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Population structure of an endangered species living in contrasted habitats: *Parnassia palustris* (Saxifragaceae)

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Abstract

In endangered species, it is critical to analyse the level at which populations interact (i.e. dispersal) as well as the levels of inbreeding and local adaptation to set up conservation policies. These parameters were investigated in the endangered species *Parnassia palustris* living in contrasted habitats. We analysed population structure in 14 populations of northern France for isozymes, cpDNA markers and phenotypic traits related to fitness. Within population genetic diversity and inbreeding coefficients were not correlated to population size. Populations seem not to have undergone severe recent bottleneck. Conversely to pollen migration, seed migration seems limited at a regional scale, which could prevent colonization of new sites even if suitable habitats appear. Finally, the habitat type affects neither within-population genetic diversity nor genetic and phenotypic differentiation among populations. Thus, even if unnoticed local adaptation to habitats exists, it does not influence gene flow between populations.

Keywords: cpDNA, gene flow, isozymes, pollen/seed migration, reproductive system

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Introduction

Habitats for plant species are naturally fragmented. Most species occur in a number of discrete populations, more or less connected by migration. The environment exhibits its own dynamics due to various stochastic disturbances (van den Meijden *et al*. 1985; Stacey & Taper 1992) and plant community succession (Harper 1977; Crawley 1997). As ecological conditions change, sites may become unsuitable for a given species, as others may become suitable and be colonized. Many species are characterized by a balance between local population extinctions and establishment of new populations, i.e. they live in metapopulations (Hanski & Gilpin 1997).

Migration to unoccupied suitable sites is the first essential step for establishment of new populations. Migration among extant local populations may increase their probability of persistence by buffering consequences of local environmental and demographic stochasticities (Stacey & Taper 1992) or by preventing inbreeding depression (Saccheri *et al*. 1998). However, migration among extant populations may also lead to outbreeding depression (Waser & Price 1989; Fenster & Galloway 2000; Quilichini *et al*. 2001) and hinder adaptation to local environmental conditions (Storfer 1999).

Metapopulation viability is determined by a balance between opposite forces: migration and selection acting at local and regional scales as well as extinction and colonization. Such balances are disrupted by human activities which often increase habitat fragmentation, reducing the size of suitable sites and increasing distances between them (Wilcox & Murphy 1985; Andrén 1994; Young *et al*. 1996). Although the complex consequences of fragmentation are not yet clearly understood (see Colas *et al*. 2002), it seems that rapid anthropogenic fragmentation affects metapopulation dynamics negatively because it tends to increase extinction rate (smaller population sizes) and to decrease colonization rate (longer distance between suitable sites) (but see Ballal *et al*. 1994).

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Parnassia palustris (Saxifragaceae) is a perennial colonizing herb experiencing anthropogenic fragmentation in Northern France. This species occurs naturally in three distinct well-defined habitats: wet dune-slacks, base-rich marshes and limestone hillsides. Wet dune-slack habitat develops on sandy soils slightly enriched with organic mater. The water level is highly variable from year to year, depending on climatic conditions. Dunes are naturally unstable and patchily distributed due to geological discontinuities. The recent expansion of seaside resorts has contributed to an increased fragmentation of this habitat. Base-rich marshes have peaty soils. The water level is more constant. Most natural marshes have been drained, the few remaining ones being managed for wildfowl hunting. Limestone hillsides present poor organic and superficial soils. As a result of declivity and sunny exposed conditions, soils are dry, even if limestone retains a certain humidity. Decreasing pasture has reduced strongly the number and surface of hillside habitats, which would otherwise be open for colonization by *P. palustris*.

The number of extinct populations on the total number of populations being recorded for the beginning of 20th century or apparent extinction rate (AER) for *P. palustris* (F. Hendoux, unpublished data) is of 42%. Great differences appear nevertheless between habitats (AER of 73%, 42% and 35% for marsh, dune and hillside habitats, respectively). No new population has been observed for 20 years. *P. palustris* is thus considered endangered in Northern France as well as in Luxembourg, Belgium and the Netherlands. The level at which populations interact (i.e. dispersal) as well as the levels of inbreeding are unknown, yet these data would be useful to set up conservation policies. Strategies for delaying population extinction rely on the degree to which populations exchange migrants and colonize new sites. One possible response of plant populations to heterogeneous environments is genetic adaptation, resulting in the formation of distinct ecotypes which continue or not to exchange effective migrants. When the establishment of migrants from other habitats is prevented, high genetic differentiation is expected between habitats and conservation strategies should not include population reinforcement by individuals from a different habitat (Van Rossum *et al*. 1997; Gauthier *et al*. 1998). The different environments in which *P. palustris* occurs can have led to different phenotypes exchanging few genes, and promoting higher level of genetic isolation between populations from different habitats than from the same habitat.

This study has three objectives:

1 To compare levels of genetic diversity and inbreeding among populations according to population size to assess a possible effect of reduction of habitat area.
2 To estimate the degree of genetic differentiation among populations using neutral markers. In plant species, genes are dispersed by both seeds and pollen. Hence, we chose to investigate genetic polymorphism for both cytoplasmic maternally inherited markers as well as nuclear markers to trace back migration by both seeds and pollen.
3 To test using genetic markers and scoring in the field characters related to fitness whether the occurrence of different habitats influences the overall pattern of genetic and phenotypic differentiation at a regional scale.

Materials and methods

Study species and populations

Parnassia palustris (Saxifragaceae) is a perennial colonizing herb, with a circumboreal distribution. The species is hermaphrodite, protandrous and probably insect-pollinated (nectaries). In Northern France, it flowers in August–September and fruits containing several hundreds of seeds ripen in September–October.

In spring 1998 young leaves were sampled in 14 populations representative of the three habitats inhabited by the species (Fig. 1, Table 1). We sampled eight hillside populations (Bonningues, Teneur, Le Molinet, Auxi, Nabringhen, Verlincthun, Alquines, Le Breuil), five dune populations (Mont St Frieux, Marchand, Perroquet 1, Stella, and Perroquet 2) but only one marsh population, as access to marshes is restricted for hunting purpose (Villiers). The total number of flowering plants was recorded in each population. In three sites (Stella, Marchand and Auxi) sampled plants were mapped for further analysis of fine spatial genetic structuring. In all populations, diploidy was confirmed by fluorescent cytometry analysis.

