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CONNECTIVITY, HABITAT HETEROGENEITY, AND POPULATION PERSISTENCE IN *RANUNCULUS*  
*NODIFLORUS* L., AN ENDANGERED SPECIES IN FRANCE.

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## ABSTRACT

- We explored the role of habitat spatial structure in the maintenance of metapopulations of *Ranunculus nodiflorus* L. This rare species grows in puddles that can be connected occasionally by flooded corridors.
- We monitored five locations in the Fontainebleau forest (France) since 2002. We recorded the presence of corridors among patches and evaluated their impact on patch demography and plant fitness.
- The presence of connections among puddles influences the structure and persistence of populations. Connections increase population size by increasing both the number of patches occupied by the species and the number of individuals within patches, but have no influence on plant fitness. We found no evidence of a persistent soil seed bank.
- The presence of natural corridors decreases the extinction probability of the populations, most probably by allowing recolonization of empty puddles after extinctions. The preservation of the corridors is therefore crucial for the conservation of *R. nodiflorus* in its natural habitat.

**Keys words:** metapopulation, *Ranunculus nodiflorus*, corridors, reproductive success, seed bank strategy, migration pattern, conservation.

## INTRODUCTION

Habitat spatial structure is an essential component affecting the dynamics and persistence of species living in fragmented landscapes (Legendre & Fortin 1989). Metapopulation models have been developed to account for such structure in populations that are discontinuous because of patchy and heterogeneous habitat (Hanski & Simberloff, 1997). These models show that metapopulation dynamics depend on the demography of each subunit (patch) and on the migration among patches (Levin, 1969). Hence, habitat fragmentation, which increases habitat structure, may strongly affect the persistence of a metapopulation over time, by (1) decreasing habitat size and therefore population size and (2) increasing isolation via decreased migration rates among patches (Young et al. 96; Thomas et al. 2001). Several theoretical approaches have confirmed this influence of habitat fragmentation by demonstrating that metapopulation persistence is governed by the number of available connected patches. From these results, Hanski *et al.* (1996) and Hanski (1997) developed the concepts of Minimum Viable Metapopulation (MVM) or Minimum Amount of Suitable Habitat (MASH). However, very few field studies have assessed how the viability of a metapopulation is actually affected by (spatial or functional) connectivity (Grasman & HilleRisLambers ; 1997) or habitat characteristics (Wahlberg et al; 2002; Murphy & Lovett-Doust; 2004).

Among the factors influencing metapopulation viability, migration is of central interest in conservation biology because (1) it can easily be artificially increased and (2) increasing migration is likely to decrease the extinction probability of the whole metapopulation. Migration among patches decreases demographic and environmental stochasticity by increasing the effective size of a patch and by enabling (re)colonization of empty patches (Brown & Kodric-Brown; 1977; Fahrig & Merriam; 1985). Migration also favors the maintenance of higher levels of genetic variability via increased local effective population sizes and decreased genetic drift (Mills and Allendorf. 96). Hence, restoring or increasing migration among small populations can improve their mean fitness, which is otherwise expected to be low because of strong genetic drift leading to loss of genetic variability and fixation of deleterious alleles (Luijten et al, 2000). Finally, migration decreases kinship among individuals within a patch and lowers biparental inbreeding depression (Richards; 2000).

To favor migration among populations in fragmented landscapes, many conservation programs promote the use of biological corridors (Simberloff et al. 1992; Haddad & Baum.

1999). Corridors are spatial structures allowing movements of individuals (plants or animals) among the habitat patches. Most studies exploring the impact of corridors on population persistence concern animal populations (Saccheri et al. 1998; Haddad, 1999; Aars & Ims, 1999). These studies show that corridors increase the mean fitness of the populations (Boudjemadi et al. 1999) and prevent metapopulation extinctions (Mech & Hallett, 2001) by enhancing migration among patches. In contrast, the impact of corridors or connections on plant population viability has been little investigated. Tewksbury et al. (2002) recently demonstrated that corridors facilitating animal movements could also promote seed dispersal; other studies investigated the role of water as a vector of seed migration among patches (Kirchner et al. 2003; Johansson et al. 1996). However, the effect of corridors and increased migration on the persistence of these plant metapopulations was not tested.

In plant populations, migration can also occur through time, via dormant seeds remaining in the soil for several generations. Hence, soil seed banks are another factor that may influence metapopulation dynamics and persistence. Seed banks are known to decrease demographic stochasticity and genetic drift in small populations. In populations with limited dispersal ability, this opportunity for “temporal migration” may limit the genetic depreciation (Levin, 1990; Kalisz et al. 1997) and decrease the extinction probability.

In this study, we explore the role of corridors and gene flow in the maintenance of small populations of the rare and endangered species *Ranunculus nodiflorus* L. This small *Ranunculaceae* lives in puddles that occur on sandstone formations in the Fontainebleau Forest (France, Arnal, 1996). The habitat is therefore naturally fragmented and strongly constrains the size of the populations. Water levels are highly variable, so that puddles can be transitorily connected, mostly on the occasion of rains, through natural water flows considered as flooding corridors. Each puddle is referred to as a patch, or, when it contains *R. nodiflorus* individuals, as a population. We define a network as a group of puddles potentially connected by water flows. A metapopulation is composed of all networks within a given site.

By collecting data on the number and size of patches, the number of empty patches, the connectivity among patches and among networks, and the extinction and recolonization rates, we were able to explore the influence of spatial and environmental characteristics of the landscape on the metapopulation dynamics. We focus here on the influence of corridors on the reproductive success of individuals and metapopulation persistence but we also searched for a possible seed bank to explain their maintenance. We estimated the viability of the metapopulation by analyzing, in every patch, vegetative and reproductive traits of plants.

Finally, we suggest management directives that could favor the maintenance of the species in its natural habitat of the Parisian region.

## MATERIAL AND METHODS

### Description of the metapopulation

#### *Study species*

*Ranunculus nodiflorus* L. (Renonculaceae) is a rare and endangered annual plant living in wet zones in Spain, Portugal and France. It has experienced a strong decline during the last century, caused by the drainage of wet lands and regression of grazing (Danton & Baffray 1995) and appears on the French Red List of threatened species (Olivier and al. 1995). The species has very strict habitat requirements, growing only in puddles with thick soil and highly variable water levels.

