genetic analyses suggested independent origins of homostyly within the heterostylous *Eichhornia paniculata* (Husband & Barrett, 1993; Ness, Wright, & Barrett, 2010), *Primula chungensis* (Zhou et al., 2017), *P. oreodoxa* (Yuan et al., 2017) and *P. merrilliana* (Shao et al., 2019). Thus, available evidence confirms that homostyly arose repeatedly from heterostylous ancestors both between and within species. However, none of these studies identified the molecular basis of such transitions, likely because the necessary genomic resources have only recently become available (Huu et al., 2016, 2020; Li et al., 2016).

The genetic underpinnings of heterostyly have been characterized in *Primula* , where recent genomic and functional studies identified *GLOBOSA2* (*GLO?*) and *CYP734A50* (*CYP?*) as the genes controlling anther and stigma positions, respectively (Huu et al., 2016; 2020). In *Primula veris* and *P. vulgaris*, *GLO?* and *CYP?* are included in a hemizygous ~280 kb heterostyly supergene (also known as S-locus) present in SS-individuals, but absent from LS-individuals (Huu et al., 2016; Li et al., 2016). It has been shown that silencing *GLO?* causes a reduction of anther height in SS-individuals, leading to the expression of short-homostyly in *P. forbesii* (Huu et al., 2020). Indeed, a transposable element insertion in *GLO?* was detected in one short-homostylous individual from a natural population of *P. vulgaris* (Li et al., 2016). Conversely, silencing *CYP?* increased stigma length in SS-morph flowers, leading to a long-homostylous phenotype in *P. veris* (Huu et al., 2016). Accordingly, two different mutations in *CYP?* were detected in two long-homostylous individuals

from natural populations of *P. vulgaris* (Li et al., 2016). Furthermore, different *CYP?* exons were likely lost in long-homostyles of the ancestrally heterostylous *P. forbesii* and in the long-homostylous species *P. grandis, P. halleri,* and*P. scotica* (Huu et al., 2016). Moreover, the expression of*CYP?* was strongly reduced in homostylous as compared to SS-individuals of *P. oreodoxa* (Zhao, Luo, Yuan, Mei, & Zhang, 2019). In addition to style elongation, the disruption of *CYP?* might also be responsible for the loss of SI in *Primula,* but this proposed function has not yet been experimentally demonstrated (Kappel, Huu, & Lenhard, 2017). To summarize, the results presented above suggest that any genetic changes resulting in the disruption of *CYP?* function or expression are linked with transitions to long-homostyly, hence reduced herkogamy. However, comprehensive analyses of the different types of genetic changes associated with transitions to homostyly based on extensive sampling of heterostyles and homostyles from multiple natural populations have never been performed.

The evolutionary consequences of shifts from heterostyly to homostyly have been studied only in a handful of species. At the microevolutionary level, increased selfing in homostyles should promote high homozygosity, leading to a reduction of effective population size and genetic diversity within populations and increased genetic differentiation among populations (Hamrick & Godt, 1996). In accordance to these expectations, genetic diversity was lower in semi-homostylous populations of the ancestrally tristylous*Eichhornia paniculata*(Husband & Barrett, 1993; Ness et al., 2010). Similarly, increase in the frequency of homostylous individuals within populations of *Primulachungensis* and *P. oreodoxa* was associated with reduction of population genetic diversity (Yuan et al., 2017; Zhou et al., 2017). Similar patterns of decreased genetic diversity were documented in homostylous vs. heterostylous taxa of *Primula*section *Obconicolisteri*(Zhong et al., 2019). At the macroevolutionary level, phylogenetic analyses in Primulaceae indicated that extinction rates are higher in homostylous than heterostylous taxa (de Vos, Kalisz, & Slotte, 2014), validating predicted long-term effects of selfing (Goldberg et al., 2010; Takebayashi & Morrell, 2001; Wright et al., 2013).

Primula vulgaris Huds. (the common primrose) represents a classical model for the study of heterostyly and intraspecific transitions to homostyly. While fully heterostylous populations of*P. vulgaris* occur across Eurasia (Richards, 2003), intraspecific variation in the frequency of long-homostyles has been reported in England (Crosby, 1940; Jacquemyn, Endels, Brys, Hermy & Woodel, 2009). Short-homostyles in this species have been rarely documented in nature (Charlesworth & Charlesworth, 1979; Li et al., 2015). Previous work shows that homostylous individuals of *P. vulgaris* are self-compatible (Piper, Charlesworth, & Charlesworth, 1986) and have a higher reproductive success (i.e., higher fruit and seed set) than heterostylous individuals in years of pollinator scarcity (Boyd et al., 1990; Piper, Charlesworth, & Charlesworth, 1984). Additionally, studies of the mating system of homostylous individuals based on segregation analyses and allozymes estimated a range of outcrossing rates from 0.05 (Crosby, 1959; Piper et al., 1984) to 0.8 (Bodmer, 1958). However, no studies have quantified whether increased frequencies of homostyles within populations correspond to decreased genetic diversity in *P. vulgaris* .

The present study on the ancestrally heterostylous *P. vulgaris* is aimed at elucidating the phenotypic and molecular origins of homostyly within species, its effects on mating system, and its consequences at the population genetic level. Specifically, we ask: 1) Do frequencies of homostyles vary across natural populations? 2) Did the loss of heterostyly occur only once or not? 3) If homostyly originated repeatedly, are such transitions associated with the same or different molecular changes in *CYP?* ? 4) Is the change from heterostyly to homostyly accompanied by a shift from outcrossing to selfing? 5) Does the occurrence of homostyles cause an increase of inbreeding within populations and genetic differentiation among populations? To answer these questions, we: quantified the frequency of homostyly in 22 natural populations of *P. vulgaris,* including those where homostyles were first discovered in this species (Crosby 1940); inferred a tree from microsatellite data to test whether homostyles of *P. vulgaris*form a single genetic cluster or not; sequenced the *CYP?* gene to discover whether different homostylous individuals of *P. vulgaris*share the same mutations or not; estimated outcrossing rates from microsatellite analyses of progeny arrays of heterostylous and homostylous individuals to test whether the latter are more selfed than the former; and performed population genetic analyses of microsatellite data to test whether increasing frequencies of homostyles in natural populations are correlated with increasing population-level selfing rates, inbreeding coefficient and genetic differentiation. Our results

have broad implications for the general understanding of convergence at the phenotypic vs. genotypic levels and the effects of contrasting floral morphologies on reproductive strategies.

2 METHODS

2.1 Study species

Primula vulgaris is a perennial, rosette-forming diploid (2n = 22) blooming in early spring (February–April). It has pale-yellow fused corollas reaching up to 40 mm in width formed by broad, overlapping, v-notched lobes (Richards, 2003). The species reproduces mainly sexually, while clonal reproduction, thought to be uncommon in the wild, has been reported under cultivation (Boyd, Silvertown, & Tucker, 1990). *Primula vulgaris* has a patchy distribution with populations ranging from dozens to hundreds of individuals mostly found in woodlands and hedgerows next to agricultural fields and less frequently in grasslands (Jacquemyn et al., 2009). Pollination in natural populations of *P. vulgaris* is effected by long-tongued bees, bumblebees and bee-flies, but butterflies, pollen-gathering bees and moths could also play role (Boyd et al., 1990; Keller et al., 2020; Woodell, 1960). Seeds, produced in capsules, are dispersed by ants and rodents (Valverde & Silvertown, 1995). The reproductive ecology (Bodmer, 1958; Crosby, 1959; Piper et al., 1986), genetics (Cocker et al., 2015, 2018; Huu et al. 2016, 2020; Li et al., 2016), demography (Cahalan & Gliddon, 1985) and evolutionary biology (Crosby, 1949, 1960; Bodmer, 1960; Piper & Charlesworth, 1986) of *P. vulgaris* have been intensively studied, making it a classic biological model system.