Phenotypic data collection

The life-cycle traits involved in fitness and adaptation are not ascertained in *P. palustris*. We chose to measure four morphological characters thought to be related to survival ability and reproductive success. The neck diameter (DIAM) is likely to be a good indicator of resource availability for survival because *P. palustris* survives as a branched rhizome in winter. Other measured traits are related to reproductive success: the total number of flowering stems (HAMP), the proportion of stems with capsules (PCAPS) and the proportion of abortive seeds without embryo (PGRV). Unless these traits are neutral, they are likely to be involved in ecological specialization to the habitats.

At the peak of flowering, in nine of 14 populations (Table 1), the neck diameter, the total number of flowering stems and the proportion of stems with capsules were measured on 30 randomly chosen plants per population. Seeds were collected in nine populations. Six of these

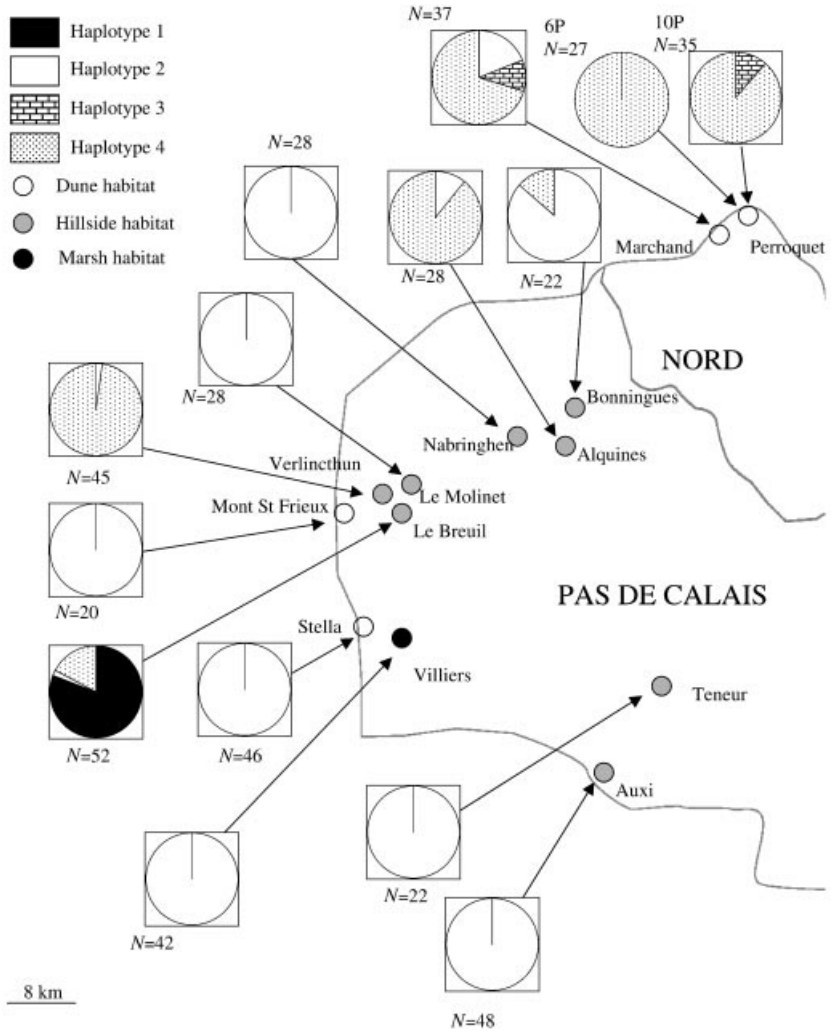

Fig. 1 Distribution of sampled populations in Northern region of France. Sample sizes and cpDNA haplotypes are shown for each population.

Table 1 Sampling design. The number of flowering plants ($N_{flowers}$) is indicated for each population. Populations which were measured for phenotypic traits are indicated by X. DIAM, HAMP, PCAPS and PGRV stand for the neck diameter, the total number of flowering stems, the proportion of stems with capsules, and the proportion of abortive seeds without embryo, respectively

Habitat	Population (coding name)	Longitude	Latitude	$N_{flowers}$	Measure of DIAM, HAMP, PCAPS	Measure of PGRV
Hillside	Bonningues (B)	02°00′56″	50°47′37″	250		x
	Teneur (T)	02°13′09″	50°27′06″	285	x	x
	Le Molinet (M)	01°48′23″	50°39′10″	300	x	x
	Auxi (A)	02°06′56″	50°13′53″	400		
	Nabringhen (N)	01°51′46″	50°44′42″	550	x	
	Verlincthun (V)	01°40′46″	50°37′46″	1 300	x	x
	Alquines (Al)	01°59′35″	50°44′28″	2 000		
	Le Breuil (Br)	01°44′46″	50°38′25″	5 200		x
Dune	Mont St Frieux (F)	01°38′30″	50°37′10″	140		x
	Marchand (Ma)	02°29′25″	51°03′54″	1 350	x	x
	Perroquet 1 (6P)*	02°31′42″	51°04′18″	3 000	x	
	Stella (S)	01°36′54″	50°27′23″	4 000	x	x
	Perroquet 2 (10P)*	02°31′42″	51°04′18″	16 000	x	
Marsh	Villiers (Vi)	01°37′10″	50°28′39″	10 000	x	x

*10P is located 500 m apart from 6P.

populations were described for the other traits (Table 1). The proportion of abortive seeds without embryo was then calculated using 150–300 seeds per plant on average and 20 plants per population.

To test for the ability of *P. palustris* to self-fertilize, a bagging experiment was performed on 30 individuals sampled from seven populations and grown at the CRP/CBNBL. One flower per individual was bagged to allow self-fertilization. In addition, a second flower was bagged after hand pollination was performed, using pollen from other individuals of the same population. Once bagged fruits were fully ripe, the proportion of abortive seeds without embryo was estimated, and the proportion of viable seeds that germinated were also calculated on a sample of 150–300 seeds per fruit.

