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Investigating genetic factors behind the decline of a threatened plant species – Tephroseris integrifolia (Asteraceae)

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Kerstin Isaksson

A doctoral thesis at a university in Sweden is produced either as a monograph or as a collection of papers. In the latter case, the introductory part constitutes the formal thesis, which summarizes the accompanying papers. These have either already been published or are manuscripts at various stages (in press, submitted or in ms).

Front cover: *Tephroseris integrifolia* (Fältnocka), photo by Kerstin Isaksson.

Contents

This thesis is based on the following papers:

I   Isaksson K, Widén B. Inbreeding depression cannot explain the rapid decline of the self-incompatible perennial *Tephroseris integrifolia* in Sweden. – *Submitted.*

II   Isaksson K. Self-incompatibility does not explain the declining population sizes of *Tephroseris integrifolia* (Asteraceae). – *Manuscript.*

III Isaksson K, Nordström S, Widén B. No connection between population size and local genetic variation in AFLP-markers in a threatened plant species (*Tephroseris integrifolia*). – *Manuscript.*

IV   Isaksson K, Widén B. Negative association between population size and quantitative genetic variance in *Tephroseris integrifolia* (Asteraceae) – evidence for a recent bottleneck? – *Manuscript.*

The participation of the author in the papers:

Paper I: Responsible for planning and carrying out cross-pollinations, for measuring plants in common garden, and for analysing the data. Co-responsible for manuscript.

Paper II: Responsible for planning and carrying out cross-pollinations, for counting seeds, for data analysis and manuscript.

Paper III: Responsible for collecting material in Estonia, for cultivation of plants, for laboratory work, main responsible for data analysis and manuscript.

Paper IV: Responsible for collecting plant material in Britain, Denmark, and Estonia, and for cultivation of plants. Co-responsible for selecting characters to investigate. Main responsible for measuring. Responsible for data analysis. Main responsible for manuscript.

Investigating genetic factors behind the decline of a threatened plant species – *Tephroseris integrifolia* (Asteraceae)

Background

Land use change is one of the major threats to global species diversity (Pimm & Raven 2000, Sala et al. 2000, Foley et al. 2005), and in remaining habitat fragments, species keep going extinct long after fragmentation has taken place (Pimm & Raven 2000, Foley et al. 2005). One of the habitat-types which are loosing most species diversity is grassland (Sala et al. 2000). In Europe, nutrient-poor grasslands have been associated with human management for thousands of years (Richards 1990), and they have harboured a lot of species (Pärtel, Bruun, & Sammul 2005), of which many are now threatened with extinction (Hodgson et al. 2005, Schleuning et al. 2009). Though most of the loss of grassland cover in Europe took place before 1850 (Richards 1990), the more severe changes occurred after 1940, through the introduction of tractors and artificial fertilisers (Ratcliffe 1984, Bernes 1994). However, the general process towards a more efficient and static land use, such as a more rigid division between fields and pastures, and the cessation of rotation of crops started much earlier than that (Emanuelsson 2009).

Genetic depletion in small populations

As habitats have become fragmented, plant and animal populations have been fragmented as well. Small population size leads to genetic depletion through genetic drift (Wright 1931), to inbreeding (Gaggiotti 2003), and to reduced fecundity as a consequence of a lower supply of suitable mates (e.g. Kolb & Lindhorst 2006, Levin, Kelley, & Starkar 2009).

Several empirical studies have reported indications of genetic depletion in small and isolated populations – e.g. there is often a positive relationship between population size and genetic variation in molecular marker genes (Lammi, Siikamäki, & Mustajärvi 1999, Landergott et al. 2001, Kang, Jiang, & Huang 2005, Coppi, Mengoni, & Selvi 2008), furthermore fitness of offspring often increases with distance between parents (Morán-Palma & Snow 1997, Byers 1998), and lack of self-incompatibility alleles has been found to reduce seed-set in small populations of self-incompatible species (Byers 1995, Wagenius, Lonsdorf, & Neuhauser 2007). A decrease of variation in potentially adaptive phenotypic characters with decreasing population sizes has mostly been found in experimental populations (Van Buskirk & Willi 2006).

However, in some cases, an unexpectedly high genetic variation has been documented in small populations (Young, Boyle, & Brown 1996, Young, Brown, & Zich 1999, Marquardt et al. 2007, Williams et al. 2007, Yates et al. 2007, Yoshioka et al. 2007, Gaudel &

Till-Bottraud 2008), and this is sometimes explained as the result of gene-flow from unknown sources outside the population (Lawrence et al. 2008, Williams et al. 2007), but populations with unexpectedly high genetic variation are often also believed to be relicts of larger populations which have suffered from a very recent decrease in population size, so that not much genetic variation has yet been lost (Young, Boyle, & Brown 1996, Williams et al. 2007, De Almeida Vieira & De Calvalno 2008, Luijten et al. 2000, Van Geert, Van Rossum, & Triest 2008). Surprisingly, for quantitative characters, variation has sometimes been found to be higher in small populations than in large populations (e.g. Widén & Andersson 1993, Waldmann 2001) and in several bottleneck experiments, quantitative variation has been found to increase during the first generations (Van Buskirk & Willi 2006). The most plausible explanation for a negative relationship between population size and variation in quantitative characters is that during the first generations of inbreeding, variation in quantitative characters can sometimes increase as the result of an increased homozygosity for previously rare alleles, leading to the expression of novel phenotypes (Willis & Orr 1993).

According to a meta-study by Spielman, Brook, & Frankham (2004) and a review by Frankham (2005) species do not go extinct before they are impacted by genetic factors. E. g. 77 % of 170 investigated taxa listed as threatened by the IUCN (i.e. globally red-listed species) had lower heterozygosity than non-threatened relatives (Spielman, Brook, & Frankham 2004).

If there is a difference in genetic variation between globally red-listed and non red-listed species, we ought also to be able to detect differences in genetic variation in different populations of the same species, depending on the size of the populations, and depending on whether we sample from a region where the species is suffering from severe decline (and consequently has been put on national red-lists of threatened species), or from a region where it is not rare, and not regarded as threatened.

A threatened grassland species

Tephroseris integrifolia (L.) Holub (Asteraceae) is a species which grows in nutrient-poor, calcareous grasslands and which in Sweden has suffered a lot from the destruction and fragmentation of these habitats (Regnéll 1976, Widén 1987). The species has sporophytic self-incompatibility (see paper II), it rarely flowers during two successive years, in the field first flowering does not occur until the fourth year, and it has no seedbank (Widén 1987). This species has continued to decrease in population size and number even after fragmentation has ceased and in spite of the implementation of a number of conservation measures (Widén & Wetterin 1999). Of totally 42 populations identified since the 19th century, only six remain today, and they are still decreasing in size (see paper I). In this thesis, I examine if the decline in Swedish *T. integrifolia* has had any effect on local genetic variation in the remaining populations, and discuss whether genetic depletion might be a contributory cause of the continuing decline of the species.

Methods

In this thesis, different aspects of local genetic variation are studied, with focus on the declining Swedish *T. integrifolia* populations, but also including Estonian (papers III and IV), British (paper IV), and Danish (paper IV) populations. In all, 23 contemporary, North European populations are included in these studies, together with herbarium data and notations from about 30 historical Swedish populations.

All of the studied populations seem to be dependent on grazing or other human-induced activities – with the exception of one Danish population, Hamborg, where the plants grew on a steep slope where erosion minimises competition

(Isaksson pers. obs.). It might also be possible that at least part of one Estonian population, Vohilad does not depend on grazing for its persistance – we found most of the plants on a beach with chalk gravel, suffering relatively little competition from other plants, but it is reasonable to believe that the species had been more common further inland when the grasslands were still grazed (Isaksson pers. obs.).

In Sweden (Gärdenfors 2005) and Britain (Cheffings & Farrell 2005), *T. integrifolia* is listed as endangered according to the IUCN classification. In Denmark it is regarded as rare (Ingelög et al. 1993), but it is not present on the Danish national red-list (*Den danske rødliste 2004*), and Ingelög et al. (1993) report it as present, but not threatened, in Estonia.

In all of our experimental studies we investigated local genetic variation (sometimes within 10 metres distance and sometimes within a 10 m² area) to make it possible to compare populations of different sizes.

The history of Swedish populations from 1820 till present times (paper I)

A survey of historical records of the species together with measures of herbarium specimens in the Botanical Museum at Lund University were performed to get an estimate of when the main extinctions of the Swedish populations occurred, and whether it is possible to observe any signs of inbreeding depression just prior to population extinctions, such as decreased plant sizes or a decreased number of flower heads.

A 30-year census of eight Swedish populations (paper I)

The eight Swedish populations remaining in 1980 have been studied more or less thoroughly since this time (e.g. Widén 1987, 1991, 1993, Widén & Andersson 1993, Widén & Wetterin 1999) and previously unpublished data on number of flowering plants, regeneration, and deaths of seedlings and adult plants are included in the first study in this thesis, to give a more detailed picture of population fluctuations and extinctions.

Inbreeding experiment (paper I)

The main focus of the first study is an inbreeding experiment on plants cultivated from seeds collected in three of the Swedish populations, Benestad, Grödby, and Råby. Plants were crossed in different categories, depending on where their field mothers had grown in the populations – between-sib cross (i.e. between plants that had the same field mother), within patch cross (i.e. between plants whose field mothers had grown less than 10 metres away from each other), cross between patches (i.e. between plants whose field mothers had grown between 25 and 400 metres away from each other, but within the same population), and in some cases cross between populations. We studied the survival and flowering rates of the offspring, and we measured characters typically related to fitness in plants.