2.2 Frequency variation of floral morphs in natural populations of Primula vulgaris

To determine whether the frequency of homostyles varies among populations of *P. vulgaris* , we estimated floral morph composition in 22 natural populations of the Somerset region (England) in the spring of 2019 (Figure 2A). These populations were selected based on Curtis and Curtis (1985). The frequency of floral morphs was estimated by randomly scoring approximately 100 individuals in sufficiently large populations (Table 1). Flowering plants were visually assigned to SS-, LS- and HO-morphs (Figure 1). To determine whether SS- and LS-morphs segregated at equal frequencies in both dimorphic heterostylous (i.e., populations consisting of SS- and LS-individuals) and trimorphic populations (i.e., populations consisting of SS-, LS- and HO-individuals), we tested whether morphs ratios deviated significantly from isoplethy using G-tests of goodness-of-fit as implemented in the R package 'DescTools' v0.99.38 (Signorell et al., 2020).

We used a ternary plot to assess whether the increase in frequency of HO-morphs occurred at the cost of only one or both heterostylous (HE) morphs. Ternary plots simultaneously display the frequencies of the three floral morphs in each population as a single point within a triangle, facilitating the detection of trends in changes of floral morph frequencies among populations (Crosby, 1949). Specifically, a trajectory following a straight line from the mid-part of the base (i.e., equal frequencies of LS- and SS-individuals) to the opposite vertex (i.e., monomorphic homostyly) suggests an equal reduction in the frequency of both SS- and LS-morphs. Conversely, an increase of HO-individuals with a skewed trajectory towards any of the sides of the triangle indicates the predominant loss of either LS- or SS-floral morphs, respectively. The ternary plot was estimated using the R package 'ggtern' v3.1.0 (Hamilton & Ferry, 2018).

2.3 Sampling for genetic analyses

In the spring of 2019, we randomly collected leaf tissue from 11–30 individuals in each of the 22 natural populations described above for a total of 613 individuals. Leaf tissue was dried in silica gel. We sampled the same number of each floral morph where possible; the sampled individuals were at least 1–2 meters apart from each other. We additionally collected leaf tissue and 2–3 capsules with seeds from nine individuals (four pins and five thrums) of one dimorphic heterostylous population (D07) and seven individuals from the fully homostylous population (M01). Collected seeds of each individual were sowed in a single pot; seedlings from a single individual formed a family. All pots were placed in water containing gibberellic acid (70 ppm) for 24 hours and later placed in growth chambers at 18 °C with 12:12 h light-dark period. The proportion of germinated seeds per family was recorded after eight weeks and leaf tissue from a total of ten seedlings per family was collected whenever possible and dried in silica gel. DNA of individuals from natural populations

and progeny arrays was extracted with a modified CTAB protocol (Doyle & Doyle, 1987) and used for all subsequent genetic analyses.

2.4 Genetic relationships of homostyles and heterostyles

Data generation - We genotyped all 613 individuals collected from the 22 natural populations using 12 microsatellites previously developed for *P. vulgaris* (Triest, Sierens, & Van Rossum, 2015). Briefly, the loci were amplified in two multiplex reactions with a QIAGEN Multiplex PCR kit. Detailed conditions for the amplification of microsatellites are included in Supplementary Material. Fragment size estimations were performed using an ABI Prism 3130xl genetic analyzer (Applied Biosystems) with GeneScan 500 LIZ (Applied Biosystems) as an internal standard. Alleles were scored using GeneMapper v.4.1 (Applied Biosystems). Presence of null alleles was estimated with Micro-checker v.2.2.3 (Van Oosterhout, Hutchinson, Wills, & Shipley, 2004).

Analyses – For the analysis of genetic relationships, populations were divided in groups as follows: each dimorphic, heterostylous population (i.e., only SS- and LS-morphs) was defined as a single HE- group, whereas each trimorphic population was split into into a HE- (SS- and LS-morphs together) and a HO- (homostyles) group. Genetic distances between HO- and HE-groups were then estimated from the proportion of shared alleles among groups using the program MSA v.4.05 (Dieringer & Schlotterer, 2003) with 1000 bootstraps. To infer the genetic relationships among HO- and HE-groups, a Neighbor-Joining tree (NJ) was inferred with PHYLIP v.3.697 (Felsenstein, 2005) using the majority rule consensus from 1000 replicated bootstraps.

2.5 Molecular basis of the transition to homostyly

Data generation - To determine if homostylous phenotypes are associated with mutations in *CYP?,* we Sanger-sequenced all five exons of *CYP?* from 38 individuals (27 HO-individuals from 10 populations and 11 SS-individuals from five populations, corresponding to 2–3 individuals per population). Additionally, we obtained the*CYP?* sequences from whole genome sequencing data of six SS-individuals from two populations (three individuals per population; EMC, unpublished data). Finally, we complemented our data set with previously published sequences from two HO-individuals (Li et al., 2016). In total, we thus analyzed*CYP?* sequences from 46 individuals (29 HO- and 17 SS-morphs).

For Sanger sequencing, we used previously designed primers of all five exons of *CYP?* (Huu et al., 2016). Reverse primers for exons 1, 2 and 3 were newly designed to obtain longer sequences (Supplementary material Table S1). Detailed PCR conditions for the amplification of all*CYP?* exons are included in Supplementary Material. Sanger sequencing was performed in an ABI Prism 3130 genetic analyzer (Applied Biosystems). Forward and reverse sequences were visually inspected and aligned with MUSCLE, as implemented in MEGA X (Kumar, Stecher, Li, Knyaz, & Tamura, 2018). All exon sequences were concatenated with Mesquite v3.61 (Maddison & Maddison, 2019). Library preparation and high throughput sequencing for the whole genome sequence data was performed by RAPiD GENOMICS (Gainsville, Florida, USA) using paired-end sequencing (˜150bp sequence reads) in NovaSeq 6000 (Illumina). We used HybPiper v1.3.1 (Johnson et al. 2016) with default parameters (except for the coverage-cutoff level for assemblies set to 4) to target and extract the sequence of *CYP?* from whole genome sequencing data. To identify synonymous and nonsynonymous mutations we used the Open Reading Frame (ORF) from *P. vulgaris**CYP?* sequence deposited in GenBank (KT257665.1; Li et al., 2016).

Analyses - To determine the relationships among *CYP?*sequences from HO- and SS-individuals, we first estimated a haplotype network with the R package 'pegas' v0.12 (Paradis, 2010). In addition to single nucleotide substitutions, we also scored each insertion or deletion (*i.e.,* indel) as a character following the "simple indel contig" guidelines by Simmons and Ochoterena (2000). Briefly, each indel of any length was scored as a presence/absence character in every individual. The resulting presence/absence matrix was coded as pseudo-nucleotides using 'A' as absence, 'T' as presence and '-' as unknown, and was concatenated to the sequence alignment of *CYP?* . Secondly, we estimated the*CYP?* phylogeny using a partitioned Maximum Likelihood (ML) analysis in RAxML v8.01 (Kozlov, Darriba, Flouri, Morel, & Stamatakis, 2019) with a

GTR-GAMMA substitution model for nucleotide substitutions and a binary (BIN) model for indels. Branch support was estimated with 1000 standard bootstrap re-samplings. A publicly available *CYP?* sequence from *P. veris*(KX589238; Huu et al., 2016) was used as outgroup to root the*CYP?* tree.

2.6 Evolutionary consequences of transition to homostyly

Using microsatellites, we tested whether the transition to homostyly is associated with a shift of mating system by estimating outcrossing rates from the progeny arrays and selfing rates from the 22 natural populations with variable frequencies of HO-morphs. Moreover, we tested whether the frequency of homostyles is positively correlated with levels of inbreeding within populations and of genetic differentiation among populations.