Isozyme and cpDNA typing

Enzyme extraction was performed on frozen leaves ground in 50 µL of germanium extraction buffer (Mitton *et al*. 1979). Horizontal starch gel electrophoreses of the homogenates were carried out using a Tris-citrate pH 7.2 buffer (Soltis *et al*. 1983). Of 13 enzyme systems tested, five were found polymorphic and allowed unequivocal genetic interpretation. Enzyme systems stained as described in Soltis *et al*. (1983), were phosphoglucomutase (PGM, EC 5.4.2.2), glucose-phosphate isomerase (PGI, EC 5.3.1.9), 6-phosphogluconate dehydrogenase (PGD, EC 1.1.1.44), malate dehydrogenase (MDH, EC 1.1.1.37) and shikimate dehydrogenase (SDH, EC 1.1.1.25).

Total genomic DNA was extracted from 30 mg of leaves, sampled from the same individuals used for isozyme assays, following Edwards *et al*. (1991). Universal primers located in coding regions of cpDNA were used to amplify intergenic variable noncoding regions (Demesure *et al*. 1995; Dumoulin-Lapegue *et al*. 1997). Of 15 pairs of primers tested, four gave a unique well-amplified product, corresponding to the amplification of one intergenic region. Polymerase chain reaction (PCR) amplification was performed in a DNA thermal cycler Perkin-Elmer PT9700. Reactions were carried out in a total volume of 15 µL using 1× PCR buffer, 3.5 mM $MgCl_2$, 200 µM dNTPs, 0.2 µM of each primers, 0.625 U of Perkin-Elmer *Taq* DNA polymerase, 200 µg/mL of bovine serum albumin and ≈ 20 ng of genomic DNA. An initial 5-min denaturation step at 94 °C was followed by 36 cycles of denaturation at 94 °C for 45 s, annealing at 57.5 °C or 60 °C depending on the primer for 45 s, and elongation at 72 °C for 4 min. A final elongation step was performed at 72 °C for 10 min. Polymorphism (small insertions/deletions or substitutions) was revealed using eight restriction enzymes (*Hin*fI, *Alu*I, *Cfo*I, *Hin*dIII, Ecor I, *Mse*I, *Taq*I, *Hpa*II from Boehringer Mannheim). Amplified products were digested for 2 h at 37 °C. Restriction digests were separated by electrophoresis in 8–10% polyacrylamide gels using Tris-borate EDTA buffer (0.5×) at 100 V for 19–24 h and silver-stained. Polymorphism due to small insertions/deletions was observed for one couple of primers/enzyme: two fragments of different sizes were found overall and encoded 1 and 2. For two other couples of primers/enzyme, polymorphism was due to substitution: the absence and the presence of the restriction enzyme site was encoded 1 and 9, respectively.

Statistical analyses

Genetic diversity and structure at the within-population level. For the isozyme loci, allele frequencies, percentage of polymorphic loci, mean number of alleles per locus, gene diversity (H_E, expected heterozygosity, Nei 1978) and [values $\hat{f}$ i.e. F_{IS} estimates according to Weir & Cockerham (1984)] were estimated within each population using FSTAT 2.9.3 (Goudet 1995). Because of large differences in sample size among populations and loci, we applied a rarefaction method to estimate within-population allelic richness (El Mousadik & Petit 1996) in FSTAT 2.9.3. Deviation from Hardy–Weinberg expectations at each locus and statistical genotypic linkage desequilibria among loci were tested using GENEPOP 3.3 (Raymond & Rousset 1995). Tests for $\hat{f}$ values were also computed for every population by a permutation procedure of alleles within sample using FSTAT 2.9 (Goudet 1995).

For the cpDNA analysis, due to the nonrecombining nature of the chloroplast genome, alleles observed at polymorphic loci were combined into haplotypes (chlorotypes). cpDNA haplotype frequencies and diversity values (according to Nei 1987) were estimated for each population using FSTAT 2.9.

To test whether genetic diversity is related to population size, correlations between the log-transformed number of flowering plants and the isozyme gene diversity (H_E), the percentage of polymorphic loci (%*P*), the mean number of alleles (n_{all}), the allelic richness (*All. rich*) and the cpDNA haplotype diversity (H_{ecp}), respectively, were calculated using SAS (SAS Institute 1992).

Tests based on permutation procedures were carried out to test for differences among hillside and dune populations for a number of variables (allelic richness, observed heterozygosity, enzyme diversity, F_{IS} and cpDNA haplotype diversity) using FSTAT version 2.9.3.

Fine within-population structure was examined within the three populations with individual mapping using AUTOCORF 2 (Hardy & Vekemans 1999). This software calculates different pairwise statistics between individuals located at the same geographical distance to each other. We estimated first kinship coefficients as described in Loiselle *et al*. (1995) and Ritland (1996). Second, we analysed the pairwise statistics a(*r*)/[1 – a(*r*)] proposed by Rousset (2000), which is somewhat analoguous to the pairwise $F_{ST}/(1 - F_{ST})$

parameter but adapted to the individual level (see Rousset 1997; 2000). Under an isolation by distance model, those statistics are expected to vary linearly (negatively for kinship coefficients and positively for the estimator of Rousset 2000) with the logarithm of the distance in a two-dimensional space (Rousset 1997, 2000; Hardy & Vekemans 1999). The average values per distance class are given. Significance of all parameters was tested by random permutations of individuals among existing positions.

