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Genetic consequences of habitat fragmentation in an agricultural landscape on the common *Primula veris*, and comparison with its rare congener, *P. vulgaris*

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Abstract

In Flanders (northern Belgium), the common (but locally rare) *P. veris* and its rare relative *Primula vulgaris*, two self-incompatible perennials, mainly occur in fragmented habitats of the intensively used agricultural landscape. We investigated genetic variation and structure for respectively 30 and 27 allozyme loci in 24 and 41 populations of *P. veris* and *P. vulgaris*, in relation to species (local) abundance and to population size and plant density, and compared populations of linear landscape elements (verges) with non verge populations. The rare *P. vulgaris* was genetically depauperate compared to the commoner *P. veris*. Both species showed a geographical structure of genetic variation and isolation by distance. The scattered populations of *P. veris* from the Westhoek region (polder habitats) were of smaller size, genetically depauperate and showed higher interpopulation divergence compared to the Voeren region, where populations are densely distributed and habitats less fragmented. Verge and non verge populations showed similar populations sizes and levels of genetic variation. For both species, small populations showed a loss of genetic variation, but still maintained high levels of observed heterozygosity. Within-population plant density negatively affected allelic richness in *P. veris*. Ours results indicate that common species can also be negatively affected by habitat fragmentation (perhaps even more so than rare species) and that verge populations can have a high potential conservation value. Both verge and non-verge populations should be included in conservation efforts.

Introduction

Due to habitat destruction, increased urbanisation and intensive agricultural practices, many rare as well as widespread plant species occur in highly fragmented habitats. Consequences of habitat fragmentation often consist of reduced population size and increased isolation. These can lead to genetic erosion and increased genetic divergence among populations, through random genetic drift, increased levels of inbreeding and reduced gene flow (e.g., Barrett and Kohn 1991; Ellstrand and Elam 1993; Oostermeijer et al. 1996). Erosion of genetic diversity, because it may promote genetic load and decrease the species potential to respond to environmental changes, is expected to increase extinction probabilities (Young et al. 1996). Habitat fragmentation and degradation also lead to changes in within-population plant density (Kwak et al. 1998). By changing pollination patterns and pollinators' behaviour, plant density may affect gene flow and outcrossing rates (Loveless and Hamrick 1984; Van Treuren et al. 1993; Richards 1997; Franceschinelli and Bawa 2000), population heterozygosity, inbreeding levels (Tarayre and Thompson 1997; Coates and Sokolowski 1999), and genotypic composition (Gram and Sork 1999).

Being common is not necessarily a guarantee for long-term survival in fragmented habitats (e.g.,

Lienert et al. 2002; Van Rossum et al. 2002). Common species may be important components of ecosystem functioning and productivity (Lienert et al. 2002; Lienert and Fischer 2003) and may be a source of new taxa through speciation (Fréville et al. 1998). Preventing their decline is therefore essential for a sustainable conservation of biodiversity. However, only a few studies have investigated the genetic consequences of habitat fragmentation on populations from widespread species (Weidema et al. 1996; Van Rossum et al. 2002; Tomimatsu and Ohara 2003). They generally have focused on (locally) rare, endangered or declining species (e.g., Raijmann et al. 1994; Fisher & Matthies 1998; Young et al. 1999; Luijten et al. 2000; Van Rossum and Triest 2003) and more recently on common habitat specialists (Lienert et al. 2002; Lienert and Fischer 2003). It is unclear if the same responses to habitat fragmentation are expected for common species and for their rare relatives having similar life-history traits. It is also unsure whether we might use models built for rare or already declining species to predict long-term survival probability of their widely-distributed congeners. In fact, only a very few studies have compared common species with their rare relatives from this conservation perspective (Fréville et al. 1998; Gustafsson and Sjögren-Gulve 2002).

Protected areas (e.g., natural reserves, EU Natura 2000 network in Europe) mainly concern habitats with a high biodiversity value, like heathlands, dry grasslands, fens or forests (e.g., European Community 2002), and priority in conservation strategies is often given to the preservation of large-sized populations (e.g., Neel and Cummings 2003). In these protection schemes, the habitat value of linear landscape elements like road verges and field and pasture boundaries, i.e., grass banks, ditches and hedgerows, is often neglected (e.g., Kleijn and Verbeek 2000). Despite the existence of agri-environment schemes in the European Union for protecting biodiversity in the farmland, the intensification of the agricultural land use has led to larger field sizes associated with the removal of many hedgerows and the destruction of many field boundaries, and to an increased application of pesticides and fertilisers (e.g., Baudry et al. 2000; Endels et al. 2002). However, these linear landscape elements constitute important habitat refuges that insure survival for many species, sometimes endangered (e.g., Hogbin et al. 1998; Kleijn and Verbeek 2000; Endels et al. 2002). They may also function as natural corridors between habitat patches in fragmented landscapes (e.g., Kwak et al. 1998; Tewksbury et al. 2002). If the linear elements formerly consisted of wide and continuous networks in the traditional agricultural landscape, they have become highly fragmented over the last 50 years in small habitat patches often characterised by low habitat quality (Baudry et al. 2000; Kleijn and Verbeek 2000). Few studies have investigated genetic variation in plant populations occurring in small linear elements of the agricultural landscape (Berge et al. 1998) and in road verges (Godt et al. 1997; Hogbin et al. 1998). As a consequence, it is not known if the verge populations can help to conserve genetic diversity in addition to those populations located in larger habitat patches.

We studied population genetic variation and structure of the common *Primula veris* (Primulaceae) and of its rare congener *Primula vulgaris* in an intensively used agricultural landscape. These two species have similar life-history traits: both are long-lived perennial, insect-pollinated, self-incompatible (distylous) herbs. However, they also differ by their phenology and seed dispersal patterns and have different habitat requirements (Weeda et al. 1985; Valverde and Silvertown 1995; Richards 1997). In Flanders (northern Belgium), *P. veris* is commoner than *P. vulgaris*: respectively 250 and 90 populations were found after a very detailed field survey of their distribution (Endels et al. 2002; Brys et al. 2003). *Primula veris* locally shows either a scattered or a widespread distribution (Van Rompaey and Delvosalle 1979). Both species mainly occur in verges of the intensively used agricultural landscape (Endels et al. 2002; Brys et al. 2003). These linear elements have become highly fragmented over the last decades (Van Steertegem 2000).

In this paper, we addressed the following questions: (1) Are the measures of population genetic variation and structure related to the abundance (common and rare, respectively) of *P. veris* and *P. vulgaris*, and within the common *P. veris*, to local abundance as expressed by population density (regions with scattered or widespread distribution)? (2) Do the verge populations show similar levels of genetic variation compared to populations from large habitat patches? (3) Are population genetic variation and structure related to habitat fragmentation, population size and plant density? (4) What are the implications of our results for conservation strategies and management of common species?

