Perspectives for genetic rescue of the extremely fragmented *Primula vulgaris* populations in The Netherlands: reflecting the future of Belgian populations?

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Background and aims – Due to habitat fragmentation and degradation many plant species have been led to near extinction, remaining as a few extremely small and isolated populations. Preserving such remnant populations requires the most appropriate management strategies to apply in complementarity with the usual habitat restoration. These require an adequately evaluated genetic and demographic status.

Methods – We investigated population genetic variation of three remnant populations of *Primula vulgaris* (Noord-Drenthe, The Netherlands) using six microsatellite loci, for comparison with fifteen populations from Flanders (Belgium), showing similar fragmentation history and population sizes.

Key Results – Noord-Drenthe populations show lower genetic variation and higher between-population genetic differentiation, but lower F_{IS} values than populations from Flanders. This suggests stronger genetic erosion and gene flow disruption than in Flanders, but also a substantial genetic diversity retained by old-aged individuals, contributing to maintain low levels of inbreeding. Such a situation might await *P. vulgaris* in Flanders in the future.

Conclusions – Given the very small number and size of populations, a genetic rescue programme is needed through active reinforcements of the existing populations and creation of new populations. Seedlings used for rescue can be obtained from seeds collected in the field, but also from outcrosses between remnant populations, to maximize genetic diversity.

Key words – Fragmentation, genetic erosion, genetic rescue, reinforcement.

INTRODUCTION

Habitat fragmentation and degradation have been so severe in Western Europe that many plant species only remain as a few, often small and isolated, populations. These populations are susceptible to genetic, environmental and demographic stochasticity and are likely to express low reproductive success, reduced gene flow, inbreeding depression, and genetic erosion, threatening their long-term persistence (e.g. Wilcock & Neiland 2002, Oostermeijer et al. 2003, Aguilar et al. 2008, Angeloni et al. 2011). Plant species with a self-incompatible mating system are even more sensitive to habitat fragmentation. Indeed, for these species successful seed production depends on cross-pollination, and on the availability of compatible mates, which can be diminished in small populations (Aguilar et al. 2006, Berjano et al. 2013). When self-incompatible plant species show flower heteromorphism, such as heterostyly, pollination must occur between the different genetically inherited floral morphs: in case of distyly, between the long-styled (pin) and the short-styled (thrum) morphs. An optimal seed production in a population therefore requires an equal proportion of the morphs. Skewed morph ratios, often observed in small populations (e.g. Endels et al. 2002), may lead to a lack of compatible pollen, and reduce the effective size of the already small populations, reinforcing the negative effects of habitat fragmentation (Kéry et al. 2003, Brys et al. 2004, Van Rossum & Triest 2006a, Brys et al. 2007).

The usual ecological management practices that aim at restoring habitat quality may not be sufficient for ensuring sustainable population persistence. Preserving these last remaining populations needs to take their particularly critical genetic and demographic situation into account, by applying complementary restoration measures, such as genetic rescue (e.g. Van Geert et al. 2008, Weeks et al. 2011, Berjano et al.

2013). However, to determine the most appropriate management strategies (e.g. allo- or auto-reinforcements, restoration of connectivity by corridors), it is essential to adequately evaluate the genetic and demographic status of the relictual populations, e.g. whether they still hold genetic diversity, and show seed production and seedling recruitment (e.g. Weeks et al. 2011).

