Biased morph ratios and skewed mating success contribute to loss of genetic diversity in the distylous *Pulmonaria officinalis*

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INTRODUCTION

Heterostyly is a sexual polymorphism that evolved independently in at least 28 flowering plant families (Barrett, 2002). In contrast to dioecious plants, heterostylosous plants are characterized by hermaphroditic flowers that show marked differences in floral design between two (distylly) or three (tristylly) distinct mating types. Short-styled (S), long-styled (L) and mid-styled (M) plants (in the case of tristyly) coexist in one population with their reproductive organs reciprocally placed so that cross-pollination between mating groups is facilitated. Flowers exhibit herkogamy and in accordance to the style morph, their stigmas are placed beneath (short), above (long) or between anther whorls (mid) (Darwin, 1877; Barrett, 2002). In most heterostylosous species other ancillary polymorphisms can be observed as well, often related to features of the stigma and pollen (e.g. Ganders, 1979).

Besides these morphological differences, most heterostylosus species are characterized by a sporophytically controlled, diallelic, heteromorphic incompatibility system (Ganders, 1979; Barrett, 1988, 2002; Barrett and Shore, 2008). This physiological barrier restricts mating opportunities to crossing between plants of the opposite style morph (legitimate pollination). According to Darwin (1877), heterostyly and the associated self-incompatibility system should function as an effective outcrossing mechanism because of pollen segregation on the insect pollinator’s body and precise deposition of this pollen on the stigma of the opposite morph. Due to this outcross mating system, populations of heterostylosous species can be expected to have high levels of neutral genetic diversity compared with species with a mixed mating system or selfing species (Awadalla and Ritland, 1997; Liu et al., 1998; Jacquemyn et al., 2004). Between-population genetic diversity (F_{ST}), on the other hand, is expected to be low in heterostylosus species compared with selfing species because of higher levels of pollen flow and less genetic drift (Hamrick and Godt, 1996; Liu et al., 1998; Ingvarsson, 2002; Jacquemyn et al., 2004; Duminil et al., 2009).

Disassortative mating in heterostylosus species, enforced by reciprocal herkogamy and associated ancillary polymorphisms...
(morphology) as well as by the heteromorphic incompatibility system (physiology), is expected to lead to equal morph frequencies (isoplethy) in optimal populations, due to negative frequency-dependent selection and the simple inheritance of heterostyly (Finney, 1952; Ganders, 1979; Heuch, 1979; Barrett and Shore, 2008). However, in several heterostylovous species, the heteromorphic incompatibility system has been shown to be subject to leakage, and in some genera breakdown of heterostyly through the loss of self-incompatibility has been observed (Ganders, 1979; Barrett, 1989, 1993). Breakdown of self-incompatibility often occurs asymmetrically among style morphs, and may cause asymmetric patterns of mating and seed-set when one morph is capable of producing more seeds after selfing and intra-morph (illegitimate) pollination than the other(s) (Kéry et al., 2003; Brys et al., 2008b; Hodgins and Barrett, 2008). In this case, illegitimate mating and morph-specific differences in reproductive success can lead to biased morph ratios, even if the population is at equilibrium (Barrett and Hodgins, 2006). Besides resulting from asymmetric mating, deviations from equal morph ratios in heterostylovous populations may also arise from demographic stochasticity or founding events. In both cases, morph bias is often, but not necessarily, related to population size (Endels et al., 2002; Jacquemyn et al., 2002; Brys et al., 2003, 2007, 2008b; Kéry et al., 2003; Wang et al., 2005; Van Rossum and Triest, 2006b).

