Abstract: Plant biodiversity has declined seriously because of both habitat deterioration and habitat fragmentation. As a result, many species have been forced into small, fragmented, and isolated populations and are believed to suffer from higher extinction risks. Genetic reinforcement and the establishment of new populations are now widely used to prevent extinction. However, the genetic background of transplants may seriously affect the long-term success of these populations because increased genetic variation may reduce the risk of inbreeding or lead to better performance by restored heterozygosity levels (heterosis). Introduced transplants, however, may be poorly adapted to the new local conditions. We tested the initial success of alternative introduction strategies. We evaluated the potential for inbreeding, heterosis, and/or local adaptation after introduction of artificial populations of *Succisa pratensis*. We introduced individuals from local and distant artificial populations that were created from either small or large populations. We created the artificial populations with the same census population size but varying effective population sizes by adjusting the relatedness of individuals. We analyzed the demographic consequences of inbreeding, heterosis, and/or local adaptation of these artificial populations. Reduced performance after selfing was manifested by a reduction in seed production, seed weight, germination, and flowering percentage. Seed production, seed weight, flowering percentage, and number of flowerheads were negatively affected by small population size. Local adaptation increased biomass and flowering percentage for local individuals. Seed weight and seed production exhibited significant heterosis. Our results demonstrate that threatened populations can benefit from introduction and genetic reinforcement of individuals from related populations. Significant differences among the artificial populations for several measured performance components suggest that introduction or reinforcement is best achieved through material from a local population or, when unavailable, from several large populations.

Key Words: multisource introductions, outbreeding, single-source introductions, *Succisa pratensis*
Introduction

Fragmentation and deterioration of habitats, changes in land use, and numerous direct and indirect effects of human activities are considered some of the major reasons for the decline in biodiversity. Due to fragmentation and deterioration of habitats, species are forced into small and isolated populations, which are likely to suffer from loss of genetic variation and fixation of alleles due to genetic drift (Charlesworth & Charlesworth 1987; Barrett & Kohn 1991; Boyce 1992; Ellstrand & Elam 1993). Eventually this could lead to a decreased capability to adapt to environmental changes and thus to a reduced evolutionary potential (Ouborg & Van Treuren 1994). These effects may be exacerbated by fragmentation and isolation (Wright 1969). Smaller and isolated patches therefore run a larger risk of inbreeding, which could affect fitness traits such as longevity and dispersal capacity (Olivieri & Gouyon 1996).

The dynamics of small populations can also be affected by demographic processes. This has been demonstrated by Menges (1991), who found a dramatic decline in seed germination when isolated prairie populations of Silene regia fell below 150 plants. Moreover, demographic and genetic factors can act simultaneously on small and isolated populations (Vergeer et al. 2003a). Ågren (1996) showed that small populations may experience low visitation rates and reduced pollinator success, reducing the level of outcrossing and increasing the rate of self-fertilization and subsequently of inbreeding (Jennersten 1988; Menges 1991; Lamont et al. 1993; Van Treuren et al. 1994).

To preserve these small and inbred populations, several conservation measures can be taken. One possibility is to increase dispersal, and thus alleviate isolation, by creating corridors via introduction. Another possibility is to introduce individuals from related populations. These measures might reinforce the genetic composition of populations through recombination among variable parental genomes, which may result in increased fitness of progeny (i.e., heterosis) (Hedrick 1995; Westemeier et al. 1998; Madsen et al. 1999). Therefore, small, and semi-isolated populations might experience a “genetic rescue effect” (Ingvarsson 2001).

Populations may also be negatively affected by these measures. Introduced transplants may be poorly adapted to the new local conditions. Moreover, input of new genes might disrupt local adaptation. Both processes reduce progeny fitness (Templeton 1986; Rhymer & Simberloff 1996; Montalvo & Ellstrand 2001). This dilemma is of serious concern in conservation biology because it remains unclear how these processes affect small and isolated populations. It is therefore important to estimate the contribution of each of these processes and to assess the balance for the design of introduction strategies. Introduction strategies should focus on the long term: one single introduction should be sufficient for long-lasting conservation. The origin, variation, and relatedness of source material (i.e., single-source or multisource introductions) used for transplantation or introduction should be considered to reduce the risk of creating disadvantages such as inbreeding and poorly adapted transplants.