Self-incompatibility experiment (paper II)

In the second study of this thesis, I made use of the cross-pollinations performed to produce the plants in the first study. Since *T. integrifolia* is self-incompatible, it is not only unable to produce seeds after self-pollination, but pollinations between different plants which are genetically similar for the self-incompatibility locus (S-locus) do not produce any seeds either. This is a system which decreases the risk of inbreeding, since close relatives are often incapable of producing offspring together (De Nettancourt 2001, Castric & Vekemans 2004). If the number of S-alleles within a population is very low, the chances of seed-set decreases for individual plants (Demauro 1993). The purpose was to evaluate whether, and to what extent, the occasionally observed low seed-set in small patches of this species (Widén 1993) could be related to a decreased genetic variation at the S-locus.

Variation in AFLP markers (paper III)
In the third study we investigated local (within ~10 m^2) genetic variation in AFLP markers, to assess the relationship between population size and local genetic variation, and to determine whether local genetic variation differed between Swedish (i. e. red-listed) and Estonian (not red-listed) populations. Two Swedish and nine Estonian populations were used.

Quantitative variation (paper IV)
In the fourth study we investigated the local quantitative variation in thirteen North European *T. integrifolia* populations – three from Britain, where it is red-listed (Cheffings & Farrell 2005), three from Denmark, where it is rare (Ingelög et al. 1993), but not red-listed (*Den danske rødliste 2004*), three from Sweden, where it is red-listed (Gärdenfors 2005), and four from Estonia, where it is neither red-listed nor rare (Ingelög et al. 1993). We use the term broad sense heritability for the phenotypic expression of genetic variation.

Results
Historical populations: The survey of historical Swedish populations showed that the most drastic decrease in population number during the investigated period probably occurred between 1880 and 1900 (paper I Fig. 2–3). Of 42 identified populations, 15 remained at the turn of the 20th century, and only six at the turn of the 21st century. There are no indications of populations suffering from inbreeding depression before they went extinct – rather, plants seem to have grown taller with time (paper I Fig. 4), while the number of flower heads has been relatively constant. This could be a result of habitats being overgrown by bushes before they were destroyed – since growing taller as a response to light shortage has been documented for *T. integrifolia* (Widén & Andersson 1993).

Census: The 30-year census of the Swed-

ish populations remaining after 1980 showed strong fluctuations in number of flowering plants between years, with minima after dry summers (paper I Fig. 5). During 1980–2009 two populations went extinct, and another two are now close to extinction. After 1990, the number of flowering plants has fluctuated between much lower values than during the previous years.

Inbreeding: The results of the inbreeding experiments (paper I) varied between different kinds of traits. I will here make a short summary of the effects of inbreeding on seed germination, survival, flowering, and on morphological traits:

Seed germination (inbreeding): For the Benestad and Grödby populations, there were significant differences between crossing categories for most traits related to seed germination (paper I Fig. 7a–c). Cross-pollinations between plants from different patches resulted in seeds with higher germination rate and shorter germination times than within patch cross-pollinations, as well as a higher survival rate for seeds that had been stored for 1.5 years, while seeds resulting from sib crosses had the lowest germination rates, the longest germination times, and the lowest survival rate after storage. For the Råby population, there were hardly any differences in seed germination between crossing categories, although the trends seemed to be the same. However, the Råby seeds had generally much lower germination rates than the Benestad and Grödby seeds regardless of crossing category.

Flowering and survival (inbreeding): For survival and flowering, both for plants transferred to a common garden and for plants transplanted to the site of an extinct population (paper I Fig. 8–9) we found more significant differences between crossing categories for Grödby and Råby plants than for Benestad plants – generally, the further away the field parents had grown in the natural popu-

lations, the higher the fitness of the offspring.

Quantitative characters (inbreeding): For quantitative characters, we found no significant differences between crossing-categories, with the exception of height, for which we found significant differences for the Benestad plants – with between-patch crosses resulting in the tallest offspring and sib-crosses in the shortest (paper I Fig. 10). There were no significant differences between crossing categories for Grödby or Råby. However, the Benestad plants in cultivation were generally taller than the plants from the other populations, regardless of crossing-category. On the other hand, the Benestad plants were significantly shorter than the Grödby plants in the natural populations.

Self-incompatibility: In natural populations, there was a clear relationship between number of flowering stems in a patch and seed-set (paper I Table 1, Fig. 6). Benestad generally had smaller patches, and consequently lower seedset. After cross-pollination in the greenhouse, the relationship between crossing-category and seed-set was the strongest for the Benestad plants (paper II Fig. 4–5). However, for plants that set seeds, seed-set was higher for the Benestad plants than for the others, except for cross-pollinations between sibs (paper II Fig. 6). For all three populations, more than 60 % of the cross-pollinations between sibs gave a seed-set above 2 %, and for within-patch cross-pollination ~80 % of the Benestad plants and ~90 % of the Grödby and Råby plants had a seed-set above 2 %, which was my definition of a compatible cross, i. e. a cross between individuals which are genetically different at the S-locus.

Inbreeding and self-incompatibility – concluding remark: Generally, both for the inbreeding experiment and the self-incompatibility study, the strongest differences were between sib-crosses and

the rest, rather than between within patch and between patch crosses.

Molecular variation: The comparison of local genetic variation in AFLP markers (paper III) in eleven populations (two Swedish and nine Estonian) indicated no difference in variation depending on population size, and no difference between Swedish and Estonian populations (paper III Fig. 1). However, the Swedish populations were genetically different from the Estonian populations, and one isolated population from an Estonian island was genetically different from the rest of the Estonian populations (paper III Fig. 2a–c).

Quantitative variation: In the fourth study (paper IV), we found that heritabilities were higher for the Swedish populations than for the rest, with the Estonian populations having the lowest heritabilities (paper IV Fig. 3). Of 18 characters, the Swedish and the Danish populations had significant heritabilities for 14, the British for ten, and the Estonian for seven characters.

Discussion

The results of the inbreeding experiment (paper I), the self-incompatibility experiment (paper II), and the AFLP analysis (paper III) are relatively consistent with one another. There is no evidence for genetic depletion in the small Swedish populations (papers I, II, and III). Most differences in fitness for different levels of inbreeding are between sib-crosses and the rest, rather than between within patch and between patch crosses (papers I and II). There is no relationship between population size and local genetic variation in eleven investigated populations (paper III) and there is no difference between Swedish and Estonian populations in local genetic variation (paper III).

However, in the fourth study (paper IV), we found a negative relationship between population size and the level of variation in quantitative characters. And

the Swedish populations had significant heritabilities for more characters than the Estonian populations. A possible explanation of these 'reverse' results is that the Swedish populations are in the early stages of a bottleneck, since an initial effect of decreased population size could be an increase in heritability as a result of homozygosity for previously rare recessive alleles, which might become more frequent through genetic drift (Willis & Orr 1993). This phenomenon has been observed in several empirical studies, according to a meta-study by Van Buskirk & Willi (2006).

Given the results of the studies presented in this thesis, it is not likely that genetic depletion has played any important part in the decrease of *T. integrifolia* in Sweden during the past decades. And given how rapidly the populations are decreasing in size, it is likely that they will reach extinction before they are impacted by genetic factors. In their study on genetic variation in threatened and non-threatened species, Spielman, Brook, & Frankham (2004) compared closely related taxa in different animal and plant groups. They found that 77 % of the studied red-listed species had less genetic variation than related common species. That decrease in population size leads to genetic depletion is further confirmed in a meta-analysis by Leimu et al. (2006), which covers 41 species and also by studies of single species (e. g. Lammi, Siikamäki, & Mustajärvi 1999, Landergott et al. 2001, Kang, Jiang, & Huang 2005, Coppi, Mengoni, & Selvi 2008). In the four studies presented in my thesis, populations of only one species are studied in regions where it is red-listed and in regions where it is not red-listed. Spielman, Brook, & Frankham (2004) hypothesise that of the 23 % of the studied rare species which had not lower heterozygosity than closely related non-rare species, many will be genetically affected by the decreased population sizes before they go extinct, which might speed up the extinction process. How-ever, they do not present any theories about possible differences in the importance of genetic factors for different species extinctions.

Why is there so little effect of fragmentation on genetic variation in T. integrifolia *populations?*
Is there a difference between extinction events driven by genetic factors, and extinction events where genetic factors are negligible? If, for instance, we compare my study of self-incompatibility in *T. integrifolia* with that performed by Wagenius, Lonsdorf, & Neuhauser (2007) on the North American species *Echinacea angustifolia*, we can conclude that the two species have many similarities. *E. angustifolia* (Asteraceae) is a grass-land species with sporophytic self-incompatibility, longevity is about 16–44 years (compared with 12–58 years for *T. integrifolia* (Widén 1987)), first flowering occurs after 2–9 years, it does not flower every year, and fragmentation of its natural habitats started between 1870 and 1950 (Wagenius, Lonsdorf, & Neuhauser 2007). Their results indicate that the low seed-set in fragments of *E. angustifolia* populations is due to the loss of S-alleles through drift, while in *T. integrifolia*, though self-incompatibility seems to play a part in the lower seed-set in Benestad, most within patch cross-pollinations gave at least some seed-set, indicating high local genetic variation. Are there any factors – intrinsic or extrinsic – which make different species react differently to decreasing population sizes, or is it just a matter of time (as Spielman, Brook, & Frankham (2004) suggest) before hitherto unaffected species are genetically affected by their rareness?

