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Reproductive success and clonal genetic structure of the rare Arnica montana (Compositae) in The Netherlands

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Abstract: In a medium-sized population of Arnica montana, a threatened species in The Netherlands, the breeding system, reproductive success and genetic clonal structure were studied. Pollination experiments suggested that A. montana is largely self-incompatible. Inbreeding depression was observed for seedling weight but not for fruit weight and germination rate. Although genetic variation is rather low in this population, the data suggest an outcrossing mating system. Analysis of the genotype of all mapped rosettes in a plot of 100 m² indicated that dense clusters often consist of identical genotypes, suggesting a clonal structure. Open clusters frequently contained several different genotypes. This may be caused by limited fruit dispersal, since seedlings were found mainly within or in the near surroundings of the clusters.

Numerous plant species have become rare or endangered as a result of the destruction and deterioration of their natural habitats. Populations of these species are often forced to persist in often small and isolated nature reserves. In these fragmented habitats, the populations frequently have reduced numbers of individuals, and are therefore prone to genetic drift and inbreeding (ELLSTRAND & ELAM 1993) both of which decrease genetic variation and fitness (BARRETT & KOHN 1991). Loss of genetic variation is thought to reduce the ability of populations to adapt to changing environments, to decrease levels of performance and to increase their susceptibility to pest and disease pressures (BEARDMORE 1983).

Levels and spatial structure of genetic variation in plant populations are influenced by breeding systems and mating patterns (LOVELESS & HAMRICK 1984, RICHARDS 1986). In self-compatible plants, pollination events generally lead to offspring that are partly self- and partly cross-fertilized. In selfed offspring, heterozygosity is reduced and deleterious recessive alleles are exposed, leading to inbreeding depression (CHARLESWORTH & CHARLESWORTH 1987, MITTON 1989).

Many flowering plant species possess a self-incompatibility system (DE NETTANCOURT 1977). Inbreeding in such strictly outcrossing species is avoided because
self- and cross pollination between individuals sharing the same incompatibility(S)-alleles is genetically prevented. A large number of S-alleles is required to maintain high levels of cross-compatibility in a population. After bottlenecks, a loss of compatible mating types is expected to reduce reproductive success and this will increase the probability of dissolution of the self-incompatibility system (Reinartz & Les 1994).

Inbreeding may still occur in self-incompatible species, however, through mating between close relatives that have partly different S-alleles. Especially in small populations, the probability that such matings occur may be relatively high (Ellstrand & Elam 1993). The spatial structure of the population will also affect mating patterns. It has been demonstrated that neighboring individuals in a population are often closely related as a result of restricted pollen and seed dispersal (Schaal 1980, Waser & Price 1983).

Arnica montana L. is a member of the family Compositae, in which self-incompatibility is a common phenomenon (Brewbaker 1957, Richards 1986). However, to our knowledge no particular data on A. montana are available. The species has become quite rare in The Netherlands (Mennema & al. 1985) and in other parts of its distribution area (Hansen 1976). Its decrease is mostly the result of land reclamation, excessive use of fertilizers, and the abandoning of formerly more widely-used agricultural practices, such as mowing and haymaking, sod-cutting and moderate grazing. It has also been shown that atmospheric acidification and deposition of nutrients affect the vitality of individual plants (Fennema 1990, Dueck & Elderson 1992). Besides these environmental factors, collecting of A. montana for medicinal purposes has also caused the disappearance or reduction in size of several Dutch populations (Mennema & al. 1985). Because of its present status, A. montana has been placed on the Dutch Red List of threatened and vulnerable vascular plant species (Weeda & al. 1990).

The present study examines the breeding system and spatial genetic structure of this threatened clonal species in a medium-sized population. In clonal species, the vegetative reproduction may lead to difficulties in estimating the actual (effective) population size. A population can consist, for instance, of numerous intermingled ramets belonging to only a few genets, or it may comprise many discrete genets that each produces a small cluster of ramets. Given the recently proposed monitoring program for this species in The Netherlands (Boekeloh & Van Zanten 1993), a good insight in its population structure is necessary to enable a more accurate estimate of population size and viability. Besides investigating clonal genetic structure, special attention was also given to the putative self-incompatibility system of the species and to the effects of various pollination treatments and inbreeding on the reproductive success, i.e., the quantity (seed-set) and quality (seed weight, germination and seedling weight) of the offspring produced. In this way, we hope to gain more insight into the breeding system of this rare species, to enable a better assessment of the specific problems of its small and isolated populations.