Primula vulgaris Huds. (Primulaceae) is a long-lived perennial, insect-pollinated, distylous herb with a North Atlantic and Mediterranean European distribution, reaching its northern margin in northern France, Belgium and The Netherlands, up to Denmark and southern Norway (Hegi 1975, Richards 1997, Jacquemyn et al. 2009). Belgium and The Netherlands are among the most densely populated regions in Europe; they are highly urbanized and industrialized, and farming practices are particularly intensive. Due to these high anthropogenic pressures, plant diversity in these regions has been greatly impacted (Noordijk et al. 2010, Hautekèete et al. 2015). The population history of *P. vulgaris* is similar in The Netherlands and Flanders (northern Belgium): this forest species shows a relictual distribution, and has incurred destruction and fragmentation of its habitats. It has become very rare and declining, only surviving in small forest fragments, and along hedges and linear landscape elements traditionally planted with willows (Weeda et al. 1985, Van Landuyt et al. 2006). These habitats nowadays are embedded in an intensively-used agricultural landscape matrix, which usually consists of fertilized pastures, however increasingly replaced by arable fields. In Flanders, less than ninety populations still exist, spread in four fragmented areas, most of them being below the minimum viable size threshold, i.e. ninety flowering individuals (Endels et al. 2002, Van Landuyt et al. 2006). Small populations display a depleted reproductive success as a result of pollination disruption, signs of genetic erosion, and higher inbreeding and genetic differentiation in recently germinated seedlings (e.g. Van Rossum & Triest 2003, Brys et al. 2004, Van Rossum et al. 2004, Van Geert et al. 2008). In The Netherlands, the situation is more dramatic: it is a truly relict species, with very few native populations left in Noord-Drenthe (and maybe also in the Dune phytogeographic district), most of the other records of the species (Floron 2011) corresponding to naturalized populations of plants of cultivated origin (Weeda et al. 1985). In Noord-Drenthe (The Netherlands) only three very small native populations subsist. In this study, we compare population genetic variation of these remnant populations, using microsatellite markers, with nearly similarly fragmented populations in Flanders and discuss on management measures to be applied to save these remnants from extinction.

MATERIALS AND METHODS

Population studied and sampling

The three populations from Noord-Drenthe (53°00′N 6°37′E, fig. 1) were visited in 2008: Geelbroek (*N* = 21 flowering individuals, located in a ditch along a pasture), Gasteren (*N* = 11 flowering individuals, situated in a forest fragment surrounded by pasture), and Eldersloo (three pin individuals, located in a ditch along a road and an arable field; table 1). Fruit set (the proportion of flowers setting fruits) was 0.96, 0.49 and 0.06, respectively (A. Vos, Werkgroep Florakartering Drenthe, pers. comm.). Young leaf material (only 1–2 leaves) was collected during the spring 2008 from all 35 flowering individuals. Fifteen populations from a similar context in Flanders (51°13′N 3°21′E, fig. 1), with *N* varying from 4 to 86 (table 1), for a total of 405 individuals sampled during the spring 2005, were included for comparison.

Microsatellite analysis

Genomic DNA extraction was performed on dried leaf material (7 mg dry weight) using the NucleoSpin® Plant extraction kit (Macherey-Nagel). Polymorphism was assayed on each DNA sample at six microsatellite loci (*PRIV4*, *PRIV6*, *PRIV7*, *PRIV13*, *PRIV15*, *PRIV17*) isolated from *P. vulgaris* according to Van Geert et al. (2006) and Triest et al. (2015). These were highly polymorphic (4–14 alleles per locus; 70 alleles in total), showed no linkage disequilibrium and gave interpretable patterns (Triest et al. 2015; GenBank accession numbers DQ858205-207 and KM538958-960). They were labeled and amplified (Tm = 57°C) by Multiplex Polymerase Chain Reactions (PCR) with a fluorescently labeled forward primer and an unlabelled reversed primer, using the QIAGEN Multiplex PCR Plus kit. The PCR products were separated by capillary poly-acrylamide gel electrophoresis (for details, see Van Geert et al. 2006). Amplification products were analyzed on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems). Allele sizes were assessed with the software GeneMarker® version 1.70 (SoftGenetics LLCÒ) by comparison with a GeneScan™ 500 LIZ® internal size standard. Potential null alleles and scoring errors were verified using MICRO-CHECKER (Van Oosterhout et al. 2004). None of the six loci showed evidence for either scoring errors due to stuttering or for large allele dropout. Only two deviations could be potentially caused by a null allele (*P* < 0.05) for PRIV4 (in population M) and PRIV6 (in Geelbroek). For PRIV4 this deviation can be attributed to a larger proportion of homozygotes than expected, only for the most common allele, and for PRIV6 only two alleles
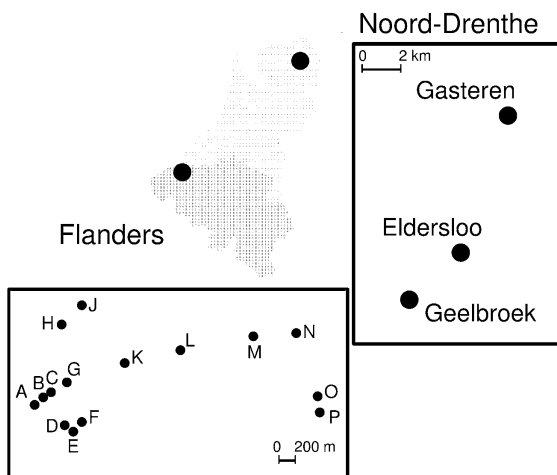

Figure 1 – Location of the studied populations of *Primula vulgaris* in Noord-Drenthe (The Netherlands) and in Flanders (Belgium).