Skewed morph ratios may affect population genetic diversity for several reasons. First, deviations from equal morph ratios may lead to high levels of genetic drift by reducing the number of available mates and thus the effective population size (Husband and Barrett, 1992; Ingvarsson, 2002; Charlesworth, 2003; Duminil et al., 2009). Moreover, because morph bias and population size are often related, morph-biased populations are expected to suffer from reduced genetic diversity due to their small size (Leimu et al., 2006; Aguilar et al., 2008). Second, in distylovous plant species in which successful mating is asymmetric and outcrossing is restricted to one of the morphs, within-population genetic diversity is expected to decrease asymmetrically in more biased populations. Populations biased towards the self-incompatible morph will suffer more from mate scarcity whereas populations with a higher proportion of the self-compatible morph will become more affected by inbreeding. However, there are only a few studies that have elucidated the relative importance of morph bias in affecting population genetic diversity in heterostylovous species, particularly in species with a weak self-incompatibility system (e.g. Van Rossum and Triest, 2006a).

In this study, we investigated the impact of skewed mating success and morph bias on population genetic diversity of the distyloous Pulmonaria officinalis (Boraginaceae). Previous research showed that 27 of 35 (77%) surveyed populations in northern Belgium were L-biased as a result of the weakened heteromorphic incompatibility system of the L-morph (Brys et al., 2008a). More specifically, we asked the following questions: (1) Do the observed differences in mating success between L- and S-morphs lead to asymmetric patterns of within-population genetic diversity? (2) To what extent is within-population genetic diversity affected by population size and morph bias? (3) How do differences in self-incompatibility and mating success between L- and S-morphs affect patterns of genetic differentiation among populations?

**MATERIALS AND METHODS**

**Study species**

Pulmonaria officinalis L., common lungwort, is a semi-evergreen, long-lived, clonal herb that grows in the understorey of species-rich mixed and open forests on relatively humid, wet and loamy soils (Hegi, 1927; Bennett, 2003). This forest species has a central European distribution, occurring from southern Sweden and Denmark in the north to Italy and Bulgaria in the south (Merxmüller and Sauer, 1972). The fragmented populations in Belgium and Britain are located outside the native distribution range of the species and have become naturalized, most probably after the species’ introduction during medieval times. Recent development of eight polymorphic microsatellite markers revealed that P. officinalis is tetraploid as all loci contained more than two and a maximum of four alleles (Molecular Ecology Resources Primer Development Consortium, 2011). Moreover, as the microsatellite profiles showed no differentiation of allele sets and allele combinations were completely random (no fixed heterozygosity), this also indicates that P. officinalis is an auto- rather than an allotetraploid (Catalán et al., 2006).

From March until the end of April, the species produces purple, tube-shaped, distylovous flowers characterized by a range of ancillary polymorphisms in corolla tube shape, anther size and pollen size. Flowers of the L-morph have smaller pollen grains and produce significantly more pollen than those of the S-morph (Brys et al., 2008a). The stigmatic surface also shows morph-specific differences in papillae size, crown area and inter-papillae distances. Short-styled pollen showed higher germination rates and higher in vitro pollen tube elongation, which is needed for traversing the long styles of the L-morph (Brys et al., 2008a).

In Belgium, flowers of P. officinalis are mainly pollinated by generalist insect species, including Bombus terrestris, B. pascuorum, B. pratorum and Bombylis major, whereas in the centre of its distribution range (Central Europe) flowers are mainly pollinated by specialist Anthophora species. These species have long proboscises that perfectly fit within the deep corolla tube of P. officinalis flowers (Oberrath et al., 1995; Brys et al., 2008a). Within the study populations, only one Anthophora species (A. plumipes) was sporadically observed on P. officinalis flowers. Comparative analyses of pollen transport showed that this species was a much more efficient pollinator than the generalist Bombus species as it transported proportionally more S-pollen on its head and more L-pollen on its proboscis (Brys et al., 2008a).

Because of higher pollen deposition rates and a weaker heteromorphic incompatibility system in the L-morph, reproductive success tends to be higher in L- than in S-morphs (Brys et al., 2008a). Higher pollen deposition in the L-morph was attributed to a better accessibility of their stigmatic surface for generalist pollinators with limited proboscis lengths as well as to the floral hairs that grow at the long-styled corolla entrance and in which L-pollen are captured as pollinators pull back their proboscises. This, in combination with the
fact that illegitimate pollination yielded approximately 2-5 (intra-morph pollination) to 10 (selfing) times more seeds in the L-morph than in the S-morph (seed-set after selfing = 1.6 %), results in skewed mating success among morphs (Brys et al., 2008a).