Material and methods

Study species. Arnica montana L. is a rare, herbaceous, rosette-forming perennial of unmanured, mown or grazed grasslands and dry heathlands in The Netherlands and other
lowland areas (Westhoff & Den Held 1969, Hansen 1976). In mountainous regions the species is much more common. Two subspecies are distinguished: subsp. montana and subsp. atlantica A. Bolòs, the former having larger capitula and wider leaves than the latter (Ferguson 1976). Subsp. montana occurs throughout the entire range of the species, from S Norway and Latvia southwards to the Appenines and the S Carpathians, but is absent from Portugal, while subsp. atlantica occurs from SW France to S Portugal (Ferguson 1976). In The Netherlands, only subsp. montana is present.

A. montana overwinters with large buds at the end of its short, thick rhizomatous stems. In spring new rosettes are formed, some of which may produce an erect stem, bearing 1 to 5 solitary capitula. In The Netherlands, flowering takes place in early summer, from June through July. Flowerheads are orange-yellow and measure 4–8 cm across. The ligulate florets are female and the tubular florets hermaphrodite. The plumed achenes (cypselas) are wind-dispersed. The seeds of A. montana show no dormancy and mostly germinate immediately following dispersal which occurs from late summer through autumn.

**Study site.** The study site is located in the south-east of the province Friesland in the northern part of The Netherlands. The site, named Schoapedôbe, is under the management of the nature conservation organisation 'It Fryske Gea'. The population is fragmented into a relatively large and two smaller subpopulations located in an area of 50 acres with grass-heath and heathland vegetation types and sand-drifts. Arnica montana is found mostly in grass-heath communities [alliance Violion caninae (Schwicker 1941 em. Preisig 1949); see Westhoff & Den Held 1969], which generally exhibit a rather open vegetation structure. Approximately one-third of the study vegetation is made up of Festuca ovina subsp. tenuofolia (Sibth.) Celak. Other conspicuous species are Calluna vulgaris (L.) Hull, Molinia caerulea L., Danthonia decumbens (L.) DC., Galium saxatile L. and Carex pilulifera L. In autumn the vegetation is mown and the hay is removed. Pollination experiments were carried out with plants from the whole population whereas the mapping and the genetic analysis were conducted in the largest part of the population. In total, 1100 rosettes were found, either growing singly, or in clusters of various sizes and densities.

**Pollination experiments.** The breeding system of A. montana was studied by means of different pollination treatments. Fifteen plants were selected that had at least four flowering rosettes in a dense cluster, so that it was very likely that all stems belonged to a single genet. On each individual plant, four different pollination treatments were conducted, each treatment on a single capitulum on a separate flowering stem. For three pollination treatments, individual capitula were placed in a metal cage, covered with fine mesh gauze to prevent insects from visiting. One caged capitulum per plant was left untouched to investigate the possibility of spontaneous self pollination (‘spontaneous selfing’ treatment). On the other caged flower heads, flowers were pollinated by hand, using either pollen from the same flower head (‘hand selfing’ treatment) or cross pollen from another plant in the same population (‘hand outcrossing’ treatment). Hand selfing was carried out by gently transferring pollen from the anthers to the receptive stigmata with a thin wooden pin. Hand outcrossing was done by brushing the anthers of one or more male flowers (from a single capitulum) against the stigmas of flowers in the female stage. These pollinations were carried out every second day until all florets in a capitulum had been in the female stage. Up to five pollen applications per flowerhead were conducted. The fourth pollination treatment (‘open pollination’) occurred on an uncaged stem, labeled with a small metal rod, which was open to natural pollinators. After all florets of these capitula had withered, they were also caged to prevent the mature fruits from blowing away.