Materials and methods

The species

Primula veris L. and *Primula vulgaris* L. (Primulaceae) are diploid, long-lived perennial rosette-forming herbs. Both are obligate outcrossers as they have a self-incompatibility system (distyly). They flower in early spring (with a longer flowering period for *P. vulgaris*), and are primarily pollinated by Hymenoptera (mostly bumblebees), but also by Diptera and moths (Woodell 1960; Hegi 1975; Boyd et al. 1990). *P. veris* can be found from Spain to Eastern Asia and is related to calcareous soils, occurring in dry to mesic grasslands, forest edges, and coastal sand-dune, but also in field and pasture boundaries (Hegi 1975; Weeda et al. 1985). It is common in Belgium, but can locally show a scattered distribution (Van Rompaey and Delvosalle 1979). Seeds are dispersed by movement of the scapes, and fall, in general, close to the parent plants. Vegetative propagation was reported under certain conditions (Tamm 1972).

P. vulgaris has a North Atlantic and Mediterranean European distribution (Hegi 1975). In Belgium, at the north-eastern border of its range, *P. vulgaris* is a very rare, declining species restricted to four fragmented areas in Flanders (Endels et al. 2002). It occurs in moist habitats, comprising deciduous forests and ditch banks along forestedges, hedgerows, intensive pastures and arable fields (Endels et al. 2002). Seed dispersal is often restricted to the site of the mother plant, but because of the presence of an elaiosome (which is not found in *P. veris*), seeds can occasionally be further dispersed by ants and rodents (Valverde and Silvertown 1995).

Populations studied and sampling procedure

In this study, a population consisted of a group of individuals separated from the others by unsuitable habitats for the species (e.g., roads, intensive pastures and fields, houses ...). For *P. veris*, young leaf material was sampled during the spring 2000 from 866 individuals distributed in 24 populations representative of two regions in Flanders (Westhoek and Voeren) (Figure 1), where the species showed contrasted abundances (Brys et al. 2003). In the Westhoek (area of 260 km^2, 14 sampled populations), an intensively used polder landscape, populations are scattered and species density is 0.33 population/km^2 (Brys et al. 2003). In Voeren (area of 50 km^2, 10 sampled populations), habitats tend to be less fragmented and mainly consist of calcareous grasslands, pastures, orchards and forest fragments (Van Steertegem 2000), and the species is widely and densely spread. Species density is at least 1 population/km^2. Population size ranged from 10 to 987 flowering individuals in Westhoek and from 27 to 20000 flowering individuals in Voeren. The geographical distance between two populations varied from 0.31 to 13.29 km within Westhoek and 0.20 to 7.78 km within Voeren, and from 215 to 234 km between the two regions. For *P. vulgaris*, 926 individual plants were sampled during the spring 1999 in 41 populations (Figure 1). They consisted of 40 populations which covered the fragmented distribution range of the species in Flanders (4 regional groups) and ranged from 7 to 700 flowering individuals, and of one very large population ($>$ 2500 flowering individuals) from northern France (close to the Belgian border), still located in same part of the distribution range (population 41). The geographical distance between populations ranged from 0.009 to 90 km. Depending on population size, 6 to 76 individuals were sampled across the whole population area. To avoid destructive sampling, material was only collected from adult plants. Population size corresponds to the number of flowering individuals. Within-population plant density was estimated as the number of flowering plants per square metre, and varied from 0.003 to 15.20 in *P. veris* and from 0.05 to 5.8 in *P. vulgaris*. For the very large populations, i.e., population V3, V8 to V10 of *P. veris* and 41 of *P. vulgaris*, plant density was measured for five 4 m^2 plots (Brys, unpublished results), and the number of flowering individuals was estimated from plant density and total population area. For the other populations, all the individuals that flowered were counted and the area occupied by the population measured.

Allozyme electrophoresis

Extraction and electrophoretic procedures followed those described in Van Rossum and Triest (2003). Fifteen enzyme systems, representing 27 putative loci were resolved for *P. vulgaris*: acid phosphatase (*Acp-1* to *-3*), aconitase (*Aco-2*), alcohol dehydrogenase (*Adh*), esterase (*Est*), glutamate dehydrogenase (*Gdh*), glucose-6-phosphate-isomerase (*Pgi-1*), glutamate-oxaloacetate transaminase (*Got-1* to *-4*), isocitrate dehydrogenase (*Idh*), leucine aminopeptidase (*Lap-1* and *-2*), malate dehydrogenase (*Mdh-1* to *-4*), phosphoglucomutase (*Pgm-1* and *-2*), 6-phospho-D-gluconate (*Pgd-1* and *-2*), shikimate dehydrogenase