Genetic structure among populations and habitats. Only dune and hillside populations were tested for an habitat effect as only one marsh population has been sampled. Genetic structure among populations within habitat and among habitats was analysed by hierarchical analysis of variance, partitioning $\hat{\Theta}$ (estimator of F_{ST} as described by Weir & Cockerham 1984) into $\hat{\Theta}_{wh}$ and $\hat{\Theta}_{bh}$ indicating the genetic differentiation of samples between populations within habitat and between habitats using ARLEQUIN version 2.000 (Schneider *et al*. 2000). For the cpDNA haplotypes, molecular information, i.e. the number of differences between haplotypes, was taken into account in calculating $\hat{\Theta}$, $\hat{\Theta}_{wh}$ and $\hat{\Theta}_{bh}$ using an AMOVA procedure (Excoffier *et al*. 1992; Michalakis & Excoffier 1996) in ARLEQUIN version 2.000. No sequence of mutational events was assumed. The same mutational weight was given to the substitutions and to the insertions/deletions. This use of molecular information did not modify genetic differentiation estimates. Statistical significance of $\hat{\Theta}_{wh}$ and $\hat{\Theta}_{bh}$ was computed by nonparametric permutational procedures of genotypes or haplotypes among populations within habitat and among populations among habitat. To test for genetic isolation of the Villiers population (marsh habitat), pairwise estimates were computed for each pair of populations regardless of the habitat using GENEPOP 3.3 (Raymond & Rousset 1995). Significance of pairwise $\hat{\Theta}$ values was determined by Fisher's exact tests in GENEPOP 3.3.

Isolation by distance patterns among populations (Slatkin 1993) were tested considering all populations and then considering dune and hillside populations separately. The correlation coefficient between geographical and genetic distances was compared with the distribution of correlation coefficients obtained from Mantel-like permutations of the genetic $[\hat{\Theta}/(1-\hat{\Theta})]$ and geographic [ln (geographic distance)] matrices as described by Rousset (1997) and included in GENEPOP 3.3.

Phenotypic traits analyses. Arcsine transformation was applied to the proportion of stems with capsules and to the proportion of abortive seeds to achieve a better fit to normal distribution (Sokal & Rohlf 1995). A nested analysis of variance (habitat, population within habitat) was performed in SAS (SAS Institute 1992) excluding the marsh habitat. To test whether the marsh population is phenotypically different from others, a one-way ANOVA (population) was conducted on the whole data set. Phenotypic correlations were calculated for each pair of characters, as well as between-population means of DIAM, HAMP, PCAPS and PGRV and the log-transformed number of flowering plants.

Considering the bagging experiment, arcsine transformation was applied to the proportion of abortive seeds without embryo, and the proportion of viable seeds that germinate, to achieve a better fit to normal distribution. A one-way analysis of variance was performed on these characters to see the influence of the pollination method (self-, open or hand pollination) on offspring viability.

Results

Within-population analyses

Isozyme study. Six polymorphic isozyme loci were identified (MDH1 and 2, PGD1, PGM1, PGI1 and SDH1) and 16 alleles recorded (Appendix 1). Expected heterozygosity (H_E) varied between 0.05 (PGM1) and 0.52 (SDH1). SDH1 could be scored for a few individuals only, due probably to enzyme degradation in frozen leaves of *Parnassia*. Given that heterozygote deficiencies within population and population structure at this locus did not differ from the other loci, it was included in further analysis. All alleles were observed in several but not all populations. No significant linkage disequilibrium between loci was found whatever the population suggesting independence among loci.

The percentage of loci polymorphic at the 95% criterion (*%P*) varied between 17% (population 6P) and 67% (populations F, 10P and S). The mean number of alleles (n_{all}) varied between 1.67 (populations N, V, F and Ma) and 2.67 (population Vi). However, the allelic richness was poorly variable among populations (Table 2). Gene diversity (H_E) varied between 0.10 for population 6P and 0.30 for population S, both from the dune habitat (Table 2).

No relationship was found between population size and any population genetic diversity estimates: $P = 0.862$, 0.669, 0.078 and 0.943 for correlation analyses between the log-transformed number of flowering plants and H_E, *%P*, n_{all} and *All. Rich*, respectively.

No differences between dune and hillside populations were observed for allelic richness ($P = 0.835$), heterozygosity ($P = 0.237$), gene diversity ($P = 0.383$) and F_{IS}-estimate ($P = 0.888$) calculated over all loci. This trend holds when excluding population Ma (which showed the lowest sample sizes at a locus) or when excluding locus SDH1 for which the lowest sample sizes were recorded.

Whereas no significant heterozygote excess was observed, heterozygote deficiencies over all loci were found to be significant for six of 14 populations (Table 2). Significant $\hat{f}$ values could result from either null alleles, selection, inbred reproductive system (e.g. selfing) or a Walhund

Table 2 Allozyme diversity at the within-population level. N, $\%P$, H_E and n_{all} stand for the mean sample size per population, the percentage of polymorphic loci at 95% criterion, the mean gene diversity and the mean numbers of alleles per locus, respectively. Allelic richness (*All. Rich.*) was calculated following the rarefaction procedure described in El Mousadik & Petit (1996). The number of individuals used to apply this rarefaction method is given in parenthesis (it corresponds to the smallest number of individuals typed for a locus in a sample; see Appendix 1). Three calculations were made: over all loci and samples, over all loci but excluding the population Ma (for which the smallest number of individuals typed for a locus was recorded) and excluding the locus SDH1 (for which only few genotypes were recorded). Heterozygote deficiencies (as measured by $\hat{f}$ values) parameters within population are also indicated

	Hillside								Dune					Marsh
	B	T	M	A	N	V	Al	Br	F	Ma	6P	S	10P	Vi
N	17.67	20.33	19.83	43.33	21.17	38.00	27.00	27.83	19.33	30.33	23.17	31.50	26.50	31.50
$\%P$	33.33	33.33	50.00	50.00	33.33	33.33	50.00	33.33	66.67	50.00	16.67	66.67	66.67	50.00
H_E	0.19	0.23	0.16	0.16	0.16	0.13	0.17	0.19	0.28	0.14	0.10	0.30	0.14	0.28
n_{all}	2.00	2.00	2.00	2.17	1.67	1.67	2.17	2.17	1.67	1.67	1.83	2.50	2.00	2.67
All. rich														
Overall (2)	1.43	1.46	1.31	1.31	1.32	1.24	1.37	1.38	1.49	1.25	1.20	1.62	1.28	1.58
Without Ma (8)	1.87	1.81	1.71	1.74	1.58	1.41	1.91	1.83	1.66	—	1.53	2.20	1.76	2.22
Without SDH1 (11)	1.08	1.19	1.23	1.30	1.11	1.07	1.20	1.27	1.33	1.39	1.05	1.59	1.38	1.51
$\hat{f}$	0.30*	0.06	0.48***	0.14*	0.00	0.00	0.04	0.13	0.02	0.23*	0.00	0.21*	0.05	0.17*