2.6.1 Outcrossing rates from progeny arrays

Outcrossing rates were estimated from nine heterostylous families with a mean number of 10 seedlings per family from D07 (dimorphic heterostylous population) and seven homostylous families with 7.86 ± 1.06 (mean ± SE) seedlings per family from M01 (fully homostylous population). Both maternal and seedling plants were genotyped with the same microsatellites as in section 2.4. Multi-locus (t_m) and single-locus (t_s) outcrossing rates and their associated standard errors were estimated with MLTR v.3.4 (Ritland, 2002), using 10000 bootstrap resamplings of maternal families. We used a one-sided Wilcoxon signed-rank test to asses whether homostylous individuals have a lower outcrossing rate than distylous individuals. Moreover, the germination rate of heterostylous and homostylous families was used as a fitness proxy to estimate the strength of inbreeding depression (δ) as 1 – (W_{HO} / W_{HE}) (Goodwillie, Kalisz, & Eckert, 2005) where W_{HO} and W_{HE} are the mean germination rates of homostyles and heterostyles, respectively.

2.6.2 Population-level estimates of selfing rates

Selfing rates were estimated using three methods [*i.e.,s(g2), s(ML) and s(BES)*] from the microsatellite data of 613 individuals of 22 natural populations (see section 2.3). First, selfing rates and associated 95% CI were estimated using the reciprocal of the two-locus heterozygosity disequilibrium (*s(g2)*) and a Maximum Likelihood (*s(ML)*) estimator, both implemented in RMES (David, Pujol, Viard, & Goudet, 2007) by running 10000 iterations. Both *s(g2)* and *s(ML)* estimate correlation among loci, providing complementary information about identity disequilibrium and selfing rate. Given that estimation of selfing using *s(g2)* and *s(ML)* can be biased due to the removal of homozygous loci (David et al.,2007), we compared these selfing rates with a model-based Bayesian estimation of selfing that makes use of all loci regardless of heterozygosity (BES v0.1.3; Redelings et al., 2015). Standard deviations were estimated using 10000 bootstrap iterations. To test if population-level estimates of selfing rates are positively correlated with the frequency of homostyles in natural populations, we used generalized additive models where frequency of homostyles was used as independent variable and specifying a Beta distribution for the estimates of selfing rates as implemented in the R package 'gamlss' v5.1.6 (Rigby, Stasinopoulos, & Lane,, 2005).

2.6.3 Population genetic analyses

Allelic richness (Ar), observed (H_o) and expected heterozygosity (H_e), and inbreeding coefficient (*Fis*) were calculated with the R package 'diveRsity' v.9.90 (Keenan, Mcginnity, Cross, Crozier, & Prodöhl, 2013) from microsatellite data of 613 individuals from 22 populations (see section 2.3). Each locus and population was tested for Hardy-Weinberg equilibrium using the R package 'genepop' v.1.1.7 (Rousset, 2008). To assess if *Fis* is positively correlated with homostyle frequency we used a standard linear model with homostyle frequency as independent variable. Given that population size can influence *Fis,* we included census size as covariable with no interaction among independent variables.

Population specific and pairwise genetic differentiation measured as*Fst*(Weir & Cockerham, 1984) and its normalized counterpart *G'st*(Meirmans & Hedrick, 2011) were estimated with the R package 'diveRsity' v.9.90 (Keenan et al., 2013). To discover whether increasing homostyle frequencies correspond to increased genetic differentiation, we tested whether pairwise differences in homostyle frequencies between populations

are positively correlated with pairwise *G'st* values. To test the significance of association between these two matrices, we used a Mantel test as implemented in the R package 'adegenet' v2.1.3 (Jombart, 2008).

2.7 Genetic structure and gene flow

In order to estimate genetic structure and patterns of gene flow among populations of *P. vulgaris* , we used InStruct v3.2.09 (Gao, Williamson, & Bustamante, 2007), which extends the Bayesian model implemented in Structure (Pritchard, Stephens, & Donnelly, 2000) to situations of partial self-fertilization and recurrent inbreeding. We ran the analyses with 100000 burn-in iterations and 20 independent MCMC chains per run from 2 to 22 k as suggested by Gilbert et al. (2012). The most likely number of clusters (k) was detected with the $\Delta\kappa$ method (Evanno, Regnaut, & Goudet, 2005) implemented in CLUMPAK (Kopelman, Mayzel, Jakobson, Rosenberg, & Barrett, 2015). Additionally, a Discriminant Analysis of Principal Components (DAPC) was performed with the R package 'adegenet' v2.1.1 (Jombart, 2008). In order to determine if there is Isolation By Distance (*IBD*) we performed a Mantel test implemented in 'adegenet' using both *Fst* and *G'st* values of genetic differentiation.

Finally, to determine whether patterns of genetic structure are explained by gene flow or genetic divergence after recent fragmentation, we estimated the size corrected effective number of migrants per generation (Nm) using the frequency of private alleles with the R package 'genepop' v.1.1.7 (Rousset, 2008). Moreover, we used Approximate Bayesian Computations (ABC) as implemented in popABC v1.0 (Lopes, Balding, & Beaumont, 2009) to compare models of genetic divergence with and without gene flow using the coalescent framework developed by Nielsen and Wakely (2001) and Hey and Nielsen (2004). Specifically, we compared a scenario of isolation without gene flow (i.e., proportion of migrants per generation, $m = 0$) with seven scenarios of increasing migration rates ($m = 0.001$, 0.01, 0.1, 0.2, 0.3, 0.4 and 0.5). Priors used for this analysis are specified in Supplementary Material (Table S2). Each model was simulated with 100000 iterations and tolerance for the rejection step was set to 0.01. Model selection was based on the posterior probability estimated by categorical regression (Beaumont, Zhang, & Balding, 2002) using custom R scripts included in the popABC documentation. A major assumption of the models implemented in popABC is that there is no genetic structure within populations. Given that HO-individuals may cause within population structure due to selfing, we decided to use only the ten dimorphic heterostylous populations for popABC analyses because no genetic substructure is expected in populations consisting entirely of outcrossing individuals. No differences in pollen and seed dispersal have been reported among HO-, SS- and LS-individuals, thus excluding populations with HO-individuals from popABC analyses should not affect estimates of migration between populations.

3 RESULTS

3.1 Frequency variation of floral morphs in natural populations of Primula vulgaris

The frequency of homostyles ranged from 0 to 100% across the 22 sampled populations of *P. vulgaris* in Somerset, England (Table 1; Figure 2A). Ten populations were dimorphic heterostylous (LS- and SS-individuals; D01–D10), one was dimorphic with LS- and HO-individuals (D*11), ten were trimorphic (LS-, SS- and HO-morphs; T01–T10), and one was monomorphic with only the HO-morph (M01). Morph ratios in dimorphic heterostylous populations did not deviate from isoplethy except in D02 and D08, which had an excess of LS- and SS-plants, respectively ($G = 7.13$, $P = 0.007$ and $G = 6.11$, $P = 0.01$, respectively; Supplementary material Table S3). In trimorphic populations, LS-individuals were significantly more frequent than SS-individuals except in T03, where SS- and LS-morph frequencies did not differ significantly ($G = 0.13$, $P = 0.71$; Supplementary material Table S3). Congruently, the ternary plot suggested a skewed trajectory towards a reduction of SS-individuals when HO-individuals were present (Figure S1).

3.2 Genetic relationships of homostyles and heterostyles

The NJ tree indicated homostyles were genetically closer to heterostyles of the same population than to homostyles of other populations (Figure 2B), implying independent origins of homostyles.