This species displays small (5 mm) and odorless yellow flowers on which insects have never been observed during field works (F. Noel, personal observation). According to Kirchner et al. (2003), it reproduces mainly by selfing. Plants produce small oval akenes (2 mm long) with a tiny tail. These akenes contain up to 14 seeds and are able to float (Kirchner et al, 2003). Some of the seeds germinate in autumn and others in spring (F. Noel, personal observation and see below). Flowering and seed production occur between April and May. The plants die rapidly after the last akene is ripe. As all annual species, *R. nodiflorus* is unable to reproduce vegetatively.

#### *Study area*

The study was carried out in the Fontainebleau forest, 50 km south of Paris, in five sites where *R. nodiflorus* was previously observed (Fig. 1): Coquibus (site 1), Meun (site 2), subdivided into four sub-sites (sites 2A, 2B, 2C and 2D), Coulevreux (site 3), De Oliviera (site 4) and Belle-Croix (site 5). Each site is a “platière”, characterized by a sandstone ground and the occurrence of temporary puddles during rainy periods. Water levels, which depend chiefly on the rainfall intensity, are highly variable and puddles can transitorily be connected by water corridors. Other vegetation (mostly heather, *Calluna vulgaris*) is rather low, but birch trees (*Betula pendula*) and pines (*Pinus sylvestris*) tend to invade the sites between clearings operated by the French National Forest Office (ONF). The total surface of each “platière” is around 148 m<sup>2</sup> in site 1 (28 puddles occupied by the species), around 525 m<sup>2</sup> in site 2 (55.5 m<sup>2</sup> in site 2A, 16 puddles; 125 m<sup>2</sup> in site 2B, five puddles; 26 m<sup>2</sup> in site 2C, four

puddles; 31.25 m<sup>2</sup> in site 2D, three puddles), 4 m<sup>2</sup> in site 3 (one puddle), 0.5 m<sup>2</sup> in site 4 (one puddle), and 32 m<sup>2</sup> in site 5 (three puddles). The two most distant sites (site 2 and 5) are 17.5 km apart (Fig. 1).

### *Demographic data*

A total of 60 permanent quadrats (30\*30 cm<sup>2</sup>) were randomly placed in every puddle where *R. nodiflorus* was observed in autumn 2002 and/or in spring 2003 (one quadrat per puddle; 28 in site 1, 28 in site 2, one in site 3, and three in site 5). In site 4, we monitored the total number of individuals in the unique puddle. From fall 2002 to spring 2003, the number of individuals ( $N_{ind}$ ) within each quadrat (or puddle for site 4) was monitored monthly. We also recorded the plant phenology, as juvenile (J) or adult bearing flowers and/or fruits (A).

### *Germination tests*

To study the ability of *R. nodiflorus* to build a soil seed bank, we performed several germination tests in experimental garden.

In spring 2003, about 375 seeds were collected in the field, directly on plants. They were bulked and sown immediately in pots with compost (¼ of Fontainebleau sand and ¾ of mould) and placed in experimental garden. The germination rate was monitored during 5 weeks.

In spring 2004, after flowering but before seed production, soil was sampled in every quadrat of all sites (57 samples i.e the number of quadrat on the field was increased in 2004 except for four quadrats where the surrounding soil had been turned over by wild boars). The soil samples were placed under germination conditions (in an incubator with a 12h day / 12h night photoperiod, 10°C night and 15°C day) to evaluate the number of seeds that were produced before 2003, did not germinate in 2004, but were still viable. The aim of this experiment was to detect a possible soil seed bank

We also performed *in situ* germination tests to assess the quality of puddles where *R. nodiflorus* was not observed between 2001 and 2004 (“empty puddles”). We measured water pH in four empty puddles of two empty networks. In each puddle, we sowed 50 seeds and mapped them with a high-precision GPS (1 millimeter accuracy).

### *Vegetative and reproductive data*

To evaluate the fitness components of plants, we measured vegetative and reproductive traits of four plants per quadrat. We systematically examined the four plants located at each corner of a quadrat. Measures were performed during the 2003 flowering season, on May, 28 and May, 30. For each plant, we measured the maximum height (Hind),

the stem diameter at the soil level (Diam), the number of leaves (Nleav), the number of buds (Nbud), the number of flowers (Nflw), the number of akenes (Nak), and the number of seeds per akene (Ns/a). We also calculated the mean number of seeds per akene (s/a) and per individual (s/I). We collected a total of 49 akenes, one akene per quadrat on average, and we sowed the resulting 567 seeds. Nevertheless, it was not possible to us to evaluate their germination rate.

To study the spatio-temporal patterns of seedling emergence in *R. nodiflorus*, we calculated coefficients of aggregation, CA, on the demographic data. This coefficient is defined as  $CA = \sigma^2 / X$ , where  $\sigma^2$  and X are the variance and mean of the number of seedlings per quadrat, respectively (Apparicio, (Cybergeog) 2000 ; Gonz  les-Astorga & N  n  ez-Farf  n, 2000). A CA of one indicates random distribution across space or time; larger values indicate aggregation. We calculated one CA per date for the spatial analysis, by computing the mean and variance over all quadrats at a given date, and one CA per quadrat for the temporal analysis, by computing the mean and variance over all dates in a given quadrat. Deviations of the CA from 1 were tested by means of a *t*-test.

#### *Environmental data*

At each monthly quadrat examination, we also recorded several environmental variables: water depth (WDepth), number of potential corridors converging to the puddle (Ncorr), sunlight intensity (Sun = high, low and medium), vegetation coverage (Veget = high, low and medium density), and evidence of disturbance such as animal or human tracks (Tracks = 0 or 1). Soil samples were collected near each quadrat; we evaluated the texture (sandy, sandy-organic, organic-sandy and organic) and measured the color (by picture analysis with the software Pixie 2.0, <http://natty.port5.com>). The water pH was also measured with a portable pHmeter (once during the study).

In February 2004, we mapped each puddle (containing plants or not) in the largest study site (site 1) with a high-precision GPS system (1 millimeter accuracy). We measured the length and width of each puddle, as well as the water depth (WDepth) and pH. When possible, we counted the total number of individuals. For large populations ( $N > 200$ ), we counted individuals in a fraction of the puddle and extrapolated to the whole surface. We also estimated the surface of the puddles occupied by *R. nodiflorus*.



### Statistical analysis

Statistical analyses were performed with JMP.5.0.1.2 (ANOVA, Wilcoxon test, Kruskal-Wallis test and correlations, SAS Institute©, 2003) or with the freeware R 1.8.0 (spatial statistical tests, mixed-model and Mantel tests, R, a language and environment©, 2003).

