

Relationship between genetic structure and seed and pollen dispersal in the endangered orchid *Spiranthes spiralis*

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Summary

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Received: 4 September 2002
Accepted: 15 November 2002

- We studied how the genetic structure helps in understanding the demographic history of a species and predicting its future, using the endangered orchid *Spiranthes spiralis*.
- A Parisian population was mapped during three consecutive years. Its genetic structure was assessed using isozymes and compared with several populations from southern France. Seed dispersal distances were experimentally evaluated. Computer simulations were performed to reconstruct the population history.
- Unlike southern France populations, the Parisian population exhibited two genetically distinct patches. Seed dispersal was limited to the neighbourhood of the fruiting plants. The simulations showed that the best explanation for the observed genetic structure was a recent colonization event by two small groups of founders.
- Current patterns of gene flow should result in a rapid genetic and demographic homogenization. New populations of *S. spiralis* could be successfully established by few founders and exhibit significant growth rates without any detrimental effects of inbreeding. This offers insight into the successful management of endangered plant species in urban areas.

Key words: genetic structure, inbreeding, *Spiranthes spiralis*, gene flow, foundation, population differentiation.

© *New Phytologist* (2003) **157**: 677–687

Introduction

Extinction and recolonization of populations are frequent events in the histories of species. When extinctions are more frequent than colonization events, species are locally endangered. In highly urbanized regions, where plant populations are particularly subject to habitat destruction, their distribution can become highly fragmented, putting them at risk of local extinction. Species' abilities to avoid extinction by maintaining stable, isolated populations and by establishing new colonies at unoccupied sites contribute critically to the maintenance of biodiversity in urban regions. Thus, conservation plans for the maintenance of isolated

populations will depend on detailed study of the breeding system of the species and the genetic structure and the demographic history of the populations (Holsinger & Gottlieb, 1991).

The orchid *Spiranthes spiralis* is widely distributed in southern Europe, in the Mediterranean region, and in north Africa. In southern France it is also relatively widespread. In northern France, however, it is a rare and endangered species; it is legally protected in 31 of the 95 administrative regions of France. In the Parisian region (within a 100 km radius of central Paris), the number of populations has dramatically declined during the last two centuries, from 30 populations distributed around Paris in the eighteenth century (Arnal, 1996) to only

two currently remaining populations, located in the Fontainebleau forest, 60 km from Paris. In this region the species is classified as on the brink of extinction (Arnal, 1996).

The decline in population number is probably the result of habitat destruction, changes in forest management and secondary succession (resulting in closed canopy forests). The two remaining populations in Fontainebleau forest are located in very unusual sites. The first is located on an aqueduct that supplies the city of Paris with potable water. In 1999, this population was comprised of about 600 rosettes, some of which flowered (N. Machon, pers. comm.). The second site is occupied by about 20 plants, and is located in the lawn of a botanical research institute (Station de Biologie Végétale, Fontainebleau, created in 1889). The plants in the second site were likely introduced from natural populations by the French botanist Gaston Bonnier between 1889 and 1922 (unpubl. data). In the Parisian region, extensive surveys have discovered no other extant populations. The nearest populations are located more than 100 km from the populations in the Fontainebleau forest.

To date, there has been little information available concerning the demography of natural populations of *S. spiralis* or on the life history parameters that may affect their stability. Such information is essential for the proper design of conservation strategies. The aims of this paper are: (1) to describe the population genetic structure of the unique extant Parisian population of *S. spiralis*, in comparison with populations from southern France (in Ardèche); (2) to try to explain this genetic structure based on the observed life history traits of the species; (3) to use a model to infer historical events that affected the Parisian population; and (4) to suggest guidelines for the management of endangered plants in urbanised areas.

Materials and Methods

The species

Spiranthes spiralis (L.) Chevall. is widely distributed in southern Europe and in the Mediterranean region. It is a self-compatible geitonogamous species that depends on insect pollinators for seed production (Willems & Lahtinen, 1997). It is a long-lived orchid species: individuals may live more than 50 yr. According to Wells (1981), the first rosette appears about 11 yr after seed germination and the first flowering stalk 2–4 yr later. Individual plants consist of rosettes and one or more small underground tubers that may live for several decades. New tubers are formed during the winter and spring. The rosette leaves emerge in autumn and die off in late spring or early summer. Inflorescences emerge in late summer just before the emergence of the rosettes.

The white and green nectar-producing flowers (Willems & Lahtinen, 1997) are less than half a centimetre long. The flowers form a single row that spirals up the inflorescence. Each plant can produce up to three inflorescences, each bearing up

to 25 flowers. The inflorescences are visible from August to September. There is no bending of the caudicle of the pollinia to reduce self pollination but outcrossing is promoted by sequential flowering, by protandry (Summerhayes, 1951; Pijl & Dodson, 1966) and by the viscidium that adheres to visiting bumblebees (Catling, 1983). In October to November the capsules become ripe and seeds are wind-dispersed.

The sites

Two regions in France were sampled: one near Paris, in Fontainebleau forest, the second in southern France, in Ardèche.

Fontainebleau forest (60 km south of Paris) The only remaining forest population in the vicinity of Paris (thereafter denoted Population F1) was sampled at the 'de la Vanne' aqueduct. The aqueduct is 110 km long and 3 m wide. It is covered by a heliophytic and herbaceous vegetation and the soil tends to be wet but well-drained. The vegetation is mowed every autumn.