Table 1 – Population details and estimates of within-population genetic variation for three *Primula vulgaris* populations in Noord-Drenthe (The Netherlands) and for fifteen *Primula vulgaris* populations from Flanders (Belgium).
N, flowering population size (number of flowering adults); n, sampling size; H_o, observed heterozygosity; H_e, expected heterozygosity; F_{IS}, Wright's inbreeding coefficient; ns, not significant; *, $P < 0.05$ after Bonferroni correction. Population codes followed Van Geert et al. (2014).

Population	N	Pin proportion	n	Allelic richness	H_o	H_e	F_{IS}	
Noord-Drenthe								
Geelbroek	21	0.71	21	2.22	0.379	0.400	0.052	ns
Gasteren	11	0.71	11	2.42	0.467	0.486	0.035	ns
Eldersloo	3	1.00	3	1.67	0.444	0.333	-0.371	ns
Flanders								
N	4	0.25	4	2.03	0.250	0.411	0.382	ns
F	8	0.50	7	3.62	0.548	0.718	0.233	ns
L	11	0.36	11	2.94	0.470	0.618	0.238	ns
B	12	0.33	11	2.77	0.555	0.568	0.015	ns
K	13	0.46	13	2.72	0.526	0.614	0.142	ns
E	15	0.80	14	3.30	0.384	0.683	0.402	*
D	25	0.40	23	3.60	0.498	0.750	0.310	*
H	26	0.52	23	3.27	0.558	0.666	0.160	*
C	30	0.80	25	4.24	0.577	0.834	0.305	*
G	32	0.50	31	3.37	0.636	0.682	0.064	ns
A	41	0.56	37	3.40	0.548	0.722	0.229	ns
J	48	0.46	43	3.33	0.467	0.703	0.328	*
P	51	0.54	36	3.50	0.472	0.748	0.369	*
O	54	0.54	48	3.29	0.546	0.705	0.221	*
M	86	0.46	79	3.07	0.523	0.648	0.191	*
Mean				3.23	0.504	0.671	0.239	*

were recorded, the commonest allele showing a higher frequency of homozygotes than the other genotypic combinations. These deviations can be explained as an effect of local recruitment from crosses between related individuals rather than by the presence of null alleles.

Data analysis

The following measures of within-population genetic variation were calculated per population as averages over loci: allelic richness (with $g = 6$ genes), observed (H_o) and expected (H_e) heterozygosity, and Wright's inbreeding coefficient (F_{IS}), corrected for small sample size (Kirby 1975, Nei 1978). Allelic richness was calculated using FSTAT version 2.9.3.2 (Goudet 2001) and the other variables using GENSURVEY (Vekemans & Lefèbvre 1997). The significance of the F_{IS} values (over all loci) estimated for each population was tested by randomisation tests using FSTAT and Bonferroni correction (Rice 1989).

To examine the patterns of genetic differentiation between populations, pairwise F_{ST}-values between populations were computed according to Weir & Cockerham's (1984) estimator (θ), and their significance tested by randomization tests using FSTAT and Bonferroni correction.

A test of comparison between groups of populations was performed using FSTAT to test for difference in genetic variation between Flanders (divided in two groups: for $N \leq 25$, similar to Drenthe's size range, and > 25; table 1) and Noord-Drenthe. The significance of the tests was assessed using a randomised permutation scheme of the populations between the groups (1000 permutations).

RESULTS AND DISCUSSION

Compared to Flanders populations ($N \leq$ and > 25; comparison tests: $P \geq 0.137$), levels of allelic richness, H_o (except when Flanders population $N \leq 25$: $P = 0.346$) and H_e were significantly lower in Noord-Drenthe populations ($P = 0.001$–0.042; fig. 2). This suggests stronger genetic erosion due to genetic drift effects in Noord-Drenthe. Surprisingly, F_{IS} values were significantly lower ($P = 0.001$–0.005) than in Flanders. Moreover, H_o and H_e were still high (table 1). Such high local genetic diversity, still allowing low levels of inbreeding might represent historical variation retained by old individuals, as observed for small populations in Flanders

(Van Geert et al. 2008). Indeed, plants may live for several decades (Boyd et al. 1990).