To analyze population structure, we tested the effects of site, quadrat and network on the variation of morphological traits using a nested ANOVA with the model:

$$Y_{ijkl} = \mu + site_i + net(site)_{ij} + quad(net(site))_{ijk} + R_{ijkl}$$

where  $Y$  is the value of a morphological trait (Nleav, Nflw, Nbud, Nak, Ddiam, Hind, or s/I),  $\mu$  is the mean value of the trait,  $site$  is the site effect ( $i = 1 \dots 5$ ),  $net$  is the network effect ( $j = 1 \dots 7$ ),  $quad$  is the quadrat effect ( $k = 1 \dots 9$ ), and  $R$  is an error term. For this analysis, we did not declare any random effect because, according to the experimental design, we had placed a quadrat in all the puddles containing the species for the entire site.

We also analyzed the spatial structure of a morphological trait by calculating standardized distances for the number of akenes per individuals (Nak/Ind) among all pairs of quadrats within each site, as  $m_{ij} = \sqrt{(x_i - x_j)^2} / \sigma$  where  $x_i$  and  $x_j$  are the mean number of akenes per individual in quadrats  $i$  and  $j$ , and  $\sigma$  the standard deviation of the number of akenes per individual over all quadrats. We assessed the relationship between morphological (similarity) and geographical distance among individuals by testing the correlation between the geographical distance matrix and the morphological distance matrix, using Mantel tests (2000 simulations; Mantel, 1969).

To evaluate the influence of corridors connecting puddles on population dynamics in site 1, we tested the effect of connexions and puddle size on the density of individuals using a mixed model analysis of covariance with puddle size and water depth as covariates:

$$Y_{ijk} = \mu + connect_i + Puddle_j + \beta(size_{ijk} - \bar{s}) + \gamma(WDepth_{ijk} - \bar{W}) + R_{ijk}$$

where  $Y$  is the population density (number of plants per  $m^2$ ),  $\mu$  is the mean population density over all puddles,  $connect$  is the connection status of a puddle (connected or isolated;  $i = 1, 2$ ),  $Puddle$  is an individual puddle effect ( $k = 1 \dots 41$ ),  $size$  is the puddle size ( $m^2$ ), with mean  $\bar{s}$ ,  $WDepth$  is the water depth, with mean  $\bar{W}$ , and  $R$  is an error term.  $Puddle$  was regarded here as a random effect because the experimentation was limited to a subsample of all puddles (site 1). This analysis to detect the effect of connections on population dynamics was conducted on

population density rather than total population size, because the latter is primarily constrained by puddle size.

Finally, we evaluated the impact of connections among puddles on individual fitness, using an analysis of covariance, with the model:

$$Y_{ijk} = \mu + connect_i + site_j + \alpha(Hind_{ijk} - \bar{H}) + \beta(Diam_{ijk} - \bar{D}) + \gamma(Hind * Diam_{ijk} - \overline{HD}) + R_{ijkl}.$$

Most abbreviations are defined above;  $Y$  is the value of individual fitness traits (Ns/I, Nak and Ns/a);  $Hind$  and  $Diam$ , the two covariates, correspond to plant height and stem diameter.

## RESULTS

We monitored the populations of *R. nodiflorus* using 60 permanent quadrats. The number of corridors potentially connecting the quadrats to one another varied between zero (completely isolated quadrats) and four. Quadrats were thus grouped into 25 networks, defined either as a single isolated quadrat or as a set of quadrats with each at least one potential connection to another quadrat (Fig. 2). Networks contained between one and nine quadrats and ranged between 0.5 m<sup>2</sup> and 72 m<sup>2</sup>. Over all sites, 7.5% of quadrats were not connected, 31% had only one connection, 40% had two, 7.5% had three and 14% had four connections.

### Distribution of plants

As previously shown by Kirchner et al (2003), the species is not uniformly distributed among puddles in the sites. The number of individuals was highly variable among quadrats (Student test on the spatial coefficient of aggregation,  $t = 3.16$ ,  $P = 0.008$ ), with total number of individuals per quadrat varying from 2 to 197 seedlings and 138 flowering plants. The distribution of density of *R. nodiflorus* was L-shaped, with a majority of quadrats exhibiting low densities (< 10 plants per quadrat) and a few quadrats with high densities (> 100 plants) (Fig. 3). In some networks, plants were present in every puddle, whereas all puddles were empty in other networks. The plant density was higher on average in the largest sites (sites 1 and 2).

Table 1 presents the mean and standard deviation, or the distribution, of environmental variables for all sites. We explored all relationships between these environmental variables (including connectivity) and the presence / absence of the species in the puddles of site 1 (“Coquibus”). *R. nodiflorus* appears to be unable to germinate and grow in too acid puddles (pH < 4.5), as shown by a strong positive relationship between pH and the probability that a puddle is occupied by the species (logistic fit,  $P < 0.0001$ ). The mean pH of occupied puddles

was 5.85 (ranging from 4.5 to 7.2, with a standard deviation of 0.75) and the mean pH of empty puddles was 4.8 (ranging from 3.5 to 7.3, with a standard deviation of 0.74) (One-way test, Chisquare = 32.07,  $P < 0.0001$ ). In addition, none of the 200 seeds sown in the empty puddles of empty networks germinated. The pH of these puddles ranged from 4 to 4.5. We therefore classified the empty puddles into “suitable” ( $\text{pH} > 4.5$ ) or “unsuitable” ( $\text{pH} < 4.5$ ) for *R. nodiflorus* growth. In the following, we considered suitable puddles only.

The presence and density of *R. nodiflorus* was also strongly influenced by connectivity. Table 2 presents the different types of puddles in site 1 and figure 5 maps all suitable puddles (unsuitable puddles are not drawn). Empty puddles were more numerous than occupied ones. Isolated puddles were generally empty and networks tended to be either completely empty or (almost) completely occupied. Among the 25 occupied puddles, three (12%) were isolated; the other ones were parts of networks. Among the suitable puddles, the plants were more likely found in the connected puddles. In network containing plants, most (if not all) puddles were occupied. Three puddles only were empty but connected to occupied puddles. Finally, the density of *R. nodiflorus* was significantly larger in connected puddles than in isolated ones (ANCOVA on plant density, significant connection effect,  $F_{1,40} = 4.946$ ,  $P = 0.0318$ ). In contrast, puddle size, water pH or water depth had no significant effect on plant density.