Study area and population sampling

The study area is situated in the north of Belgium. In this region, 35 P. officinalis populations were identified during previous research (Brys et al., 2008a, b). Most populations (n = 26) are located in the north-western part of Belgium near Brakel, six are located in the centre of northern Belgium and three in eastern Belgium near Voeren. Distances between populations vary between 478 m and 159 km (mean: 31 km). During the flowering season of 2010 (March–April), we visited a subsample of 27 P. officinalis populations. For each population, population size was determined by counting the total number of flowering ramets. However, because this species reproduces partially by vegetative propagation it was impossible to estimate the total number of genets per population. For each population, the style morph (L/S) was determined for at least 400 individuals (if possible) and morph bias was calculated as the difference in the number of individuals of the L- and the S-morph, divided by the total number of individuals (Brys et al., 2008b). Hence, morph bias varied between −1 (only S-morph) and 1 (only L-morph) with 0 for isoplethymy. Table 1 summarizes population sizes and morph bias of the 27 sampled populations.

In each of the selected populations, 20 plants were sampled for genetic analysis. Individuals were sampled from the entire area occupied by each population to avoid the effects of population substructure. To investigate the effect of morph type on population genetic diversity, leaves of ten S-plants and ten L-plants were collected. In small and/or biased populations with fewer than ten individuals per morph type, we collected leaves from all individuals available. Young leaf material was collected and dried in silica gel. Before DNA extraction, leaves were homogenized with a mill (Retsch MM 200, Haan, Germany) to a fine powder. Total DNA was extracted from 20 mg silica-dried leaf tissue of 526 plants in total following the NucleoSpin Plant II protocol (Macherey-Nagel, Düren, Germany). DNA quality and concentration were estimated using a NanoDrop ND-2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Microsatellite analysis

Eight recently developed microsatellites (Molecular Ecology Resources Primer Development Consortium, 2011) were amplified in two multiplex PCRs in a 2720 Thermal Cycler (Applied Biosystems, CA, USA) in a total volume of 10 μL containing 5 μL Qiagen Multiplex PCR Master Mix, 3 μL RNase-free water, 1 μL template DNA and 1 μL of

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A, allelic richness; A1, allelic richness within individuals; G, genotypic richness; H0, observed heterozygosity; HE, expected heterozygosity under random chromosome segregation (Thrall and Young, 2000); FIS, fixation coefficient calculated as 1 − HE/H0.

Table 1. Characteristics of 27 Belgian Pulmonaria officinalis populations sampled for microsatellite analysis during the flowering season of 2010: six different measures of genetic diversity were calculated for each population.
one of the two multiplexed primer combinations. The two multiplexes had equal thermocycler profiles with initial denaturation of 15 min at 95 °C; 25 cycles of 30 s at 95 °C, 1.5 min at 59 °C and 1 min at 72 °C; and a final elongation of 30 min at 60 °C. Then, 1 μL of the PCR reaction was added to a solution of 8-8 μL formamide and 0.2 μL of the Applied Biosystems’ GeneScan 500 LIZ size standard. Fragments were sized on an ABI Prism, 3130 Genetic Analyzer (Applied Biosystems) and scored with GeneMapper Software v4.0 (Applied Biosystems).