Ardèche sites The sites are located about 600 km south of Paris. Six populations (denoted A1 to A6) were studied. They were all located along a 2-km transect on a west facing slope of a mountain named 'Travers de l'Outre' (400 m elevation at its summit). A1, A3 and A4 are located in garrigues (dominated by *Juniperus communis* and *Quercus ilex*), A2 and A6 occupy grazed lands, and A5 inhabits an orchard.

Exhaustive sampling was conducted in five of the six southern populations (119 individuals in A1, 42 in A2, 10 in A3, 50 in A4 and 27 in A6). In population A5, more than 200 individuals were observed but only 65 were randomly sampled. In the Parisian population (F1), all of the 544 individuals were sampled.

Demographic data

The Parisian population (F1) covered a surface of approximately 20 m × 3 m on the aqueduct. Individual plants were mapped using a grid of 50 cm × 50 cm quadrats. The coordinates on the grid of each plant were recorded. To show the demographic structure of the population, we subdivided the population arbitrarily along the entire width of the aqueduct into 10 segments of 2 m width each.

Electrophoretic data

From each individual plant, a leaf sample comprising 1 cm² was obtained from the apex of one rosette leaf. These samples were stored in Eppendorf tubes that were plunged into liquid nitrogen just after being harvested in the field. The 857 individuals were analysed by starch gel enzyme electrophoresis. The leaf samples were crushed in the laboratory with a rotating glass bar under crushing buffer to solubilize and

stabilize the enzymes. The crushing buffer was composed of 60 mg of cysteine, 75 mg of dithioerythritol, and 2 g of polyvinylpyrrolidene (PVP) in 50 ml of tris HCl 0.1 M, pH 7.2.

For each sample, four enzymes were stained – peroxidase (PRX), leucine aminopeptidase (LAP), glutamate-oxaloacetate-transaminase (GOT) and acid-phospho-monoesterase (ACP) – with lithium-borate buffer (pH 8.3), for which migration lasted about 8 h at 40 mA per gel (Shikimate dehydrogenase (SKDH), diaphorase (DIA) and alcohol dehydrogenase (ADH) were discarded because of poor resolution).

We computed allelic frequencies for the PRX locus in each population. Deviations from Hardy–Weinberg expectations were examined for this locus within each population by calculating Wright's fixation index, F (Wright, 1922). Genetic parameters and statistical tests were calculated using GENEPOP (Rousset & Raymond, 1995) and TFGA (Miller, 1997). The Weir and Cockerham (1984) method is used for F -statistics, and exact tests were performed using Guo & Thompson's (1992) method.

Spatial genetic structure

The sampled individuals in two of the seven populations (populations F1 and A1) were carefully mapped, providing the spatial coordinates and the PRX genotype of all the individuals of the populations. This allowed us to compute the kinship coefficients (Loiselle *et al.*, 1995) between each pair of individuals as a function of their distance in both populations. These computations were performed with AUTOCOR (Hardy & Vekemans, 1999). For representation purposes, we subdivided the pairs of individuals into 10 distance classes of equal size and the average kinship coefficient for each class was computed. The significance level of these average coefficients was assessed through 10 000 permutations.

Vegetative reproduction

In population F1, we observed that interplant distances were frequently very short. We wanted to rule out the hypothesis that a nonnegligible fraction of these adjacent plants represented vegetatively produced clones. For that purpose, using the proportions of each genotype observed in pairs of adjacent individuals, we performed a χ^2 test to determine whether the genotypes of adjacent individuals were identical more often than expected by chance.

Seed dispersal

To evaluate seed dispersal in population F1, seed traps were placed along the top of the aqueduct at regular intervals. The traps consisted of 10-cm diameter Petri dishes containing an aqueous solution of 30% of glycerol and several drops of detergent. Three intervals of seed dispersal were monitored during the autumn of 1998: (1) from 6 to 12 October, (2)

from 12 to 16 October, and (3) from 16 to 22 October. At the beginning and end of each interval, the Petri dishes were deposited and retrieved, respectively, at 15 : 00 hours. For the first interval, the dishes were placed at 50 cm intervals along a 36-m transect that traversed the population, in the middle of the aqueduct. During the second interval (12–16 October), in addition to these 40 dishes, 33 dishes were placed every metre along the northern and the southern edges of the aqueduct. During the third interval, at one site where six inflorescences were nearly adjacent to each other (less than 20 cm apart), 40 additional dishes were placed as near as possible to each other and to the inflorescences.

The distances from the centre of each dish to the three nearest inflorescences were recorded. At the end of each observation period, the dishes were observed in the laboratory under a microscope ($\times 70$) to record the number of seeds per dish. Although it can be difficult to distinguish seeds of different species of orchids, we could assume that all the seeds that we counted were *S. spiralis* seeds because no other orchids were dispersing their seeds on the aqueduct in autumn. There were a few senescent inflorescences of *Gymnadenia conopsea*, but they had been previously removed from the top of the aqueduct.

We performed statistical analyses using SAS (SAS Institute Inc., 1990) to describe seed dispersal in population F1. First, an ANOVA was conducted to determine whether the monitoring periods differed with respect to the mean number of seeds found in each Petri dish. Second, for each dish containing seeds, we plotted the number of seeds as a function of the distance to the nearest plant dispersing seeds. We determined the best-fitting curve to explain the data using PROC NLIN.