3.3 Molecular basis of the transition to homostyly

We sequenced ˜97% of the 1,587 bp comprising the five exons of *CYP?*from 17 SS- and 27 HO-individuals of *P. vulgaris* from seven and ten different populations, respectively. Seven different *CYP?*alleles were found in our sampling (*CYP?-1* to *-7* ; Figure 3A) that differed from two previously reported alleles from long-homostyles here labelled as *CYP?-8* and *-9*(corresponding to *CYP? S^{LH1}* and*S^{LH2},* respectively, of Li et al., 2016). All 17 SS-individuals shared the same allele (*CYP?-1*), which was identical to a functional copy of *CYP?* previously reported in*P. vulgaris* (GenBank: KT257665.1; Li et al., 2016). Unexpectedly, this putatively functional *CYP?* allele also occurred in six HO-individuals. Furthermore, six *CYP?* alleles (*CYP?-2* to*-7*) found in the remaining 21 HO-individuals differed from*CYP?-1* by different kinds of mutations: four nonsynonymous mutations and two deletions. Specifically, the nonsynonymous mutation in*CYP?-2* introduced a premature stop codon in exon 2, whereas a 31 bp deletion in exon 5 (*CYP?-5*) as well as an 8 bp deletion in exon 1 (*CYP?-6*) introduced a frameshift leading to an early stop codon in the translation of *CYP?.* Moreover, a mutation in exon 5 of *CYP?-3* changed a non-polar (phenylalanine) to a polar amino acid (serine), whereas another mutation in exon 3 of *CYP?-4*changed an amidic (asparagine) to hydroxylic amino acid(serine). Finally, the arginine to histidine mutation in *CYP?-7* caused no change in amino acid side-chain polarity.

In the haplotype network, *CYP?-1* occupied a central node and was connected by a single mutational step to all other alleles except for*CYP?-5,* which differed by two mutational steps (Figure 3B). Additionally, phylogenetic analysis recovered well-supported hierarchical relationships among the different *CYP?* alleles in SS- and HO-individuals (Figure 4). Specifically, *CYP?-2* was shared among homostyles from different populations (T03, T04, T07, T10 and D*11; Figure 4);*CYP?-3* was unique to population T05 and *CYP?-5* was unique to population T09. Finally, two *CYP?* alleles were detected in each of three populations: *CYP?-4* and *CYP?-6* were found in population T06, *CYP?-2* and *CYP?-4* in population T10, and*CYP?-2* and *CYP?-7* in population D*11 (Figure 4).

3.4 Evolutionary consequences of transition to homostyly

Mating system in homostyles - Outcrossing rates estimated from progeny arrays were more variable and significantly lower in homostylous than heterostylous families (single locus [t_s]: mean ± SE; 0.24 ± 0.10 [homostylous] and 0.83 ± 0.05 [heterostylous]; multi-locus [t_m]: 0.14 ± 0.06 and 0.98 ± 0.02, respectively; Wilcoxon signed-rank test $W = 63$, $P = 0.002$ and 0.001, respectively; Figure 5). As expected, the frequency of homostyles within populations was positively correlated with population-level estimates of selfing rate *(pseudo-R^2* $= 0.439$,$P = 0.002$; *pseudo-R^2* $= 0.288$, $P = 0.007$;*pseudo-R^2* $= 0.447$, $P < 0.001$; for *s(g2)* ,*s(ML)* and *s(BES)* , respectively; Table 1, Figures 6A and S2). Seed germination rate was significantly lower for homostyles than heterostyles (0.11 ± 0.03 vs. 0.26 ± 0.05; $W = 308$, $P = 0.04$) resulting in an estimated inbreeding depression (δ) of 0.58.

Patterns of genetic diversity – 18 out of 22 populations (except for D03, D06, D08 and D10) had significant deviations from Hardy-Weinberg equilibrium due to a heterozygote deficit (Supplementary Table S4). Population-level estimates of allelic richness (*Ar*) varied from 1.58 to 3.78 (mean ± SE, 3.19 ± 0.09), expected heterozygosity (H_e) from 0.14 to 0.53 (0.42 ± 0.01), and observed heterozygosity (H_o) from 0.11 to 0.46 (0.37 ± 0.02). Inbreeding coefficient (*Fis*) ranged from -0.08 to 0.39 among populations (0.13 ± 0.02; Table 1) and was positively correlated with the frequency of homostyly ($R^2 = 0.405$, $P = 0.03$), but not with population size ($P = 0.27$; Figure 6B).

Mean values of genetic differentiation among populations were 0.083 (0.074–0.093) and 0.164 (0.147–0.181) for *Fst* and *G'st* , respectively (Table S4), and were strongly correlated with each other (*pseudo-R^2* $= 0.974$, $P = 0.001$). Pairwise population values of *Fst* and *G'st* are shown in Supplementary Material Table S5. As expected, pairwise *G'st* was positively correlated with pairwise increase of frequency of homostyles (Mantel test: $R^2 = 0.481$, $P < 0.001$).

3.5 Genetic structure and gene flow

InStruct analyses recovered two clusters (Figure 7A) and showed that T08 differs from the remaining 21 populations. Identical results were found with Structure (results not shown). Accordingly, the DAPC analyses

separated T08 from the rest of the populations (Figure 7B). Furthermore, we found no evidence of isolation by distance (Mantel test, $P = 0.457$). Together, these results suggest that genetic differentiation among populations is not strong, corroborating *Fst* results presented above. Using the frequency of private alleles we estimated that the effective number of migrants per generation (Nm) was 1.96. Comparison of different demographic scenarios lent higher support to models with inter-population gene flow than to the isolation model. Among the models with varying migration rates (m), the one with$m = 0.2$ had the highest posterior probability, indicating moderate gene flow (Figure S3).

4 DISCUSSION

Convergence is a fundamental topic in evolutionary biology, yet knowledge of the connections between genotypic and phenotypic convergence remains limited. Our study of transitions from heterostyly to homostyly provides novel insights into the molecular basis of phenotypic convergence and its evolutionary consequences. Specifically, we found that heterostyly has been repeatedly lost in *P. vulgaris* , and these independent losses are associated with nonsynonymous mutations and indels at different nucleotide positions of the same gene (*CYP?*). Additionally, we demonstrated that the transition to homostyly promotes a change of mating system from outcrossing to selfing, leading to consequences at the population genetic level. Finally, we discuss the potential role of gene flow in the spreading of homostyly among populations and the putative reasons why homostyly, despite its apparent advantages, has not become fixed in *P. vulgaris* .

4.1 Homostyles replace short-styled individuals within populations

The variation in the frequency of floral morphs among populations provides an opportunity to elucidate how homostylous phenotypes originate and spread. The homostyles of *Primula vulgaris* , typically characterized by long styles and high anthers (Huu et al., 2016; Li et al., 2016), display the stigma type of LS-morph but retain the anther position and pollen type of the SS-morph (Crosby, 1940). Therefore, the origin of HO-individuals from SS-individuals should cause an initial reduction of the latter, triggering downstream consequences that further increase the number of homostyles. Firstly, pollen of homostyles can be used for both self- and cross-fertilization of LS-individuals, but not for fertilization of SS-individuals (Figure 1). Homostyles can also be fertilized by compatible pollen of SS-morphs, but this cross is thought to occur rarely in nature due to stigma clogging with self-pollen in homostyles (Crosby, 1959; but see Bodmer, 1959). Hence, homostyles have a reproductive advantage over both LS- and SS-morphs as a result of their ability to both self-fertilize and cross with LS-morphs (Richards, 2003). Finally, self-fertilization of homostyles (Figure S4A and B) and occasional crosses from homostyles to LS-individuals produce homostylous and LS-progeny, but no SS-progeny (Figure S4C and D). In accordance with these expectations and findings of previous studies (Crosby, 1949, 1959; Curtis & Curtis, 1985), our results demonstrate that, as homostyles increase within populations, more SS- than LS-individuals are lost within populations (Table 1 and Figures 2A and S1). The fact that LS-individuals are maintained at low frequencies within populations, even in the absence of SS-individuals, suggests that they are occasionally fertilized by homostyles. Taken together, these results corroborate the hypothesis that long-homostyles originate from SS-individuals and explain why the latter occur at lower frequencies than LS-individuals across populations with all three morphs.