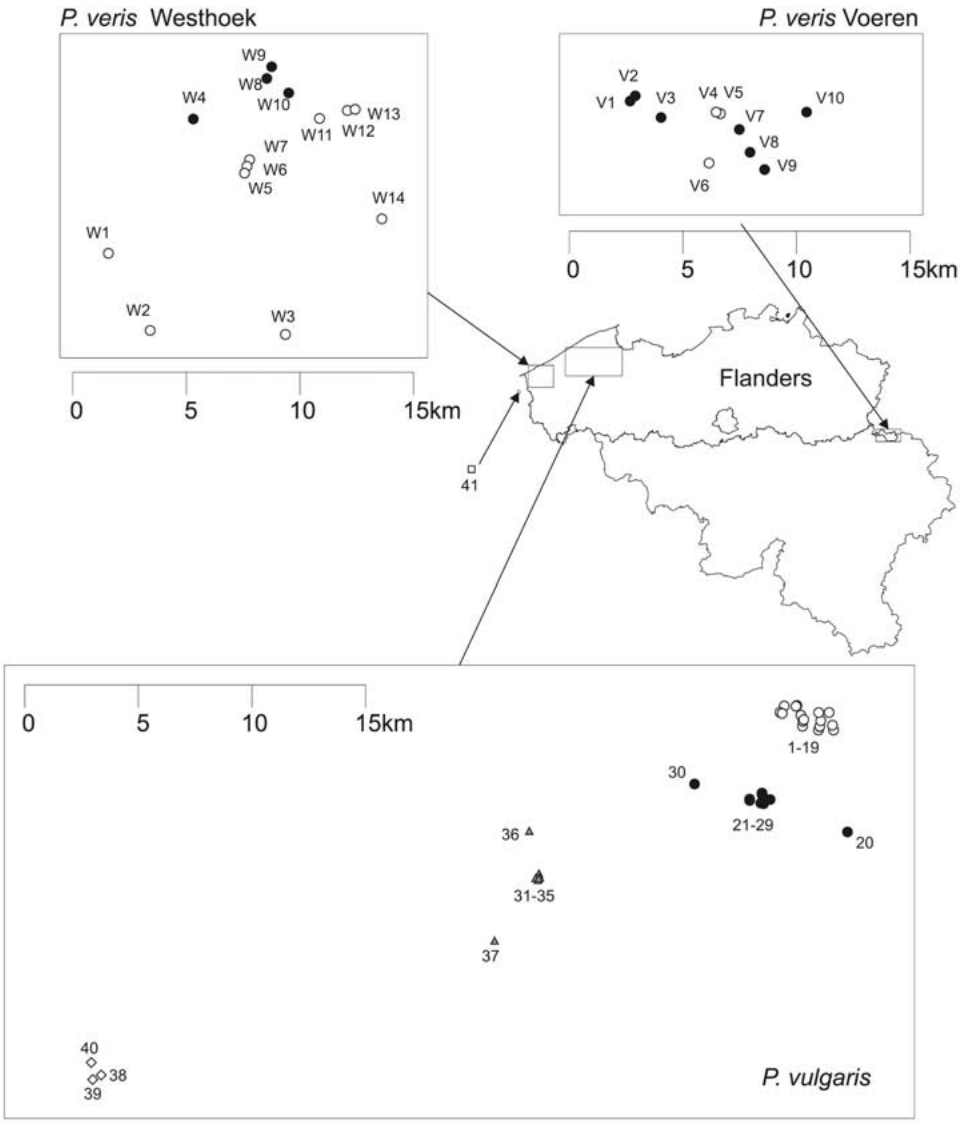

Figure 1. Location of 24 populations of *P. veris* and 41 populations of *P. vulgaris* in Flanders (Belgium) and northern France (population 41 of *P. vulgaris*). For *P. veris*, W1 to W14: populations from the Westhoek region; V1 to V10: populations from the Voeren region; ○, ●: verge and non verge populations, respectively. For *P. vulgaris*, the symbols ○, ●, ◬, ◇ and □ represent different regional group. Non verge populations: 29, 31, 33, 34, 35, 36, 37, 38, 40, 41.

(*Skd*), superoxide dismutase (*Sod*), and triose phosphate isomerase (*Tpi-1* and *-2*). For *P. veris*, malic enzyme (*Me-1* and *-2*) was also resolved (buffer systems: sodium-borate pH 8.5/Tris-citrate pH 7.8 and histidine-citrate pH 7.0 (Van Rossum and Triest 2003) for *Me-1* and *-2*, respectively; staining recipe from Soltis et al. (1983), and two loci were identified in aconitase (*Aco-1* and *-2*), giving a total of 16 enzyme systems and 30 loci.

Data analysis

For each species, a test for genotypic disequilibrium between pairs of loci was performed using FSTAT (Goudet 2001). Because multiple tests were involved, the sequential Bonferroni-type correction was applied to test for significance (Rice 1989).

All the analyses of genetic data were performed using GEN-SURVEY (Vekemans and Lefèbvre 1997), except when otherwise specified. The following measures of genetic variation were calculated for

each population: allelic richness ($r(g)$) for a fixed sample size (g genes), based on the rarefaction method described in El Mousadik and Petit (1996), observed heterozygosity (H_o), expected heterozygosity (H_e) corrected for small sample size (Nei 1978), and Wright's inbreeding coefficient (F_{IS}) corrected for small sample size (Kirby 1975). Allelic richness was calculated for each population for the smallest sample size $g = 38$ genes in *P. veris* and 12 genes in *P. vulgaris*, using FSTAT (Goudet 2001). Allelic richness and H_e were also calculated at the species level. For direct comparison between *P. veris* and *P. vulgaris*, H_o, H_e, F_{IS} and $r(12)$ were recalculated for *P. veris* after removal of ME and *Aco-1*.

Deviations from Hardy-Weinberg expectations were investigated by performing exact tests for each population and locus, using GENEPOP (Raymond and Rousset 1995). The significance of the F_{IS} values calculated for each population over all loci was tested by randomisation tests using FSTAT (Goudet 2001) and sequential Bonferroni-type correction (Rice 1989).

For comparison tests, populations were grouped into verge populations, i.e., populations located in road verges, field or pasture boundaries of the intensively used agricultural landscape (13 and 31 populations for *P. veris* (10 Westhoek and 3 Voeren) and *P. vulgaris*, respectively), and non verge populations, i.e., populations occurring in larger habitat patches (forest, grasslands) with no intensive agricultural use (11 and 10 populations for *P. veris* (4 Westhoek and 7 Voeren) and *P. vulgaris*, respectively) (Figure 1).

We performed a test of comparison among groups of populations using FSTAT (Goudet 2001) to test for differences in amount of genetic variability ($r(g)$,H_o, H_e, and F_{IS}) between the two species, between the Westhoek and Voeren regions within *P. veris*, and between verge and non verge populations within species. The significance of the test was assessed using a randomised permutation scheme of the populations among the groups (1000 permutations) and Bonferroni corrections (Rice 1989). We also tested for differences in population size between these groups using a Student t-test. A non-parametric Kolmogorov-Smirnov (K-S) test was also performed for comparison between verge and non verge populations within regions in *P. veris*.

To investigate whether there were relationships between within-population genetic variation (dependent variables: $r(g)$, H_o, H_e and F_{IS}), population size and plant density, we performed multiple regression analyses using STATISTICA. These examine the relationship between a dependent and a single independent variable while the other independent variable in the model is (statistically) held constant (Sokal and Rohlf 2000). Population size for both species, and plant density in *P. vulgaris* were log-transformed to achieve homoscedasticity and normality. Deviations from normality were not detected for the other variables. For *P. veris*, separate analyses were performed for the regions: multiple regression analyses for the Westhoek, and Spearman correlation analyses for Voeren, because of a small number of populations (10) and of colinearity in the multiple regression analysis (Variance Inflation Factor = 3.31). Population size and plant density were indeed highly correlated ($r_s = 0.988$). The relationships between the predictor variables (population size and plant density) were also examined using Pearson's (or Spearman for analysis within regions in *P. veris*) correlation coefficients.

Population genetic structure was investigated on polymorphic loci using Nei's total genetic diversity (H_T) and mean within-population genetic diversity (H_S), corrected for small sample size according to Nei and Chesser (1983), and using Weir and Cockerham's (1984) estimator of F_{ST} (θ). Averages for all populations (with their over-locus standard deviations and 95% confidence intervals) were obtained by bootstrapping over loci. An analysis of molecular variance was performed using ARLEQUIN (Schneider et al. 2000). The between-population component of the total genetic diversity is given by F_{ST}. F-statistics were also used to partition the genetic diversity into its between-region component (F_{RT}), i.e., Westhoek versus Voeren for *P. veris*, and among the five geographical groups for *P. vulgaris* (Figure 1), and into its between-population (within region) component (F_{SR}). The F-statistics are related by the equation $(1 - F_{ST}) = (1 - F_{SR})\ (1 - F_{RT})$ (Weir 1990). The significance of F_{ST}, F_{RT} and F_{SR} was tested using permutation tests (Excoffier et al. 1992). We performed a test of comparison among groups of populations using FSTAT (Goudet 2001) to test for differences in F_{ST}-values between the Westhoek and Voeren regions for *P. veris*. The significance of the test was assessed using a randomised permutation scheme of the populations among the regions (1000 permutations).