Changing dispersal mechanisms and 'colonization deficit'
Several plant species which have traditionally been characterised as wind-dispersed, among them Asteraceae species with achenes with pappi, have been found to been epizoochorous, i. e. dis-

persed in the fur of animals (Courvreur et al. 2004, Römermann, Tackenberg, & Poschlod 2005, De Pablos & Peco 2007). Experimental studies of putatively wind-dispersed seeds have also showed that the achenes of some *Senecio* species (closely related to *Tephroseris* and with similar pappi) only disperse a few metres at normal wind-speeds (Sheldon & Burrows 1973). Apart from the destruction and fragmentation of semi-natural grasslands in Europe during the past centuries, isolation seems to have been further enhanced as livestock became more immobile, being enclosed within smaller pieces of grass-land, and not moving so much between pastures (e.g. Bruun & Fritzbøger 2002). As pointed out by Wessels et al. (2008) and Ozinga et al. (2009), red-listed or declining species have been found to be over-represented among epizoochorous plants, and Ozinga et al. (2009) identify what they call a 'colonization deficit' as responsible for the decline of many grassland species. In paper III, we hypothesise that the land use changes which have led to the extinctions of *T. integrifolia* populations have also disrupted the dispersal dynamics of this species – even if movements of cattle and sheep might have been modest in southern Sweden compared with areas where transhumance (seasonal movement of live-stock) was widely practiced (e.g. described by Fischer, Poschlod, & Beinlish 1996).

What will happen to *T. integrifolia* in Sweden?

In a meta-study of 100 vertebrate taxa, O'Grady et al. (2008) find a strong negative correlation between generation length and the minimum population size which gives a 90% chance of population persistence, but no relationship between generation length and the minimum population size that gives a 90% chance of persistence for a fixed number of generations. They conclude that the reason why the results of estimations of extinction risks varies so much between taxa is that extinction risks are usually calcu-

lated for a certain time-period, rather than a certain number of generations, and that, as a result, extinction risks of long-lived plants and animals are severely underestimated. It would, they also conclude, be easier to predict the extinction risk of a taxon if it is calculated for a certain number of generations, rather than a certain time-period (O'Grady et al. 2008). It might be added though, that for long-lived species, since the extinction process is slower, we have more time to rescue them.

The average life-length of *T. integrifolia* plants is approximately 40 years, given the calculations of Widén (1987), though it varies between populations. About 120 years have passed since most of the Swedish *T. integrifolia* populations disappeared (paper I) – a time-period during which the plants in a *T. integrifolia* population would be replaced three times. The introduction of tractors and artificial fertilisers – which implied a radical change of the remaining semi-natural grasslands – started about 70 years ago (Ratcliffe 1984, Bernes 1994), slightly less than the time it would take for the plants in a population to be replaced twice. According to the census described in paper I, the number of flowering *T. integrifolia* plants has decreased to about one sixth during the past 30 years, i.e. in less than the average life-length of an established plant. It is therefore probably not too bold to assume that *T. integrifolia* in Sweden is one of the species which might be driven to extinction before it is affected by genetic factors (cf. Spielman, Brook, & Frankham (2004)).

What is the conservation value of T. integrifolia *in Sweden?*
The conservation value of a species is often estimated on a national basis – a species being rare in a state gives it a high conservation value there, regardless of the species' status in neighbouring countries (Lesica & Allendorf 1995). The result is that conservation efforts are often spent on species in the periphery of their distribution, where the gen-

etic variation is the lowest, rather than in the centre (Lesica & Allendorf 1995). The Swedish *T. integrifolia* populations are certainly peripheral compared to the overall distribution (cf. Smith 1979). What measures would make the populations increase? How much would it cost? And would it be worth the effort? If, as I suggest, *T. integrifolia* is one of the species which suffer from 'colonization deficit' (cf. Ozinga et al. 2009), then it might be favoured by activities which lead to an enhanced transportation of seeds, e.g. the movement of hay or animals between fragments of semi-natural grassland. It is likely that such activities might favour other grassland species as well, something which would increase the value of such measures. However, if there is some method through which *T. integrifolia* could be rescued in Sweden, it must be implemented very soon, or it will be too late.

Fragmentation and population dynamics – alternative scenarios

In all of the studies in this thesis, local genetic variation was investigated, to make it possible to compare populations of different sizes, as well as to find indications of loss of genetic variation *after* population sizes have decreased. What does local genetic variation tell us about the condition of a population? And what does it tell us about the possibilities for populations to recover, should they ever get the chance to increase in size? Let us assume three plant populations, A, B, and C, of equal sizes, densities, dispersal dynamics, and mortalities. The number of new plants which can be established each generation is density dependent:

Populations A and B go through a fragmentation process, while population C remains large and thriving:
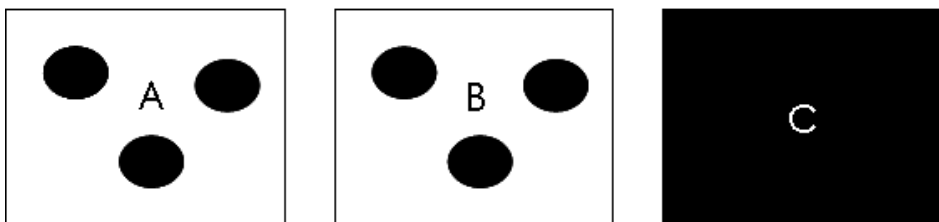
In population A, due to some additional environmental changes, all establishment of new individuals ceases, but mortality of already established individuals is the same, while in population B, the plants continue to reproduce, and offspring can be established within the habitat fragments, but no dispersal occurs between fragments. Consequently, plant density will remain the same within the fragments in population B, while density in population A will decrease as plants die, and no new seedlings are established:
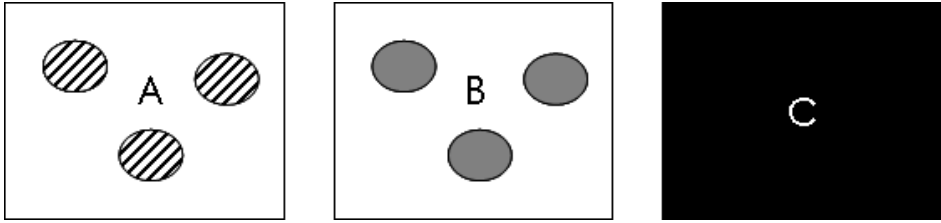
After a few years, half of the plants present when habitats A and B were fragmented have died. Twenty random individuals from each population are sampled for genetic analysis within an area corresponding to the fragment-sizes of populations A and B. This corresponds to the analyses of local genetic variation described in this thesis.
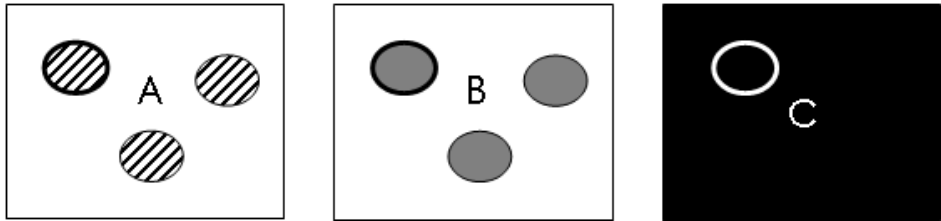
In population A, all of the sampled individuals are old – having been successfully established before the habitat was fragmented – while in populations B and C half of the sampled individuals are old and half are young. In population B, all of the younger plants are the offspring of the older plants within the fragment, while in population C, parents of young plants might be found both within and outside the sampled area. If we know nothing about the change of dynamics in populations A and B, and if we do not compare the plant densities of the three populations, we might draw the conclusion that population A is 'healthier' than population B, and possibly more valuable for conservation. For neither population A nor population B has loss of genetic variation contributed to the decrease in population sizes. The local genetic variation might not have changed much since fragmentation – however, all the genetic variation outside the fragments was lost during the fragmentation process.

Let us assume that the habitats of populations A and B are restored, so that new plants can again be established both within and between the fragments:
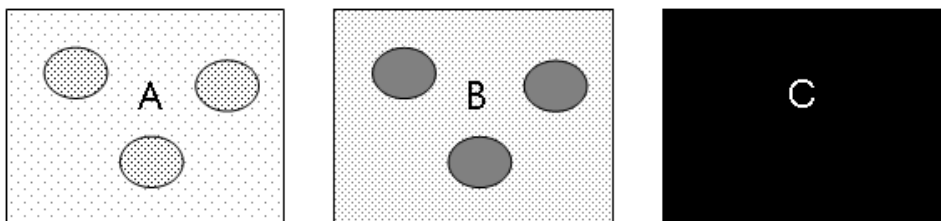
After another few years, a new sample is taken from each of the populations of the same size as at the previous sampling, but in populations A and B from an area outside the fragments that were preserved during the fragmentation:
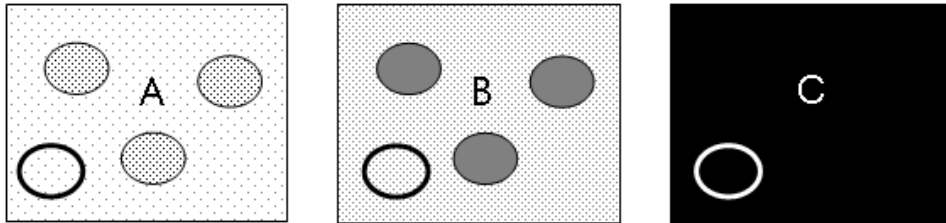

How much genetic variation will we be able to detect? And how will the three populations differ?

How much genetic variation is detected at the two sampling events in the three populations A, B, and C, will depend very much on genetic structure and population dynamics prior to the fragmentation events. Leblos Estoup, & Streiff (2006) demonstrated in their simulations where different sized populations suffered an instantaneous loss of individuals and habitat area, that local genetic variation decreases more quickly in panmictic populations than in populations with strong isolation by distance, and that this effect was stronger for very small sampling areas.