Three weeks after the last pollination treatments were carried out, the individual infructescences were collected and the number of developed and undeveloped achenes per capitulum was counted. Developed achenes could easily be distinguished from undeveloped ones because the former are hard and black and the latter soft and whitish. The sum
of both types of achenes was taken as the total number of florets initially present in the pertaining capitulum.

**Determination of reproductive success.** Seeds collected from the different pollination treatments were used to measure different parameters of reproductive success, viz., achene (fruit) weight, number of days to germination and estimated seedling weight after four weeks. One hundred developed achenes were studied from all those produced after each pollination treatment. Achenes were sampled from each of the harvested infructescences, making sure that each flowerhead (individual) was represented in the total sample weighted for the relative number of viable achenes per head. The weight per individual achene was determined on a microbalance. The achenes were placed on wet filter paper in a Petri-dish at 25 °C and in a light regime of 12 h day/12 h night. Linear placement of the achenes in the dishes made it possible to follow the germinating seeds individually. The germination date of each individual achene was recorded. Four days after germination, seedlings were planted in soaked peat pellets. After four weeks of growth, the above-ground biomass of each seedling was estimated using a non-destructive method. This estimation is based on the product of (1) the total number of leaves per rosette (minus the cotyledons) including the already vegetatively formed side rosettes, (2) the length and (3) the width of the largest rosette leaf present at that moment. Regression between this estimated seedling weight and the actual dry weight was found to be highly significant in earlier experiments ($R^2 = 0.707$, $P \leq 0.0001$). Because not all seedlings germinated at the same time and some plants thus had a longer growth period, the estimated seedling weight was standardized to a growth period of exactly four weeks for each individual.

**Spatial distribution of genotypes in the study population and sampling.** In the larger subpopulation of the Schoapedöbe, a plot with an area of $10 \times 10$ m$^2$ was installed in an area where the density of rosettes was relatively high, so the sample size achieved by this plot would be substantial. All vegetative and flowering rosettes of *A. montana* occurring within this plot were mapped. At the end of the flowering season, all seedlings that had emerged in the plot were also recorded. A leaf sample was taken from each of the 450 mapped rosettes in the plot. Seedlings could not be sampled because they did not have enough leaf tissue for electrophoresis. Collected leaves were put in plastic bags, placed on ice and returned to the laboratory for electrophoresis. Leaf samples were kept refrigerated overnight and were prepared within 24 hours after sampling, during which enzyme activity remained satisfactory.

Approximately 90 mg of leaf tissue, devoid of the midrib, were ground in 100 ml of pH 8.3 extraction buffer. This extraction buffer contained 0.1 M tris, 1% glutathion in reduced form (w/v), 5% sucrose (w/v), 20 mM NaNO$_3$, 0.14 M NaCl, 10 mM MgCl$_2 \cdot 6$H$_2$O, 10 mM dithioerythritol and 0.1% β-mercaptoethanol (v/v). Following centrifugation, one part of the supernatant was adsorbed onto 4 × 12 mm filter paper wicks and stored in Petri-dishes at −70 °C until it was used for horizontal starch gel electrophoresis. The other part of the supernatant was used directly for polyacrylamide slab gel electrophoresis.

Prior to this study, 26 different enzymes and 4 buffer systems were tested, using plants from six different populations including the Schoapedöbe study population. Although many enzymes were screened, only 17 enzymes showed activity. For the final analysis, only 6 enzymes were selected: aspartate aminotransferase (AAT, E.C. 2.6.1.1), esterase-β, (EST-β E.C. 3.1.1.--), NADH dehydrogenase (NADH.DH, E.C. 1.6.99.3), phosphoglucomutase (PGM, E.C. 5.4.2.2) and shikimate dehydrogenase (SKD, E.C. 1.1.1.25). Theoretically, more enzymes could have been screened, but the rapid decrease in enzyme activity and the large distance of the study population from the laboratory limited the analysis to those enzymes that were suitable for both electrophoresis on a single buffer system and a reliable interpretation of the banding patterns.
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For the actual analysis a pH 8.1 lithium-borate buffer system and 12%/6% (w/v) starch/sucrose gels were used for AAT, EST-β, NADH.DH, PGI, and PGM, following the procedures described in SOLTIS & SOLTIS (1989). SKD was resolved on polyacrylamide gels using a modified tris-glycine high pH discontinuous buffer system (HAMES & RICKWOOD 1981). For the preparation of the stacking gel buffer phosphoric acid was used instead of HCl to adjust the pH. For the stacking gel we used ammonium persulfate instead of riboflavin with a 3.65% acrylamide concentration. The final acrylamide concentration in the resolving gel was 8.6%.