Data analysis

Measures of genetic diversity. Due to the tetraploid nature of *P. officinalis* (Molecular Ecology Resources Primer Development Consortium, 2011), standard genetic analyses cannot be used to estimate measures of genetic diversity. Therefore, we used the software AUTOTET to estimate measures of population genetic diversity (Thrall and Young, 2000). For each population six measures of genetic diversity were calculated: allelic richness (*A*), allelic richness within individuals (*A*)(i.e. the average number of alleles per individual), genotypic richness (*G*), observed (*H*) and expected (*H*) heterozygosity, and the fixation or inbreeding coefficient (*F*)). Observed heterozygosity was calculated by weighting the five possible classes of genotypes (AAAA, AAAB, AABB, AABC, ABCD) inversely to the probability of any of their alleles being identical by descent. AUTOTET computes expected heterozygosity and fixation coefficients under the assumption of both random chromatid segregation where sister chromatids can segregate into the same gamete (i.e. double reduction, with a maximum probability of *α* = 1/7) and random chromosomal segregation (Thrall and Young, 2000). For all eight loci, chi-square goodness-of-fit tests for observed-to-expected genotype frequencies were used to test whether populations showed significant deviation from Hardy–Weinberg equilibrium. These six measures were calculated for each population but also separately for both morphs within the population. A paired-sample *t*-test was then used to test whether one morph is genetically more diverse than the other morph. Finally, we also tested for linkage disequilibrium by using the program LD4X run in R (R Development Core Team, 2010), which is especially developed for autotetraploid species (Julier, 2009). Linkage disequilibrium or allelic association implies a non-random association of alleles at different loci. First, the software builds 5 × 5 contingency tables for each pair of alleles with allelic dosages from 0 to 4. Secondly, Fisher’s exact tests are performed to detect linkage between pairs of alleles from the eight microsatellite loci applied in the analysis. The program applies a Bonferroni correction by adjusting the probability threshold to the number of comparisons tested for each pair of loci.

Relationship between morph bias and genetic diversity. To assess the relationship between measures of genetic diversity (*A*, *A*, *G*, *H*, *H*, *F*), morph bias and population size, multiple regressions were conducted in SAS v9-1 using the ‘reg’ procedure. Population size was log-transformed to satisfy the normality assumption. Morph bias, ranging from –1 to 1, was preferred as an independent variable to the absolute value of morph bias (Van Rossum and Triest, 2006a), because we were particularly interested in the distinction between L-morph-biased and S-morph-biased populations in our dataset. Both the linear and the quadratic terms of morph bias were entered in the models. For all models tested, Akaike’s information criterion (AIC) was used to check statistical model fit. The competing model with the smallest AIC value was considered the best model. In case the variation in the data was best described by the quadratic function, the morph ratio for which genetic diversity was at its maximum was determined by calculating the first derivative of the curve equation.

Genetic differentiation. Pairwise genetic distances (*F*) among the 27 populations were estimated using POLYSAT, a newly developed package in R that calculates *F* values from polymorphic microsatellite data scored in GeneMapper (Clark and Jasieniuk, 2011). POLYSAT provides a separate set of functions which are applied depending on the species’ inheritance mode (autopolyploidy versus allopolyploidy). Allele frequencies were estimated using the ‘simpleFreq’ method, which assumes that all alleles in partial heterozygotes have an equal chance of being present in more than one copy (Clark and Jasieniuk, 2011). On the basis of these allele frequencies, pairwise *F* values were calculated between all 27 populations for all individuals, and for L- and S-morphs separately. To investigate whether pairwise *F* values were significantly different between L- and S-morphs, a paired-sample *t*-test was used. Finally, a Mantel test in GenAlEx 6 (Peekall and Smouse, 2006) was used to test the hypothesis that pairwise genetic distances of S-morphs were significantly related to those of L-morphs. The deviation from equal pairwise *F* values for both morphs was tested statistically by calculating the 95% confidence interval around the slope of this relationship.

RESULTS

Measures of genetic diversity

The total number of alleles observed per locus in the overall sample of 526 individuals ranged from three to 15, with an overall total of 53 alleles scored over eight loci. For loci Puof-04, Puof-12 and Puof-15, we found only 15, three and six alleles, respectively, which is less than reported by Molecular Ecology Resources Primer Development Consortium (2011) because no German populations were included for this analysis. For the loci Puof-08, Puof-13 and Puof-19, by contrast, we found more alleles (seven, eight and four, respectively) due to our larger sample size. Significant departures from Hardy–Weinberg equilibrium were observed in all single-locus exact tests of the pooled population data. Chi-square goodness-of-fit tests for observed-to-expected genotype frequencies showed for all loci except for one (Puof-12), a negative deviation from Hardy–Weinberg equilibrium. The *χ*-values of all single-locus exact tests calculated for each population separately are summarized in Supplementary Data Table S1 (available online).