Reynolds' genetic distances, corrected for small sample size (Reynolds et al. 1983), were computed between all pairs of populations. A distance based on the coancestry coefficient, θ, was preferred over Nei's

Table 1. Mean values of within-population genetic variation for 24 populations of *P. veris* and 41 populations of *P. vulgaris* (from Van Rossum and Triest 2003) from Flanders and northern France

Population	$r(g)$	H_o	H_e	F_{IS}
Primula veris				
Mean (30 loci)	1.42	0.075	0.103	0.257
SD	0.11	0.020	0.022	0.147
Species level	1.45	—	0.121	—
Mean (27 loci)	1.32[a]	0.074	0.097	0.226
SD	0.07	0.018	0.024	0.179
Primula vulgaris				
Mean (27 loci)	1.18	0.055	0.062	0.101
SD	0.03	0.012	0.011	0.178
Species level	1.23	—	0.076	—
p	0.001	0.001	0.001	0.003

$r(g)$ = allelic richness, with g = 38 genes for *P. veris* except ([a]), and with g = 12 genes for *P. vulgaris* and ([a]) of *P. veris*; H_o = observed heterozygosity; H_e = expected heterozygosity; F_{IS} = Wright's inbreeding coefficient; SD = standard deviation; p: significance level tested by a 1000 permutations test. p is significant after Bonferroni correction when < 0.012.

distance, because in this system population divergence was assumed to have occurred through drift only (Weir 1990). To summarize the patterns of differentiation between populations, a cluster analysis using Ward's method (1963) was performed on Reynolds' distance matrix using STATISTICA.

In order to test for isolation by distance, $F_{ST}/(1 - F_{ST})$ ratios were computed for each pair of populations and regressed on the logarithm of the geographic distance using GENEPOP (Raymond and Rousset 1995). The significance of the regression was tested by performing a 1000-permutation Mantel test, associated with Spearman rank correlation coefficient (r_s) as test statistics.

Results

Genetic variation within populations

In *P. veris*, 20 out of the 30 loci scored (66.7%) were polymorphic in at least one population for a total of 69 alleles detected. In *P. vulgaris*, 14 of the 27 loci (51.9%) were polymorphic in at least one population for a total of 50 alleles. For both species, no linkage disequilibrium was found between pairs of loci after sequential Bonferroni correction. Mean values for the estimates of within-population genetic variation are given in Table 1. At the population level, allelic richness ($r(g)$) ranged from 1.13 to 1.64 for *P. veris* with g = 38 genes, and from 1.11 to 1.25 for *P. vulgaris* with g = 12 genes. In *P. veris*, observed heterozygosity (H_o) and expected heterozygosity (H_e) corrected for small sample size ranged from 0.042 to 0.115 and from 0.037 to 0.133, respectively, and in *P. vulgaris*, from 0.031 to 0.080 and from 0.036 to 0.087, respectively. Wright's inbreeding coefficient (F_{IS}) values ranged from –0.154 to 0.454 in *P. veris* and from –0.451 to 0.429 in *P. vulgaris*.

In *P. veris*, exact tests for Hardy-Weinberg proportions at individual loci showed significant deviations ($p < 0.05$) in 92 out of 237 tests (38.8%) for positive F_{IS} values (heterozygote deficiency), and in only 3 out of 237 (1.3%) tests for negative F_{IS} values (heterozygote excess). Twenty-two out of 24 populations showed significantly positive F_{IS} values and one population significantly negative F_{IS} value. In *P. vulgaris*, 45 out of 202 tests (22.3%) of the exact tests for Hardy-Weinberg proportions showed significant deviations ($p < 0.05$) for positive F_{IS} values, and 4 out of the 202 tests (2.0%) for negative values. Eleven out of 41 populations showed significantly positive F_{IS} values and two populations significantly negative F_{IS} values.

Comparisons between species, regions within P. veris*, and verge and non verge populations*

The widespread *P. veris* had significantly larger populations than the rare *P. vulgaris* (t = 3.51, $p <$ 0.001). Similarly, for all the estimates of within-population genetic variation, *P. veris* showed significantly higher mean values than *P. vulgaris* at the population level after Bonferroni corrections (Table 1). The same observation for higher values of genetic variation for *P. veris* than *P. vulgaris* can be made at the species level.

Within *P. veris*, tests of comparison between the "scattered" Westhoek and "widespread" Voeren regions showed that population size (t = 2.29, p = 0.031), as well as the mean values of allelic richness and H_e for Voeren were significantly higher (the two last variables after Bonferroni corrections) than the values for Westhoek (Table 2). No significant difference after Bonferroni corrections ($p > 0.012$) was found between the two regions for H_o and F_{IS}.

No significant difference in population size ($t <$ 1.51, $p > 0.10$; K-S test: $p > 0.10$) and in genetic variation after Bonferroni corrections ($p > 0.012$)

Table 2. Mean values of within-population genetic variation for verge and non verge populations of *P. vulgaris* and of *P. veris*, and for the Westhoek and Voeren regions. For abbreviations, see Table 1; n = number of populations; *p*-values only given after Bonferroni correction

Population	n	$r(g)$	H_o	H_e	F_{IS}
Primula vulgaris					
verge populations	31	1.18	0.057	0.064	0.116
non verge populations	10	1.18	0.055	0.064	0.138
Primula veris					
verge populations	13	1.38	0.066	0.096	0.311
non verge populations	11	1.46	0.082	0.111	0.258
Westhoek	10	1.37	0.068	0.094	0.260
Voeren	14	1.49	0.086	0.115	0.252
p		0.009		0.011	

was found between verge and non verge populations, neither for both species (Table 2), nor for the Westhoek and Voeren regions in *P. veris* (not shown).