The Swedish *T. integrifolia* populations might, after the initial habitat destructions during the late 19th century, have encountered a situation similar to what happened in population B above, managing to reproduce for a few generations, but with lower effective population sizes than before, which would explain the greater variation in quantitative characters in the small Swedish populations than in the large Estonian populations, indicating an early bottleneck (paper IV). However, since the 1980s the Swedish *T. integrifolia* populations seem to have been in a situation more similar to that of population A, with almost no successful reproduction. We do not know why this happened, but based on the studies presented in this thesis, we can assume that the recent rapid decline in *T. integrifolia* was not caused by genetic factors.

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20

I

Inbreeding depression cannot explain the rapid decline of the self-incompatible perennial *Tephroseris integrifolia* in Sweden

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Abstract

Habitat loss, change, and fragmentation are serious threats to many species. Historical documents indicate that the rare field fleawort, *Tephroseris integrifolia* (L.) Holub (Asteraceae), in Skåne, southern Sweden, has decreased from about forty populations in the mid 19th century to six populations in the early 21st century. This decrease in population number can to a large extent be explained by the conversion of its natural habitat, nutrient-poor calcareous grassland, to arable fields and forest plantations. A census of extant populations from 1980 to 2009 revealed a dramatic decrease in population sizes during the early 1990s, despite far-reaching conservation measures, and even extinction of two populations in the late 1990s. Here we investigate if this decline can be explained by inbreeding depression as a consequence of reproductive isolation. However, we found only weak signs of inbreeding depression, which consequently cannot explain the drastic decline of the species. We discuss possible alternative causes of the decline. Seedling deaths and a low seedling-recruitment seem to be strongly connected with drought, grazing, and mollusc herbivory, and are probably connected to a restricted seed-dispersal. We thus conclude that the most probable causes of the decline are a combination of lack of suitable habitat and of stochastic external threats.

Keywords: Population census, inbreeding depression, population structure, threatened species, extinction

Introduction

The loss of suitable habitats as a consequence of human activities has led to decreasing population sizes of many formerly common species, as well as local extinctions of whole populations (Fischer & Stöcklin 1997, Luijten et al. 2000, Matthies et al. 2004). Habitat fragmentation has also led to increasing isolation of populations and individuals, which in the case of sessile organisms, such as plants, often causes complete or partial reproductive isolation (Snaydon & Davies 1976, Ouborg 1993, Willi et al. 2006). Habitat fragmentation might also lead to an increased exposure to diseases (Groppe et al. 2001, Mbora & McPeek 2009), to the invasion of new species (Cillers, Williams, & Barnard 2008), and to altered water and soil conditions (Camargo & Kapos 1995, Cramer & Hobbs 2002) as a consequence of a larger edge-to-surface ratio. Habitat change, isolation and edge-effects together affect species composition (Kiviniemi & Eriksson 2002) as well as population de-

mography and reproductive success (e.g. Widén 1993), and might in the end cause genetic depletion (e.g. Van Rossum et al. 2002).

The spatial genetic structure of natural populations is influenced by processes such as genetic drift (Shah et al. 2008), gene flow (Young, Brown, & Zich 1999, Gaudeul & Till-Bottraud 2008), and natural selection (Snaydon & Davies 1976). In plant populations, low genetic variation within fragments and high variation between fragments have been observed, and are believed to be the results of genetic drift and restricted gene-flow (Shah et al. 2008, Liu et al. 2009). However, in many studies, an unexpectedly high genetic variation has been found in supposedly isolated population fragments or small populations, and often also an unexpectedly low genetic variation between fragments or relatively isolated populations (Young, Boyle, & Brown 1996, Young, Brown, & Zich 1999, Marquardt et al. 2007, Williams et al. 2007, Yates et al. 2007, Yoshioka et al. 2007, Gaudeul & Till-Bottraud 2008). One explanation is a high gene-flow between fragments or populations, particularly in wind-pollinated species (Williams et al. 2007), or gene-flow from undetected individuals of the same species (Lawrence et al. 2008). Under some circumstances, fragmentation even seems to increase gene-flow (Young, Brown, & Zich 1999). However, if the time since fragmentation is short compared to the generation-time of the studied species, it might not yet be possible to observe the genetic consequences of fragmentation (Young, Boyle & Brown 1996, Williams et al. 2007, De Almeida Viera & de Calvalno 2008). This is further confirmed by studies where the offspring have a lower heterozygosity than their parents (Lawrence et al. 2008, Van Geert, Van Rossum, & Triest 2008), indicating that fragmentation will take its toll on genetic diversity in the near future. However, a large discrepancy in genetic variation or heterozygosity between established individuals and seedlings may also indicate that a large number of homozygotes do not survive until the adult stage, or even that no or extremely few seedlings survive, regardless of their genetic constitution, if the environment is so altered that recruitment is made impossible. The remaining adult population may then consist of old individuals, which means it is a relict of a formerly large and thriving population (Luijten et al. 2000, Van Geert, Van Rossum, & Triest 2008). Excess variation in alleles under balancing selection such as self-incompatibility-alleles or, to a certain extent, major-histocompatibility-complex-alleles, over putatively neutral genes are also an indication of loss of variation through genetic drift, but at the same time examples of mechanisms counteracting the detrimental effect of drift (Luijten et al. 2000, Mable & Adam 2007, Vandepitte et al. 2007). If small populations consist mainly of adult flowering plants, they might be survivors from a much larger population and hence contain an unexpectedly large genetic variation (e.g. Luijten et al. 2000).

In studies of the effects of habitat fragmentation, it is important to correctly estimate population sizes. Population size seems to be crucial for survival of rare and threatened species (Matthies et al. 2004, Münzbergová 2006). To estimate past plant population sizes is difficult. Historical development of individual populations is inherently difficult to assess, but the general trends in population development can be inferred from past and present distribution maps (e.g. Luijten et al. 2000). Site area (Ouborg 1993) or mean cover (Fischer & Stöcklin 1997) are often substitutes for population size. Direct counts of numbers of individuals in plant populations are almost lacking before the 1980s (Matthies et al. 2004).

In the present study we investigate possible inbreeding depression in the rare plant *Tephroseris integrifolia* – a species which has continually declined in Sweden during the past 140 years as a consequence of habitat loss and fragmen-

tation, as well as of decreased grazing, but which is still declining, despite considerable conservation measures. We use a combination of historical records (herbarium specimens and literature data) and empirical data from the last 30 years to show the decline in population number and size of this rare and endangered plant species in Sweden. We performed crossing experiments to explore if inbreeding depression could be a key factor explaining the rapid decline in population size and number. The questions we want to answer are:

1. Is inbreeding depression a key factor in the decline of *T. integrifolia* in Sweden?
2. Could inbreeding depression be a threat to *T. integrifolia* in the future?

Material and methods

Study species

Tephroseris integrifolia (L.) Holub (Asteraceae) is a perennial plant, 10–50 cm high, with 3–10 leaves in the basal rosette, and with 1–3 leaves on the flowering stem. Usually one major and a few minor rosettes develop on an almost vertical rhizome. The shoot producing the flowering stem dies after seed dispersal, and one of the minor rosettes becomes dominant, or adventitious buds on the rhizome develop into new rosettes. The flower heads (1–4, sometimes more) are arranged in a corymb. The plant flowers in May to June, and the number of flowering plants per population varies considerably between years (Widén 1993). Plants rarely flower in two successive years, and flowering is particularly poor the year after a heavy drought. The primary pollinators are Diptera (mostly syrphid flies). The plant is self-incompatible and has wind-dispersed fruits (achenes, hereafter called seeds) with pappi (Widén 1987). Seed set is density dependent, increases with population size, and shows an optimum for individuals flowering during the peak of the flowering period (Widén 1991,

1993). No soil seed bank is accumulated (Widén 1987). In an experimental study Widén (1993) found a weak trend of inbreeding depression on early stages of the life cycle (germination percentage and germination speed), but no correlation between population size and levels of inbreeding depression. The plant grows in calcareous, grazed semi-natural grasslands. It is sensitive to competition from other plants, thus grazing increases seed germination and seedling survival – however, grazing early in the season tends to reduce reproduction, since both flowers and seeds are grazed (Widén 1987).

T. integrifolia has a disjunct distribution with the westernmost populations occurring in Britain and the easternmost in Japan (Smith 1979).

Tephroseris integrifolia in Sweden

In Sweden *T. integrifolia* is recorded only in the southernmost province, Skåne, and the first record is from 1623 (Andersson 1944). The decline of the species began already in the late 19th century (Nilsson & Gustafsson 1977). The basic threat to the plant has been habitat fragmentation caused by changes in land-use, but the present-day decline in population size may also be connected with deterioration of habitat quality. Many habitats of the species have been exposed to artificial fertilization, ceased grazing, or impact from invasive species (Widén 1987 and unpublished).

In the present study we use plant material from three populations representing the two geographical areas with extant populations (NE and SE Skåne, respectively).