**Reproductive success and measurements of fitness-related parameters.** The data were tested for normality with Kolmogorov-Smirnov one sample tests. When the data did not fulfill the assumptions of parametric statistics, and transformations did not improve this, non-parametric tests were used. Differences among treatments in the total number of florets per capitulum and the different parameters of reproductive success were tested using an unpaired t-test. Differences in achene (seed) production and the ratio between developed achenes to the total initial number of florets (seed set) between the different pollination treatments were tested by separate Mann-Whitney U-tests following Kruskal-Wallis ANOVA.

**General genetical analysis.** Standard measures of genetic variation were calculated for the study population: the proportion of polymorphic loci (P, 99% criterion), the mean number of effective alleles, averaged over all loci (Ae = 1/∑p_i^2), and the observed (H_e) and expected (H_e) heterozygosity. Genetic diversity was calculated over all loci by H_e = 1-∑p_i^2. A measure for multilocus genetic diversity that has been used in other studies of clonal species (ELLSTRAND & ROOSE 1987) was also calculated for this population: D = 1-∑[(n_i(n_i-1))/(N(N-1))]. Wright’s inbreeding coefficient (WRIGHT 1922) was determined and genotype frequencies per locus were tested for Hardy-Weinberg equilibrium using G-tests for goodness-of-fit (SOkal & ROHLF 1981).

**Spatial patterns in the distribution of rosettes and multilocus genotypes.** The spatial distribution of the rosettes mapped was tested for deviation from a random pattern using the method of contiguous quadrat analysis (BOOTS & GETIS 1988).

Spatial structure in the distribution of the multilocus genotypes was analysed by two different methods:

1. The percentage of nearest neighbours with an identical multilocus genotype was determined for all rosettes.

2. The spatial co-ordinates of all rosettes in the plot were specified with an accuracy of 1 cm. Each of the mapped rosettes was characterized with respect to the number of copies it carried of a certain allele (0, 1 or 2). Spatial autocorrelation analysis (SOkal & ODEN 1978) was performed on transformed individual allele frequencies (0, 0.5 or 1, respectively). Only polymorphic loci (99% criterion) were used in the analysis. Spatial autocorrelation is present whenever individual allelic frequency is correlated with the presence (positive correlation) or absence (negative correlation) of the same allele in directly neighbouring or nearby individuals. Moran’s index I (MORAN 1950) was used as a statistical measure of spatial autocorrelation. The index was calculated by the following formula using the computer program SAAP 4.3 (WARTENBERG 1989): I = N 1/ (z_i - x) 2 - 1, where N is the number of rosettes, w_ij is a join matrix where w_ij is one if rosettes i and j are in the same distance class and zero otherwise, z_i = (x_i - x) and z_j = (x_j - x). The variables X_i and x_j are the genotypic scores for rosette i and j, respectively, and x is the mean score for all rosettes in the plot. Based on the actual distribution of rosettes in the study plot, the following distance classes (in cm) were used for the calculation of Moran’s I: 0–10, 11–20, 21–30, 31–40, 41–50, 51–60, 61–70, 71–80, 81–90, 91–100, 101–200, 201–300, 301–400, 401–500, 501–750, 751–988 (max. distance).