Effect of population size and density on genetic variation within populations

Population size and plant density were positively correlated: Pearson's correlation coefficient was 0.686 ($p < 0.001$) for *P. veris* and 0.394 ($p < 0.05$) for *P. vulgaris*. Variance inflation factors were close to 1 (1.89 and 1.18, respectively), so that we could assume no colinearity effect and reliable regression coefficients (Sokal and Rohlf 2000). For both species, significant multiple regression models ($p < 0.05$) were found for allelic richness and H_e (Table 3). No significant model ($p > 0.05$) was found for H_o ($R = 0.361$, $F = 1.58$, $p > 0.10$ for *P. veris*; $R = 0.349$, $F = 2.64$, $p = 0.085$ for *P. vulgaris*) and F_{IS} ($R = 0.292$, $F = 0.98$, $p > 0.10$ for *P. veris*; $R = 0.205$, $F = 0.84$, $p > 0.10$ for *P. vulgaris*). In *P. veris*, the standard partial regression coefficient (β) indicated that allelic richness was positively related to population size (Figure 2a) and negatively related to plant density ($\beta = 0.829$ and –0.404, $p < 0.001$ and = 0.037, respectively). H_e was positively related to population size ($\beta = 0.584$, and $p = 0.015$). No significant relationship was found between H_e and plant density, in the multivariate (Table 3) and univariate regression ($R = 0.0456$, $p > 0.05$). In *P. vulgaris*, the standard partial regression coefficient (β) indicated that H_e was positively related to population size ($\beta = 0.404$, $p = 0.014$). A similar trend was found for allelic richness ($\beta = 0.310$, $p = 0.060$) (Figure 2b). No significant effect ($p > 0.05$) of plant density was observed for *P. vulgaris*, either in the multivariate or in the univariate regression (results not shown).

When analysing the relationships within each regions, population size and plant density were significantly correlated for Voeren ($r_S = 0.988$, $p < 0.001$), but not for the Westhoek region (0.253, $p > 0.10$). For the Westhoek ($n = 14$ populations), variance inflation factor was close to 1 (1.13), indicating no colinearity effect and reliable regression coefficients (Sokal and Rohlf 2000). A significant multiple regression model ($p < 0.05$) was found for allelic richness (Table 3). The standard partial regression coefficient (β) indicated that allelic richness was positively related to population size ($\beta = 0.803$, $p = 0.003$) (Figure 2a). No significant model ($p > 0.10$) was found for H_o ($R = 0.234$, $F = 0.32$), H_e ($R = 0.471$, $F = 1.57$), and F_{IS} ($R = 0.565$, $F = 2.58$). However, when applying a univariate (Spearman) correlation analysis, a significant, positive relationship was found between F_{IS} and population size ($r_S = 0.618$, $p = 0.020$). No significant effect ($p > 0.05$) of plant density was observed, either in the multivariate regression or in the univariate correlation analysis (results not shown). For Voeren, there was no significant correlation ($p > 0.10$) between the estimates of genetic variation and population size or plant density ($r_S < 0.467$).

Population genetic structure

The gene diversity analysis (Table 4) indicated that in *P. veris*, $H_T = 0.184$, $H_S = 0.155$, and $F_{ST} = 0.150$ and in *P. vulgaris*, $H_T = 0.143$, $H_S = 0.120$, and $F_{ST} = 0.165$. For all loci combined, F_{ST} values ranged from 0.004 (*Got-3*) to 0.325 (*Lap-2*) in *P. veris*. They were low (< 0.150) in *P. vulgaris*, except for *Est* ($F_{ST} = 0.599$). The Ward's dendrogram based on Reynolds' genetic distances revealed that *P. veris* populations clustered according to their regional origin, Westhoek or Voeren, with two exceptions: W4 and V7 (Figure 3). The analysis of molecular variance gave a value of 0.075 for the between-region component of genetic diversity (F_{RT}) and 0.118 for F_{SR} (between populations within regions). The value for the overall F_{ST} was 0.184. The permutation tests showed significance ($p < 0.001$) for the three *F*-statistics. The majority of the total variance (81.6%) was attributable to the within-population component of variation (variance = 1.54). A further 10.9% of the total variance

Table 3. Significant multiple regression analysis of population size and plant density on the measures of within-population genetic variation for 24 populations of *P. veris*, 41 populations of *P. vulgaris*, and for the Westhoek populations of *P. veris*

Variable	β	t	p	Source	ANOVA			
					df	*MS*	F *ratio*	p
P. veris								
$r(38)$ ($R = 0.707$)								
Population size	0.829	4.58	<0.001	regression	2	0.0748	10.51	<0.001
Plant density	–0.404	–2.23	0.037	residual	21	0.0071		
H_e ($R = 0.502$)								
Population size	0.584	2.63	0.015	regression	2	0.0014	3.53	0.048
Plant density	–0.369	–1.67	0.111	residual	21	0.0004		
P. vulgaris								
$r(12)$ ($R = 0.419$)								
Population size	0.310	1.94	0.060	regression	2	0.0141	4.06	0.025
Plant density	0.186	1.16	0.254	residual	38	0.0035		
H_e ($R = 0.457$)								
Population size	0.404	2.57	0.014	regression	2	0.000504	5.00	0.012
Plant density	0.106	0.68	0.503	residual	38	0.000101		
P. veris, Westhoek								
$r(38)$ ($R = 0.761$)								
Population size	0.803	3.86	<0.003	regression	2	0.0999	7.56	0.009
Plant density	–0.377	–1.81	0.098	residual	11	0.0132		

$r(g)$ = allelic richness, with $g = 38$ genes for *P. veris* and 12 genes for *P. vulgaris*; H_e = expected heterozygosity; R = multiple correlation coefficient; β = standardised partial regression coefficient; t = t-statistic (two-tailed test of whether the partial regression coefficient differs from zero); *df* = degrees of freedom; *MS* = mean square; p = significance probability.

(0.21) was explained by variation between populations within regions, and 7.5% by variation between regions (variance = 0.14). Interpopulation gene differentiation expressed by average F_{ST}-values, is significantly higher within the Westhoek (0.150) than within the Voeren region (0.073) as tested by permutation tests ($p = 0.015$).

In *P. vulgaris*, the Ward's dendrogram based on Reynolds' genetic distances showed a geographical structure, the populations tending to cluster according to their regional origin (Figure 4). The analysis of molecular variance gave a value of 0.115 for the between-region component of genetic diversity (F_{RT}) and 0.087 for F_{SR} (between populations within regions). The value for the overall F_{ST} was 0.192. The permutation tests showed significance ($p < 0.001$) for the three F-statistics. The majority of the total variance (80.8%) was attributable to the within-population component of variation (variance = 0.86). A further 11.5% of the total variance (0.12) was explained by variation between populations within regions, and 7.7% by variation between regions (variance = 0.08).

Table 4. Genetic diversity statistics at 20 and 14 polymorphic loci in *P. veris* and *P. vulgaris*, respectively

Locus	H_T	H_S	F_{ST}
P. veris			
Mean	0.184	0.155	0.150
SD	0.181	0.155	0.032
95% CI	0.112; 0.262	0.092; 0.222	0.099; 0.211
P. vulgaris			
Mean	0.143	0.120	0.165
SD	0.180	0.160	0.067
95% CI	0.056; 0.234	0.048; 0.200	0.085; 0.329

H_T = total genetic diversity; H_S = genetic diversity within populations; F_{ST} = interpopulation gene differentiation; SD = standard deviation; CI = confidence intervals.