Benestad (SE Skåne)

The Benestad population is situated on a south-west facing slope within the Benestads backar nature reserve with grazed, unfertilised pastures on calcareous soils. Several hundred years of limestone quarrying has created the present-day topography, which consists of a mosaic of dry hills with species-rich grassland ve-

getation separated by wet-land vegeta-
tion fed by ground-water on plateaus and
in valleys between the hills (Regnéll

1976, 2003). *T. integrifolia* grows on the
top and the slopes of the hills in rather
well-defined patches of a few to 50 m[2],
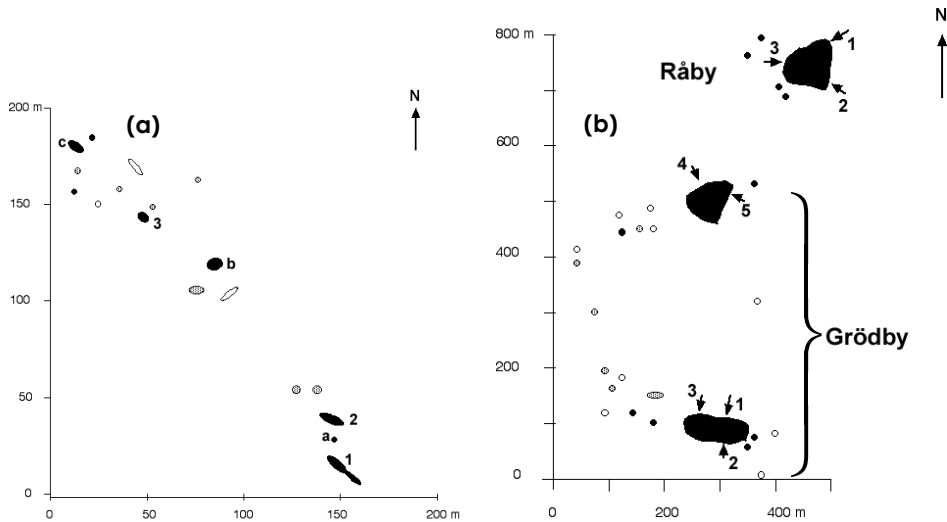
Fig. 1. Schematic map of the spatial distribution of patches occupied by *Tephroseris integri-folia* at Benestad (a) and Grödby-Råby (b). Patches with plants recorded for the last time during the 1980s have been marked with open symbols. Gray indicates extinction during the 1990s and black indicates records of flowering plants 2000–2009. Figures indicate the position of seed samples and letters denote patches mentioned in Table 1.

separated from each other at a distance
of 10 to 50 m (Fig. 1a).

Grödby–Råby (NE Skåne)
Grödby: Until the late 1950s, when a
large part of the Grödby area was plan-
ted with pine, the Grödby population
was one of the largest known *T. integri-folia* populations in Sweden (Nilsson &
Gustafsson 1977). The plantation divi-
ded the Grödby population into two
parts, referred to as Grödby S and
Grödby N, about 400 metres apart. In
the early 1980s plants were scattered
around the pine plantation (Fig. 1b). The
area south of the pine plantation (Gröd-
by S) is a species-rich grassland with sev-
eral rare species indicating a long con-
tinuous grazing management. To the
east the pine plantation borders on
arable fields, and to the west and north
there are grazed pastures with a trivial
vegetation (Widén, personal observa-
tion), with scattered patches with *T. in-*

tegrifolia (Grödby N).
   Råby: The Råby population is situated
300 m further to the north-east of the
Grödby site. The vegetation is trivial and
the area has obvious remains from old
arable fields (piles of stone). According to
the local inhabitants, potato was culti-
vated here during the 1950s.
   Grödby N is closer to the Råby popu-
lation than to Grödby S (Fig. 1b). It could
be argued that it is more logical to regard
Grödby S, Grödby N, and Råby as three
sub-populations of the same population,
than to consider Grödby and Råby as two
separate populations. However, due to
the relatively recent connection between
Grödby S and Grödby N, we have chosen
to regard Grödby as one population
which has recently suffered severe frag-
mentation, and the Råby population as a
separate population – since we are not
interested in the genetic similarities or
differences between populations, but
rather the relationship between physical

Table 1. Data from samples in natural populations 2000. Mean (SD) and sample size, n. Patch number refers to Figure 1. Number of stems refers to the number of flowering stems within the sampled area (patch).

Population	Patch	No of stems	Plant height	Number of heads	No of florets /head	Fruit set	Achene weight 10^{-3} g	Germination rate
Benestad	1	33	14.1 (2.97) 15	2.9 (0.74) 15	52.9 (25.66) 15	0.29 (0.295) 15	0.54 (0.147) 12	0.63 (0.443) 12
	a	1	21 (-) 1	3 (-) 1	65 (-) 1	0.02 (-) 1	0.62 (-) 1	1.0 (-) 1
	2	18	23.2 (5.91) 14	3.9 (1.73) 14	75.9 (19.26) 14	0.47 (0.209) 14	0.62 (0.116) 14	0.77 (0.328) 14
	b	5	20.0 (1.41) 2	3.0 (0) 2	72.0 (21.21) 2	0.07 (0.098) 2	0.67 (-) 1	0.67 (-) 1
	3	20	16.5 (4.37) 11	2.6 (1.17) 12	62.2 (21.6) 12	0.17 (0.159) 12	0.53 (0.126) 11	0.72 (0.354) 11
	c	3	18 (-) 1	5 (-) 1	65 (-) 1	0 (-) 1	-	-
Grödby S	1	132	20.3 (5.42) 27	4.2 (1.67) 27	76.9 (13.59) 27	0.64 (0.231) 27	0.47 (0.072) 26	0.88 (0.244) 26
	2	43	24.2 (4.92) 13	5.3 (1.75) 13	87.2 (16.62) 13	0.47 (0.227) 13	0.47 (0.054) 13	0.96 (0.259) 13
	3	82	20.5 (6.68) 22	4.6 (1.81) 22	82.1 (20.06) 22	0.42 (0.223) 22	0.48 (0.103) 21	0.87 (0.181) 21
Grödby N	4	16	15.2 (2.60) 11	5.2 (1.66) 11	85.1 (21.51) 11	0.13 (0.117) 11	0.42 (0.071) 10	0.86 (0.204) 10
	5	24	20.8 (2.75) 11	4.8 (2.48) 11	79.5 (12.52) 11	0.15 (0.130) 11	0.42 (0.071) 9	0.80 (0.304) 9
Råby	1	153	19.2 (4.64) 14	4.6 (1.78) 14	85.2 (17.04) 14	0.49 (0.256) 14	0.47 (0.065) 14	0.90 (0.109) 14
	2	26	20.8 (5.02) 11	6.1 (3.02) 11	75.0 (15.24) 11	0.34 (0.212) 11	0.55 (0.079) 10	0.80 (0.326) 10
	3	20	21.0 (6.29) 10	5.4 (1.71) 10	86.7 (14.11) 10	0.27 (0.287) 10	0.57 (0.128) 9	0.82 (0.319) 9

population structure and genetic variation.

Investigation of historical populations

To estimate the number of *T. integrifolia* populations in Sweden at different times in the past, we recorded date and locality of findings accounted for in published records collected in the floristic inventory of Skåne (Tyler et al. 2007) as well as of herbarium specimens in the Botanical Museum at Lund University. Since the exact positions of earlier findings are

often vague, whereas parish is always given, we record presence and absence of *T. integrifolia* in parishes for this investigation. We make the following two assumptions: 1. As *T. integrifolia* is a relatively slow colonizer; we assume that findings of *T. integrifolia* in a certain parish during this period (1800–2009) indicate presence of the species in that parish since 1800. 2. When findings are no longer made in a parish, we assume *T. integrifolia* to have gone extinct in that parish. We believe that this gives a rather good estimate of when the species has disappeared from different parts of Skåne. We also measured the height of all herbarium specimens and counted the flower heads, in order to find signs of inbreeding depression prior to extinction. Height and number of flower heads were analysed separately with ANCOVA, testing for the effects of administrative county district (Swedish härad) and collection-year.

30 year census

From 1980 onward one of us (BW) estimated population sizes by counting the annual number of flowering stems in natural populations. The counting was always performed early in the flowering season, before any grazing had reduced the number of flowering stems. If grazing had occurred, attempts were made to include grazed stems.

Sampling of experimental plants

Populations of *T. integrifolia* often have a patchy structure, with a varying distance between the patches. In this study we use three populations with contrasting population structure and patch dynamics (Fig. 1). As is clear from Table 1, plants growing alone, or very few together (patches a, b, and c at Benestad) failed to set seed. These plants grew more than 10 metres away from other plants, indicating that the maximum pollination distance is less than 10 metres. We therefore defined a patch as a group of plants which grow more than 10 metres away from other plants. For the

experimental plants in this study, samples were rarely collected more than 10 metres apart within the same patch. The positions of patches occupied by *T. integrifolia* have been indicated on maps (Fig. 1). GPS was used to determine the centre of small patches and the centre and outline of large patches. Because of the irregular flowering of the species it is difficult to determine year when the plant has gone extinct in a patch. Here we report the decade when the last flowering individual was observed in a patch.

The sampling design was based on the structure of the Benestad population, which has a clear, patchy structure. From the other two populations we sampled from areas (»artificial patches») of the same size as the Benestad patches, demarcated within more continuous areas or larger patches, and approximately on the same distances from each other as the Benestad patches. From Benestad we collected seeds in three well-defined and well-separated patches (1, 2, and 3). The positions and areas of these patches have been rather stable since 1980. In Grödby S, three artificial patches (or more strictly, sampling areas), 1, 2, and 3, were demarcated and sampled from. Grödby N has a patchy structure, although not as clear as at Benestad – the structure varies between years and scattered plants appear between patches. Seeds were sampled from two patches (4 and 5). From the Råby population, which mostly consists of three large patches (with scattered, irregularly flowering individuals between the patches), we demarcated and collected seeds from one artificial patch within each of the larger patches.

Seeds were collected from one flower head per plant during the fruiting period in mid June. We determined seed set as the proportion of developed achenes of the total number of florets per flower head. The positions of the sampled plants were determined to the nearest dm in a coordinate system for each population. Plant size, and seed weight (aver-

age weight of 20 seeds per mother) were also recorded (Table 1). The main sampling was done in 2000, and an additional sampling was done at Råby and at Grödby N in 2001. Since plants rarely flower during two successive years, we assume that we have not sampled from the same plant twice. Seeds were sown indoors in the autumn the same year as sampled, and the progeny plants were kept outdoors during the winter.