Since genotypic scores do not follow a normal distribution, Moran’s I was tested for significance under the randomization assumption as a standard normal deviate (CLIFF &
Results

Pollination treatments. The initial number of florets per capitulum did not differ significantly between pollination treatments. The type of pollination did result in very significant differences in seed set, however (Fig. 1). The open-pollinated heads had a relatively high seed set (78%). In comparison with open pollination, the cross-pollinated flowerheads showed a significantly lower seed set (55%). After self pollination, either spontaneous or by hand, a dramatically reduced average seed set of no more than 10% was observed. Spontaneous self pollination resulted in an average seed set of 1.3% which was significantly lower than the seed set after hand selfing (8%).

Although the average seed set of hand self-pollinated flowerheads was very low, there was considerable variation in seed set among individual plants, ranging from 0 to 42% (Fig. 2). The success of hand selfing appears to be somewhat higher than that of spontaneously selfed capitula, as seed set in the latter group was never higher than 10% (Fig. 2).

Measurements of reproductive success. The mean individual achene weight did not differ significantly between the four pollination treatments (Fig. 3 A). The low number of viable achenes per capitulum in the selfing treatments did not result in an increase in the individual fruit weight. Although the average achene weight was equal for the different treatments, the mean number of days to germination showed significant differences. Seeds developed after spontaneous selfing
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Fig. 2. Frequency diagram of the percentage of seed set per capitulum for the hand selfing treatment and for spontaneous selfing in the Schoapedöbe population of *Arnica montana*.

Fig. 3. Fruit weight (A), number of days to germination (B) and seedling weight (C) in relation to different pollination treatments in the Schoapedöbe population of *Arnica montana* (cross cross pollination; open open pollination; hs hand selfing; ss spontaneous selfing). Values that have no letter in common are significantly different (t-test, P < 0.05).

Germinated significantly slower than seeds obtained from all other pollination treatments. Also, seeds from cross-pollinated flowerheads germinated significantly faster than those from free-pollinated heads. Seeds obtained by hand selfing showed no significant difference in germination rate with seeds from cross- or free-pollinated flowerheads (Fig. 3 B). Despite the obvious differences in germination rate, the percentage of seeds germinating was nearly 100 for each pollination treatment.

The estimated weight of seedlings from the cross pollination treatment was significantly higher than that of the other treatments (Fig. 3 C). However, no differences in seedling weight were found between hand selfing, spontaneous selfing and free pollination.

**Genetic variation.** Out of the 450 mapped rosettes, 428 were suitable for genetic analysis. The remaining 22 rosettes had already withered and did not show any enzyme activity. Overall allozyme variation was low in the study population,
Fig. 4. A map of the distribution of the 17 multilocus genotypes and seedlings in the Schoapedöbe population of Arnica montana. Uppercase letters present flowering and lowercase letters vegetative rosettes. ▲ seedling, ● vegetative rosette with unknown genotype, ■ flowering rosette with unknown genotype

with a proportion of polymorphic loci of $P = 0.333$ (based on 6 loci). The actual proportion of polymorphic loci in this population is probably considerably lower than presented here. No variation in another 11 allozymes (that were not used further in this study) was observed during the preliminary screening, so a more accurate estimate of $P$ would be 0.111. In the study population, only two polymorphic loci were found, Skd and Est β. The mean number of alleles per locus ($A$) was 2.5
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(3 for *Est*-β and 2 for *Skd*) and the effective number of alleles per locus (*A_e*) was 1.14. The mean genetic diversity (*H_e*) in this population was 0.17 for the six assayed loci. Again, when the 11 monomorphic loci are taken into account as well, *H_e* would be 0.0600. The mean observed diversity in this population (*H_o* = 0.0597) was quite similar to the mean expected value. Genotype frequencies did not significantly depart from Hardy-Weinberg equilibrium in this population [G-test: G(*EST*) = 2.264, ns; G(*SKD*) = 0.412, ns]. Wright’s inbreeding coefficient *F_is* was +0.024 and −0.025 for *Est*-β and for *Skd*, respectively, with an average of +0.004.

Out of the 18 possible multilocus genotypes (*N_g*) in this population, 17 were found among the 428 analysed rosettes. This resulted in a mean multilocus genotype diversity (*D*) of 0.90 in this population.