A significant association was found between $F_{ST}/(1 - F_{ST})$ and geographic distances matrices by performing a Mantel test, for *P. veris* ($r_S = 0.277$, $p = 0.001$) and for *P. vulgaris* ($r_S = 0.577$, $p < 0.001$). When considering populations of *P. veris* for

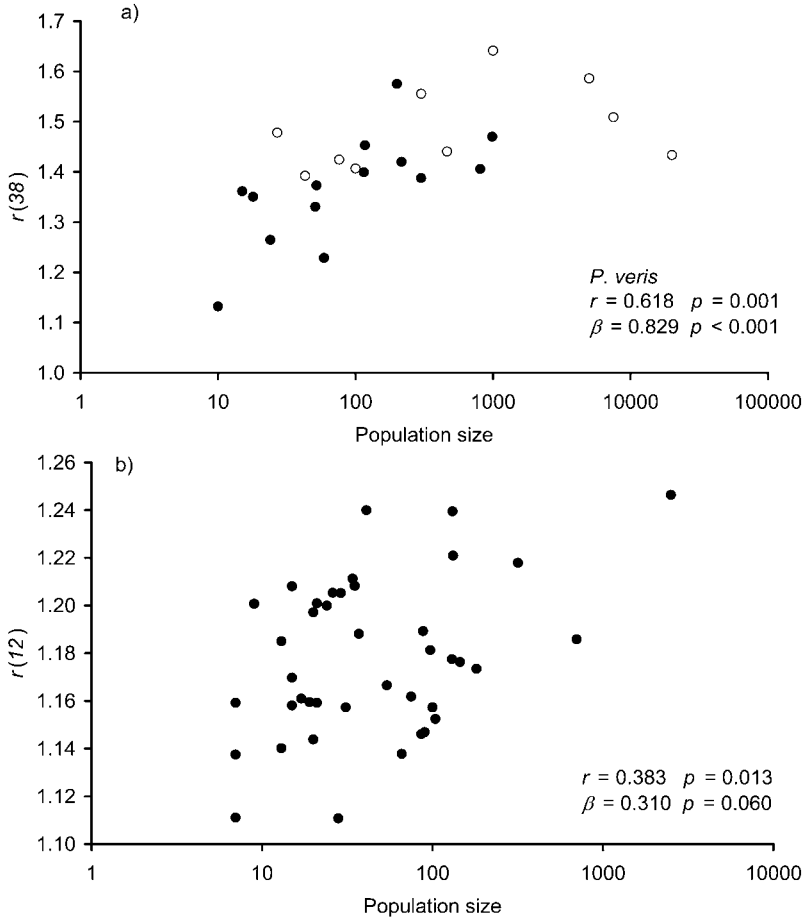

Figure 2. Relationship between population size (log-transformed) and allelic richness ($r(g)$) in (a) 24 populations of *P. veris* with $g = 38$ genes, and (b) 41 populations of *P. vulgaris* with $g = 12$ genes. In figure a): (○), Voeren populations ($r_S = 0.455$, $p > 0.10$); (●), Westhoek population ($r_S = 0.780$, $p < 0.001$; $\beta = 0.803$, $p = 0.003$). r and r_S = Pearson's and Spearman correlation coefficient, respectively; p-value significant after Bonferroni correction when <0.012; β = partial regression coefficient.

the two regions separately, no significant association ($p > 0.05$) could be found, neither for Westhoek (r_S = –0.030) nor for Voeren ($r_S = 0.101$).

Discussion

Genetic variation within populations in relation to species (local) rarity or commonness

Long-lived perennial, outcrossing plant species, like *P. vulgaris* and *P. veris*, are expected to maintain high levels of variation (Hamrick and Godt 1990). In *P. veris*, the levels of within-population genetic diversity fall within the range of those reported by Hamrick and Godt (1990) for species with similar life-history traits. By contrast, the rare *P. vulgaris* in Flanders shows little genetic variation and is genetically depauperate compared to the commoner *P. veris*. This may be partly due to the lower population sizes of *P. vulgaris* than of *P. veris*. It is also consistent with the results reported by Gitzendanner and Soltis (2000) in their review for rare and widespread congeners. Cahalan (1983) also found low values of genetic variation based on 15 loci in nine British populations of *P. vulgaris* from North Wales and Somerset (P = 25.9%, $A = 1.3$, $H_e = 0.074$) in the main range of the species. This suggests that the results found for the Flemish populations are not related to their marginal geographic position.

P. veris showed higher F_{IS} values than *P. vulgaris*. Most populations of *P. veris* show a heterozygote deficiency whereas most populations of *P. vulgaris* are at Hardy-Weinberg equilibrium. Low inbreeding

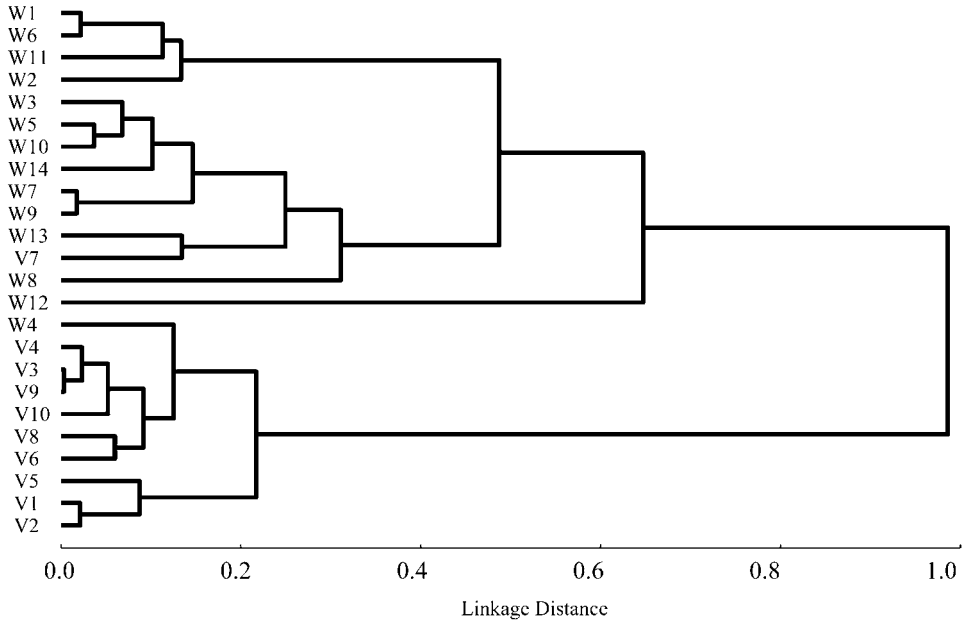

Figure 3. Ward's dendrogram based on Reynolds' genetic distances among 24 populations of *P. veris.* W1 to W14: populations from Westhoek; V1 to V10: populations from Voeren. Linkage distance = Reynolds' distance.