Here we tested the initial success of alternative introduction strategies. We tested the effects of variation and relatedness of source material on the initial success of introduction by introducing artificial populations of Succisa pratensis with the same census population size but varying effective population size by adjusting the relatedness of individuals. We analyzed the effects of origin and original population size of the source populations by examining local as well as different distant source populations. We analyzed the risk of inbreeding with a crossing experiment in which different levels of inbreeding were created. We hypothesized that introduction with local
Introducing populations would be advantageous because of local adaptation and that introduction of small populations would increase the risk of disadvantages such as inbreeding.

Methods

Study Species and Site

*Succisa pratensis* Moench (*Scabiosa succisa* L.) is an iteroparous, hermaphroditic, long-lived (>25 years) perennial herb that flowers from late July until October (Adams 1954). Although the flowers are self-compatible, insect pollination is presumed to be necessary because flowers that were not visited produce few viable seeds (personal observation). The plant is visited by many different insect groups but predominantly by hoverflies and various bee species (personal observation). In the Netherlands, it occurs in open patches of different types of nutrient-poor grasslands, such as heathlands and unfertilized hay meadows. It is still quite common in the Netherlands, but due to changes in land use, to habitat fragmentation, and to deterioration, its distribution area has decreased 74% since 1935 (van der Meijden et al. 2000). The remaining populations are isolated from one another, with population sizes ranging from approximately 10 to 30,000 flowering individuals; however, many of them are very small and threatened with extinction.

We performed our field experiment in the nature reserve de Schaopedobbe in the northern part of the Netherlands (52° 57'N, 6° 16'E). We created experimental plots in a sod-cut, nutrient-poor grassland, next to a heathland, that was colonized by grass and heathland species, including *Succisa pratensis* (considered a local population). The turf in the experimental area was cut at least 1 year before the start of the field experiment to reduce competition and increase establishment probabilities.

Experimental Design

During the growing season of 1999, we collected seeds of *Succisa pratensis* on 15 randomly selected flowering individuals, regularly spaced across the vegetation, in the local and 12 distant population (6 small and 6 large populations). Small populations consisted of <100 flowering individuals and large populations of >10,000 flowering individuals. We classified the local population as small. Only populations occurring on comparable vegetation types on nutrient-poor heathlands were selected. All populations occurred in the northeastern part of The Netherlands, in the same region. The distance between the local and all distant populations was at least 25 km.

We germinated seeds in a greenhouse and subsequently planted individuals in plastic pots (diameter 14 cm) filled with a 1:1 mixture of potting compost and sand. Based on various possible introduction strategies, different artificial populations were created with the same census population size (12 individuals) but varying effective population sizes ($N_e$) by adjusting the relatedness of the individuals.

We analyzed the effect of origin of the individuals by combining different source populations (origin effect). We created artificial populations of distant small and distant large populations to test the effect of population size of the source populations (original population size effect). Artificial populations created from the local population were used to test whether it is advantageous to be adapted to local conditions, and artificial populations derived from a mix of only different distant populations were used to analyze the effects of heterosis. The effect of relatedness of the individuals was analyzed by varying the number of individuals used per parent plant within an artificial population (relatedness effect). This resulted in an experimental design of 12 artificial populations (Fig. 1). Four of these were derived from distant small populations, which were randomly selected from the 6 distant small populations. Two of them (“unrelated small”) were made up of 12 unrelated individuals of 1 population (1 individual per parent plant); the other two (“related small”) were made up of 12 related individuals of 1 population (6 individuals per parent plant). Similarly, 2...
artificial populations with 12 unrelated individuals and 2 artificial populations with 12 related individuals of 4 randomly selected, distant large populations were created (“unrelated large” and “related large,” respectively). In addition, 2 artificial populations were composed of 12 unrelated individuals (1 individual per parent plant) originating from the local population (“unrelated local”), and 2 artificial populations with 12 unrelated individuals (12 distant populations, 1 individual per population) were made as well (“unrelated mix”).

Plants were randomized throughout the greenhouse. We watered the plants three times a week with tap water, corresponding to the annual amount of rainfall in The Netherlands of 1000 mm. During summer the temperatures varied between 20° and 25° C during the day and between 15° and 18° C during the night. In winter the temperatures ranged from 5° to 10° C during the day and to 0° to 5° C at night. The relative humidity in the greenhouse varied with day and night temperatures between 60% and 90%, respectively.