4.2 Multiple losses of heterostyly within species are associated with different molecular changes in the CYP? gene

Phenotypic convergence implies independent origins of the derived character state (Rosenblum et al., 2014). Testing for convergence thus requires an explicit framework of relatedness to determine whether taxa characterized by the derived state share a single origin or not (Lee & Coop, 2019; Stone, Nee, & Felsenstein, 2011). The NJ tree inferred from microsatellites indicates that homostyles of the ancestrally heterostylous *P. vulgaris*(Mast et al., 2006) are genetically closer to heterostyles of the same population than to homostyles of other populations (Figure 2B), implying that homostyles of *P. vulgaris* do not share a single origin. This finding confirms previous results in *Primula chungensis* (Zhou et al., 2017), *P. oreodoxa* (Yuan et al., 2017), *P. merrilliana* (Shao et al., 2019), and *Eichhornia paniculata*(Barrett et al., 2009; Husband & Barrett, 1993). While analyses of relatedness among homostyles and heterostyles suggest independent origins of the

former in *P. vulgaris* , they cannot reveal the molecular basis of such transitions. Therefore, we investigated whether independent shifts from heterostyly to homostyly were driven by the same or different molecular changes in the gene that controls stigma position (Huu et al., 2016; Li et al., 2016).

Recent advancements in genomics have enabled the identification of loci responsible for phenotypic convergence at the intraspecific level (Lee & Coop, 2019; Sackton & Clark, 2019). For instance, multiple loss-of-function mutations were detected in *SCR* or a *SCR* -like gene, suggesting independent losses of SI in *Arabidopsis thaliana* and *Laevenworthia alabamica* (Chantha, Herman, Platts, Vekemans, & Schoen, 2013; Shimizu, Shimizu-Inatsugi, Tsuchimatsu, & Purugganan, 2008). In the case of homostyly, recent studies indicated that disruption of *CYP?* can cause a shift to homostyly (Huu et al., 2016; Kappel et al., 2017; Nowak et al., 2015). Accordingly, all short-styled individuals of *P. vulgaris* analyzed in the present study share the same functional *CYP?* allele (*CYP?-1*), whereas alleles with nonsynonymous mutations or indels (*CYP?-2* to *-9*) are found exclusively in homostyles (Figures 3A, B and 4). These mutations cause either changes in the polarity of amino acid side chain (*CYP?-3* and *-4*) or premature stop codons (*CYP?-2* , *-5* and *-6*), both types of mutation presumably inducing loss or reduction of function (Zhang 2000). In one case (*CYP?-7*), a nonsynonymous mutation did not change the amino acid side-chain polarity, thus this mutation may or may not affect protein function (Figure 3A). Notably, the alleles found in our study differ from the ones previously reported in two long-homostyles of *P. vulgaris* (*CYP?-8* and *-9* , originally named *CYP? S^{LH1}* and *S^{LH2}* , respectively; Li et al., 2016). Thus, a total of eight different putative loss-of-function mutations in *CYP?* have been identified so far in homostylous individuals of *P. vulgaris* . Our haplotype network analysis shows that all disrupted *CYP?* alleles are independently derived from the *CYP?* allele of SS-morphs *via* different mutations (Figure 3B). Overall, our results reveal that a diversity of changes in the same gene underlies independent origins of homostyly, providing new evidence on the genetic basis of phenotypic convergence within species.

Convergent phenotypes can be linked not only to mutations in coding regions of specific genes, but also to mutations in promoter regions and/or structural rearrangements. For instance, some individuals of the self-compatible *A. thaliana* were characterized by mutations in *cis* -regulatory regions, inversions or splicing variants causing loss of function in the *SCR* or *SRK* genes that control self-incompatibility (Dwyer et al., 2013; Shimizu et al., 2008; Shimizu & Tsuchimatsu, 2015). In *P. vulgaris* , a surprising result is that six out of 27 HO-individuals have the same *CYP?* allele as the SS-individuals (*CYP?* -1; Figures 3A, 3B and 4). These six homostyles occur in two geographically and genetically close populations (M01 and T08; Figures 2A and 2B). The occurrence of a *CYP?* allele with no mutations in HO-individuals suggests that other mechanisms might be responsible for the inactivation of *CYP ?* , including mutations in its promoter region and/or structural rearrangements that cannot be detected *via* exon sequencing. In general, this result underscores that mutations outside genes controlling specific traits might represent important sources of phenotypic convergence.

4.3 Consequences of shifts to homostyly on mating system and population genetics

Changes in floral morphology can have profound effects on mating system (Opedal, 2018). Specifically, the reduction of anther and stigma separation and loss of self-incompatibility of homostylous morphs should increase selfing when compared to heterostyles. Evidence of this mating system transition in homostyles has been reported in *Eichhornia* (Husband & Barrett 1993), *Turnera* (Belaoussoff & Shore, 1995), *Amsinckia* (Schoen et al., 1997) and some *Primula* species (Yuan et al., 2017; Zhou et al., 2017; Zhong et al., 2019). In the case of *P. vulgaris* , previous work provided varying estimates of the mating system of homostyles (Bodmer, 1984). On the one hand, analyses of floral morph segregation in the progeny of open-pollinated plants concluded that outcrossing rates of homostyles were as high as 0.80 (Bodmer 1958). Conversely, similar analyses under both controlled and natural field conditions (Crosby 1958), as well as allozyme analyses of progeny arrays (Piper et al., 1986), reported outcrossing rates for homostyles ranging from 0.05 to 0.10. Our results from progeny arrays indicate that outcrossing rates in homostyles are significantly lower than in heterostyles ($t_m = 0.14$ vs. 1.0, respectively; Figure 5), corresponding to the definition of selfers for homostyles (i.e., t_m [?] 0.2; *sensu* Schemske & Lande 1985; Goodwillie et al., 2005). Additionally,

the outcrossing rates of homostyles are more variable than those of heterostyles (Figure 5), suggesting that occasional outcrossing can occur in the former. This is in accordance with previous results in *P. halleri* , a homostylous species with variable herkogamy which also displayed variable outcrossing rates (de Vos, Keller, Zhang, Nowak, & Conti, 2018). Furthermore, the influence of homostyly on mating system was supported by a positive correlation between the frequency of homostyles within populations and population-level estimates of selfing rates (Figure 6A). Overall, our findings clarify previous conflicting results about the effects of homostyly on mating system in *P. vulgaris* , confirming that transitions to homostyly increase selfing.

Mating systems have important effects on how genetic diversity is partitioned both within and among populations (Barrett, 2010b; Wright et al., 2013). The higher selfing rate of *P. vulgaris*homostyles (Figures 5 and 6A) should increase homozygosity within populations, leading to an increment of inbreeding and genetic differentiation among populations (Hamrick & Godt, 1996). In accordance with these expectations, we found that inbreeding coefficients are positively correlated with the frequency of homostyles within populations (Figure 6B). Moreover, pairwise genetic differentiation among populations is correlated with pairwise increase in the frequency of homostyles (Mantel test, $P = 0.001$). These results are in accordance with previous comparisons among homostylous and heterostylous taxa in *Primula* section *Obconicolisteri* (Zhong et al., 2019) and populations in *P.chungensis* (Zhou et al., 2017), *P. oreodoxa* (Yuan et al., 2017), and *Eichhornia paniculata* (Husband & Barrett, 1993; Ness et al., 2010). Therefore, our findings confirm the population genetic consequences predicted for the transition to homostyly as a transition to increased selfing.