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Table 3 Chloroplast DNA polymorphism. The size of the amplified products, the definition of the variants and haplotypes are given together with the haplotype frequencies over the whole data set. HK and K1K2 stands for the primers pairs trnH-trnK1 and trnK1-trnK2 (Demesure *et al*. 1995). Size of amplified products and size variants are given in base pairs (bp)

	Primer code			
	HK 1500	K1K2 2500		
Size of PCR products (bp)				
Enzyme	*Alu*I	*Hpa*II	*Hin*fI	Overall frequency
Variants (bp)	1: 500 2: 550	1: 2500 9: 1700 + 800	1: 160 9: 80 + 80	
Haplotype code				
1	1	9	1	9%
2	1	9	9	55%
3	2	1	9	2%
4	2	9	9	34%

effect. Given that these values were not due to the same locus in all populations, we can exclude the null allele and selection hypotheses. The spatial autocorrelation analyses in A, S and Ma, all of which exhibited significant $\hat{f}$ values, showed no significant kinship coefficient values among individuals whatever the class of distance considered. For example, Loiselle's kinship coefficients (Loiselle *et al*. 1995) for the shortest distance classes were not significant and equal to −0.028 ($P = 0.489$), 0.012 ($P = 0.904$) and 0.015 ($P = 0.05$) in A, S and Ma, respectively. Moreover, the slope of the regression of the pairwise $a(r)/[1 - a(r)]$ statistics with the class distances was not significant: $r = -0.003$ and $P = 0.506$, $r = 0.006$ and $P = 0.512$, $r = -0.005$ and $P = 0.768$ for population A, S and Ma, respectively. It is thus likely that the observed heterozygote deficiencies are due to partial selfing rather than to a spatial Wahlund effect in these populations. Assuming genetic equilibrium under a mixed-mating model and no spatial structure, the population selfing rate s can be estimated from the value of $\hat{f}$ using the relationship $\hat{f} = s/(2 - s)$. Selfing rate was thus estimated to 0.25, 0.35 and 0.37 in population A, S and Ma, respectively.

cpDNA study. Of 32 enzyme-primer combinations tested (eight enzymes and four primer pairs), only three revealed variability at the species level (Table 3). Four haplotypes were detected over the whole studied area (Table 3). Haplotype 2 is most frequent over the whole dataset (55%). It was found in all populations except 6P and 10P, two neighbouring populations inhabiting dunes from Nord. Haplotype 2 is unique in seven populations (Fig. 1).

Table 4 Pairwise population differentiation estimates with allozymes (below the diagonal) and cpDNA haplotypes (above the diagonal) data sets. Significance of $\hat{\Theta}$ values was tested by randomizations of multilocus genotypes between populations

	B	T	M	A	N	V	Al	Br	F	Ma	6P	S	10P	Vi
B	—	0.095	0.116	0.174*	0.116	0.865***	0.720***	0.559***	0.087	0.521***	0.871***	0.169*	0.771***	0.158*
T	0.228***	—	0.000	0.000	0.000	0.970***	0.877***	0.609***	0.000	0.670***	1.000***	0.000	0.887***	0.000
M	0.199***	0.228***	—	0.000	0.000	0.972***	0.889***	0.628***	0.000	0.692***	1.000***	0.000	0.897***	0.000
A	0.158***	0.312***	0.072	—	0.000	0.978***	0.917***	0.681***	0.000	0.749***	1.000***	0.000	0.920***	0.000
N	0.133***	0.297***	0.081	0.121***	—	0.972***	0.889***	0.628***	0.000	0.692***	1.000***	0.000	0.897***	0.000
V	0.391***	0.460***	0.178***	0.219***	0.299***	—	0.039	0.788***	0.969***	0.115**	–0.012	0.978***	0.084*	0.977***
Al	0.087*	0.277***	0.032	0.072**	0.046**	0.210***	—	0.713***	0.872***	0.017	0.072	0.914***	0.081*	0.910***
Br	0.182***	0.283***	0.021	0.110***	0.116***	0.063**	0.045*	—	0.601***	0.650***	0.767***	0.676***	0.749***	0.667***
F	0.222***	0.371***	0.184***	0.104***	0.208***	0.212***	0.176***	0.149***	—	0.661***	1.000***	0.000	0.883***	0.000
Ma	0.064*	0.295***	0.122***	0.002	0.149***	0.298***	0.091**	0.161***	0.104***	—	0.113**	0.744***	0.066*	0.734***
6P	0.223***	0.377***	0.028	0.072***	0.061	0.288***	0.041	0.111***	0.241***	0.119**	—	1.000***	0.072	1.000***
S	0.139***	0.280***	0.133***	0.116***	0.174***	0.179***	0.119***	0.110***	0.103***	0.126***	0.181	—	0.919***	0.000
10P	0.168***	0.334***	0.042**	0.027*	0.085***	0.237***	0.047	0.093***	0.177***	0.036	0.024	0.127***	—	0.915***
Vi	0.112**	0.293***	0.228***	0.094***	0.233***	0.347***	0.167***	0.234***	0.076***	0.051	0.238***	0.127***	0.175***	—

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Table 5 Hierarchical analysis of variance for allozyme frequencies and cpDNA haplotypes

Source of variation	Degree of freedom	Sum of squares	Variance component	Percentage of total variance	*P*-value*
Enzymatic loci					
Among habitats	1	4.29	0.004	1.55	0.234
Among populations, within habitats	11	27.81	0.034	14.90	< 0.001
Within population	909	170.86	0.187	83.54	< 0.001
cpDNA					
Among habitats	1	9.68	–0.004	–1.21	0.433
Among populations within habitats	11	106.27	0.289	78.98	< 0.001
Within population	425	34.55	0.081	22.22	< 0.001

*Nonparametric randomization test (1000 permutations).