Crossing Design

In June 2000 we regrouped plants in bud stage according to their experimental population to facilitate the crossings. Each plant of each artificial population received two treatments replicated twice: two flowerheads were self-pollinated by hand (“self”), and two flowerheads were hand-pollinated with a fresh pollen mix (collected daily) consisting of pollen of all other individuals within the same artificial population, excluding pollen of the individual itself (“wpc”). This resulted in a total of 576 pollinated flower heads. Prior to wpc pollination, flowerheads were emasculated with a small pair of scissors to prevent self-pollination. We pollinated the plants by brushing the open stamens (male stage) of a flowerhead across the receptive stigmas (female stage) of another flowerhead. Pollinations were repeated every day until all flowers in a capitulum had been in the female stage. We self-pollinated the plants by manually brushing pollen over the stigmas within the flowerhead. After 6 weeks, we collected ripened, intact, seeding flowerheads. Seeds with endosperm development (thick and hard) could easily be distinguished from seeds without endosperm development (small and soft). The sum of all “developed” seeds was used to calculate total seed set. We did not analyze the percentage of seed set because it appeared that emasculation occasionally might have damaged or removed pistils. To break dormancy, we stored the developed seeds in the dark at 4° C for 6 weeks.

Analysis of Plant Performance

Seeds produced by each treated flowerhead were counted and weighed on a microbalance in the laboratory. We placed seeds on two layers of wet Whatman paper (no. 1) in petri dishes (one seed family per dish) under a day/night regime of 16/8 hours, at a temperature of 25–20° C and a light intensity of 150 µE/m/second. Seeds were placed in a grid pattern (1 seed/cm) to reduce the risk of fungal spread to neighboring seeds. We checked germination every 2 days and calculated the germination rate for the period for which the slope of the regression line through the germination curve was steepest (days 8–15 after sowing).

Eight weeks after the start of the experiment, when no further germination occurred, the total number of seedlings was counted (total germination). To check for viability of ungerminated seeds, we squeezed seeds that did not germinate with a pair of tweezers. If the embryo was soft or slimy, we assumed that it had been attacked by a fungus or died in another way; otherwise, we assumed that seeds were dormant or not viable. The seedlings (one per crossing treatment per individual) were subsequently individually planted in plastic pots (14-cm diameter) filled with a sandy, slightly acidic and nutrient-poor soil collected near the local population. In March 2001, the 8-week-old seedlings were transplanted into the field.

Survival was monitored regularly, but only final survival was included in the statistical analysis because we were mainly interested in the success of alternative introduction strategies. In September 2002, we measured final survival; at that stage all surviving individuals were well established and mortality rates per census were almost zero. In August 2002, we estimated the aboveground biomass with a nondestructive method based on the product of the number of leaves of the rosette and the length and width of the largest leaf. Regression of the estimated aboveground biomass and the actual dry weight was highly significant ($R^2 = 0.909, df = 73, p < 0.0001$). In September 2002, we determined flowering percentage by counting the number of flowering individuals and the number of flowerheads per individual.

Data Analysis

We started the experiment with 576 individuals, with 192 individuals per original population size treatment, 96 individuals per origin treatment, 96 individuals per relatedness treatment, and 48 individuals per crossing-type treatment, of which, 96, 48, 48, and 24 individuals, respectively, were introduced per treatment.

We tested the effect of origin of source population (distant large, distant small, local, and a mix of distant populations) and crossing type (within-pollination cross and self-pollination) on the initial success of introduction by using a general linear model procedure (procedure general linear models, SAS for windows, version 8.2, SAS 1999–2001), with origin and crossing type as fixed factors. These analyses only included unrelated individuals (Fig. 1) and were analyzed according to a factorial design. To test the effects of origin of source population (local, distant large, distant small, and local and a mix of...
Results

Seed Production and Seed Weight
Seed production and seed weight were significantly affected by origin, original population size, crossing type, and level of relatedness (Table 1). Artificial populations made up of local or distant small populations produced fewer and lighter seeds than did artificial populations made up of distant large or several distant populations (Figs. 2 & 3). Furthermore, artificial populations derived from several distant populations (unrelated mix) produced more and heavier seeds than did artificial populations derived from (unrelated) distant large populations. Seed production decreased significantly when artificial populations were composed of related individuals, and selfing resulted in significantly lower seed production. Similar results were observed for seed weight (Table 1; Fig. 3).