Plant size and seed set in natural populations

We compared plant-height and seed-set in the field in the three populations with ANOVA (with population as a fixed factor and number of flowering plants per patch as a covariate). In the same way we analysed weight and germination rate of the collected seeds. Since seed-set and germination rate are both ratios, we transformed them with the logit-function (see below under Germination). We also analysed the relationship between number of flowering plants and the portion of plants which had received compatible pollen – counting seed-set below 0.02 as 0, and seed-set above 0.02 as 1 (cf Isaksson paper II). This analysis was performed with a generalised linear model, with seed-set given a binary-probit distribution.

Crossing experiments

Most of the cross-pollinations were performed in 2002 between plants from the 2000 seed collection. Additional pollinations were performed between the Råby plants in 2003, both from the 2000 and 2001 seed collections. However, we never cross-pollinated two Råby plants from different collecting years. Additional cross-pollinations were done on the Benestad and Grödby S and N plants in 2004.

Crossing categories and distances

The cross-pollinations of the plants were divided into different categories based on the positions of the field parents:

1. Between sibs crosses (i.e. the plants had the same field parent).
2. Within patch crosses (i.e. field parents were not identical, but grew within the same patch).
3. Between patch crosses (i.e. maternal parents grew in the same population but in different patches). In some analyses we separated between crosses 3a. between close patches (less than 150 metres apart) and 3b. between distant patches (400-500 metres apart) – i.e. crosses between Grödby S and Grödby N.
4. Between populations crosses. Crosses between plants from different populations (Benestad and Grödby). These offspring were only analysed with respect to germination, survival, and flowering.

The plants to be pollinated were kept in an insect-free greenhouse environment. We performed the cross-pollination by gently rubbing two flower heads against each other on two different occasions with a one- to three-day interval. The first cross-pollinations were carried out when ray florets and the outer tubular florets were receptive, and the second when most florets in the two flower heads were fully developed.

Cultivation of offspring

The rapidly declining viability of seeds makes it necessary to sow seeds within 1.5 years after harvest. The seeds from the 2002 and 2003 cross-pollinations were sown in October 2003. Plants derived from this sowing are referred to as cohort 2003 below. Only plants of cohort 2003 were used for all experiments. The seeds from the 2004 cross-pollinations were sown in July 2004 (cohort 2004). We sowed 20, or all seeds, if they were less than 20, from each harvested flower head on Petri dishes. After germination they were transferred to 5×5 cm^2 pots and planted in standard soil.

In all c. 8175 seeds from 503 crosses were sown in 2003. Of the surviving offspring, plants from about 300 crosses were saved for morphological analyses.

Five seedlings from each family were transplanted to 10×10 cm plastic pots (one seedling per pot) in the greenhouse. After about a month, the pots were transferred to a common garden outdoors where they were kept during the winters.

In July 2004 about 5500 seeds from 320 crosses were sown. Of the surviving offspring, plants from about 100 crosses were saved for morphological analysis.

The plants in cohort 2003 had by September 2004 developed new rosettes, and from each genet, one or two rosettes of about the same size as the 2004 seedlings were transplanted to separate 10×10 cm^2 pots. At the same time the seedlings in cohort 2004 were also transplanted to 10×10 cm^2 pots (five from each family). All the plants were kept in the green house for two months. Where two rosettes from the same genet were available, one was selected for field-transplantation, and transplanted to the site of an extinct population (Lövhall, see Fig. 5) in November 2004. All other plants were at the same time transferred to a common garden.

Germination

Germination in 2003 was monitored from October 14, when the seeds were sown, till November 19. Germination in 2004 was monitored from July 5, when the seeds were sown, till August 2. Some seeds were sown in the same year when they were harvested and some in the following year. Since germination decreases drastically with time, we made a strict separation between seeds sown soon after harvesting, and those stored from the previous year (hereafter called young and old seeds, respectively).

We analysed the germination data in three different ways:

1. *Germination or not* – that is, whether any seeds from one flower head germinated. Flower heads with less than 5 filled seeds were excluded from the analysis. At least one germinated seed gave the value 1, and no germinated seeds gave the value 0. All cases were pooled within population. This was analysed for the populations separately with χ^2 with respect to crossing-category.

2. *Germination rate* – that is, the number of germinated seeds divided by the number of sown seeds from one flower head. Since germination rate is a ratio, it was transformed with the logit function:

    Logit(germination rate)
    $= log((germination\ rate/(1-ger-mination\ rate))$

    Germination rate=0 was excluded from this analysis. Germination rate was analysed with ANOVA, where field paternal grandmothers were nested within population, and crossing-category, population, and interaction between crossing category and population were analysed.

3. *Germination time* – that is, average germination date of the germinated seeds. This was analysed with ANOVA, where field paternal grandmothers were nested within population, and crossing-category, population, and interaction between crossing-category and population were analysed.

Flowering and survival

Survival was recorded on two occasions for plants in the common garden experiment, in April 2005, and in November 2005 (i. e. before and after flowering). Flowering was recorded in summer 2005. For the field experiment (plants transplanted to the site of the extinct population Lövhall), survival, flowering, and number of flowering stems were recorded in spring 2005. Both flowering and survival were analysed with χ^2-tests. For the common garden experiment, we analysed survival of winter and survival of summer, separately and together, as well as survival after flowering.

Morphology

The following morphological variables were scored on plants in different cros-

sing-categories: height of flowering stems, number of stem-leaves, number of flower heads, number of ray-florets, length of ray-florets (average based on three florets), width of ray-florets (average based on three florets), and width of flower head disc. Flower-characters were scored on first flowering flower head. All morphological characters were analysed separately with one-way ANOVAs, where field-grandmother was nested within population. We checked the effect of crossing-category, population, field grandmother, the interaction of crossing category and population, and the interaction of crossing category and field grandmother.

Statistics

All statistical analyses were performed with SPSS 16.0 (SPSS Inc., Chicago, IL).

Results

Historical populations

A maximum number of 42 local populations in Skåne can be identified from herbarium material and literature data. The number of parishes with records of *T. integrifolia* decreased from 26 in the early 19th century to 4 in the early 21st century (Fig. 2 and 3). The decline in number of records was most pronounced in the late 19th century (Fig. 3). The estimated number of local populations was 15 at the turn of the 20th century, and six at the turn of the 21st century. In the 19th century, the species was recorded in both the western and the eastern parts of Skåne, but it has now disappeared from the west, and from most of the original eastern localities.

According to the measures of the herbarium specimens, sizes of *T. integrifolia* plants have fluctuated a lot. However, there is a general trend that the plants are taller the later they were collected (p < 0.05, Fig. 4), while at the same time the number of flower heads does not change with time (p > 0.05, data not shown).

Fig. 2. Map of the province Skåne with parishes that have been reported to harbour populations of *Tephroseris integrifolia* since the middle of the 19th century. Light grey indicates extinction before 1900, dark grey indicates extinction during the 20th century and black indicates extant populations after 2000. (Two dubious findings have been excluded.)
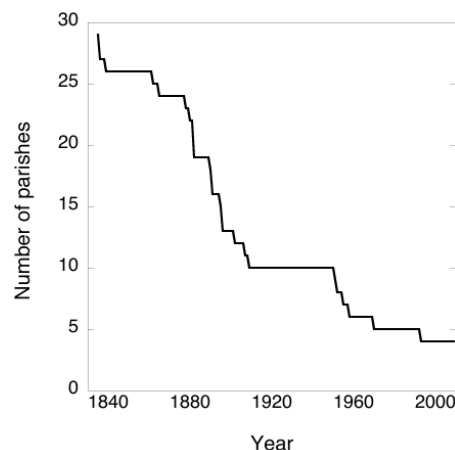
Fig. 3. Number of parishes in Skåne with known populations of *Tephroseris integrifolia* from 1835 to 2009.

30 year census

When the census of populations started in 1980, seven populations were known, most of which were included in the annual census. The seventh population, Edenryd, was checked most years, but a regular count of number of flowering stems did not start until 1999. At one site, Grödby, the spatial distribution of *T. integrifolia* was unclear in 1980, and new

Fig. 4. Plant size of herbarium specimens in Lund Botanical Museum sampled since the early 19th century in different districts (härad). Plant height (cm), differences between years and districts are significant at p≈0.01. Interaction between year and district is significant at p=0.012.
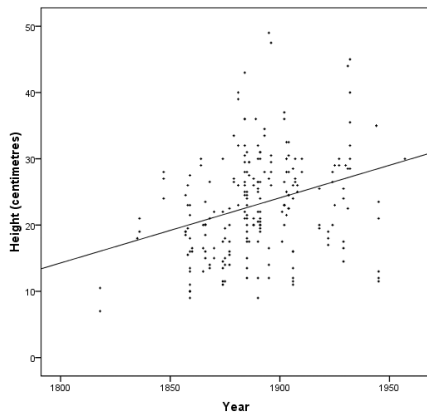
patches of the plant were detected during the following years. These are presented as Grödby N (see Fig. 1b) in Fig. 5. The eighth population, Råby, was rediscovered in 1983 but a regular counting of flowering stems did not start until 1999.

The number of flowering stems varied much between populations, with maxima ranging from 50 at Åby to 8000 at Tosteberga (Fig. 5). The numbers fluctuated dramatically between years in all populations. In the largest population in the 1980s, Tosteberga, a general trend could be found that can also be traced with some modifications in the other populations. From 1980, the numbers increased and reached a maximum in 1982, and then decreased to a minimum in 1984, after which a new increase started with a maximum in 1988, and again a minimum in 1991. After 1990, the number of flowering plants fluctuated between much lower levels than observed during the 1980s in all populations. Two populations, Lövhall and Åby, have gone extinct with the last record of flowering stems in 1997 (Fig. 5).