4.4 Does homostyly spread among populations and why has it not become fixed in *Primula vulgaris?*

Gene flow plays a central role in the spreading of mutations (Morjan & Rieseberg, 2004; Ralph & Coop, 2010), potentially contributing to the migration of homostylous alleles among populations. In *P. vulgaris,* previous hypotheses proposed that homostyly could have migrated from initial places of origin to neighboring populations through transport of pollen or seed, the former being more likely (Crosby, 1949, 1960). Estimates of gene flow through pollen and seed in this species indicated that dispersal is restricted to a maximum of a few hundred meters from the parental plant (Cahalan & Gliddon, 1985), but occasional pollen flow over 1–3 kilometers has also been reported (Van Geert, Van Rossum, & Triest, 2010). Our population genetic analyses support a model of moderate levels of gene flow among populations ($Nm = 1.96$; Figure S3), while rejecting the hypothesis of a recent fragmentation of an ancestral, widespread population. Accordingly, our results do not support strong genetic differentiation among populations (Figures 7A and B) or isolation by distance (Mantel test, $P = 0.457$), further favoring the hypothesis of gene flow among populations. Moreover, the geographic distribution of *CYP?* alleles in our study indicates that 12 homostylous individuals from five populations (D*11, T03, T04, T07 and T10) separated by 3-18 Km shared the same*CYP?* allele (*CYP?* -2; Figure 4). This suggests that homostylous alleles could have migrated between neighboring populations, as the independent origin of exactly the same mutation should be unlikely. Altogether, these results favor the conclusion that gene flow might have facilitated the migration of homostylous alleles among geographically close populations of *P. vulgaris* , confirming the importance of gene flow for the spread of potentially advantageous mutations.

A crucial question remains as to why homostyly has not become fixed in*P. vulgaris* despite automatic selfing advantage (Fisher, 1941, 1949) and reproductive assurance (Crosby, 1949). In fact, the opposite has been found, since previous studies recorded the loss or decrease of homostyles in revisited populations (Curtis & Curtis 1985; Boyd et al., 1990). A possible explanation for the failure of homostyly to completely replace heterostyly is that the negative effects of inbreeding depression (Richards 1984; Goodwillie et al., 2005) can outweigh the advantages of homostyly (Piper et al., 1984; Boyd et al., 1990). Two recent studies investigated the relationship between inbreeding depression and the spread of self-compatibility, finding contrasting results. In experimental populations of *Linaria cavanillesi* , self-compatible individuals with no inbreeding depression displaced self-incompatible individuals in just three generations (Voillemot & Pannell, 2017; Voillemot, Encinas-Viso, & Pannell, 2019). In contrast, inbreeding depression values of 0.54 prevented the spread of SC plants within experimental patches of SI plants in*Laevenworthia alabamica* (Layman,

Fernando, Herlihy, & Busch, 2017*)* . Our estimate of inbreeding depression (0.58) in *P. vulgaris* is closer to the latter study, suggesting that inbreeding depression might be sufficiently high to prevent the fixation of homostyly within populations. While theory proposes that increased selfing should purge inbreeding depression over time by eliminating recessive deleterious alleles from populations (Schemske & Lande, 1985), transitions to homostyly in *P. vulgaris* may be too recent for the purging process to have occurred. Moreover, recessive deleterious alleles could have been re-introduced into populations *via* gene flow, thus slowing the decrease of inbreeding depression. Our study estimated moderate levels of gene flow among populations of *P. vulgaris* ($Nm = 1.96$; Supplementary Figure S3), implying that re-introduction of genetic load is feasible. Overall, our findings suggest that inbreeding depression plays a key role in maintaining heterostylous morphs within populations despite the reproductive advantages of homostyly.

5 CONCLUSIONS

By combining microsatellite and sequence data we demonstrate that heterostyly has been lost repeatedly in association with independent mutations in the same gene (*CYP?*) of *P. vulgaris* , indicating that any disruption of this gene causes style elongation resulting in convergent homostylous phenotypes. However, some homostyles have no mutations in *CYP?* , suggesting that mutations outside a gene, e.g., in promoter regions, and/or structural rearrangements, might also cause convergent phenotypes, as found in *Arabidopsis thaliana* for the genes underlying self-incompatibility. Finally, we show that the transition to homostyly is associated to a shift of mating system from outcrossing to selfing, increasing inbreeding within populations and genetic differentiation among populations, as expected. Future comparisons of whole genome sequencing data between heterostyles and homostyles will test the predicted reduction of effective recombination rate, genome-wide diversity, and efficiency of purifying selection in homostyles. Furthermore, this kind of data will enable the investigation of potential differences in the accumulation of transposable elements in the hemizygous heterostyly supergene vs. the rest of the genome in both outcrossing heterostyles and selfing homostyles.

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AUTHOR CONTRIBUTIONS

EMC, BK, JdV, PS and EC designed the research. EMC collected the data. EMC, RS and BK performed laboratory work. EMC, RS, EL-B and BK analyzed the data. EMC and EC wrote the paper and all authors provided feedback on the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

-Microsatellite genotypes from the 613 individuals from the 22 natural populations analyzed in this study, as well as DNA alignment for CYP^T haplotype network and phylogeny will be made available in Dryad.

-Scripts for analyses and figures in R are being curated and will be made available in Github.

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Figure 1. Heterostyly and homostyly in *Primula vulgaris* . Heterostylous floral morphs (HE-morphs) can be either short-styled (SS-morphs) or long-styled (LS-morphs); they are characterized by spatial separation of anthers and stigmas (i.e., herkogamy) and self- and intramorph-incompatibility, enforcing outcrossing.

Homostylous floral morphs (HO-morphs) are characterized by self-compatibility and reduced or no herkogamy, facilitating selfing; most homostyles have both stigma and anthers at the mouth of the corolla tube (i.e., long-homostyly); short homostyly is rare (not shown). Anthers are represented in yellow and stigmas in green. Red arrows indicate compatible pollinations between floral morphs.

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Figure 2. A) Geographic distribution of 22 populations of*Primula vulgaris* from Somerset, England. Pie charts represent intra-population frequencies of long-styled (LS; white), short-styled (SS; black) and homostylous (HO; grey) floral morphs; dimorphic (D) populations consist of LS- and SS-morphs, except for population D*11, which consists of LS- and HO-morphs; trimorphic (T) populations consist of LS-, SS- and HO-morphs; the single monomorphic (M) population consists of HO-morphs. **B)** Unrooted Neighbor-Joining tree inferred from 12 microsatellite loci based on the proportion of alleles shared among 33 groups defined as follows: one monomorphic group (M01); 10 dimorphic groups (D01–D10) of heterostylous individuals (HE), plus two groups from D*11, which was subdivided into HE (only LS-individuals) and HO groups; and 20 groups from trimorphic populations (T01–T10), each subdivided into HE and HO groups; HO groups are boldfaced and groups of the same population are in the same color. Bootstrap support values [?] 80 are indicated next to the branches.