We analyzed the soil characteristics near 18 puddles and tested their impact on the reproductive and vegetative performances of the plants. We found a significant negative relationship (linear fit,  $P = 0.015$ ) between soil pH and the number of akenes produced by the plants (the number of seeds per akene was not affected by pH). We also observed a positive relationship between the soil depth and the number of seeds per akene ( $P = 0.045$ ). No relationship was found between pH and soil depth ( $P = 0.67$ ), so that the fitness of *R. nodiflorus* was optimized in acid puddles with a good soil depth.

Finally, analyses on all sites pooled together showed no significant relationship between environmental variables (soil depth, Ncorr, Sun, Veget, Tracks, texture of soil) and the log-transformed maximum density of the quadrats (the maximum density observed during the study period for each puddles).

## Demographic data

In 2003, we observed a significant temporal variation in the number of individuals per quadrat (Student test on the temporal coefficient of aggregation:  $t = 3.36$ ,  $P = 0.001$ ). Figure 4, reporting the dynamics of the number of plants from October 2002 to June 2003, shows

two distinct germination periods, which is consistent with this temporal aggregation. The first period of germinations occurred in fall and winter 2002; the resulting plants remained at the seedling stage until spring, when a second period of germinations occurred. Between March and May 2003, the seedlings grew up, flowered and produced akenes. For sites 1 and 2, the maximum population size occurred in May whereas for the sites 3 and 5, it occurred in November.

Figure 4 also reports rainfalls during the same period. On the whole period, there was a significant positive correlation between demography and pluviometry for all sites. Within seasons, this correlation was significant in fall-winter only (Spearman correlation coefficient,  $r = 0.52$ ,  $P = 0.0023$  for all sites on one year;  $r = 0.73$ ,  $P = 0.0018$  in fall-winter (September to March); and  $r = 0.44$ ,  $P = 0.072$  in spring (April to June)). Moreover, no correlation was observed between demography and temperature (Spearman correlation coefficient,  $r = 0.05$ ,  $P = 0.77$  for all sites on one year;  $r = -0.01$ ,  $P = 0.95$  in fall; and  $r = 0.33$ ,  $P = 0.17$  in spring). These correlations suggested that germinations were favored by rainfall in autumn, whereas spring germinations tended to occur when the puddles dried (although the negative relationship between demography and pluviometry in spring was not significant).

### **Variability of vegetative and reproductive components of the plants**

A large fraction of the variation in vegetative and reproductive traits was attributable to differences among networks, as indicated by significant network effects for all traits (Nleav, Nflw, Nak, Ddiam, Hind, s/I) (Table 3). To a lesser extent, site and quadrat effects also explained the distribution of the variation among puddles, but not for all traits. The role of network isolation in phenotypic variation was confirmed by analyzing the standardized morphological distance, calculated on the number of akenes per individual (Nak) between all pairs of quadrats. Distances were significantly smaller for pairs of quadrats within a same network than for pairs of quadrats from different networks ( $P = 0.012$ , Wilcoxon test, Fig. 6 and Table 4). In contrast, the number of akenes per individual did not depend on sites, as indicated by non-significant differences between pairs of morphological distances within and among-sites.

A Mantel test showed an isolation by geographic distance for reproductive traits (Nak and Nflw) in sites 1 and 2 ( $P = 0.0045$  and  $0.026$ , respectively, with a 2000 simulations test). For these sites, individuals further apart exhibited larger differences in their number of akenes and flowers.

Finally, the fitness of individuals, measured as seed or akene production, did not depend on connectivity (ANCOVA on reproductive traits), with similar values of total number of seeds and total number of akenes ( $N_s/I$ ,  $N_{ak}$ ) in connected and isolated puddles (Table 5).

### **Germination tests and seed bank**

Of the 375 seeds collected in the field in May 2003 and sown in June 2003, 250 (67%) had germinated in July. An appreciable fraction of the seeds can therefore germinate just after being produced if they encounter favorable conditions and do not appear to suffer any dormancy. We observed only 13 germinations of *R. nodiflorus*, occurring in eight of the 57 soil samples collected in spring 2004 before seed production. This suggests that there were very few viable seeds left in the soil samples, most probably because the majority of seeds had germinated earlier. We therefore conclude that this species has little opportunity to build a persistent seed bank in the soil.

## **DISCUSSION**

We described the dynamic of a metapopulation of a small and threatened plant in the Parisian region to identify the major factors influencing the dynamics of the metapopulation and thus playing a key role in the persistence of the species in its natural environment. We specifically evaluated the role of the water corridors as connections among populations, but we also investigated the effects of other environmental variables. Habitat quality can explain the absence or presence of the species in the temporary puddles (Dupré & Ehrlén; 2002). Our results highlight three main factors affecting the dynamics of *R. nodiflorus* populations: (a) the presence of corridors connecting puddles, influencing spatial migration (b) the chemical properties of the puddles (soil and water), controlling their suitability for *R. nodiflorus* and (c) the dispersal of the species in time (seed bank and variation in germination time). We detail these three factors below.

### Corridors, Connectivity and Population dynamics

#### **1- Connectivity increases population density**

We showed that *R. nodiflorus* was frequently present in networks and rarely observed in isolated puddles. In addition, the majority of networks were either entirely empty or full (except for 3 puddles that were empty among the occupied puddles of a network). We also demonstrated that the plant density was higher in connected than in isolated puddles. Thus,

corridors seem to enhance the probability of puddles to be filled with plants, by promoting seed dispersal as detailed below. We observed that water levels are generally higher in networks than in isolated puddles ( $\chi^2 = 4.0169$ ,  $P = 0.045$ ,  $df=1$ ); this may also explain the higher density in connected puddles, which provide a more favourable habitat. However, an analysis of covariance on plant density showed that water level did not explain the observed differences between connected and isolated puddles, so that connectivity was the main factor controlling plant density. To our knowledge, this is the first experimental demonstration that connectivity may actually decrease the extinction probability of a plant metapopulation.

Previous experiments have shown that *R. nodiflorus* seeds are able to float during a few weeks (three to four, Kirchner et al, 2003). In addition, seeds are produced in early summer, leaving ample time for dispersal before germination, which occur in autumn or in spring. Therefore, between June and March of the following year, when rainfalls raise water levels in puddles, small streams of water connect puddles and disperse seeds. The direction of the flow may depend on the relative elevation of the puddles but also on the direction of the wind because most sites are relatively flat. In connected puddles, corridors therefore bring in new seeds each year and help prevent population extinctions. In contrast, isolated puddles probably experience repeated bottleneck events.