Among the 27 sampled populations, 1163 of the 29 926 allelic combinations were significantly correlated at *P* < 0.05. After Bonferroni correction, only 339 combinations (1.1%) remained significant in 23 of 27 populations, illustrating that linkage disequilibrium is very limited among the developed microsatellite markers. Allelic richness (*A*) and average allelic richness per
individual ($A_i$) ranged from 1.88 to 4.38 and 2.66 to 3.25, respectively, whereas genotypic diversity ($G$) varied between 1.88 and 7.38 (Table 1). Expected heterozygosity ($H_E$) varied between 0.35 and 0.71 (Table 1). Negative overall $F_{IS}$ values were observed in all populations and varied between –1.00 and –0.15 (Table 1). None of these six measures differed significantly between the two morphs ($0.145 \leq P \leq 0.823$). A significant difference between morphs could have biased the overall population genetic diversity measurement as both morphs were equally sampled.

**Morph bias and genetic diversity**

Consistent with previous analyses (Brys et al., 2008b), the average morph bias of the studied populations was 0.17 and deviated significantly from zero ($t = 2.216, P = 0.036$), indicating that the majority of the studied populations were dominated by individuals of the L-morph. Comparison of regression models with linear and quadratic terms of morph bias and population size showed that, for all measures of genetic diversity used, morph bias$^2$ was the only significant factor ($P > 0.05$) associated with within-population genetic diversity and thus was a better descriptor of the variability in genetic diversity than population size (Fig. 1, Table 2). AIC fit values for all five competing models are reported in supplementary Table S2 (Supplementary Data). Variation in five of six genetic measures was best explained by the multiple regression model that included the linear and quadratic term of morph bias. Only in the case of genotypic richness ($G$) did the best model include population size. Genetic diversity was low in strongly morph-biased populations, and was highest in populations that showed slight L-morph excess. Interestingly, calculation of the morph ratio corresponding to maximum genetic diversity from the obtained regression equations showed that, on average, genetic diversity was highest in populations with a morph bias of 0.213 ($\pm 0.013$), corresponding to an L-biased population with 60.7% ($\pm 0.7$) L-morph individuals. However, the shift of the curve translated in the coefficient of the variable ‘morph bias’ did not significantly differ from 0 (Table 2).
Table 2. Regression coefficient estimates and the level of significance (* P < 0.05, ** P < 0.01, *** P < 0.001) obtained by multiple polynomial regressions for six measures of genetic diversity (A, A, G, H, H, F) as dependent variables and morph bias, morph bias, and the logarithm of population size as covariates

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>G</th>
<th>A</th>
<th>H</th>
<th>H</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morph bias</td>
<td>0.06</td>
<td>0.16</td>
<td>0.01</td>
<td>0.00</td>
<td>0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>Morph bias</td>
<td>-0.26***</td>
<td>-0.50***</td>
<td>-0.05*</td>
<td>-0.013**</td>
<td>-0.04***</td>
<td>-0.08***</td>
</tr>
<tr>
<td>Population size</td>
<td>0.12</td>
<td>0.48</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Skewed morph frequencies are not unusual in populations of heterostyly species and variable deviations from isoplethy have been observed before in several species. Figure 3 shows the average morph frequency and variation in morph ratios for 12 distylos species. Although the exact causes of skewed morph ratios are not always easy to untangle (Kéry et al., 2003), it has been shown that skewed morph ratios can have a pronounced negative effect on overall reproductive success within heterostylous populations and thus can contribute to patterns of genetic diversity. Kéry et al. (2000) and Brys et al. (2007), for example, found a marked decrease in sexual reproduction in the distylos species Primula veris and Hottonia palustris when populations became more biased in either morph. Wang et al. (2005) and Brys et al. (2008b) disentangled the effects of morph ratio and population size on reproductive success by manipulating morph ratios in experimental populations and confirmed a significant negative effect of morph bias on population fecundity, especially in individuals belonging to the morph type in the majority.