Overall, cpDNA haplotype diversity (H_{ecp}) is equal to 0.57, although eight populations of 14 were monomorphic (Fig. 1). For polymorphic populations, cpDNA haplotype diversity varied between 0.04 for V and 0.47 for Ma. cpDNA haplotype diversity did not differ significantly between hillside and dune habitats ($P = 0.15$). No relationship was observed between cpDNA haplotype diversity and the log-transformed number of flowering plants ($P = 0.403$).

Among-population and among-habitat analyses

Isozyme study. Considering the 14 populations, $\hat{\Theta}$ was significant ($P < 10^{-5}$) and equal to 0.16. Population pairwise $\hat{\Theta}$ values are shown in Table 4. The overall genetic isolation between populations was not due to geographical isolation, as shown by a nonsignificant test of isolation by distance over all populations ($P = 0.347$), or across populations of each habitat ($P = 0.103$ and $P = 0.120$ for hillside and dune habitats, respectively). Overall genetic differentiation is not due to a habitat effect (nonsignificant $\hat{\Theta}_{bh}$ (Table 5)). Marsh population Vi was not less differentiated from one habitat than the other. It was highly significantly differentiated from all other populations except from Ma, a population from the dune habitat located 89 km away.

Because of increased genetic drift in small populations, higher population differentiation among populations of small size is expected, unless they are close enough to other populations for gene flow to counteract drift (see Fréville *et al.* 1998). In *Parnassia palustris*, no correlation could be detected between population size and the number of significant pairwise $\hat{\Theta}$ values ($r = -0.13$, $P = 0.66$). For example, the large population V (1300 flowering

Table 6 Results of the analysis of variance excluding the population from the marsh habitat (a) or including this population (b). Means which are followed by the same letter are not significantly different. The proportion of abortive seeds (PGRV) was not scored on the same individuals and populations as the neck diameter (DIAM), the total number of flowering stems (HAMP) and the proportion of stems with capsules (PCAPS)

(a)

Trait	*F* habitat	*F* population (habitat)	Dune habitat (*N* = 120 or 55†)	Hillside habitat (*N* = 137 or 90†)
DIAM	NS	40.19***	1.35a	0.41a
HAMP	6.51*	20.36***	16.34a	4.40b
PCAPS	NS	2.66*	0.51a	0.32a
PGRV†	NS	4.56***	0.37a	0.43a

(b)

Trait	*F* population									
DIAM	71.6***	6P	10P	Ma	M	S	V	Vi	T	N
		2.42a	1.28b	1.17b	0.60c	0.54cd	0.44cde	0.31de	0.28e	0.27e
HAMP	38.23***	6P	10P	Ma	M	S	V	Vi	T	N
		28.80a	15.70b	14.80b	7.13c	6.07c	3.87c	3.63c	3.30c	3.17c
PCAPS	4.81***	Ma	6P	M	S	10P	N	T	Vi	V
		0.63a	0.53ab	0.45abc	0.44abc	0.43bcd	0.39bcde	0.30cde	0.23de	0.20e
PGRV	5.28***	Vi	T	F	B	V	S	M	Br	Ma
		0.64a	0.54ab	0.54ab	0.51abc	0.42bc	0.41bc	0.40bc	0.32cd	0.16d

NS: not significant, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, †PGRV measured on a different number of individuals from other measures.

individuals) exhibited as many significant $\hat{\Theta}$ values (13 of 13) as the smallest populations, B, T and F (Table 4).

cpDNA study. The trend observed on isozymes holds for cpDNA markers: (i) a high and significant $\hat{\Theta}$ value (0.79, $P < 10^{-5}$) was observed over all populations; (ii) no genetic differentiation was found between habitats when a hierarchical analysis of variance was performed (Table 5); (iii) population size was not related to the number of significant pairwise $\hat{\Theta}$ values ($r = 0.39$, $P = 0.16$). However, among population genetic differentiation on isozymes was not correlated with genetic differentiation obtained for cpDNA markers (Mantel test, $P = 0.634$). For cpDNA haplotypes, maximum values of $\hat{\Theta}$ (1.00) were observed between populations which were composed of different exclusive haplotypes, e.g. between population 6P (100% of haplotype 4) and population F (100% of haplotype 2) (Fig. 1). Population Br which showed an original haplotype composition (Fig. 1) was highly differentiated from every other population (Table 4).

Conversely to the isozymes, an isolation by distance pattern was found to be significant overall ($P = 0.025$). This result was explained by the presence of population 6P only ($P = 0.137$ when 6P was excluded from the analysis). This population is the only one showing both a nonsignificant value of $\hat{\Theta}$ at the shortest distance (0.55 km apart from population 10P where haplotype 4 was observed at high frequency) and the highest possible values (i.e. 1.00) at longer distances. When considering each habitat separately, no isolation by distance was found to be significant ($P = 0.078$ for the dune habitat with 6P and $P = 0.213$ without 6P, and $P = 0.794$ for the hillside habitat).

Phenotypic study. Habitat differentiation was found to be significant on the total number of flowering stems only (HAMP), the dune habitat showing more flowering stems than the hillside habitat (Table 6a). Within-habitat population effect was, however, higher than habitat effect for this character. The three other phenotypic traits (DIAM, PCAPS and PGRV) were significantly differentiated among populations, but not among habitats (Table 6a). When including the marsh population Vi, populations were highly differentiated for all characters (Table 6b). Population Vi was not significantly different from hillside populations V, T and N for HAMP, DIAM and PCAPS, showing low values for these characters. The highest proportion of abortive seeds was observed in Vi and it was not significantly different from some hillside (T and B) and dune (F) populations (Table 6b). Interestingly, plants from dunes from Nord (6P, 10P and Ma) showed the largest neck diameter, the highest number of flowering stems, the highest proportion of fruits and the lowest proportion of abortive seeds.