Another hypothesis to explain the significant density difference between connected and isolated puddles is that the isolated ones provide inbreeding depression or fixation of deleterious mutation by genetic drift that could act on the seeds viability and/or on the germination rate. Thus, the reproduction success rate of individuals may be strongly reduced in isolated puddles.

## 2 – Connectivity, migration and spatial structure

The analysis of the spatial structure of vegetative and reproductive traits of the plants revealed that individuals from the same network were more similar than individuals belonging to different networks. This does not directly confirm the role of flooding corridors in seed migration, because isolation by distance seems to occur also among quadrats for the reproductive traits. Unfortunately, it was not possible to distinguish between the effect of distance within and among networks and the effect of corridors (usually connecting neighboring puddles).

The similarity of plants within a network could be due to genetic and/or environmental homogeneity. Using a limited number of isozymes, Kirchner et al (2003) demonstrated that puddles are not genetically differentiated within networks, whereas genetic differentiation

among networks is marginally significant. In our study, we did not detect any difference among networks in soil composition and insolation but maximum water depth, number of days under water, and disturbances were variable across networks. Therefore, the observed differentiation of quantitative traits among networks is likely to have both a genetic and environmental basis. We intend to use microsatellite markers to verify that genetical exchange does occur within networks and to assess the role of corridors in the migration process.

### 3 – Connectivity does not increase mean fitness

The analysis of akene and seed production, two traits likely associated with plant fitness, showed that populations growing in isolated puddles did not suffer from decreased fitness compared to populations growing in connected puddles. We originally thought that isolated populations of *R. nodiflorus* would suffer from the fixation of deleterious alleles by genetic drift or a stronger inbreeding depression. However, our results did not highlight any alteration of reproductive success in isolated puddles. The breeding system of the species, presumably predominant selfing, could help to regularly and efficiently purge strongly deleterious alleles. Alternatively, the fixation of slightly deleterious alleles, which are not efficiently purged by selfing, could be undetectable on the traits we measured.

## Habitat suitability and occupation

### 1 – Influence of pH on habitat suitability

Analyzing the differences between empty puddles and puddles occupied by the species allowed us to identify the biotope of *R. nodiflorus*. Empty puddles had a significantly lower pH than puddles where the species was found. *R. nodiflorus* therefore appears to have a strict habitat requirement in terms of water pH, which must exceed 4.5 for seed germination to occur. The difference of pH among puddles is likely caused by the presence of calcareous stones brought in when the road running across the “platière” was built. These stones were dropped in puddles close to the road, which thus exhibit a higher pH. Puddles more distant from the road have a more acidic pH, unless they benefit from a flow of calcareous water (or presence of calcareous noddles). *R. nodiflorus* does not grow in all suitable (pH > 4.5) habitats. The population is relatively stable through time, with a single extinction and two new colonisations observed in two years. The species seems to have opportunities to extend to empty suitable networks, except if its dispersal capacity is limited for biological or environmental reasons or if the suitability of the puddles was not correctly assessed. This can be tested by germination tests in empty puddles considered as suitable.

## 2 – Extinction threshold

In a fragmented population, habitat suitability and habitat occupancy can inform on the probability of extinction. Eriksson & Kiviniemi (1999) define the “quasi-equilibrium extinction threshold” of a fragmented population as  $h'_c = 1 - s/h$ , where  $h$  is the fraction of suitable patches and  $s/h$  the occupied fraction of suitable patches; if  $h/h'_c < 1$ , the population is threatened. This definition assumes that a species may persist for a while under unfavorable conditions, via dormant seeds for example, and thus incorporates a delay before extinction when the fraction of suitable habitats decreases. It was therefore more appropriate for our study metapopulation than the classical extinction threshold ( $h_c$ ), based on equilibrium between colonization and extinction rates.

The data on *R. nodiflorus* yielded  $h = 84/128 = 0.65$ ,  $s/h = 25/84 = 0.30$ , so that the population is just below the threshold ( $h/h'_c = 0.93$ ) and, according to the authors, does not have a high risk of extinction. This prediction however strongly depends on our ability to discriminate empty suitable and unsuitable puddles, and the extinction risk might be higher if some puddles have been incorrectly classified as suitable. The process of extinction (caused by environmental and demographic stochasticities), although greatly reduced within a network thanks to corridors, still occurs at a low rate. If this rate of extinction is higher than the presumably low rate of recolonization via migration between “isolated” puddles, the population is experiencing a slow decline eventually leading to extinction. Our data do not cover a sufficiently long period of time to detect such decline, but the present “empty” networks could have been “occupied” in the past. Only a thorough monitoring on several decades could confirm this hypothesis.

### Temporal dispersal and seed bank

#### 1 - The soil seed bank of *R. nodiflorus*

Very few viable seeds were detected in the soil after the spring episode of germination, which suggests that most viable seeds germinate in the year following their production. We conclude that *R. nodiflorus* has not developed a persistent soil seed bank strategy. Note however that the presence of a soil seed bank may depend on the climatic conditions and can fluctuate over years (Leck; 1996; Gutiérrez & Maserve; 2003). Therefore, it is possible that *R. nodiflorus* usually forms a soil seed bank but that, by chance, environmental conditions favored the germination of all seeds in 2003. This hypothesis is consistent with the observed increased population sizes in the puddles of 2004 (data not shown).



In annual species, the absence of a seed bank may greatly increase extinction rates (Pavone & Reader; 1982; Levin; 1990; Kalisz et al; Baskin& Baskin; 98), especially in patches with small population size such as those observed here, because of increased demographic and environmental stochasticity. However, the production of seeds that germinate simultaneously may enhance the ability of *R. nodiflorus* to compete with other species. This strategy is observed in perennial plants with vegetative and sexual reproduction or in animal species (Winkler & Stocklin; 2002; Arendt & Wilson; 1997). Moreover, autumn germinations that survive the winter can be more vigorous and have a greater fitness than the spring germinations (Arthur et al, 1973).

## 2 - The germination dynamics of *R. nodiflorus*

The population dynamics of *R. nodiflorus* are complex, and seem highly connected with climatic variation. First, our results suggest that population dynamics depend on the water conditions and their temporal variation in autumn and spring (whereas no relation with temperature have been demonstrated). We could not exclude others factors like sunlight intensity, vegetation coverage or stamping, which may be correlated with variations in water levels and could also be responsible for the observed dynamics of germination. *R. nodiflorus* germinates during two periods: in autumn when puddles are filling up and in spring when puddles are drying up. Therefore, germination of *R. nodiflorus* seems to require an alternation of wet and dry periods. This requirement explains that individuals mainly grow on the edge of puddles where variations of water level are larger. Our observations showed that most germination occurred in the drying puddles where the water level had been particularly high, although this observation might simply indicate a better survival of plants in the preceding generation.