In line with our expectations, we showed that morph bias had a pronounced negative effect on within-population genetic diversity. All six measures of genetic diversity were significantly affected by morph bias (Table 2), and AIC values confirmed the higher predictive value of morph bias over population size in five of six response variables (Table S2). Although previous meta-analyses have shown a clear positive correlation of population size and genetic diversity in many plant species (Honnay and Jacquemyn, 2007), our results show that morph bias is a more important demographic variable to explain population genetic diversity in this distylos species. In contrast to our results, Van Rossum and Triest (2006a) did not find a negative relationship between morph bias and each of four genetic diversity parameters (A, H, H, F) in 24 populations of the distylos Primula veris. In Primula elatior, however, a similar significantly negative effect of morph bias on allelic richness (A) was found in seedlings during an early stage of population fragmentation, which the authors attributed to limited availability.
of compatible mates and increased inbreeding (Van Rossum and Triest, 2006b).

The effect of morph bias was best described by a negative quadratic function, which showed a decrease in genetic diversity with an increasing deviation from the isoplethic situation (Fig. 1). Interestingly, maximum genetic diversity was found in populations in which 60-7% of all plants belonged to the L-morph, which is very close to the equilibrium morph ratio reported by Brys et al. (2008b). Due to the strict heteromorphic self-incompatibility system in most heterostylous species, the dominant morph in biased populations is generally characterized by a fitness disadvantage because of fewer mating opportunities (fewer pollen donors/recipients) compared with the rare morph. Such negative frequency-dependence will lead to equilibrium morph ratios in which all morphs have an equivalent fitness (Fisher, 1930; Thompson et al., 2003). However, in species that are lacking strict self-incompatibility and are characterized by asymmetric illegitimate pollination patterns, equilibrium morph ratios may significantly deviate from a 1:1 ratio due to the higher reproductive success of one of the two morphs. In P. officinalis, for example, significantly higher rates of illegitimate pollination in the L-morph individuals resulted in a 1.36-fold higher female fecundity compared with the S-morph, which resulted in an equilibrium L/S-morph ratio of 1.94:1 (i.e. 66% L-plants) (Brys et al., 2008b). This L-biased equilibrium ratio was also reflected in patterns of genetic diversity, which showed that maximum genetic diversity occurred at a morph ratio of 60% L-plants, and decreased with increasing deviations from the equilibrium morph ratio (Fig. 1).

In species with strict self-incompatibility, where only mating between plants of the opposite morph is possible, an increasing morph bias leads to smaller effective population sizes (Husband and Barrett, 1992), which, in turn, leads to higher genetic drift and lowers genetic diversity. However, in species in which partial selfing is possible, reductions in genetic diversity may also result from selfing, as it further lowers the effective population size (Ingvarsson, 2002; Charlesworth, 2003; Siol et al., 2007; Duminil et al., 2009). This may have contributed to the lower genetic diversity in the studied P. officinalis populations, particularly in L-biased populations. However, the inbreeding coefficient decreased rather than increased with increasing morph bias (Fig. 1F), implying an increase in the proportion of heterozygotes as morph ratios became more skewed. Moreover, because patterns were more or less similar for L- and S-biased populations, this does not correspond to the observation that selfing can only occur in the L-morph. Therefore, partial selfing is unlikely to have contributed substantially to the reduced genetic diversity in morph-biased populations.