The Model

The simulations were conducted using a stochastic, spatially explicit model of a metapopulation consisting of 10 demes distributed in a linear array. Demographic and genetic evolution of the metapopulation were simulated using the software METAPOP (Machon *et al.*, 1995). This software simulates the combined effects of migration, drift, selection, mutation and demography. In the present study, selection and mutation were assumed to be absent. Migration is modelled by using seed and pollen flow matrices (Appendix 1). Seed migration was assumed to follow an isolation by distance model (Wright, 1943; Malécot, 1948) with a low migration rate. Pollen dispersal followed a model in which the probability of migration from one deme to another decreased with the distance between them, except for the within-patch fertilization. We compared four models of pollination (matrices P1 to P4 in Appendix 1), for which within-patch fertilization was (1) impossible, (2) low (the proportion of within-patch pollen in the pollen cloud of each patch is $P = 0.02$), (3) medium ($P = 0.11$) and (4) high ($P = 0.22$). The choice of these pollen dispersal models was based on the particular reproductive characteristics of this species: pollinia

adhere to visiting bumblebees and are eventually deposited on another plant (Heinrich, 1975). Bumblebees optimize foraging by visiting adjacent flowers (or plants) until they receive no more nectar, at which point they move to the next nearest plant. Given that pollinia remain stuck to a given bumblebee for only a short period of time, pollinia typically travel between neighbouring plants before being deposited.

Because *S. spiralis* has overlapping generations and a long pre-reproductive phase, we used the demographic model with several classes of individuals and density-dependence developed by Austerlitz *et al.* (2000) for tree species. This allowed us to evaluate the impact of the juvenile phase on population structure. For this, we used a model with 30 age classes. In the first 15 age classes, plants did not reproduce. In the last 15 classes, fecundity was $f = 20$ in the absence of density dependence (i.e. each plant produces 20 offspring per year). We assumed that all individuals of all classes (except for the last one) survived and progressed to the next class each year and that all the individuals of the last class died after their final reproductive event, after 30 yr of life. We compared this simulation with the evolution of a metapopulation of annual plants using the same gene flow matrix. In that case, we assumed that the demes grew logistically with a growth rate of 2 towards a carrying capacity of 100.

We followed the evolution of population sizes and excess heterozygosity (F_{IT}) in the population for a biallelic nuclear locus. F_{IT} represents the reduction in heterozygosity relative to what is expected under Hardy–Weinberg equilibrium at the whole population level. The more highly negative the value of F_{IT} , the greater the excess heterozygosity. This parameter is denoted F_{IT} because it is measured over the whole set of demes in the model. It is however, equivalent to the F_{IS} measured experimentally in each population, this F_{IS} being computed without any assumption of population subdivision. The initial conditions were as follows: all the demes were empty except demes 3 and 7, which contained one juvenile (non-flowering rosette) individual (class 10) with AA and BB genotype, respectively. We also computed allelic frequencies in each deme 23 yr after the colonization event.

Results

Spatial distribution

The Parisian population (F1) is approximately 20 m long and 3 m wide. In 1997, 1998 and 1999, respectively, 96, 136 and 575 plants were counted in this population. The plants were not regularly distributed, as two patches of individuals could be distinguished, centred on the third and seventh subpopulations (Fig. 1) (according to the arbitrary divisions described above). No apparent microenvironmental structure can explain this aggregation.

The area occupied by population A1 in southern France is approximately 30 × 20 m. The individuals were also patchily

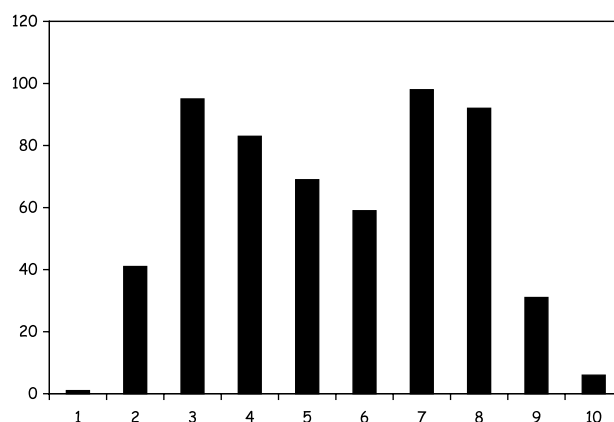


Fig. 1 Number of plants found in each subpopulation of the Parisian population F1 of *Spiranthes spiralis*. This population was arbitrarily subdivided along the entire width of the aqueduct in to 10 segments of 2 m each.

Table 1 Sample size and allelic frequencies for peroxidase (PRX) locus in the seven studied populations

Population	Allelic frequencies for PRX	F_{IS}
F1	0.53 / 0.47	-0.136**
A1	0.70 / 0.30	-0.060
A2	0.50 / 0.50	-0.036
A3	0.55 / 0.45	-0.370
A4	0.44 / 0.56	-0.281*
A5	0.57 / 0.43	-0.374**
A6	0.59 / 0.41	-0.201

*, **Significant at $P < 0.05$ and $P < 0.01$, respectively.

distributed there, but the demographic structure coincided with the ecological structure of the site: *S. spiralis* does not grow where there were sandstone outcrops. Elsewhere, individuals were regularly distributed.

Enzymatic markers

The four systems revealed four loci. Three of the loci were monomorphic: ACP, GOT, and LAP, with the same allele found in all populations. Peroxidase was polymorphic, and its expression appeared to be disomic. This locus had two alleles (A and B). Table 1 shows the allelic frequencies for this locus, calculated for the seven populations sampled. Weak but statistically significant differentiation among these populations was detected, with a global F_{ST} of 0.0255. Significant differentiation was also detected between F1 and the southern France populations ($F_{ST} = 0.022$).