values can be expected in obligate outcrossers like *P. vulgaris* and *P. veris* (Loveless and Hamrick 1984). Only a few individuals were found to be homostylous (self-fertile) in Flanders (Van Rossum, unpublished data). Leptokurtic pollen carryover and seed dispersal restricted to the vicinity of the mother plant, as found for *Primula* species (Richards 1997), can result in the subdivision of populations into neighbourhoods of genetically related individuals (Cahalan and Gliddon 1985; Barrett and Kohn 1991), and for self-incompatible species in biparental inbreeding (Ellstrand and Elam 1993). The higher F_{IS} values observed in *P. veris* compared to *P. vulgaris* can partly be ascribed to a higher microgeographical genetic structure (Van Rossum and Triest 2003; unpublished). This may be explained by different life-history traits: for *P. vulgaris*, a seed dispersal over longer distances, by ants and rodents (Valverde and Silvertown 1995), and a very long flowering period (more than three months) compared to *P. veris* (Van Rossum, personal observations). Moreover, *P. vulgaris* is the only flowering, nectar-producing species in most of the sites where it occurs in Flanders (Van Rossum, personal observations). This increases its chances to be visited by any insect foraging for nectar and pollen, and can favour long-distance events of pollen dispersal. For *P. vulgaris* vegetative spread only occurs within very short distances, through the production of lateral rosettes (Valverde and Silvertown 1997). By contrast, *P. veris* has been reported to be clonally propagated by rhizome branching under certain conditions (Tamm 1972). Clonal propagation can also contribute to microgeographical genetic structure and increase inbreeding (Charpentier 2002). However, our sampling scheme was not designed to investigate clonality.

Only a very few populations showed a significantly negative F_{IS} value: one for *P. veris* and two for *P. vulgaris*; 11 out of 41 populations for *P. vulgaris* showed a high heterozygote deficiency ($F_{IS} > 0.200$). For most of these populations, high levels of disturbance were observed, such as partial destruction of the population by land or ditch redevelopment, herbicides application, digging up of flowering plants (for *P. vulgaris*), and extremely high flower predation by mammals (for *P. veris*).

Genetic variation of verge populations

For both species, verge populations were found to have similar population sizes and be equally diverse than the non verge populations. There is a trend to higher values of heterozygosity in non verge populations for *P. veris*, but this is not observed when the test is performed within regions. The linearity of these landscape elements does not seem to strongly influence

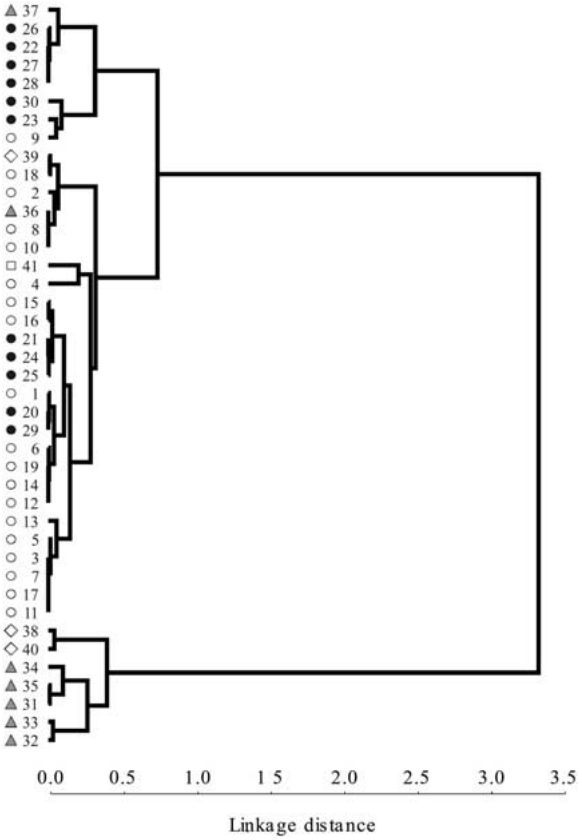

Figure 4. Ward's dendrogram based on Reynolds' genetic distances among 41 populations of *P. vulgaris*. The symbols ○, ●, ▲, ◇ and □ represent different regional groups (see Figure 1). Linkage distance = Reynolds' distance.

the processes which determine the levels of within-population genetic diversity. It might even favour seed and pollen dispersal (Godt et al. 1997). However, other factors, such as demographic structure and dynamics, need to be investigated to assess the demographic viability of verge populations (Jacquemyn et al. 2003). High genetic variation was also found for road verges populations of *Grevillea barklyana* (Hogbin et al. 1998), and gene flow was maintained within an agricultural landscape for outbreeding species like *Festuca ovina* and *Lychnis viscaria* (Berge et al. 1998).

Effects of population size and plant density

There is a positive relationship between population size and allelic richness and/or expected heterozygosity in *P. veris* and *P. vulgaris*, but population size has no effect on observed heterozygosity. This suggests that the alleles lost were initially present at low frequencies and that the main cause of genetic erosion is genetic drift. Genetic erosion in small, fragmented populations resulting from genetic drift has been reported for several self-incompatible perennial herbs, either in declining (e.g., Young et al. 1999; Luijten et al. 2000) or in widely distributed species (Weidema et al. 1996; Van Rossum et al. 2002). The still high levels of observed heterozygosity in small populations can be explained by the fact that for both species, individual plants may live for several decades (Tamm 1972; Boyd et al. 1990), which can delay the effects of fragmentation. Therefore, the observed variation may still reflect historical variation, and the process of genetic erosion may have just started. A low effect of inbreeding on genetic variation is expected in obligate outcrossers, especially if recruitment of new (inbred) individuals is absent in the populations. For both species, a low reproductive success was found in small populations, as a result of lower habitat quality and pollination disruption but also because a biased morph ratio (Brys et al. 2003, 2004). Morph anisoplethy might affect genetic variation by reducing mating opportunities and therefore effective population size (Byers and Meagher 1992; Richman and Kohn 1996). For *P. vulgaris*, small populations were shown to be ageing remnants from formerly larger populations, where little seedlings recruitment occurs (Endels et al. 2002). Moreover, a study of relationships between neighbouring populations suggested that small populations located near highly genetically variable populations might still receive moderate gene flow (Van Rossum and Triest 2003).