Spatial structure

All populations have a patchy spatial structure, and in the early 1980s the populations Åby, Edenryd, and Kverrestad had, like the present-day Grödby population, a sub-population structure with aggregations of patches separated by distances of > 100 metres. The number of patches at each site has decreased during 1980–2009. In the 1980s, many patches had high densities of *T. integrifolia* plants. In Tosteberga, Edenryd, and Grödby, patches that still had surviving plants in 2009 tended to be associated with large stones and shrubs. The distribution of plants in the core area of the eastern part at Grödby S had been more or less continuous since the early 1980s, though the irregular flowering of the plants has created a patchy annual structure of flowering individuals (Fig. 1b). The population structure in Grödby N is patchy, but patches are rarely separated by more than 10 metres in the core area of the eastern part. At Råby, plants tend to be clumped together around stones, but scattered individuals have often occurred in the gaps between patches.

At Benestad about 20 patches have been identified since 1980 (Fig. 1a). Flowering in some of the patches has occurred with several years interval between 1980 and 2009. The majority of the patches are very small and none of them cover any large area, and due to the topography at the site, they are all well circumscribed.

Plant size and seed set in natural populations

In the field, seed-set was significantly lower in the Benestad population than in the Grödby population (p = 0.023). However, the effect of population was no longer significant (p = 0.8) when number of flowering plants per patch were taken into the analysis – which was significant at p = 0.0000002. The plants in the three small Benestad patches a, b, and c (Fig. 1a) had almost zero seed-set. The plants in Benestad patch number 2 had an unusually high seed-set (on average 0.47) considering the relatively low number of flowering plants (18), com-
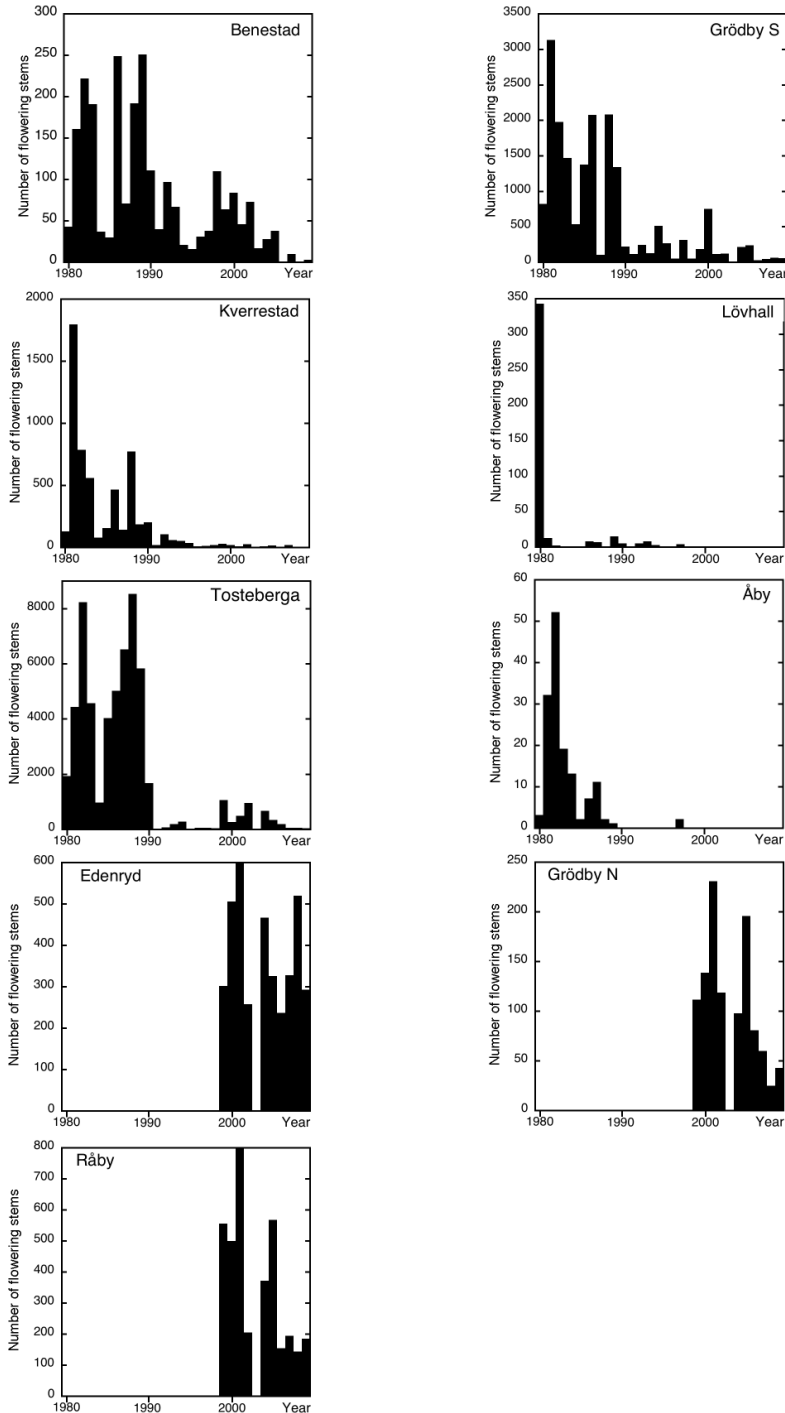
Fig. 5. The annual number of flowering stems of *Tephroseris integrifolia* at sites with extant populations in 1980 or later. Flowering was observed at Edenryd, Grödby N, and Råby since the 1980s, but a regular counting of flowering stems did not start until 1999.
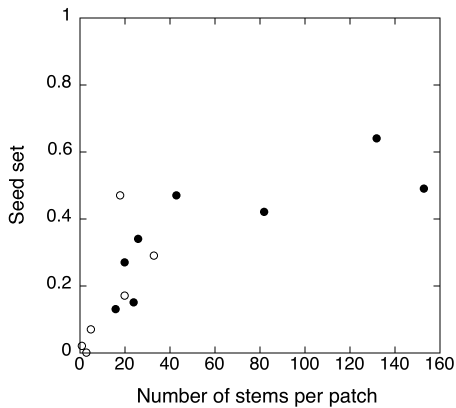
Fig. 6. Relation between seed set and number of flowering stems of *Tephroseris integrifolia* at each patch in 2000. Open symbols denote Benestad and filled symbols Grödby-Råby.

pared with other similar-sized patches in this study (Table 1, Fig. 6). Plants at Benestad were on average shorter than the others, but the differences were not significant (p = 0.083 compared with Grödby and p = 0.22 compared with Råby). There was no significant difference in seedweight (p = 0.26) or germination rate (p = 0.095) between populations, and they were not related to number of flowering individuals per patch (p = 0.125 and 0.246, respectively). There was a significant relationship between number of flowering plants per patch and portion of plants which had received compatible pollen (p = 0.008).

Germination

Germination or not
When we only investigated whether any seeds from one head had germinated, 93% of young seeds germinated (97% if only cases with at least 5 sown seeds were included) while 68% of old seeds germinated (75% if only cases with at least 5 sown seeds were included). Germination rate was high for young seeds and no difference between categories could be established. For old seeds, there was a significant difference between crossing-categories for the Benestad population, where between-patch cros-

ses rendered seeds with higher germination rate than the rest, and for the Grödby population, where seeds from between-patch crosses had the lowest germination rate. For the Råby-population, the trend was similar (p = 0.043) to that in the other two populations (Fig. 7a).

Germination rate
Germination ability of *T. integrifolia* seeds decreased rapidly with time; 78% of young seeds germinated, while 30% of old seeds germinated. Germination rate was thus relatively low for old seeds, and we could find no consistent difference between crossing categories (Benestad: p = 0.60; Grödby: p = 0.76; Råby: p = 0.14 – data not shown). However, for young seeds there was a significant difference in germination-rate between crossing-categories for the Benestad plants – between-patch crosses gave a significantly higher germination rate than sib- and within-patch crosses, and the Grödby plants – sib-crosses gave a significantly lower germination rate than the rest, but not for the Råby plants, though they seemed to show the same trend as plants from the other two populations (Fig. 7b).

Germination time
For old seeds we found no significant relationship between germination time and crossing category (Benestad: p = 0.80; Grödby: p = 0.57; Råby: p = 0.46 – data not shown). However, there was a significant difference in germination time for young seeds derived from Benestad and Grödby, with the sib-cross-seeds germinating later than seeds from other cross-pollinations, but not for seeds from Råby, which showed a similar trend, but no significance (Fig. 7c).