HAMP was positively correlated to DIAM ($r = 0.99$ at $P = 0.0001$) and PCAPS ($r = 0.67$ at $P = 0.0461$), but no relationship was found with the population size ($P = 0.3893$, 0.3720 and 0.9720 for HAMP, DIAM and PCAPS,

respectively). PGRV was negatively correlated to these characters ($r = -0.91$ at $P = 0.0110$ for HAMP, $r = -0.94$ at $P = 0.0049$ for DIAM, and $r = -0.83$ at $P = 0.0396$ for PCAPS), but not related to population size ($P = 0.7249$).

In the bagging experiment, all flowers produced fruits and all fruits produced seeds. Results of ANOVA on seed characteristics showed that in self-fertilized fruits, the proportion of abortive seeds and the proportion of germination of viable seeds were, respectively, higher ($F = 23.03$ at $P < 0.001$ level) and lower ($F = 3.89$ at $P < 0.05$ level) than in hand-pollinated or open-pollinated fruits.

Discussion

A hierarchical analysis of the distribution of the nuclear and chloroplastic genetic diversity, as well as of phenotypic traits within and among habitats inhabited by the endangered species *Parnassia palustris*, gave insights about the level of isolation between the natural populations. At equilibrium under a mutation-drift model, genetic diversity is expected to decrease with effective population size. Many empirical studies have confirmed this theoretical expectation (Lammi *et al*. 1999; Luijten *et al*. 2000). Twenty-three studies of animals and plants were reviewed by Frankham (1996). This meta-analysis showed significant associations between logarithm of population size and all measures of allozyme variation (gene diversity, observed heterozygosity, allelic diversity and percentage of polymorphic loci), although significant correlations were not found within every species. Other recent studies did not find correlations at the within species level (Hurtrez-Boussès 1996; Barrett & Husband 1997; Young *et al*. 1999; Schmidt & Jensen 2000). At least two reasons can be invoked to explain this result: (i) population sizes might have been underestimated if the studied populations are connected to other populations which were not sampled. Migration is thus likely to counterbalance drift in some populations. For instance, in *Eucalyptus albens*, Prober & Brown (1994) found a significant relationship between log population size and measures of allozyme variation, with some small populations, however, exhibiting higher genetic variation than expected. This was explained by their geographical proximity to a bigger population (see also Fréville *et al*. 1998 on *Centaurea* species); (ii) all populations of *P. palustris* occur in human managed habitats. They are likely to have experienced different histories including bottlenecks and changing degree of connection to other populations. Current population genetic diversity reflects different unknown histories of populations that might not be correlated with current population sizes.

Moderate heterozygote deficiencies were observed in six of the 14 populations studied (average $\hat{f}$ value of 0.11) whereas panmixia was observed for eight of 14 populations. A spatial Walhund effect is unlikely given the results of the spatial autocorrelation analyses. Bagging experiments suggest that *P. palustris* is self-compatible. The occurrence of significant $\hat{f}$ values in some populations could thus be explained by partial selfing. These results lead to the conclusion that neighbourhood sizes are large in populations of *P. palustris* from Northern France. They also suggest that seeds and/or pollen migrate over long-distance at a local scale, such that genetic clustering of kin is unlikely.

Within-population analyses suggest that dispersal is not limited at a local scale. This result holds for pollen dispersal at a regional scale. Isozymes showed a moderate and significant genetic differentiation which is not due to a genetic isolation among habitats. A lack of isolation by distance coupled with a lack of clear differentiation on pairwise genetic distances suggests that the overall gene flow is not strongly limited at the geographical scale of this study (i.e. regional scale). In *P. palustris*, mean F_{ST} values among populations was five times higher for cpDNA markers than for isozymes, which was not due to a habitat effect. According to Ennos (1994), supposing that populations are at migration-drift equilibrium, pollen migration contributes 20 times more to gene flow among populations than seed migration in *P. palustris*. The high limitation of seed migration may explain the significant F_{ST} for isozymes. Pollen/seed migration rates equal or higher than 20 have already been observed in *Quercus* spp. (Kremer *et al*. 1991), *Pinus contorta* (Dong & Wagner 1993) or *P. sylvestris* in Scotland (Sinclair *et al*. 1998). Although diaspores may be transported for long distances by animals (squirrels) in these species, the contribution of seed dispersal to gene flow is negligible compared to pollen dispersal by wind. This result is more surprising in *P. palustris*. The presence of nectaries suggests insect pollination and consequently a lower contribution of pollen to gene flow among populations than in *Quercus* or *Pinus* species. The tiny seeds (about 0.02 mg) possess a wing, and are a priori well adapted to wind dispersal over long distances. Potentially, they can also be transported with mud on the feet of animals (and humans). Our results show that they actually disperse very rarely among populations. Further investigations on dispersal modes would resolve this paradox between ecological observations and genetic analysis in *P. palustris*.

Seed migration in *P. palustris* seems to occur at a smaller scale than the one considered in this study, as suggested by a high mean F_{ST} found on cpDNA markers and an isolation by distance pattern mainly due to the two closest populations (0.55 km between 6P and 10P). For example, a cpDNA F_{ST} value of 0.97 was found between M and V separated by only 6 km (Table 4), and our study was conducted over about 100 km along the Channel coastline. Colonization may occur in *P. palustris* if suitable sites are separated by hundreds of metres but is very unlikely if suitable sites are separated by several kilometres.

Fragmentation of all habitats suitable for this species is thus likely to have a negative impact.