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Figure 3. Variation of *CYP?* detected in 44 individuals of *Primula vulgaris* (17 short-styled and 27 homostylous individuals) from ten natural populations sampled for this study,plus two *CYP?* alleles (indicated with *) from two homostylous individuals previously reported as *CYP?*S^{LH1} and S^{LH2} by Li et al. (2016); *CYP?* is a 1587 bp long hemizygous gene that comprises five exons (base-pair lengths of each exon are indicated in parentheses). **A)**Graphical representation of the nine *CYP?* alleles with different types of mutations in *CYP?2–9* : vertical lines, white triangles, and black triangles represent point mutations, deletions, and insertions, respectively, with positions of each mutation reported above each exon; one synonymous, and five nonsynonymous mutations, two deletions, and one insertion were found. **B)** Haplotype network of the nine *CYP?* alleles: each circle (i.e., node in the network) represents a different *CYP?* allele (*CYP?1–9* , listed next to each circle), with circle size proportional to the number of homostylous (grey) and short-styled (black) individuals carrying that allele (reported inside the circle); nodes are connected by solid lines representing the lowest number of mutational steps between alleles, in this case always a single mutational step indicated by a single dash across the line, except for *CYP?-1* and *CYP?-5* connected by two mutational steps; dashed lines represent connections with two mutational steps between alleles, indicated by a double dash across the line.

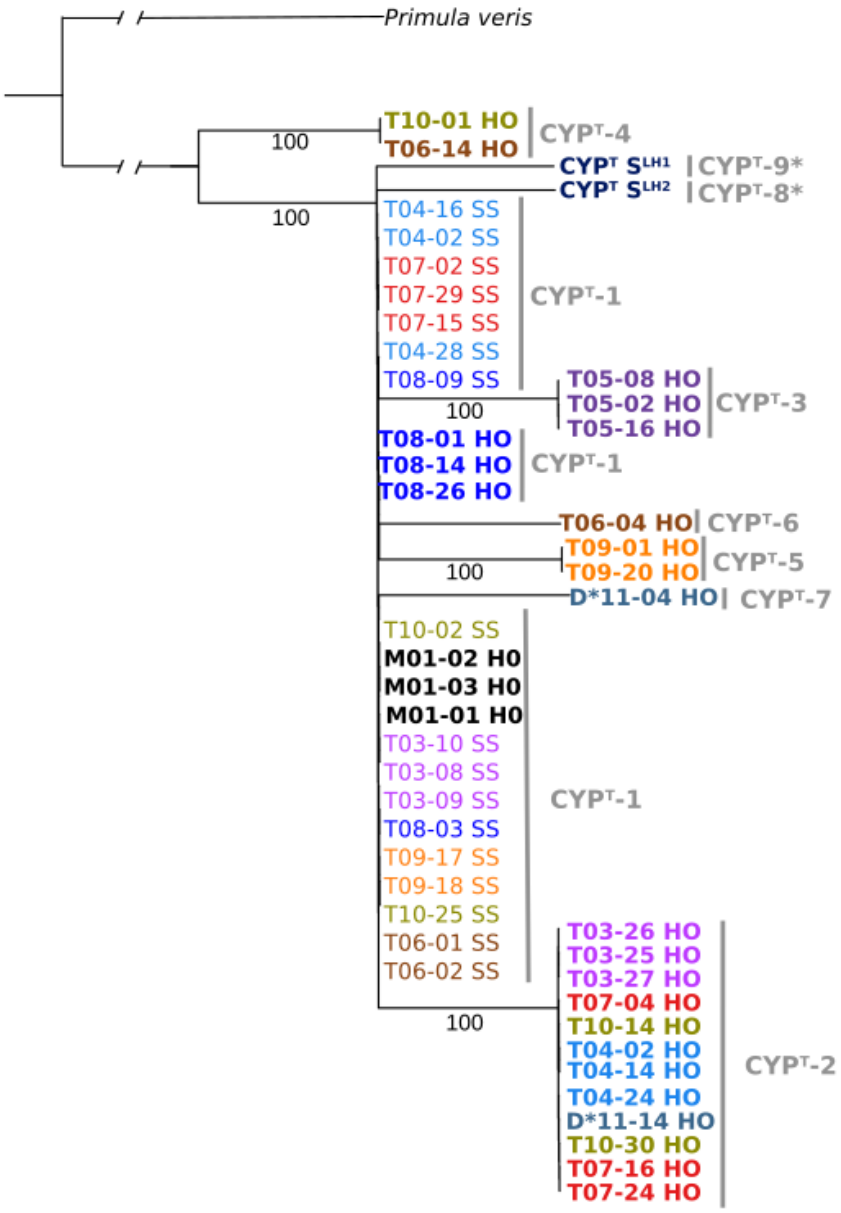

Figure 4.- Maximum likelihood tree of *CYP?* sequences from 44 individuals of *Primula vulgaris* (17 short-styled and 27 homostylous, boldfaced) sampled for this study, plus *CYP?* sequences from two homostylous individuals (indicated with *) previously reported as *CYP?*S^{LH1} and S^{LH2} (Li et al., 2016), and a *CYP?* sequence from an SS-individual of *Primula veris* (GenBank: KX589238) as outgroup. Each accession at the tips is labeled from left to right with population number, individual number, and floral morph type (SS and HO). Accessions from HO-individuals are boldfaced; accessions from the same population have the same color (see also Fig. 2). Bootstrap support values [?] 80 are indicated below to the branches.

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Figure 5.- Boxplot of outcrossing rates in *Primula vulgaris* inferred from progeny arrays of heterostyles and homostyles. Triangles represent the outcrossing rate of each family in the progeny (nine heterostylous families, black; seven homostylous families, grey). Significance levels indicate that outcrossing rates were significantly higher ($***P < 0.001$) in heterostyles than homostyles (Mean $\pm$ SE $= 0.98 \pm 0.01$ and 0.14 ± 0.05, respectively).

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Figure 6.- Correlations between population genetic parameters (y axis) and frequency of homostyles (x axis) in 22 natural populations of *Primula vulgaris* inferred from 12 microsatellites: A) Population level estimates of selfing rates based on multilocus linkage disequilibrium (See Methods) and B) Inbreeding coefficient.

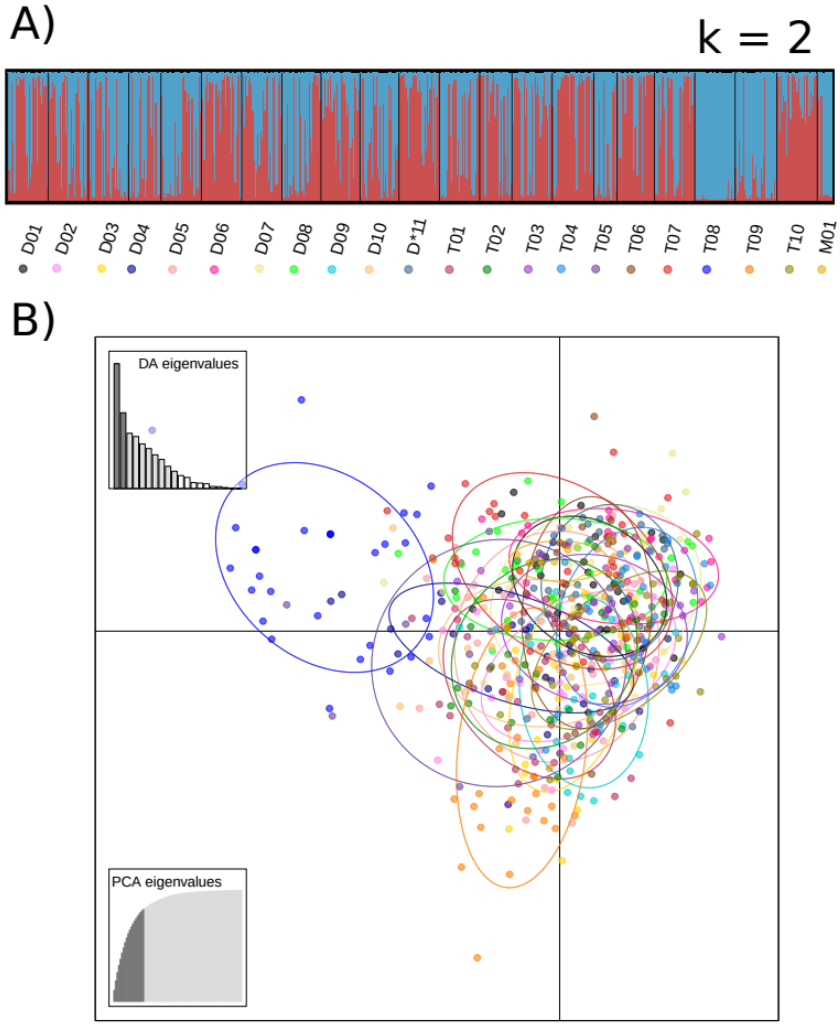

Figure 7. - Patterns of genetic differentiation for the 22 natural populations of *Primula vulgaris* . A) Results from InStruct analysis of microsatellites assigning all populations to two genetic clusters (k=2): Optimal number of clusters was selected according to Evanno et al. (2009); B) Plot of the two first PCs from Discriminant Analysis of Principal Components (DAPC) separating population T08 (blue circles) from the rest of the populations.