**Spatial pattern and genetic similarity.** In the study plot, a total of 526 rosettes (vegetative, generative plus seedlings) was mapped. 73% of all mapped rosettes were vegetative. The percentages of seedlings and flowering rosettes are quite low, 14.5% and 12.5%, respectively. Apart from occasional isolated single rosettes, most rosettes were grouped together in either small or large clusters. Not all clusters produced generative rosettes and not all individual rosettes within a flowering cluster produced an inflorescence. The distribution of the seedlings suggests a limited dispersal of achenes. Seedlings were only found in one side of the

![Moran's spatial autocorrelation I as a function of distance](image)

Fig. 5. Moran’s spatial autocorrelation *I* as a function of distance (correlogram) for (*a*) esterase β and (*b*) Skd (full symbols indicate significance, *P* ≤ 0.05)
plot, and were mostly located within loose clusters of mature flowering rosettes, or in their direct vicinity.

Mapping of the electrophoretically genotyped ramets in the Schoapedôbe population (Fig. 4) showed that most of the clustered rosettes consisted of identical genotypes. This supports the hypothesis that a group of densely clustered rosettes belongs to one single genet. However, less densely grouped rosettes often consisted of two or more different multilocus genotypes. These different genotypes were either found on the margins of the clusters or the whole group of rosettes formed a mixture of two or three genotypes (Fig. 4).

The contiguous quadrat-analysis statistically confirmed the visual observation of clustering of the rosettes in the study population. The spatial distribution of the rosettes deviated significantly (P < 0.005) from a random pattern, in the expected direction (i.e., no antagonism or overdispersion). Moreover, the multilocus genotypes appear to be significantly clustered. The percentage of nearest-neighbour ramets with identical genotypes is 86% in the Schoapedôbe population. This is very significantly different from the 6% identical neighbours that may be expected when the distribution of the 17 genotypes is random (e.g., the probability of an identical nearest neighbour is 1/17 for each pair, G = 1790, P ≤ 0.001).

Moran's spatial autocorrelation coefficients (I) for the two allozyme loci are presented as distance correlograms in Fig. 5 a, b, incorporating the overall significance per distance class as assessed by Bonferroni criteria. Overall significance is very high. In total, 57 out of 60 Moran's I-values in the correlograms were significant. Significant positive autocorrelation was observed in the shortest distance classes (0-40 cm) for each of the three alleles of Est. For Skd, significant positive associations of allele frequencies were observed up to a distance of 100 cm. Hence, the patch size, indicated by the distance class in which the value of I drops below zero for the first time, is different for the two variable loci, with Est showing a smaller patch size (41-50 cm) than Skd (91-100 cm).

Discussion

Breeding system. In the family Compositae, multiallelic sporophytic self-incompatibility is common (Brewbaker 1957, Richards 1986). In this S-I system, the behaviour of the pollen is determined by the genotype of its parent (Heslop-Harrison 1975). The major effect is the inhibition of self-fertilization and the promotion of outcrossing between genetically different individuals of the same species (Lewis 1949, Barrett 1988). The observed low seed set for self-pollinated flowerheads in A. montana in the Schoapedôbe population suggests that the species has a genetic self-incompatibility system. However, considerable variation in the expression of this self-incompatibility system was observed, given the highly variable seed set of self-pollinated flowerheads. This variation implies that some individual plants show a partial breakdown or failure of the incompatibility system. Such a breakdown has previously been reported in Taraxacum sect. Ruderalia (Jenniskens 1984); partial break-down of the self-incompatibility system may also be caused by trigger-pollen in this group (Menken & al. 1989, Morita & al. 1990).

Although some plants are more or less self-compatible, pollinators are appar-
ently essential for fertilization since flowers are not able to self-pollinate spontaneously. The significantly lower seed set for cross-pollinated flowerheads in comparison to the open-pollinated flowers is probably caused by either an insufficient number of hand pollinations or by the pollen donor sharing some S-alleles with the pollen receptor. Since open-pollinated flowerheads show a quite considerable seed set, it must be concluded that pollinator activity in the Schoapedôbe population is sufficient.