We did not follow seedlings individually, so that we could not establish whether autumn germinations died before spring and were replaced by spring germinations or survived to reproduce. Nevertheless, the population size never dropped to zero between autumn and spring (except maybe in site 5, Fig. 4), which tended to demonstrate that autumn seedlings were able to resist the winter conditions and to continue their life cycle in spring. Regardless of their germination date, all plants flowered during a short period in April-May and produced seeds in May-June

The existence of two periods of germination suggested that there are two kinds of seeds in the population: (1) seeds germinating immediately after production, when the biotope is wet and (2) seeds germinating after a cold period (winter), when the biotope is drying. We

were not able to assess whether plants produce both kind of seeds (heteromorphism) or if each type of seeds is produced by different plants (polymorphism) (Arthur et al, 1973, Onipchenko et al; 1998; Venable et al; 1998; Brändel; 2004). Assuming that the second type of seeds has more time to migrate, and because plants predominantly self, we can imagine that two populations coexist: one with a low dispersal ability, submitted to bottlenecks every winter, and one with higher migration rates, submitted to more frequent extinction and recolonization events. However, this kind of “bet-hedging” strategy (Schaffer, 1974) provides the population with two opportunities to go successfully through the critical stage of germination. If seedlings are destroyed during winter or if bad climatic conditions prevent seed germination in spring, the maintenance of the population is nevertheless insured.

### Conclusion

In summary, the presence of *R. nodiflorus* strongly depends on the environmental quality of puddles but large numbers of isolated empty puddles also suggests an additional role of connectivity. Isolated populations do not seem to suffer genetic depreciation, so that differences between isolated and connected puddles are more likely attributable to a higher demographic stochasticity and to smaller probabilities of recolonization after extinction. Differences between connected and isolated puddles are probably exacerbated by the absence of a persistent soil seed bank. Therefore, in view of the positive impact of natural corridors on the population demography, their maintenance appears to be a crucial condition for conservation of the species in Fontainebleau forest. Efficient protection of the sites requires careful management to prevent invasion of corridors by woody vegetation. In addition, population recovery might be greatly facilitated by occasional artificial migration from large populations to empty networks or isolated puddles (i.e. by sowing seeds in the empty puddles) and/or by changing the chemical characteristics of the unsuitable ( $\text{pH} < 4.5$ ) puddles.

## TABLES

Table 1: Environmental variability over all puddles in the 5 sites. Total water level is the sum of water levels observed over the year and average immersion time is the mean number of days when puddles are submerged. For vegetation coverage, sunlight intensity, and tracks, values are percentages of total number of quadrats.

Variables	Distributions		
	Mean	Standard deviation	
Maximum water level (cm)	3,12	3,18	
Total water level (cm)	4,22	4,98	
Average immersion time (days)	20,29	19,85	
	Low	Medium	High
Vegetation coverage	36,8	51,5	11,7
Sunlight intensity	5,6	17,9	76,5
	Yes	No	ND
Tracks	54,6	30,6	14,8

Table 2: Characteristics of puddles in site 1 (Coquibus)

		pH < 4.5	0			
Occupied puddles	25	<hr/>				
		Isolated		3		
		pH ≥ 4.5	25	<hr/>		
		Connected	22	Connected to occupied	19	
				Connected to empty	3	
<hr/>						
Empty puddles	103	Connected	29	Connected to empty	26	
				Connected to occupied	3	
		pH ≥ 4.5	59	<hr/>		
		Isolated		30		
		<hr/>				
		pH < 4.5	44			
<hr/>						

Table3: Analysis of variance on morphological traits. Traits are abbreviated as follows: *Nleav* = number of leaves per plant; *Nflw* = number of flowers per plant; *Nak* = number of akenes per plant; *Hind* = plant height; *Diam* = stem diameter; *s/I* = number of seeds per plant.

Variables	Effects					
	Site		Network (site)		Quadrat (network, site)	
	<i>F</i>	<i>P &gt; F</i>	<i>F</i>	<i>P &gt; F</i>	<i>F</i>	<i>P &gt; F</i>
<i>Nleav</i>	0.54	0.7	2.84	<b>0.0007</b>	1.92	<b>0.0046</b>
<i>Nflw</i>	1.41	0.23	2.14	<b>0.0108</b>	1.78	<b>0.0108</b>
<i>Nak</i>	1.66	0.16	4.18	<b>&lt;0.0001</b>	1.31	0.13
<i>Hind</i>	17.75	<b>&lt;0.0001</b>	4.65	<b>&lt;0.0001</b>	1.92	<b>&lt;0.0001</b>
<i>Diam</i>	1.04	0.38	3.07	<b>0.0002</b>	2.7	<b>&lt;0.0001</b>
<i>s/I</i>	13.17	<b>&lt;0.0001</b>	5.15	<b>&lt;0.0001</b>	2.32	<b>0.0005</b>

Table 4: Mean and standard deviation of morphological distance for fruit number over all quadrat pairs. Statistics are computed among and within site, as well as among and within networks. *N* is the number of pairs in each group and Wilcoxon statistical tests are performed for both the site and network scales.

Standardized morphological distance			Wilcoxon test
	Within sites ( <i>N</i> = 778)	Among sites ( <i>N</i> = 600)	Among/Within sites
Mean	1.094	1.131	P = 0.18
Standard Deviation	0.909	0.884	
	Within networks ( <i>N</i> = 1298)	Among networks ( <i>N</i> = 80)	Among/Within networks
Mean	1.125	0.871	<b>P = 0.0197</b>
Standard Deviation	0.906	0.729	

Table 5: Analysis of variance on fitness traits (*Nak* and *s/I*). Traits and effects are abbreviated as follows: *Nak* = number of akenes per plant, *s/I* = number of seeds per plant, *Hind* = plant height, *Diam* = stem diameter, *Site* = site, *Connect* = connection status (connected or isolated)..