Using genetic markers and scoring in the field characters related to fitness, we studied the distribution of genetic and phenotypic variation in *P. palustris* populations occurring in contrasted habitats. Matings between individuals adapted to different habitats are likely to lead to outbreeding depression. Dissolution of coadapted gene complexes would be at the origin of such a depression (Lynch 1991; Schierup & Christiansen 1996). Indeed, outbreeding depression is well known in crop science when varieties have undergone opposite selections, and in wild plants when matings occur between distant individuals from different populations (Fenster & Galloway 2000), or even from the same population (Waser & Price 1989; Quilichini *et al*. 2001). No data are available to provide evidence for outbreeding depression in *P. palustris*. However, its ecological characteristics suggest that this phenomenon is likely to occur in this species. Effective gene flow is then expected to be much higher among populations from the same habitat than among populations from different habitats. However, we found restricted gene flow among populations whatever the habitat. No habitat effect could be detected on the variation of either nuclear or cytoplasmic genetic markers, suggesting that these loci are not directly or indirectly influenced by habitat-related divergent selection. Depending on the balance between selection and hybridization, genetic differentiation can be observed in association with ecological separation (Van Rossum *et al*. 1997; Gauthier *et al*. 1998) or not (Tomas *et al*. 2000; Vijverberg *et al*. 2000). Nevertheless, in our study even ecological specialization was difficult to observe. Only one of four traits related to fitness showed moderate habitat separation: the dune habitat possessed more flowering stems than the hillside habitat. Variation between populations within-habitat was, however, higher than variation between habitat for this character. Unless studied characters are neutral, they were therefore expected to vary among habitats due to either local adaptation (genetic variation) or plasticity (environmental variation). Our results suggest that local adaptation, if any, is not strong enough to lead to differentiated ecotypes.

Our results are of relevance to the management of the endangered species *P. palustris* in Northern France. The lack of observation of new populations for 20 years coupled with the increasing extinction rates of natural populations can be explained by a limitation of seed migration. This trait prevents colonization of new sites and may endanger the conservation of *P. palustris*, especially if the availability of suitable habitats is decreasing. Nevertheless, well-established populations exchange moderate gene flow, possess high genetic diversity and do not appear to have undergone severe genetic bottlenecks in the recent past. Thus, detailed demographic studies would now be needed to assess the conservation status of *P. palustris* more effectively.

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Researchers from the laboratory Génétique et Evolution des Populations Végétales are involved in studies of dispersal and reproductive system in plant species. Bruno Colas carries out research on rare or endangered plant species. Frédéric Hendoux and Benoit Destiné are involved in the management and conservation policy of endangered plant species from Northern France.

Appendix

Allele frequencies and sampling size for each enzymatic locus within each population and overall the study

		Hillside								Dune					Marsh		
Locus	Alleles	B	T	M	A	N	V	Al	Br	F	Ma	6P	S	10P	Vi	all	H_E
MDH1		*N* = 24	*N* = 25	*N* = 27	*N* = 53	*N* = 28	*N* = 49	*N* = 30	*N* = 43	*N* = 21	*N* = 40	*N* = 26	*N* = 46	*N* = 33	*N* = 44		
	1	0.792	0.960	0.981	0.745	0.964	1.000	0.933	0.977	0.571	0.688	0.962	0.772	0.879	0.477	0.828	
	2	0.208	0.040	0.019	0.255	0.036	0.000	0.067	0.023	0.429	0.313	0.038	0.228	0.121	0.523	0.172	0.285
MDH2		*N* = 11	*N* = 17	*N* = 16	*N* = 40	*N* = 12	*N* = 26	*N* = 22	*N* = 17	*N* = 21	*N* = 28	*N* = 17	*N* = 18	*N* = 20	*N* = 23		
	1	1.000	1.000	1.000	1.000	1.000	0.981	1.000	1.000	1.000	0.893	0.971	0.917	0.875	0.978	0.970	
	2	0.000	0.000	0.000	0.000	0.000	0.019	0.000	0.000	0.000	0.107	0.029	0.083	0.125	0.022	0.030	0.058
PGI1		*N* = 23	*N* = 25	*N* = 26	*N* = 49	*N* = 24	*N* = 46	*N* = 30	*N* = 42	*N* = 21	*N* = 43	*N* = 26	*N* = 48	*N* = 36	*N* = 42		
	1	0.978	1.000	0.865	0.898	1.000	0.511	0.900	0.702	0.667	0.930	1.000	0.542	0.917	0.845	0.816	
	2	0.022	0.000	0.135	0.092	0.000	0.489	0.100	0.298	0.333	0.070	0.000	0.115	0.056	0.107	0.142	
	3	0.000	0.000	0.000	0.010	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.344	0.028	0.048	0.042	0.312
PGM1		*N* = 23	*N* = 25	*N* = 24	*N* = 47	*N* = 26	*N* = 37	*N* = 29	*N* = 37	*N* = 20	*N* = 36	*N* = 26	*N* = 39	*N* = 30	*N* = 42		
	1	1.000	0.980	1.000	1.000	0.827	1.000	0.983	0.973	0.800	1.000	1.000	0.987	1.000	0.988	0.974	
	2	0.000	0.020	0.000	0.000	0.173	0.000	0.017	0.027	0.200	0.000	0.000	0.013	0.000	0.012	0.026	0.051
PGD1		*N* = 16	*N* = 20	*N* = 11	*N* = 44	*N* = 25	*N* = 38	*N* = 30	*N* = 27	*N* = 20	*N* = 33	*N* = 25	*N* = 30	*N* = 29	*N* = 25		
	1	1.000	0.475	0.864	0.955	1.000	1.000	1.000	0.963	1.000	0.970	1.000	0.983	1.000	0.960	0.953	
	2	0.000	0.525	0.136	0.045	0.000	0.000	0.000	0.037	0.000	0.030	0.000	0.017	0.000	0.040	0.047	0.090
SDH1		*N* = 9	*N* = 10	*N* = 15	*N* = 27	*N* = 12	*N* = 32	*N* = 21	*N* = 14	*N* = 13	*N* = 2	*N* = 19	*N* = 8	*N* = 11	*N* = 13		
	1	0.111	0.200	0.733	0.833	0.583	0.859	0.524	0.643	0.769	1.000	0.737	0.563	0.864	0.462	0.665	
	2	0.389	0.450	0.033	0.074	0.250	0.125	0.119	0.107	0.000	0.000	0.079	0.125	0.091	0.154	0.131	
	3	0.222	0.100	0.000	0.000	0.000	0.000	0.167	0.000	0.000	0.000	0.000	0.125	0.000	0.154	0.046	
	4	0.222	0.000	0.200	0.037	0.167	0.000	0.095	0.179	0.231	0.000	0.158	0.188	0.045	0.077	0.104	
	5	0.056	0.250	0.033	0.056	0.000	0.016	0.095	0.071	0.000	0.000	0.026	0.000	0.000	0.154	0.053	0.525