Genetic structure of *S. spiralis* populations

Table 1 reports the F_{IS} values. It shows that the F1, A4 and A5 populations exhibit a significant excess in heterozygotes. For

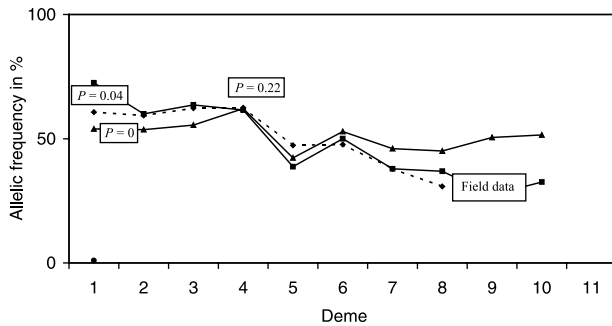


Fig. 2 Simulated (solid lines) allelic frequencies along the metapopulation 23 yr after its foundation, for various levels of pollen flow among subpopulations (P), contrasted with field data (dashed line).

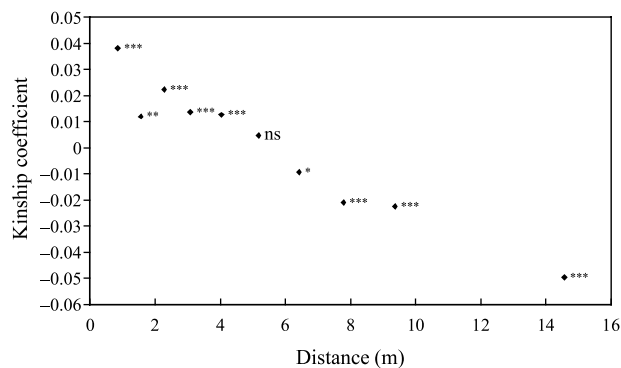


Fig. 3 Loiselle's kinship coefficient between pairs of individuals as a function of their physical distance. The pairs of individuals were grouped into 10 different classes of equal size. The values shown are the average kinship coefficients in each class, along with their significance level (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, nonsignificant).

the other populations (A1, A2, A3 and A6), the F_{IS} was not significantly different from zero. These populations can be considered to be at Hardy–Weinberg equilibrium.

For the Parisian population F1, a clear spatial pattern was observed for locus PRX; the frequency of the A allele decreased from the east to the west end of the population (Fig. 2). Kinship decreased linearly with distance (Fig. 3) as expected in a one-dimensional population but not linearly with the logarithm of distances (data not shown), as would be expected in a two-dimensional population (Rousset, 1997, 2000; Hardy & Vekemans, 1999). The slope of the regression is $a = -3.67 \times 10^{-5}$. The neighbourhood size (N_b) is deduced from (Hardy & Vekemans, 1999):

$$N_b = -(1 - F_{IS})/a$$

$$(F_{IS} = -0.136, \text{ so } N_b = 155.6).$$

In one dimension (Rousset, 1997)

$$N_b = 4D\sigma^2$$

(D is the linear density (individuals per metre) and s is the standard deviation of gene dispersal distance, which is assumed to be symmetric and thus with an expected value of 0). Here we observed 575 individuals distributed along a 20-m transect, so $D = 28.75$ individuals. m^{-1} , and thus $\sigma = 1.16$ m. Thus, gene flow is relatively high compared with the spatial scale of the population. However, this value has to be accepted with caution because the equations above are equilibrium formulae and we have no evidence that this population has reached equilibrium.

For the A1 population at Ardeche, kinship slightly decreases linearly with the logarithm of distances (data not shown), as expected in a two-dimensional population (Rousset, 1997, 2000; Hardy & Vekemans, 1999) ($P = 0.0359$). The slope of the regression is $a = -0.006468$. Here $F_{IS} = -0.060$, so $N_b = 1064$. In two dimensions (Rousset, 1997)

$$N_b = 4\pi D\sigma^2$$

(D is the spatial density of individuals and s the standard deviation of gene dispersal distance). Here we had 119 individuals in a 26.5×10.79 m rectangular area, so $D\sigma^2 = 84.7$ individuals. m^{-2} , so $\sigma = 14.3$ m. Thus, gene flow was high relative to the physical dimensions occupied by the population.

Detection of vegetative reproduction

In the F1 population, we observed 61 pairs of individuals growing adjacent to each other, including 26 aa, 75 ab, 21 bb genotypes. Within each of these pairs, each individual was less than 5 cm away from its neighbour. Given the genotype frequencies observed among these pairs, we expected to observe 27.61 pairs of genetically identical individuals (aa,aa or ab,ab or bb,bb) and 33.36 pairs of genetically dissimilar individuals (aa,ab or aa,bb, or ab,bb). We observed 31 genetically identical and 30 genetically dissimilar pairs of individuals. A χ^2 test ($\chi^2 = 0.747$, $df = 1$, $P = 0.387$) shows that the observed distribution of the genotypes is not significantly different from random so, even if vegetative reproduction occurs, it has a minor influence on the genetic structure in these populations of *S. spiralis*.

Seed dispersal

Two approaches were used to evaluate long- vs short-distance seed dispersal of *S. spiralis* in the aqueduct population (F1). The first approach focused on the Petri dishes placed along transects that traversed the population. The second approach focused on the short-distance dispersal of seeds into the Petri dishes placed very close to several inflorescences during the third interval.