Population size and plant density are correlated in *P. vulgaris* and *P. veris*: small populations tend to have also low density. We found almost no effect of plant density on genetic estimates (except with allelic richness in *P. veris*). Some of the studied populations show a patchy structure, which may not be reflected by our measure of plant density (number of flowering plants per m^2). Patchy populations compared to continuous populations are expected to show more pronounced local genetic drift and inbreeding (Williams 1994; Doligez et al. 1998). In *Thymus vulgaris*, sparse and dense populations had low fixation indices resulting from random pollinator movement, whereas populations with a clumped structure showed significant heterozygote deficiencies (Tarayre and Thompson 1997). Although no effect of plant density on genetic variation had been reported for *P. elatior* (Van Rossum et al. 2002), the fine scale study of a patchy population revealed a negative relationship between local plant density and allelic richness (Van Rossum and

Triest, unpublished results). Similarly, the present results indicate that plant density negatively affects allelic richness in *P. veris*. Plant density has thus a different effect than population size: specifically, no higher genetic diversity is found at higher densities, contrary to common expectations (Young et al. 1996; Gram and Sork 1999). In fact, pollinators were shown to move more between individuals at high densities whereas in sparse populations they spend more time on the same plant. This favours selfing by geitonogamous pollination (Van Treuren et al. 1993; Kwak et al. 1998). In the self-incompatible *P. veris*, selfing is very unlikely in natural conditions (Richards and Ibrahim 1982). Therefore only pollination among (cross-compatible) individuals, resulting from random pollinator movement over long distances, will be successful (Cahalan and Gliddon 1985; Richards 1997). In dense populations, pollinators behaviour in combination with restricted seed dispersal, can favour local biparental inbreeding (Coates and Sokolowski 1992). This may lead to a faster loss of rare alleles. Increased biparental inbreeding associated with high plant density was reported for the self-incompatible *Phlox drummondii* (Watkins and Levin 1990) and the bird-pollinated *Banksia cuneata* (Coates and Sokolowski 1992). Plant density did not affect within-population genetic variation in the self-incompatible *Rutidosis leptorrhynchoides* (Young et al. 1999). In *P. veris*, it is better for a small population to be sparse than dense to maintain higher genetic variability. However, the positive effect of low plant density on genetic variation is not high enough to prevent genetic erosion in small populations.

Genetic structure among populations

Analysis of genetic structure reveals a geographical pattern in both species. In *P. vulgaris*, this depends on the geographical origin. In *P. veris*, there is a clear differentiation between the Westhoek and Voeren regions. For both species, most of the variation (around 81%) is found within populations. Within *P. veris*, the scattered populations from Westhoek and the widely-distributed populations from Voeren (species density threefold higher than in Westhoek) show contrasted genetic patterns. The Westhoek populations have smaller population sizes and are genetically depauperate compared to Voeren populations. No isolation by distance is observed within each region, but the F_{ST} values among Voeren populations were lower than among Westhoek populations. This may be explained by the larger population sizes and higher genetic variability in Voeren than the Westhoek, but might also be indicative of a still effective gene flow in Voeren. By contrast, populations in the Westhoek might be more genetically isolated from each other, as a result of higher habitat fragmentation and smaller areas of suitable habitats (intensively used polders) (Van Steertegem 2000). This hypothesis is supported by the correlation between allelic richness and population size that is significantly positive for the Westhoek but not for Voeren, and by the slightly higher allelic richness values for the populations with population sizes < 100 in Voeren than in the Westhoek (Figure 2a). However, if populations have been recently isolated in Voeren, the F_{ST} values might still reflect historical genetic structure, rather than current levels of gene flow. They may therefore overestimate gene flow (Ellstrand and Elam 1993).

Implications for conservation

As shown for rare and declining species, still common species can be negatively affected by habitat fragmentation. Commonness is not a guarantee for long-term survival of populations in a fragmented habitat. Conservation efforts also need to consider widely distributed species so as to prevent local population collapses and consequent species decline. Moreover, our results indicate that the negative effects of fragmentation tend to be stronger in the common *P. veris* than in its rare, declining relative *P. vulgaris*, especially when comparing populations in a similar context of fragmentation (in western Flanders). The detection of the decline in genetic diversity in the fragmented populations of *P. veris* may be a signal that this species in Flanders is in a transition phase to the status of less common to rare.

Priority in conservation strategies is often given to the preservation of large populations (e.g., Luijten et al. 2000; Neel and Cummings 2003), located in protected areas, which mainly concerns habitats of high biological value (e.g., European Community 2002). Our study, like a few previous ones (Godt et al. 1997; Berge et al. 1998; Hogbin et al. 1998) demonstrates the potential value of verge populations in the intensively used agricultural landscape for holding genetic diversity and as refuges that may buffer the species against further decline, in a landscape that is often like a virtual biological desert. In Flanders, *P. vulgaris*, and *P. veris* in some regions such as the Westhoek, are almost limited to small agricultural

landscape elements. The preservation of these populations occurring in more stressful habitats (Endels et al. 2002; Brys et al. 2003) can be a further guarantee for long-term species conservation, especially if populations appear to constitute a connected network (Van Rossum and Triest 2003). It may also preserve the species evolutionary potential so that it could face environmental changes leading to less suitable habitat conditions (Young et al. 1996). However, these populations are particularly susceptible to decline because of higher exposure to habitat degradation than non verge populations (Kleijn and Verbeek 2000; Endels et al. 2002). The EU agri-environment schemes must be more widely implemented to prevent small landscape elements from being eliminated. It is essential to convince farmers to avoid directly spreading herbicides on verges by determining buffer zones (Kleijn and Verbeek 2000), and to manage field and pasture edges so that it maintains biodiversity, by e.g., mowing and removal of the litter.

Our results also suggest that a good potential for restoration still exists in the populations, and that small populations should not be neglected in the conservation programs as they still retain high levels of heterozygosity (Lesica and Allendorf 1992; Young et al. 1996). In the same way, they could act as possible relay, e.g. for pollinators, between two larger populations (Kwak et al. 1998). Management measures intending to restore larger population sizes by increasing reproductive success and subsequent seedlings recruitment through reducing vegetation height and opening gaps (Jacquemyn et al. 2003) must also take plant density into account, as high density can result in genetic loss. Gene flow from genetically diverse populations to dense populations with low genetic diversity must be effective, by active hand cross-pollinations if necessary, or by introducing seeds or seedlings from large populations located in the same geographical region and in the same type of habitat. Furthermore, as pollination processes appear crucial for maintaining gene flow and diversity, conservation programs should include particular measures for protecting or restoring suitable pollinator populations (Kwak et al. 1998).

In human-used landscapes such as urban and agricultural environments, it is quite unlikely that fragmented (semi-)natural habitats will again develop into continuous areas. One key factor to ensure sustainable conservation of widespread plant species may be the maintenance or restoration of gene flow among fragmented populations, through corridors. There is therefore an urgent need for quantitative estimation of gene dispersal in human-used environments with respect to landscape structure, to evaluate the potential role of small landscape elements as functional corridors.

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