Germination and Germination Rate
Germination was significantly affected by crossing type (Table 1) as a consequence of strongly reduced germination in self-pollinated plants (Fig. 4). For germination rate, the effects of origin and crossing type were significant (Table 1), with lower germination rates for the distant-small-population treatments compared with ones of the distant-large-population treatments. Significant interactions (origin × crossing type, original population size × crossing type, and original population size × relatedness × crossing type) were also observed.

Biomass and Survival
In the field, crossing type did not significantly affect biomass, and there was no relatedness effect (Table 1).

Effects of origin were indicated, however, by a remarkably high biomass for individuals of the local populations (Table 1; Fig. 5). Interaction effects (original population size × crossing type, original population size × relatedness, and relatedness × crossing) were significant, suggesting effects of crossing type and/or relatedness in at least some populations. In the field, mean mortality of all transplanted plants declined with increasing age, with the highest mortality occurring during the first field season. Mean mortality rates measured every 3 months during the first 1.5 years of life were low (0.05, 0.07, 0.03, 0.01 [winter], 0.00, and 0.00, respectively).

Flowering
In 2002 we measured significant effects of origin and original population size on flowering percentage (Table 1; Fig. 6). Plants from distant small populations were less likely to flower than those from other distant populations. Furthermore, plants from the local population and from a mix of distant populations showed higher flowering percentages than did plants from distant large and distant small populations. For the number of flowerheads produced, we observed significant effects of origin and crossing type (Table 1) because of decreased flowerhead production in plants originating from small populations and because of the effects of selfing.

Discussion
Our objective was to evaluate the initial success of alternative introduction strategies as a restoration measure, either for genetic restoration of small, inbred, and threatened populations or for establishment of new populations. Several potential problems may be associated with introduction, the most immediate of them being the increased risks of inbreeding depression and the disruption of local adaptation.

The risk of inbreeding in introduced populations was demonstrated by crossing experiments that showed strong reductions in seed production, seed weight, germination, germination rate, and flowering percentage after selfing. Small populations are expected to suffer from a loss of genetic variation (genetic erosion) as a result of genetic drift and a higher risk of inbreeding (Charlesworth & Charlesworth 1987; Barrett & Kohn 1991; Young et al. 1999). Effects of inbreeding in small populations have already been shown in several plant species (Jennertsen 1988; Menges 1991, 1992; Ouborg & van Treuren 1995; Ågren 1996; Fischer & Matthies 1998; Kéry et al. 2000; Luijten et al. 2000, 2002), including *Succisa pratensis* (Vergeer et al. 2003a). It is therefore likely that introduction of related or single-source material of small populations will result in an increased propensity to inbreeding in introduced populations.
Table 1. Results of a general linear model procedure on seed production, weight, germination, germination rate, biomass, and number of flowerheads and of a binary response analysis on survival; and flowering percentage as a function of origin, crossing type, and origin \times crossing type and as a function of original population size, relatedness, crossing type, and their interactions.\(^a\)

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\(^a\)Probability: \(^*\)p < 0.05; \(^**\)p < 0.01; \(^***\)p < 0.001.

\(^b\)Seed weight was log transformed to correct for non-normality.

\(^c\)Germination was arcsin-square-root transformed to correct for non-normality.

\(^d\)Germination rate was ln+1 transformed to correct for non-normality.

In addition, we observed population-size effects in the introduced offspring of distant small populations, such as reduced seed production, lower germination rate, and reduced flower capacity. These results were in agreement with earlier studies of *Succisa pratensis* (Vergeer et al. 2003\(^a\), 2003\(^b\)), in which population size was correlated with genetic variation and fitness: small populations performed more poorly, had higher inbreeding coefficients, and suffered more from deteriorating soil conditions. We also observed effects of relatedness of the source material, evident as reduced seed weight, seed production, and biomass and as increased mortality when related individuals were introduced. These results suggest an increased risk of inbreeding depression when related source material is introduced. Significant interactions of original population size \times crossing type and original population size \times crossing type \times relatedness for seed weight and germination rate (Table 1) were also measured. These
interactions may suggest that distant small populations are genetically eroded and that lack of expression of inbreeding depression may be due to a lack of genetic variation and thus limit differences between selfing and outcrossing (within population-cross treatments). Another interpretation is that both the seed weight and germination rate of seeds derived from distant small populations were less affected by selfing than were those of seeds derived from distant large populations. This might suggest that these small populations were already inbred to some degree. Because we could not analyze these assumptions, it is not possible to determine whether these results were due to a lack of genetic variation or to inbreeding depression. It is clear however, that the data suggest that small populations are more likely to suffer from a loss of genetic variation than large populations, which may affect fitness and subsequently result in a lower success of introduction.