The first approach captured very few seeds; only four, nine and five seeds were found, respectively, among the 73 Petri

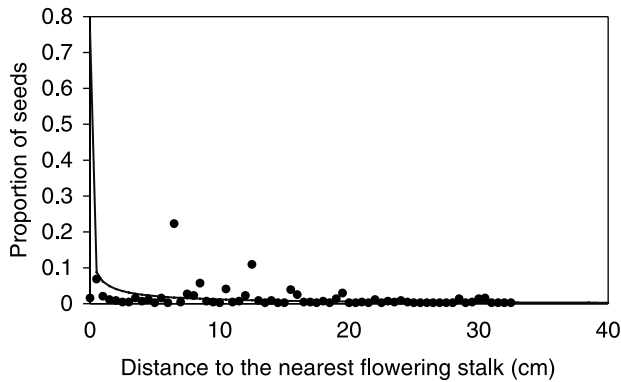


Fig. 4 Observed values (circles) and theoretical model of seed dispersal (line is theoretical distribution).

dishes observed during all three monitoring periods. During the second monitoring period, among the 33 additional dishes placed along the south and north boundaries of the population, 20 additional seeds were counted. With the second approach, a total of 439 seeds were found in the 40 dishes placed close to the inflorescences.

We then evaluated the factors influencing seed deposition by including data only from the dishes in which seeds were found ($n = 66$ Petri dishes). Using these data, we performed ANOVAS to detect differences among the three sampling periods ($P = 0.5312$). The three periods did not differ significantly with respect to mean seed deposition per Petri dish.

Given this result, we pooled the 66 Petri dishes from all sampling periods. Because the data showed a nonlinear relationship between the proportion of seeds dispersed and the distance to the nearest dispersing infructescence (Fig. 4), we used PROC NLIN to find an exponential model that could

explain the observed distribution. The mathematical form used here is

$$f(x) = a.e^{b.x^c}$$

(x is the distance to the nearest infructescence). The best-fitting curve was found for $(a, b, c) = (1.000, -2.5094, 0.2192)$. With these parameters, the sum of squares of the residuals is 0.05834.

Thus, most seeds are found in the dishes very close to the dispersing infructescences. Many dishes contained very few seeds (one or two) even at short distances from the infructescences. The dishes containing relatively high numbers of seeds were always very close to the dispersing plants; all dishes containing ≥ 10 seeds were located < 15 cm from the closest plant (Fig. 4). Seeds were found up to 11 m from the nearest plant; 95% of the seeds were found less than 135 cm from dispersing infructescences and 50% were located within 5 cm of the nearest infructescences.

The model

Assuming an annual life history, simulations show that within a few generations, the population reaches demographic (Fig. 5) and genetic (Fig. 6) equilibrium. Assuming a perennial life history, starting with the two founders, for the subsequent 15 yr the demes were occupied only by the offspring of the two founders (Fig. 5). Then, the young reached adulthood and began to reproduce; the demes were then filled by these offspring until carrying capacity was reached. During the initial phase, we observed an excess of heterozygotes. When the first offspring cohort began to reproduce, F_{IT} increased towards zero.

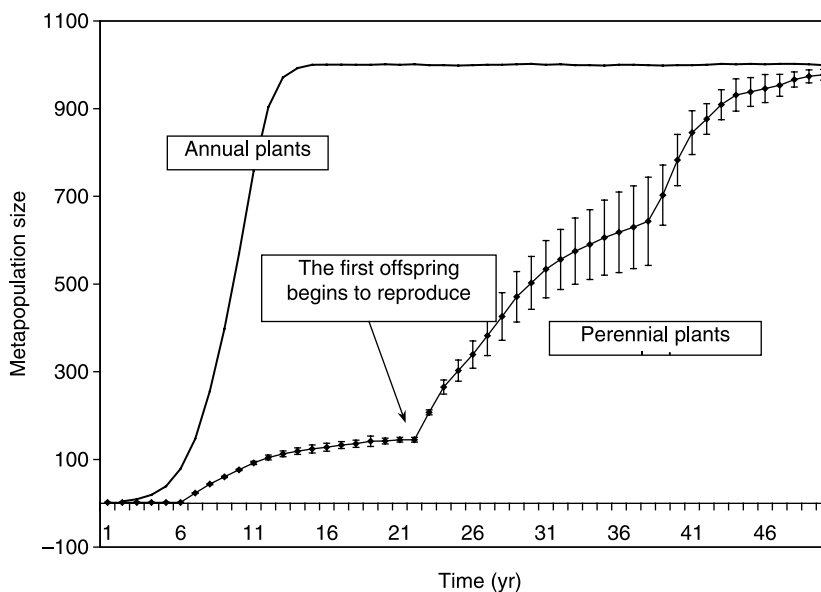


Fig. 5 Metapopulation dynamics of annual or perennial plants. Values are means \pm SD of 10 simulations.