Effects	Number of akenes per plant		Number of seeds per plant	
	F	P > F	F	P > F
<i>Hind</i>	12,63	<b>0,0005</b>	5,59	<b>0,0198</b>
<i>Diam</i>	2,18	0,1423	1,14	0,2886
<i>Site</i>	3,3	<b>0,0224</b>	1,32	0,2714
<i>Connect</i>	0,17	0,6809	0,67	0,4152
<i>Hind*Diam</i>	0,23	0,6325	0,16	0,6861

FIGURES

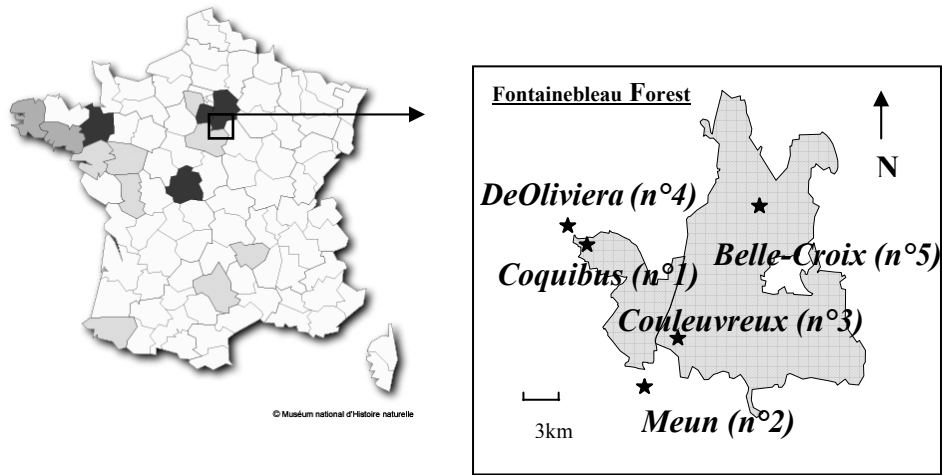


Figure 1: Study area in the Fontainebleau Forest (southeast of Paris, France) and map of the five study sites.

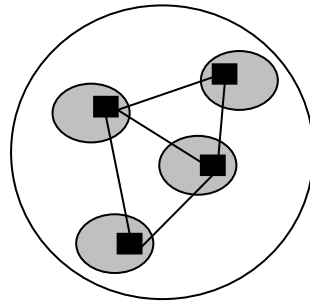


Figure 2: schematic representation of the experimental field design (*in situ*). The dark circle delimits a network, grey circles (●) represent puddles, black squares (■) represent permanent quadrats and solid lines (—) represent the temporary water corridors.

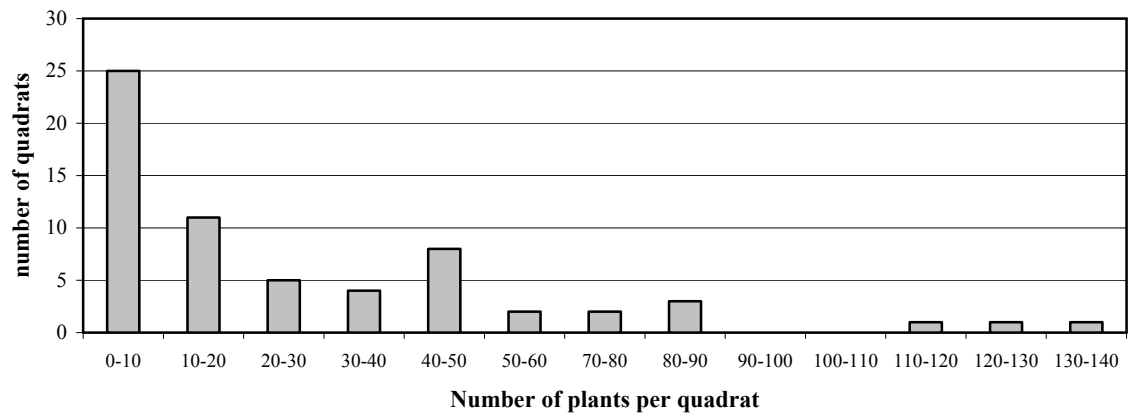


Figure 3: Distribution of flowering plant density in the quadrats

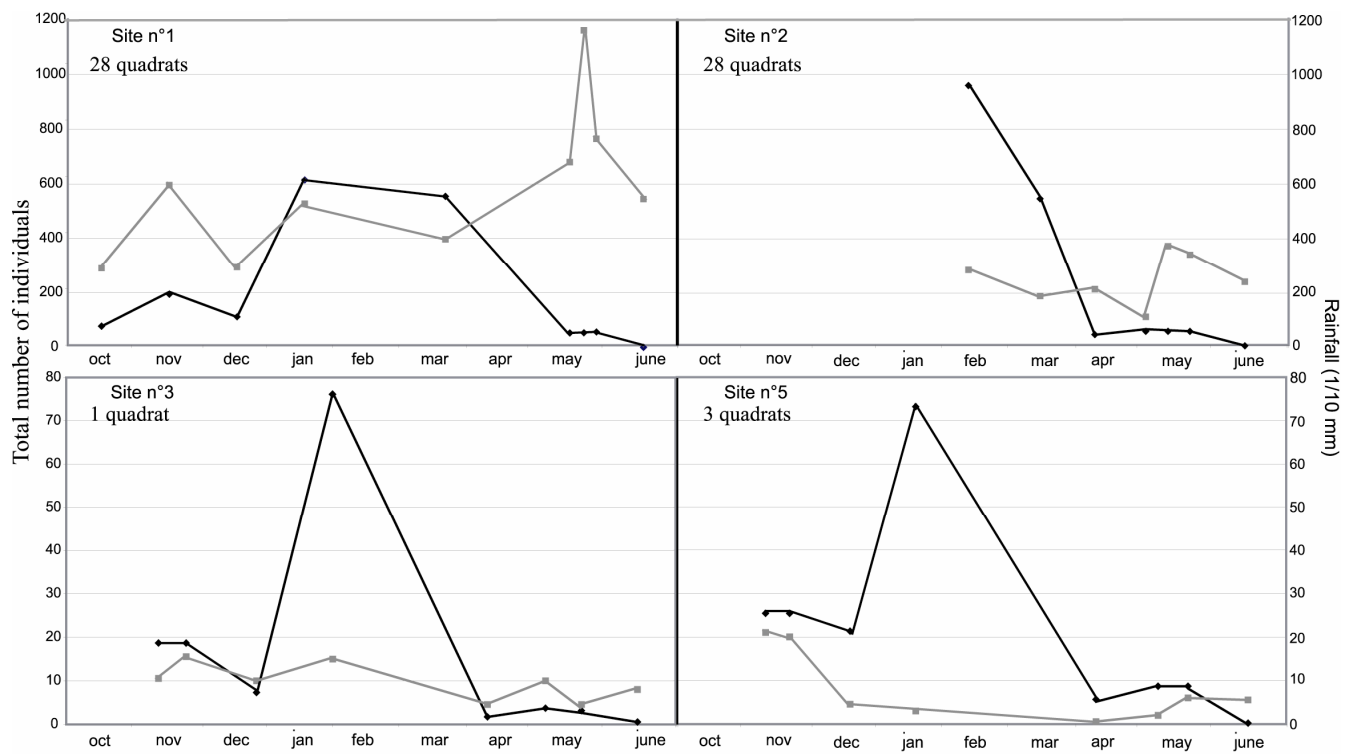


Figure 4: dynamics of rainfalls (black line) and population size (gray line) in each site. Note that the scale of the y-axis varies across sites.