Genetic differentiation between Noord-Drenthe populations was high (F_{ST} = 0.435, 0.469 and 0.508; $P < 0.05$ after Bonferroni correction). The F_{ST} values between Noord-Drenthe populations were significantly higher ($P = 0.003$ and 0.007) than between populations from Flanders (mean F_{ST} = 0.107 and 0.086 for $N \le$ and > 25, respectively; comparison test: $P = 0.859$), indicating that Noord-Drenthe populations appear genetically isolated from each other, despite geographical distances ranging 3–11 km (in Flanders: 0.07–3.18 km). As seed dispersal is restricted for *P. vulgaris* (Valverde & Silvertown 1995), and pollen (using fluorescent dye as analogue) has been found to be dispersed up to 1 km in Flanders (Van Geert et al. 2010), we can expect a stronger disruption of gene flow between Noord-Drenthe populations.

The populations in Noord-Drenthe consist of a majority of pin individuals (Eldersloo is even monomorphic; table 1). Such a lack of compatible mates for self-incompatible plant species can contribute to reinforce the negative effects of small population size, especially when the population is isolated, reducing reproductive success and increasing the levels of genetic drift and inbreeding (Wilcock & Neiland 2002, Brys et al. 2004, Van Rossum & Triest 2006a, Berjano et al. 2013). Nevertheless, some seed production and even seedling recruitment were observed (A. Van Geert, pers. obs.), even in Eldersloo, suggesting that seed development was due to intrapin outcrosses and/or selfing. In Flanders, partial pin self-compatibility was demonstrated by controlled hand-pollinations, with viable seeds showing very few signs of inbreeding depression at the early stages of seedling development (Van Geert 2010). Under pollen limitation, e.g. as a result of a lack of thrum pollen or poor pollinator service, such partial self-compatibility, by allowing intrapin outcrosses, may increase mating opportunities and somewhat mitigate the negative impact of habitat fragmentation (Van Rossum & Triest 2006b). For instance, an indication of a pin reproductive advantage in small fragmented populations has been observed in the closely related *P. veris* (Van Rossum et al. 2006). Furthermore, even if the genetic consequences of habitat fragmentation appear more severe in Noord-Drenthe than in Flanders, populations when taken together still hold substantial genetic diversity. Additionally seed set and seedling recruitment indicates the possibility for colonization of other suitable habitat patches.