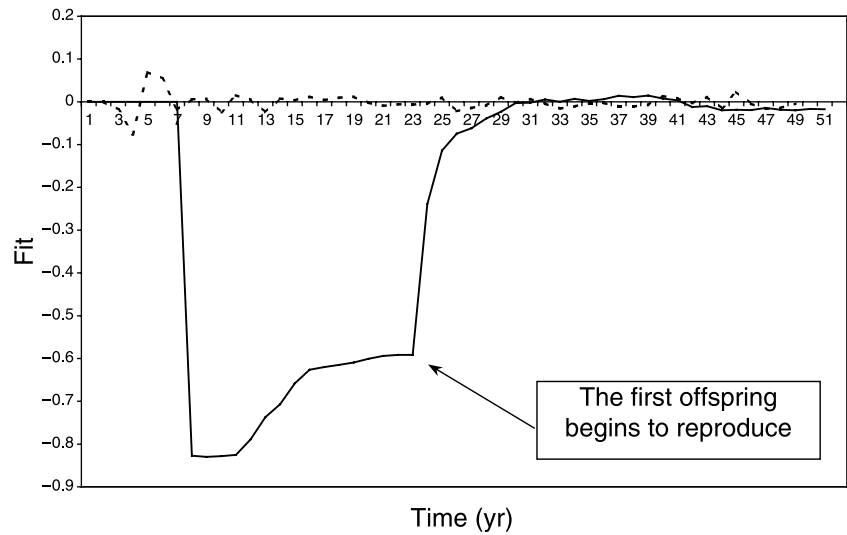


Fig. 6 Evolution of FIT for annual (dashed line) and perennial (solid line) plants. Means of 10 simulations by METAPOP software (Machon *et al.*, 1995).

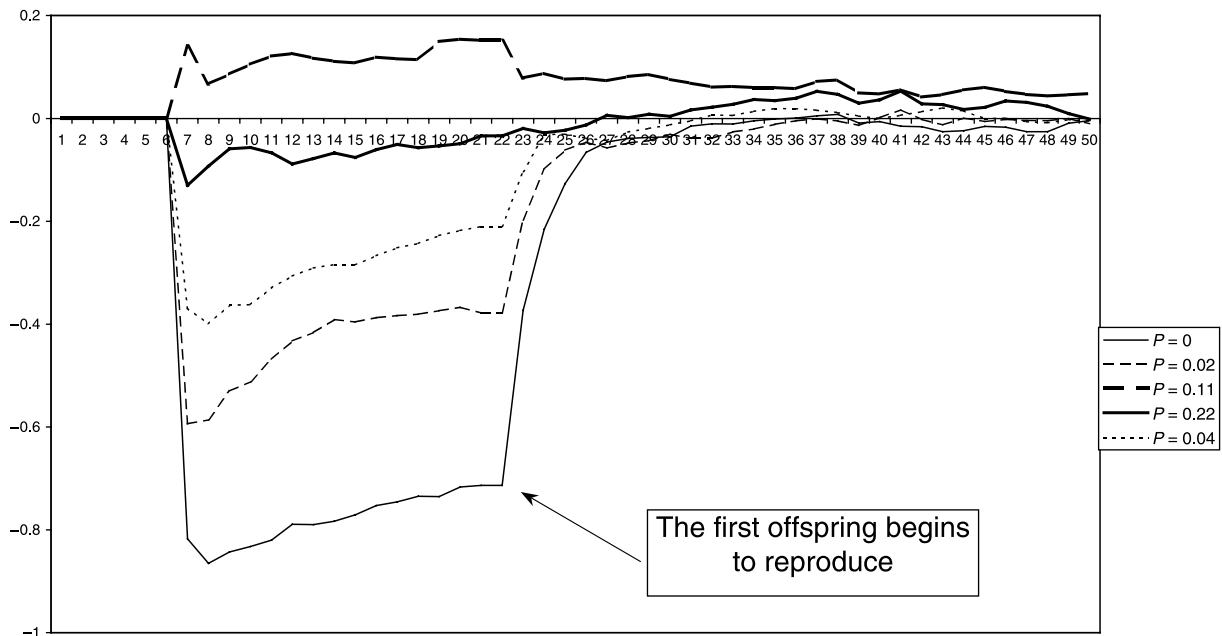


Fig. 7 Evolution of FIT according to different pollen flow models: proportion of within-deme pollen $P = 0$ (continuous thin line), $P = 0.02$ (thin dashed line), $P = 0.04$ (dotted line), $P = 0.11$ (thick solid line), $P = 0.22$ (thick dashed line).

The excess in heterozygotes (F_{IT}) decreased with the capacity of pollen to fertilize plants within the deme where it was produced (Fig. 7). When the proportion of within-patch pollen exceeded 10%, F_{IT} was not significantly different from zero. The maximum excess in heterozygotes was obtained when the proportion of intrademe pollinations was zero.

By contrast, a gradient of allele frequencies is observed across the metapopulation when short-distance pollen flow is maintained. In Fig. 2, we plotted the simulated allelic frequencies of allele A in each deme, 23 yr after foundation of the metapopulation (i.e. when the demes began to be filled and the first offspring began to reproduce). We modelled three levels of pollen flow: (1) where pollen is unable to fertilize

plants within the deme (matrix P1: $P = 0$); (2) where the proportion of within-deme pollen that fertilizes plants is $P = 0.04$ (Matrix P3); and (3) where the proportion of within-deme pollen that fertilizes plants is $P = 0.22$ (Matrix P5). Simulated results are shown in Fig. 2 along with our field observations for F1. The simulated values were most similar to the field data when we assumed relatively low levels of within patch-pollen flow.

Discussion

The aim of the present study on *S. spiralis* was to show how the use of biological, genetic and demographic data allows one

to infer the history of remaining populations of rare plants and to offer guidelines for the future management of endangered species.

Demography

We had no quantitative data before 1997 for the Parisian population (F1) and only 1 yr of demographic data for the Ardèche populations (A1–A5). Over 3 yr, the population size of the F1 population increased dramatically. From the demographic survey alone, however, we cannot determine whether this increase represents the sustainable growth of a recently founded population, an ascending portion of a demographic cycle or a response to environmental factors such as weather conditions. However, the aggregation of genotypes in two patches seems to indicate the expansion of two distinct subpopulations. Moreover, we know that this expansion is not due to vegetative reproduction because neighbouring plants were not more genetically similar than expected at random. Given that no environmental barrier exists to prevent the homogenization of this patchy population structure, we project that this distribution is transitional and will culminate in a single uniform population.