In self-incompatible species, a large number of S-alleles is necessary for maintaining a high cross-compatibility level in a population (Byers & Meagher 1992). For A. montana, the number of S-alleles was not investigated, but the data show a striking similarity to those from the rare Aster furcatus Burgess (Les & al. 1991, ReInartz & Les 1994). Both species are clonal, exhibiting extremely low genetic variation and both seem to show a partial breakdown of this self-incompatibility system. Further investigation of the self-incompatibility system of A. furcatus showed that the breakdown of the incompatibility system was associated with a low number of S-alleles and by complex overdominance relationships among S-alleles. The loss of S-alleles was most likely the result of population bottlenecks (ReInartz & Les 1994). Computer simulations of a sporophytic self-incompatibility system, performed by Imrie & al. (1972) showed that in small populations genetic drift caused rapid loss of S-alleles. Populations consisting of few individuals are not able to maintain a high diversity of S-alleles and thus show a decrease in the frequency of available mates. This either causes a reduced seed set or an increase in variation of seed set per individual due to the variance in available mates (Byers & Meagher 1992). Since open pollinated flowerheads of A. montana have a rather high seed set and the majority of the self-pollinated heads had a low number of viable seeds, it is likely that a relatively high number of different S-alleles is still present in the population. However, many of the Dutch populations are of much smaller sizes and further studies are necessary to test their reproductive performance.

**Genetic population structure.** The overall genetic variation of A. montana in the Schoapedôbe population is very low. The presence of only two polymorphic loci did not allow any firm conclusions as to the number of different genotypes in the population. Therefore, the clusters that are now classified as belonging to the same genet may still show genetic variation that could not be demonstrated with allozyme electrophoresis. Inferences made about the genetic structure on the basis of the present analysis are therefore conservative.

The suggested population structure where groups of rosettes may be or have been connected by rhizomes, so that they consist of ramets belonging to the same genet, seems to hold particularly for dense clusters of rosettes. However, the more open clusters often consisted of more than one genotype. These mixtures of different genotypes are probably the result of the limited achene dispersal of achenes observed in the population. Seedling recruitment was largely observed within, and in the near surroundings of, flowering clusters of rosettes. This agrees mostly with observations on other clonal plant species that belong to the so-called Repeated Seedling Recruitment (RSR)-type (EriKsson 1993). Despite mixing rosettes of different genotypes, it is likely that the limited seed dispersal together with the clonal spread of genets will have created neighbourhoods with closely related
rosettes. The spatial autocorrelation analysis suggests that in *A. montana* the diameter of such related patches is approx. 50–100 cm.

When all assayed allozyme loci are considered, the relatively large population of *A. montana* investigated here showed a very low proportion of polymorphic loci and low genetic diversity. For a self-incompatible, obligately outcrossing species, such a high degree of fixation is rather unusual (Loveless & Hamrick 1984). Historical population bottlenecks, accompanied by inbreeding among close relatives and lack of immigration of new variants from other populations because of increased isolation may be the most important causes for this low level of genetic variation. Populations with low levels of genetic variation may have reduced fitness (Barrett & Kohn 1991) and may be less well adapted to changes in the environment (Frankel & Soule 1981, Beardmore 1983). Many of the extant populations of *A. montana* in The Netherlands are much smaller than the one studied here, and may be at risk.

In spite of the very low level of overall gene diversity, the two polymorphic loci showed allele frequencies that are indicative of an outcrossing species. This is confirmed by the high mean multilocus genotype diversity found in the Schoapedöbe population. For the two polymorphic loci all but one of the 18 possible genotypes were observed in the study plot.

**Inbreeding depression in various parameters of reproductive success.** The average weight of the achenes was very similar after the different pollination treatments, suggesting that there is no inbreeding depression in this parameter. However, variation in fruit-weight that is not the result of inbreeding depression could also be expected. Since the pollination experiments led to considerable differences in seed set, achenes from heads (individuals) with a high seed set may have had lower weight than those from heads with very low seed set. Such negative relationships between seed set and seed weight, resulting from variation in maternal investment, have often been mentioned for other plant species (Schaal 1984, Roach & Wulff 1987). Apparently, in *A. montana* maternal investment per achene was not limited. However, it can also be concluded that no difference in achene weight for a small number of fertilized ovules may be the result of a loss in capacity of maternal investment by the individual plant.