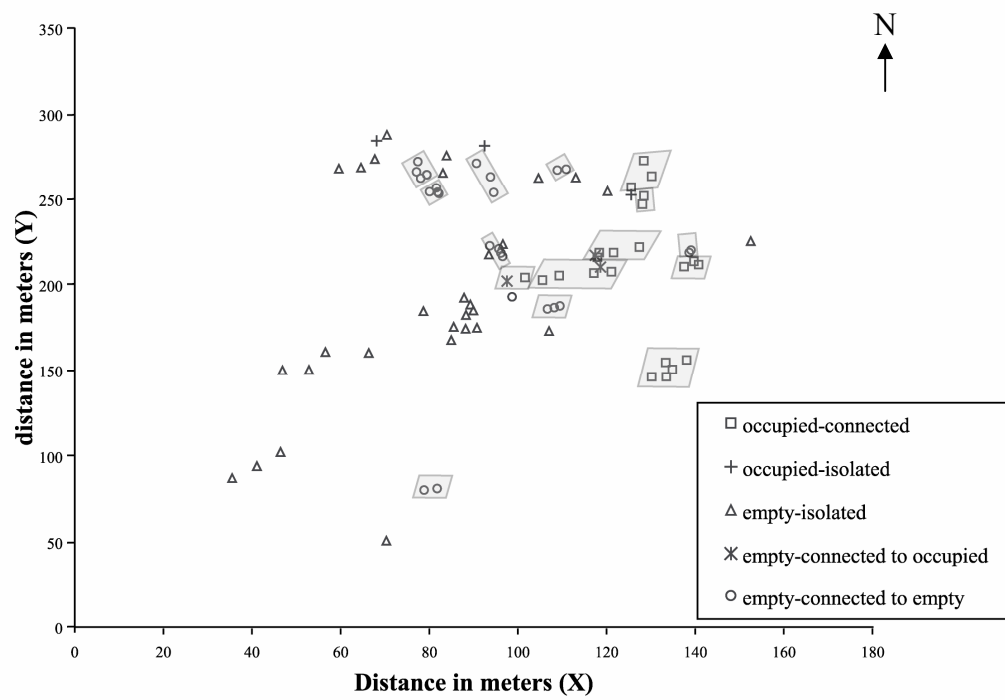


Figure 5: Cartography of all puddles suitable for the species in site 1 (occupied and empty puddles are represented). Grey polygons represent the networks.

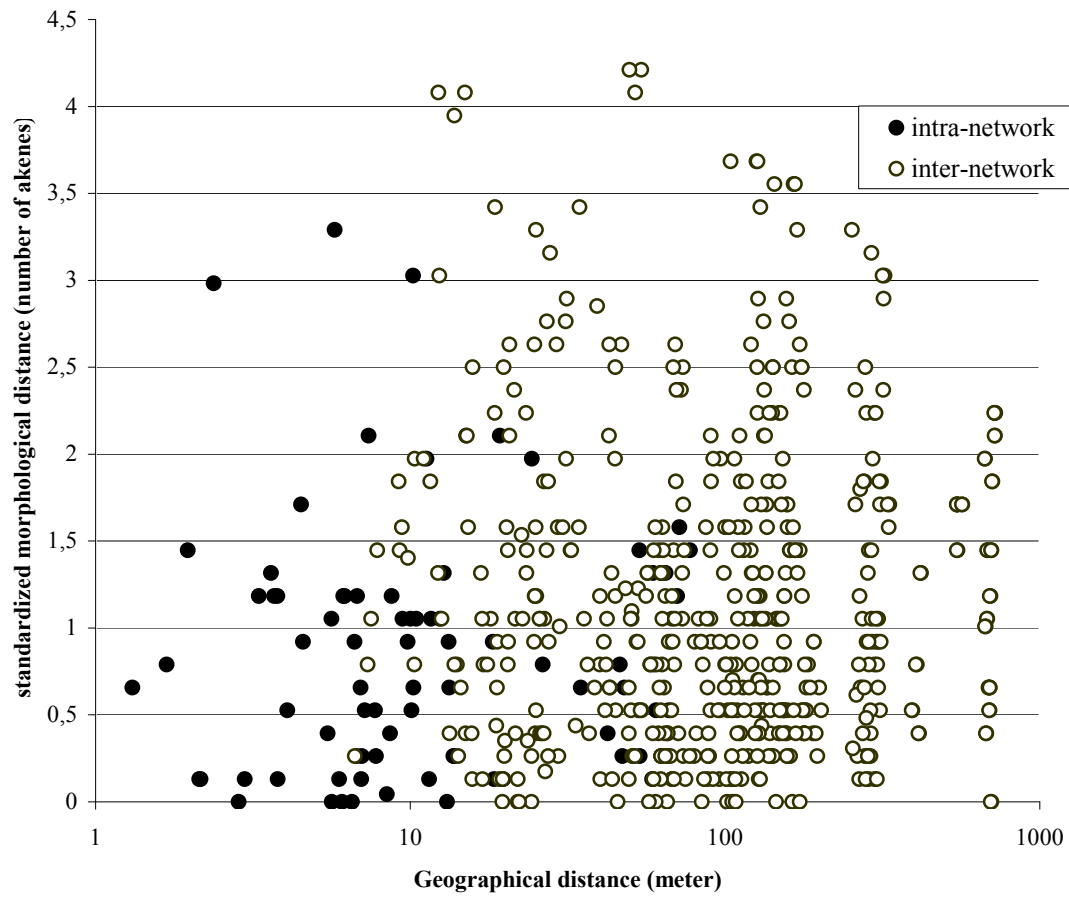


Figure 6: Relationship between standardized morphological distance for reproductive traits (number of akenes) and geographical distance. Each point corresponds to a pair of quadrats; all pairs are included. Black circles represent intra-network pairs and open circles represent inter-network pairs.

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