Our results emphasize the importance of genetic variability among introduced individuals, which is strongly influenced by the origin and relatedness of source material. Introduction of several (large) populations may introduce genetically more variable material, and introduction of multisource material may foster greater success through recombination among variable parental genomes. When high numbers of unrelated individuals from different source populations are introduced, a large and genetically variable gene pool will be introduced in which maladapted individuals will be outcompeted by the best-adapted individuals (i.e., natural selection) (Tecic et al. 1998). The importance of multisource introductions of different source populations is illustrated by our findings of increased performance of seed production, seed weight, and flowering percentage after interpopulation crosses (“unrelated mix”). This increase in plant performance suggests that at least some of the populations were already subjected to genetic erosion and that interpopulation crossing increased genetic variation by recombination among variable parental genomes and subsequently enhanced progeny fitness (i.e., heterosis). Similar results

Figure 2. Mean seed production in relation to origin (distant large, distant small, local, and a mix of distant populations), original population size (distant large and distant small), and relatedness (related and unrelated individuals) of the source material after within-population crossing and selfing. Significant differences within the level of origin are indicated with different letters (Tukey’s multiple comparisons test, \( \alpha = 0.05 \)).

Figure 3. Mean seed weight in relation to origin (distant large, distant small, local, and a mix of distant populations), original population size (distant large and distant small), and relatedness (related and unrelated individuals) of the source material after within-population crossing and selfing. Significant differences within the level of origin are indicated with different letters (Tukey’s multiple comparisons test, \( \alpha = 0.05 \)).
have been found for both animal species (Hedrick 1995, 2001; Westemeier et al. 1998; Madsen et al. 1999) and other plant species (Fenster 1991; Les et al. 1991; De Mauro 1993; Oostermeijer et al. 1995; Byers 1998; Luijten et al. 2002). Our study and the others listed suggest that at least some small and inbred populations of endangered species could be genetically restored via the introduction of individuals from related populations. Similar conclusions have been drawn by Helenurm (1998), van Groenendael et al. (1998), Fenster and Galloway (2000), Galloway and Fenster (2000) and Luijten et al. (2002).

We found indications of local adaptation. Plants from local populations had a higher biomass and produced more flowers than plants from distant populations, even though the distant seed sources were from populations whose habitat was similar to that of the experimental site. This suggests that material taken from populations from different habitats might perform worse than those taken from similar habitats, thus highlighting the importance of using local seed sources. Although these effects were pronounced, it cannot be concluded that they were an effect of local adaptation because we did not perform reverse transplantation—the local population was not tested on the site of the distant population—and we cannot exclude maternal effects. However, numerous researchers have shown the effects of local adaptation by measuring the higher performance of local relative to distant transplants (Schemske 1984; Silander 1985; Waser & Price 1985; Schmitt & Gamble 1990; Wang et al. 1997; Montalvo & Ellstrand 2000a, 2000b, 2001). This home-site advantage implies that if distant transplants are introduced, they may be poorly adapted. Further problems can arise if distant transplants cross with local plants, which may disrupt locally adapted genotypes and lead to reduced performance of the hybridized progeny (i.e., outbreeding depression) (Fenster & Dudash 1994; Knapp & Rice 1994; Montalvo et al. 1997). Outbreeding depression, however, is normally manifest in the second and later generations as a result of recombination between parental genomes, and is unlikely to be seen in the first generation (Lynch).
of unrelated individuals, provided that the population is genetically variable. In general, however, there will be either no local population or the local population will have already experienced a long history of inbreeding. In these situations we suggest the use of large numbers of unrelated individuals (multisource introductions) from a mix of different, genetically variable genotypes of several (large) populations as the most successful strategy for the creation of sustainable and viable populations. Selection of source populations with habitat conditions similar to those of the future population is advised to diminish eventual problems of local adaptation.

Acknowledgments

We thank R. Peters and L. van den Berg for their help and company in the field and during all pollination experiments. We thank J. Stuefer for statistical assistance and J. van Groenendael, L. van den Berg, and two anonymous referees for helpful comments and critical reading of the manuscript. We also thank It Fryske Gea for their permission to perform the field experiment in de Schaopedobbe. This project was funded by the Dutch Technology Foundation (STW).

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