In southern France, the 1-yr demographic data showed no spatial structure in the A1 population. This population more likely represents a phase of demographic equilibrium among a set of populations showing metapopulation dynamics.

Population genetics

The genetic diversity maintained within and among conspecific populations allows inferences concerning the historical events and recent evolutionary processes experienced by these populations (Godt *et al.*, 1996). Experimental constraints obliged us to use only four loci, among which only one was polymorphic. Thus, the observed pattern could be the result of a selective process. However, the spatial genetic structure in the A1 population is clearly an isolation by distance pattern, which is an indication of neutral behaviour.

We found very low genetic differentiation among the studied populations of *S. spiralis* ($F_{ST} = 0.022$ between F1 population and southern France populations). No qualitative genetic differences (different loci, different alleles) were observed between the regions. This result may be due to the low number of studied loci but it can also be interpreted as meaning that the disappearance of many populations near Paris is a recent event. We can assume that gene flow did occur more frequently among southern and northern France populations in the past or that the Parisian populations were founded from seeds or plants collected in southern France.

The difference observed between the southern France populations and the Parisian population is in the spatial distribution of genetic diversity within populations. In the A1

Ardèche population, a limited isolation by distance was found whereas a strong cline of allelic frequencies was observed in the Parisian population. In this population, the two demographic patches coincide with the genetic differentiation observed within the population. For the PRX locus, one allele is predominant in one patch whereas the other allele is predominant in the other patch. Since no reproductive barriers appear to exist between the two groups of plants, this structure is likely to be transitional. The expected trajectory of the population is one of disappearance of genetic differences between subpopulations by gene flow.

Seed dispersal

We used two approaches to evaluate long- vs short-distance seed dispersal. On 16 d of seed dispersal, we captured a total of 439 seeds in population F1. This value is low compared with the number of seeds released by the 30 plants dispersing during this period. According to Willems and Lahtinen (1997), the number of seeds per capsule is highly variable in *S. spiralis*, depending on the mating system, on the level of inbreeding and on pollinator visitation rates. However, seed number per fruit ranges from 0 to 2000 (Willems & Lahtinen, 1997); if we assume an average of 800 seeds per fruit, this would generate a total of over 200 000 seeds produced in the aqueduct population. The low recovery rate of these seeds in our experiment can be accounted for by the fact that the surface area covered by the dishes represents a very small percentage of the area available for seed dispersal. We placed fewer than 150 dishes of 80 cm² (i.e. a total sampled area of 1.2×10^4 cm²) within a radius of 1800 cm (i.e. an area of 10^7 cm²). The number of seeds we recovered was greater than that expected if dispersal was uniform across the whole population. We can consider that our Petri dishes captured enough seeds to represent the seed shadow produced by this population.

The results showed that a very low percentage of seeds is dispersed between 2 m and 12 m from their maternal plants. We did not detect dispersal to greater distances but the coverage of the dishes at long distances was very small in relation to the total surface upon which such seeds could fall. Also, the vast majority of the seeds fell within 15 cm of the dispersing plants (Fig. 4), which is contrary to what is often thought about dispersal distances of orchids. Orchid seeds are known to be light and easily transported by the wind. Perhaps the rainfall that characterizes the seed dispersal season of this species prevents the seeds from being transported for long distances: autumn rain could project the seeds directly onto the soil in the neighbourhood of their mother plants.

The model

The aim of the simulation was to attempt to reconstruct the history of the aqueduct F1 population from the biological

characteristics of the plants. For seed dispersal, the model of isolation by distance that we chose is consistent with our measurements of seed dispersal with the Petri dishes. It is also consistent with the aggregation of the plants in two distinct patches that we observed in F1 and that appeared transiently in the simulations before equilibrium was reached.

We observed no insect-mediated pollen dispersal, but according to Delforge (1994), *S. spiralis* is pollinated by *Bombus* sp. While bumblebees sample nectar, pollinia are attached by their viscidium to the upper surface of the insect proboscis. Outcrossing is thus possible, and facilitated by protandry and by sequential flowering within inflorescences. Fewer than 10 flowers on the same inflorescence are mature at the same time; more frequently, only three or four flowers are simultaneously mature (N. Machon, pers. obs.). Thus, allogamous reproduction is favoured. The efficiency of the viscidium, in the flowers, renders the pollinia sticky and may frequently allow mating between distant flowers. The simulations we performed showed that the model that is the most consistent with the cline of allelic frequencies and the value of F_{IS} in the population is indeed a model where within-patch pollen dispersal is restricted (low but possible) and where bumblebees preferentially carry pollinia for relatively short distances, even if long-distance pollinations would be possible.

The model also showed that an important biological characteristic that explains the demographic and genetic structure of F1 is the existence of a long, nonreproductive juvenile stage. The simulations showed that this life-history trait contributes to a period of excess heterozygosity. The length of this period exactly corresponds to the time necessary for the first offspring of the founding individuals to become reproductive adults. A population of annual plants would not exhibit such an excess of heterozygotes (Fig. 6).