Decreasing inbreeding coefficients with increasing morph bias have also been reported by Van Rossum and Triest (2006a) for the distylous Primula veris. Overdominance (i.e. higher fitness of the heterozygous genotype compared with the corresponding homozygous genotype) can result in an excess of heterozygotes in biased populations (David, 1998; Hansson and Westerberg, 2002). In small, inbreeding populations of the self-incompatible perennial Arnica montana (Asteraceae), Luijten et al. (2000) also found more heterozygotes. This ‘heterozygosity paradox’, which was first described by Brown (1979), was also found by Barrett and Husband (1990). They reported an excess of heterozygotes in small Jamaican populations of the tristylos species Eichhornia paniculata that showed low outcrossing rates. Van Rossum and Triest (2006b) found, in contrast to our results, an increasing heterozygote deficiency in skewed populations of the self-incompatible Primula elatior (FIS-values: −0.058 to 0.480), which indicates high levels of genetic drift in morph-biased populations.

Genetic diversity and its distribution within and among populations of a particular species is known to be strongly influenced by mating system (Hamrick and Godt, 1996; Ingvarsson, 2002; Duminil et al., 2009). Whereas in the studied P. officinalis populations, plants of the S-morph are more bound to outcrossing, plants of the L-morph most likely exhibit a mixed-mating system that allows certain amounts of intramorph- and self-fertilization (Brys et al., 2008a, b). Hence, when comparing genetic differentiation among populations for S- and L-morphs separately, we

Fig. 3. Average L-morph frequency and the variation in morph ratios of populations from 12 different distylous species. The dashed line indicates isoplethy (= equal morph frequencies).
expected to obtain lower $F_{ST}$-values for the outcrossing S-morph. Ness et al. (2010) found this pattern when comparing $F_{ST}$-values among selfing and outcrossing populations of the tristylist species *Eichhornia paniculata*. In *P. officinalis*, however, no significant difference was found in the average population genetic distances between the L-morph (0.107) and S-morph (0.106) (Fig. 2A). However, the relationship between pairwise genetic distance of the S- and L-morph did not coincide with the dashed line that indicates no difference in pairwise $F_{ST}$-values between the two morphs (Fig. 2B). When $F_{ST}$-values were high (>0.1), pairwise genetic distances between populations were higher for the L-morph than for the S-morph, whereas at high levels of gene flow ($F_{ST} < 0.1$), pairwise genetic distances were higher for the S-morph than for the L-morph. This difference can be explained by differences in mating system between the S-morph (outcrossing) and L-morph (mixed-mating). Anthers of the L-morph produce nearly twice as many pollen grains and long styles receive significantly more pollen than short styles, both resulting in higher gene flow among the L-morphs of different populations when populations are close to each other. However, in spatially isolated populations, for which levels of gene flow are low, pairwise genetic distances are expected to be higher for the L-morph than for the S-morph because of higher levels of inbreeding, as some of the pollen of the L-morph might be used for self-fertilization, which promotes genetic differentiation between populations (Charlesworth and Pannell, 2001; Ingvarsson, 2002).

To conclude, this study has shown that morph ratio exerted a non-negligible effect on population genetic diversity and differentiation of a distylous plant species with a weak self-incompatibility system. The effect of morph bias on genetic diversity was clearly shaped by the skewed mating success between the morphs, as shown by the shifted top of the curve towards an equilibrium morph composition with a majority of L-individuals. Because of the negative effect of morph bias on fecundity, seedling recruitment and genetic diversity in distylous populations, morph ratio imbalance should definitely be considered in conservation management as it also affects the long-term survival of rare and threatened species (Endels et al., 2002).

SUPPLEMENTARY DATA
Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: $\chi^2$-values and the level of significance per population for each microsatellite locus. Table S2: Akaike’s information criteria (AIC) for the five possible models built with ‘Morph bias’, ‘Morph bias$^2$’ and ‘Population size’ for all six genetic diversity measures.

ACKNOWLEDGEMENTS
This work was supported by the Flemish Fund for Scientific Research (FWO) [project G.0500-10 and a postdoctoral grant (R.B.)] and the European Research Council (ERC starting grant 260601 – MYCASOR) (H.J.).

LITERATURE CITED