We can hypothesize that a situation with extremely fragmented small populations will await *P. vulgaris* in Flanders in the future. In Noord-Drenthe, only two populations are located in protected nature areas. All populations in the agricultural landscape have disappeared, except one. In Flanders, none of the populations are located in protected nature areas, and only about ten populations in 2013 could be considered as viable (above the minimum viable size of ninety flowering individuals; Van Rossum, pers. obs.). Populations, whenever large or small, remain very vulnerable to high demographic stochasticity because of intensive agricultural practices (e.g. shift to arable fields with intensive use of fertilizers and pesticides, clearing of field boundaries, cutting of willow trees). In 2007, two large *P. vulgaris* populations (> 100 individuals) were created in the Damme Golf Course property using seedlings obtained from seeds harvested in the area. These populations have remained healthy, produced seedlings and may thus become an important part of the *P. vulgaris* population network. However, the same year, even more *P. vulgaris* individuals have been destroyed in the agricultural landscape than those newly installed (Van Geert, pers. obs.). It is imperative that the management recommendations obtained by the various studies on *P. vulgaris* in Flanders (e.g. Endels et al. 2002, Jacquemyn et al. 2003, Van Rossum & Triest 2003, Endels et al. 2004, Van Geert et al. 2008, 2010) should be
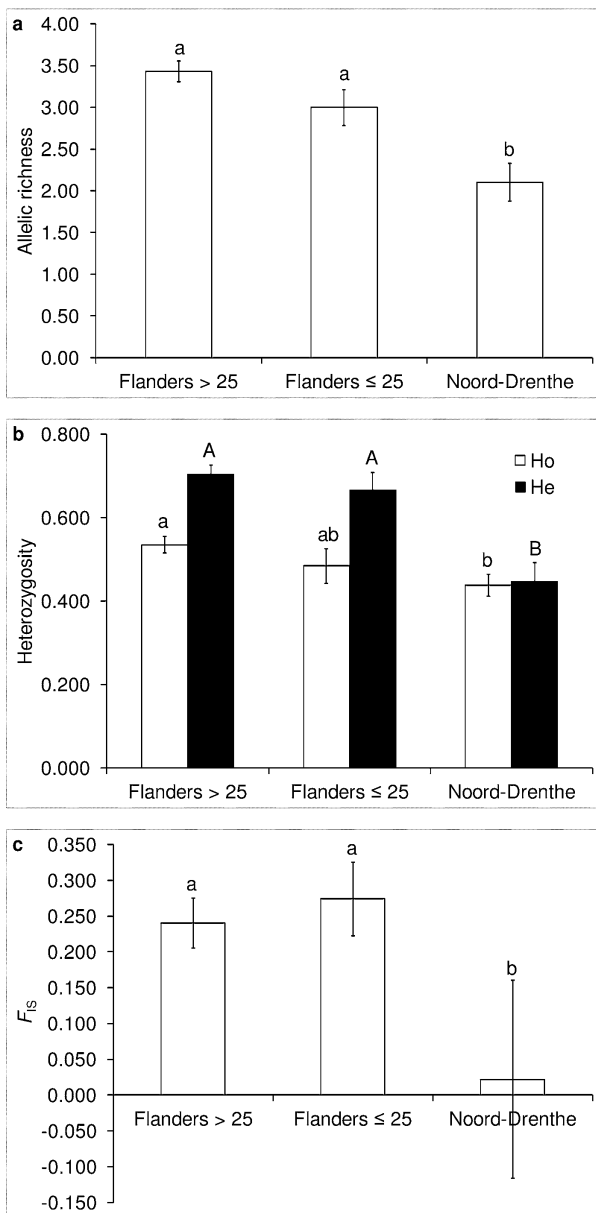

Figure 2 – Comparison between *Primula vulgaris* populations (mean ± SE) from Flanders ($\le$ and > 25 flowering individuals) and Noord-Drenthe: (a) allelic richness; (b) H_o and H_e; (c) F_{IS} values. The significant differences ($P < 0.05$) between groups are indicated with different letters (a–b).

put into practice, to prevent a similarly critical situation as in Noord-Drenthe, albeit within a few decades.

Given the very small number and size of populations in Noord-Drenthe, a genetic rescue programme is needed through active reinforcements of the existing populations and creation of new populations. Seedlings used for rescue can be obtained from seeds collected in the field, but also from outcrosses between populations, which might contribute to maximize genetic diversity in all populations (Weeks et al. 2011). The presence of linear landscape elements can facilitate pollinator movements and pollen dispersal between populations of *P. vulgaris* in the intensively-used agricultural landscape (Van Geert et al. 2010) and of the closely related *P. elatior* in urban forests (Van Rossum & Triest 2012). The effectiveness of these biological corridors also depends on the surrounding co-flowering vegetation richness (Van Geert et al. 2014) and can be increased by the presence of small stepping-stone populations (Van Rossum & Triest 2012). Creating a network of populations connected by pollen flow through biological corridors and improving the quality of the surrounding landscape matrix may therefore certainly contribute to guarantee species long-term persistence in Noord-Drenthe.

Primula vulgaris is critically endangered in Belgium and The Netherlands, at the margin of its distribution range. On the contrary, the species is still common and not threatened in the main range of its distribution, e.g. in Great Britain or in Spain (Jacquemyn et al. 2009). However, British populations were reported to have locally declined in abundance, as a result of a change in forest exploitation practices (less coppicing and pollarding) and of intense plant picking (Jacquemyn et al. 2009). Populations of common species occurring in highly fragmented habitats are also susceptible to the negative consequences of small population size and spatial isolation (Van Rossum et al. 2002, 2004, Van Rossum & Triest 2006a, Honnay & Jacquemyn 2007, Van Rossum 2008). Attention should therefore be paid to the fact that common species, in particular when they are obligate outcrossers, may rapidly decline in isolated habitat fragments that cannot support large plant populations.

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