Thus, these simulations provided insights into the history of F1. They show that it is likely that this population was founded from a very small number of individuals (perhaps as few as two genetically distinct founders) and could still be expanding. The founding event cannot be precisely dated but it is unlikely that it happened more than 50 yr ago (or the population would already be genetically homogeneous) or less than 15 yr (or the first progeny could not have begun to reproduce). The origin of the plants is unknown. It could be a successful transplantation, voluntarily performed by an orchidophile. We know that *S. spiralis* was present in a few locations 30 or 40 yr ago in the Fontainebleau forest, in the neighbourhood of the aqueduct (Arnal, 1996). With the exception of one population in the lawn of a botanical research institute a few km from the aqueduct, all populations have disappeared. Plants could have been sampled from one of those local populations. As for the current aqueduct population, genetic homogenization is expected within a few decades.

In conclusion, this study showed that the aqueduct population of *S. spiralis* appears to be relatively young and expanding, and not in imminent danger of extinction. However, the long-term survival of the species in the Parisian region is questionable. The uniqueness of this population and its habitat, and the limited seed dispersal of adult plants, raise the concern that successful natural colonization events are likely to be very rare.

Since it is likely to be the lack of seed dispersal that explains the near-disappearance of *S. spiralis* in the Parisian region, the artificial creation of new populations should decrease the risk of extinction. If new populations are established by the transplantation of seeds or juvenile rosettes to ecologically suitable sites, long-lived populations should result. The resulting set of subpopulations is likely to exhibit metapopulation dynamics through pollen flow, contributing to the perpetuation of the species in the Parisian region.

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Appendix 1: pollen and seed flow matrices

Seed flow matrix (absolute proportion of seeds moving from the source population to the target population)

Target population	Source population									
	1	2	3	4	5	6	7	8	9	10
1	1	0.02	0.01	0.005	0.005	0	0	0	0	0
2	0.02	1	0.02	0.01	0.005	0.005	0	0	0	0
3	0.01	0.02	1	0.02	0.01	0.005	0.005	0	0	0
4	0.005	0.01	0.02	1	0.02	0.01	0.005	0.005	0	0
5	0.005	0.005	0.01	0.02	1	0.02	0.01	0.005	0.005	0
6	0	0.005	0.005	0.01	0.02	1	0.02	0.01	0.005	0.005
7	0	0	0.005	0.005	0.01	0.02	1	0.02	0.01	0.005
8	0	0	0	0.005	0.005	0.01	0.02	1	0.02	0.01
9	0	0	0	0	0.005	0.005	0.01	0.02	1	0.02
10	0	0	0	0	0	0.005	0.005	0.01	0.02	1

Pollen flow matrices (in all cases the values are the relative proportions in the pollen cloud in the target population)

P1

Target population	Source population									
	1	2	3	4	5	6	7	8	9	10
1	0	9	8	7	6	5	4	3	2	1
2	9	0	9	8	7	6	5	4	3	2
3	8	9	0	9	8	7	6	5	4	3
4	7	8	9	0	9	8	7	6	5	4
5	6	7	8	9	0	9	8	7	6	5
6	5	6	7	8	9	0	9	8	7	6
7	4	5	6	7	8	9	0	9	8	7
8	3	4	5	6	7	8	9	0	9	8
9	2	3	4	5	6	7	8	9	0	9
10	1	2	3	4	5	6	7	8	9	0

P2

Target population	Source population									
	1	2	3	4	5	6	7	8	9	10
1	1	9	8	7	6	5	4	3	2	1
2	9	1	9	8	7	6	5	4	3	2
3	8	9	1	9	8	7	6	5	4	3
4	7	8	9	1	9	8	7	6	5	4
5	6	7	8	9	1	9	8	7	6	5
6	5	6	7	8	9	1	9	8	7	6
7	4	5	6	7	8	9	1	9	8	7
8	3	4	5	6	7	8	9	1	9	8
9	2	3	4	5	6	7	8	9	1	9
10	1	2	3	4	5	6	7	8	9	1

P3

Target population	Source population									
	1	2	3	4	5	6	7	8	9	10
1	2	9	8	7	6	5	4	3	2	1
2	9	2	9	8	7	6	5	4	3	2
3	8	9	2	9	8	7	6	5	4	3
4	7	8	9	2	9	8	7	6	5	4
5	6	7	8	9	2	9	8	7	6	5
6	5	6	7	8	9	2	9	8	7	6
7	4	5	6	7	8	9	2	9	8	7
8	3	4	5	6	7	8	9	2	9	8
9	2	3	4	5	6	7	8	9	2	9
10	1	2	3	4	5	6	7	8	9	2

P4

Target population	Source population									
	1	2	3	4	5	6	7	8	9	10
1	5	9	8	7	6	5	4	3	2	1
2	9	5	9	8	7	6	5	4	3	2
3	8	9	5	9	8	7	6	5	4	3
4	7	8	9	5	9	8	7	6	5	4
5	6	7	8	9	5	9	8	7	6	5
6	5	6	7	8	9	5	9	8	7	6
7	4	5	6	7	8	9	5	9	8	7
8	3	4	5	6	7	8	9	5	9	8
9	2	3	4	5	6	7	8	9	5	9
10	1	2	3	4	5	6	7	8	9	5

P5

Target population	Source population									
	1	2	3	4	5	6	7	8	9	10
1	10	9	8	7	6	5	4	3	2	1
2	9	10	9	8	7	6	5	4	3	2
3	8	9	10	9	8	7	6	5	4	3
4	7	8	9	10	9	8	7	6	5	4
5	6	7	8	9	10	9	8	7	6	5
6	5	6	7	8	9	10	9	8	7	6
7	4	5	6	7	8	9	10	9	8	7
8	3	4	5	6	7	8	9	10	9	8
9	2	3	4	5	6	7	8	9	10	9
10	1	2	3	4	5	6	7	8	9	10