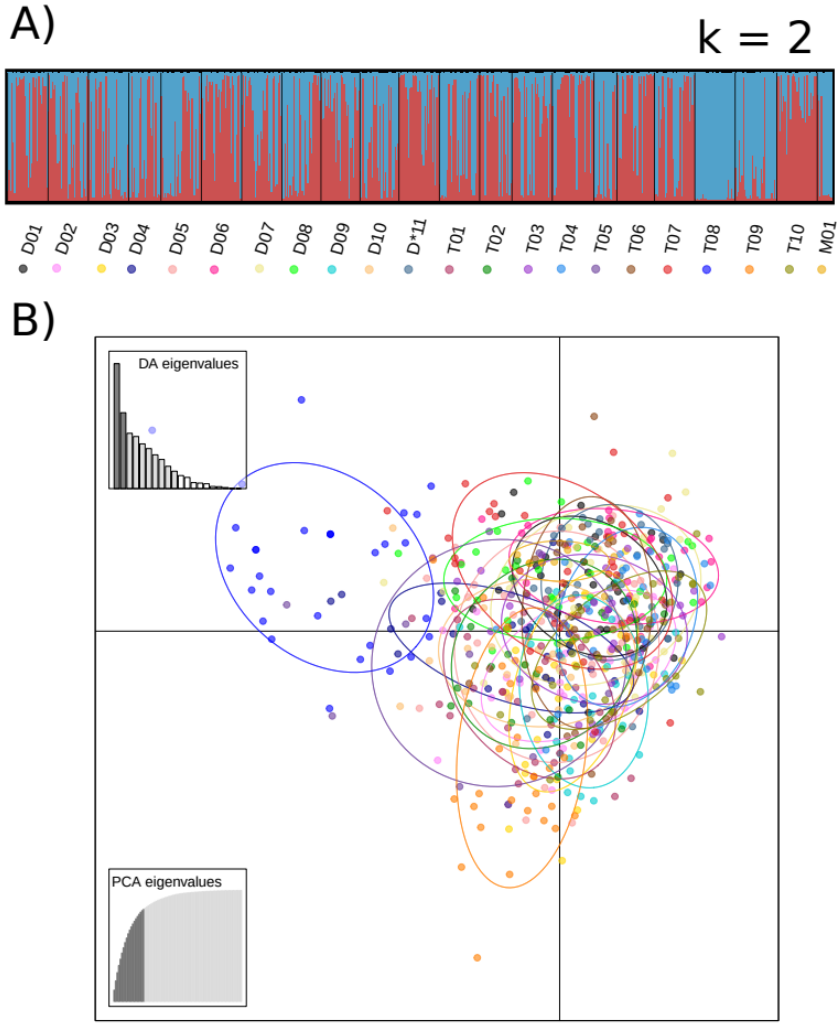

Table 1. Summary data for 22 natural populations of Primula vulgaris sampled in Somerset, England, with floral morph frequencies, levels of inbreeding (Fis), and three estimates of selfing rates (s(g2), s(ML) and s(BES)) inferred using 12 microsatellites. Dimorphic (D) populations consist of long-styled (LS) and short-styled (SS) morphs, except for population D*11, which consists of LS- and homostylous (HO) morphs; trimorphic (T) populations consist of LS-, SS- and HO-morphs; the single monomorphic (M) population consists of HO-morphs.

Population	Latitude (N), Longitude (E)	Population size	Sample size (morph freq)	Floral Morph			Sample size (pop genetics)	*Fis*	*s(g2)*	*s(ML)*	*s(BES)*
				LS	SS	HO					
D01	-2.58°, 51.18°	273	100	0.51	0.49	0	30	0.131	0.03	0.000	0.180
D02	-2.63°, 50.98°	~300	96	0.64	0.36	0	30	0.066	0.15	0.157	0.154
D03	-2.33°, 50.85°	~500	110	0.44	0.56	0	30	0.071	0.01	0.086	0.131
D04	-2.20°, 50.95°	65	65	0.48	0.52	0	24	0.143	0.00	0.000	0.212
D05	-2.22°, 51.05°	260	105	0.46	0.54	0	30	0.105	0.15	0.198	0.318
D06	-2.49°, 51.23°	~350	101	0.58	0.42	0	30	0.009	0.13	0.000	0.122
D07	-2.59°, 51.21°	~250	100	0.58	0.42	0	30	0.149	0.24	0.200	0.348
D08	-2.48°, 51.16°	150	94	0.37	0.63	0	29	-0.01	0.15	0.123	0.222
D09	-2.49°, 51.05°	~300	100	0.57	0.43	0	29	0.002	0.00	0.000	0.168
D10	-2.71°, 51.09°	~500	104	0.52	0.48	0	29	-0.085	0.09	0.068	0.052
D*11	-2.42°, 51.13°	58	50	0.34	0	0.66	30	0.065	0.11	0.070	0.218
T01	-2.44°, 51.00°	94	94	0.63	0.31	0.06	30	0.117	0.34	0.260	0.295
T02	-2.38°, 50.92°	~150	108	0.62	0.32	0.06	24	0.124	0.10	0.134	0.255
T03	-2.43°, 51.18°	88	88	0.41	0.38	0.22	30	0.052	0.13	0.144	0.219
T04	-2.40°, 51.16°	~200	104	0.36	0.16	0.48	31	0.389	0.53	0.519	0.529
T05	-2.25°, 50.95°	20	15	0.33	0.07	0.53	17	0.284	0.00	0.091	0.473
T06	-2.56°, 51.14°	79	79	0.41	0.06	0.53	28	0.225	0.21	0.219	0.422
T07	-2.30°, 51.08°	103	85	0.29	0.09	0.61	30	0.227	0.31	0.353	0.394
T08	-2.58°, 51.09°	161	95	0.36	0.02	0.62	30	0.126	0.42	0.457	0.314
T09	-2.55°, 51.05°	87	87	0.33	0.03	0.63	31	0.175	0.19	0.284	0.318
T10	-2.42°, 51.10°	~150	82	0.26	0.02	0.72	30	0.251	0.31	0.352	0.367
M01	-2.63°, 51.03°	19	19	0	0	1	11	0.272	0.78	0.644	0.461
							Mean (± SD)	0.131 (± 0.11)	0.199 (± 0.19)	0.198 (± 0.12)	0.281 (± 0.13)

Fis varies from -1 (heterozygotes excess) to + 1 (homozygotes excess due to inbreeding): values closer to 0 indicate random outcrossing under HWE; population level estimates of selfing rates vary from 0 (maximum outcrossing) to 1 (maximum self-fertilization).