No differences in the proportion of seeds germinating were found among the pollination treatments. In the early stages of the life-cycle, performance of outbred and inbred progeny is generally very similar, probably as a result of the strong maternal carry-over effects in these stages (Schaal 1984, Roach & Wulff 1987, Wolfe 1993, Oostermeijer & al. 1994). Although there were no differences in germination percentage, there were differences in germination rate. On average, the seeds from self-pollinated capitula seemed to germinate at the same time as the seeds from outcrossed and open-pollinated heads, but outbred seeds germinated significantly faster than the seeds from open-pollinated capitula. From these results, it cannot be concluded that there is any reduction in germination rate resulting from inbreeding. If this were the case, one would expect that offspring from outcrossed heads would germinate the fastest, those from selfed heads the slowest, while the offspring from the open-pollinated capitula (which are the result of a mixture of self- and cross fertilizations) would occupy an intermediate position. As this was not the case, these results are difficult to explain.
Estimated seedling biomass of the outbred progeny was significantly higher in comparison with the offspring of the other pollination treatments. After seedling establishment, the genotype becomes a more important determinant of performance than maternal carry-over effects. Inbreeding depression is therefore generally most strongly expressed in, e.g., adult size, flower production, number of ovules per flower (Roach & Wulff 1987, Dudash 1991, Johnston 1992, Oostermeijer & al. 1994). The level of inbreeding depression expressed often depends on the environment in which it is measured. In the natural habitat the expression of inbreeding depression may be higher than in the greenhouse, owing to less optimal growing conditions (Dudash 1991).

No significant difference in seedling biomass was found between selfed and open-pollinated plants. This is in contrast to our expectations, since in a self-incompatible species, open pollination should have a similar result as outcrossing. However, the observations may be explained by differences in the distance of the pollen donor between the open-pollinated and hand-outcrossing treatments. Pollen for the outcrossing treatment was taken from a donor at least 10 meters away. The uncaged flowers were most likely pollinated with pollen from a donor at a (much) shorter distance, since pollinators generally fly rather short distances (Levin & Kerster 1969). Especially when the density of flowering stalks is high, pollen and gene dispersal tend to be restricted because of the foraging behaviour of the pollinators (Schaal 1980). Since in the study population vegetative and flowering rosettes are not randomly distributed and seed dispersal is rather limited as well, any pollination between neighbouring flowering rosettes will very likely lead to a rather high inbreeding coefficient in the resulting offspring. Hence, the offspring from the open-pollinated capitula may be more similar to inbred than to fully outbred progeny.

Although self-incompatible species such as A. montana are normally prevented from self-fertilization, inbreeding may still occur through mating with closely related or genetically similar individuals which still have different S-alleles. Low genetic variation is not necessarily a good indicator of the number of S-alleles in a population. Oenothera organensis Munz, an endemic gametophytic self-incompatible species had a high allelic diversity at the S-locus, but an extremely low diversity at other loci (Levin & al. 1979).

Apart from an increased relatedness of individuals owing to the low level of genetic variation in the studied A. montana population, the possible partial breakdown of the self-incompatibility system may also have increased the ratio of inbred to outbred offspring in open pollinated capitula. Together, both factors may explain the relatively low performance of seedlings from naturally pollinated flower heads.

It can be concluded that in the present situation inbreeding depression lowers the performance of seedlings in the study population of this rare, self-incompatible species. This inbreeding depression seems to occur in spite of sufficient pollinator visitation and may be ascribed to (1) the patchy clonal structure of the population, (2) the low level of genetic variation in the population, and (3) partial breakdown of the self-incompatibility system. The latter two may be the result of a (recent?) bottleneck. These findings suggest that the perspectives for the (majority of) the small populations present in The Netherlands may be under pressure.
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References


Reproduction and genetic structure in *Arnica montana*


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