Flowering
Plants in cohort 2003 were too small when transferred to the experimental beds outdoors to be able to flower in 2004, while plants in cohort 2004 had grown large enough during their first year to flower in 2005. The overall fre-
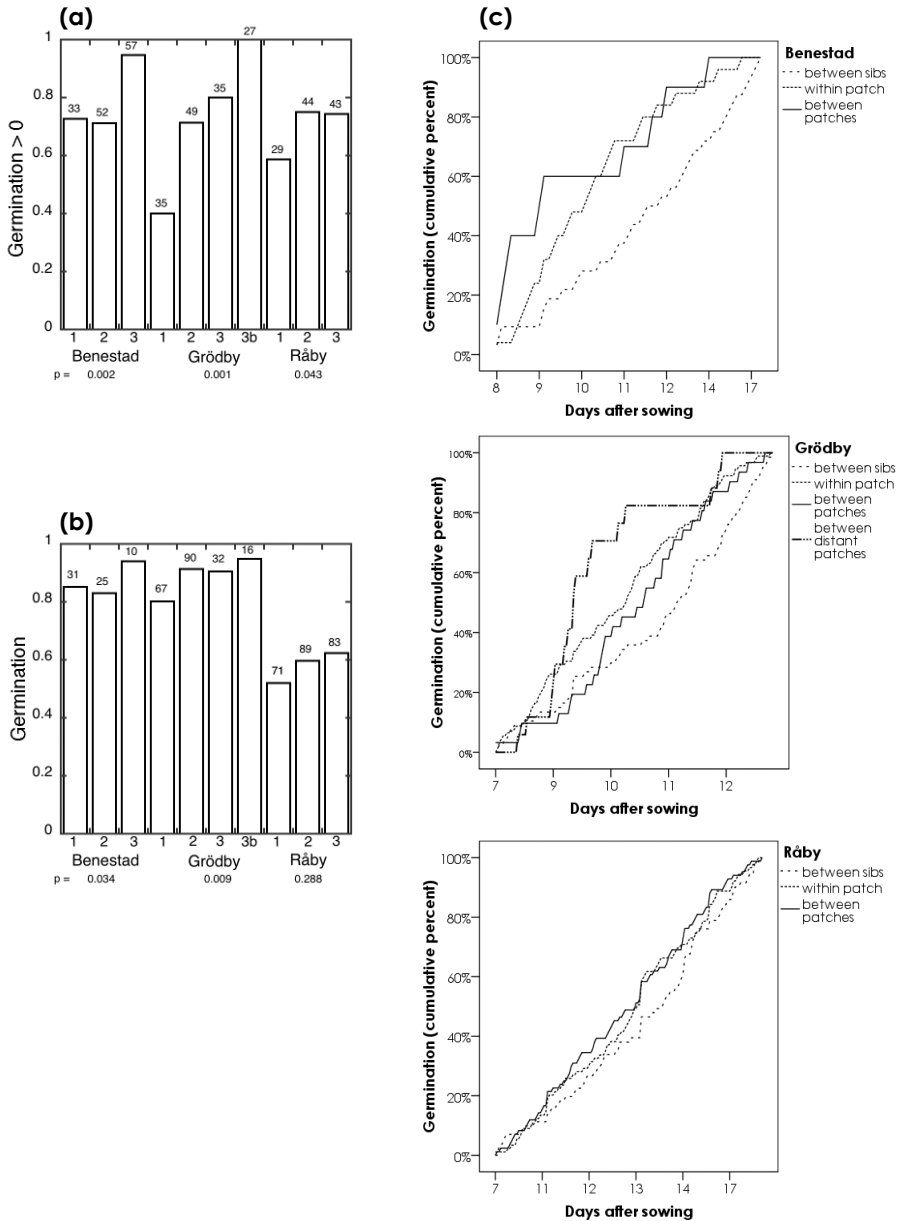
Fig. 7. Germination of *Tephroseris integrifolia* seeds for different crossing categories (1 = sib cross, 2 = within patch cross, 3 = between patches cross, 3b = between distant patches cross). Figures above bars indicate cample sizes.

(a) Germination or not for old seeds. Differences between crossing categories are significant at p = 0.002 for Benestad, at p < 0.001 at Grödby and marginally significant (p = 0.043) at Råby.

(b) Germination rate for young seeds. Differences between crossing categories are significant at p = 0.002, and differences between populations at p < 0.001.

(c) Germination time of young seeds. Differences between crossing categories are significant at p = 0.001 (for Benestad p = 0.007, for Grödby p = 0.006, for Råby p = 0.33), and differences between populations at p < 0.001.

quency of plants in cohort 2003 that flowered in 2005 was about 40 % with significant differences between crossing categories, sib-crosses showing the lowest tendency to flower (Fig. 8a). The differences in flowering frequency between crossing categories were significant at Grödby and Råby, but not at Benestad. At Råby, within-patch crosses gave higher flowering-frequencies than between-patch crosses (Fig. 8a).

The tendency to flower in 2005 was slightly lower in cohort 2004 (Fig. 8b) than in cohort 2003 (Fig. 8a). There was a significant difference in flowering between crossing-categories for the Benestad plants, with the highest flowering frequency for the between-patch crosses, and between-sibs and within-patch crosses rendering plants with almost equal flowering frequencies, but not for the Grödby plants, which had very low flowering frequencies for all crossing categories. Flowering frequency of plants transplanted to the field differed significantly ($p < 0.05$) between crossing categories (Fig. 8c).

Survival
For the plants in cohort 2003, we could detect no significant differences in survival during the winter 2004/2005 between categories, probably because survival generally was so high (data not shown). For the survival of the entire year 2005, there was a significant difference between crossing categories for the Grödby plants, where plants from between-patch crosses had a higher survival rate than plants from the other crossing categories and Råby, where plants from withinpatch crosses had a higher survival rate than plants from sib-crosses and between-patch crosses, but not for Benestad (Fig. 9a).

For the plants in cohort 2004, which experienced their first winter in 2004/2005, there was a significant relationship in survival between crossing-categories among the Benestad plants, where between-sibs crosses gave the lowest and between-patch crosses the
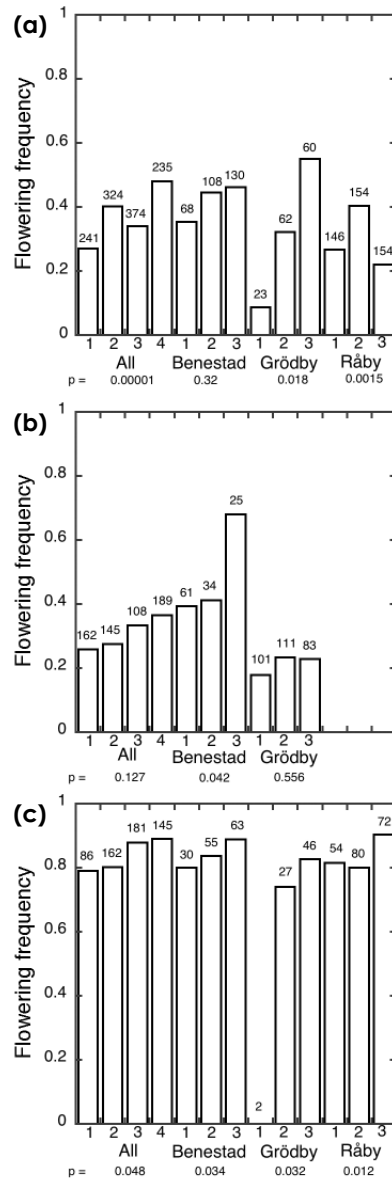
Fig. 8. Flowering frequency of *Tephroseris integrifolia* for different crossing categories (1 = sib cross, 2 = within patch cross, 3 = between patches cross, 4 = between populations cross). Cf. fig. 7

(a) Flowering frequency in cohort 2003 in cultivation 2005.

(b) Flowering frequency in cohort 2004 in cultivation 2005.

(c) Flowering frequency in the field 2005 of cohort 2003 transplanted to the field in November 2004.

Fig. 10. Height of flowering stems of *Tephroseris integrifolia* in cultivation 2005 (both cohort 2003 and cohort 2004) in different crossing categories (cf. fig. 7 & 8).

highest survival, but not among the Grödby plants (Fig. 9b).

For the plants that flowered in 2005, there was no significant difference in survival between categories, probably because the sample was too small (data not shown).

Survival of field transplants was the lowest for between-sibs crosses, and increased with distance between field-grandmothers (Fig. 9c) – though Grödby and Råby regarded separately seemed to show the same trend, the results were not significant. There was no difference in survival between plants from different crossing categories for the Benestad plants.

Morphology

The progeny in the crosses were significantly taller ($p < 0.0001$) at Benestad than at Grödby and Råby (Fig. 10) as opposed to the plants growing at their original field-sites (Table 1). There was no significant difference between the Grödby and the Råby plants ($p = 0.50$). The other morphological traits did not show any significant differences within or between populations, except for a difference between Grödby and Råby in width of flower head disc (data not shown). We

Fig. 9. Survival of *Tephroseris integrifolia* for different crossing categories (cf. Fig. 7 & 8).
(a) Survival of cohort 2003 in cultivation 2005.
(b) Survival during the first winter 2004/2005 of cohort 2004.
(c) Survival during the winter 2004/2005 of cohort 2003 in the field.

found a significant difference in height between crossing-categories within Benestad (p = 0.023), where the plants from between sibs crosses were the shortest and plants from between patch crosses were the tallest (Fig. 10). There were no significant relationships between crossing-category and height for the Grödby (p = 0.24) or the Råby (p = 0.14) populations. For the rest of the morphological traits we found no significant relationships (data not shown).

Discussion

Most Swedish *T. integrifolia* populations have disappeared since the 19th century. The remaining populations have decreased in size since 1980. The decline in number of populations represented in the herbarium material towards the end of the 19th century cannot be explained by a decreased tendency to collect plant material, since many specimens were still collected, though from fewer populations. Therefore, it is probable that most populations disappeared during the last quarter of the 19th century. Of the eight populations that remained when monitoring of *T. integrifolia* started in 1980, two (Lövhall and Åby) have disappeared altogether, and two others (Benestad and Kverrestad) are on the verge of extinction today (2009).

The main reason for the decline in number of populations of *T. integrifolia* during the 19th and 20th centuries is habitat loss caused by changing land use. Nilsson & Gustafsson (1977) listed a range of known causes of extinction for individual populations; establishment of arable fields or cessation of grazing in semi-natural grasslands, forest plantation, abandonment of limestone quarries and heavy fertilising combined with intensified grazing by cattle.

The decline of *T. integrifolia* was observed by botanists in the early 20th century (e.g. Andersson 1944), and despite legal and conservation measures to protect the species the decline has continued; the species was protected by law already in 1935 and an action plan was established in 1999 (Widén & Wetterin 1999). The action plan comprised reinforcement of extant natural populations by transplanting adult plants raised from seeds in cultivation back to their site of origin. These artificial populations are in most cases well separated from extant natural stands reported in this paper (Widén, unpublished). A series of studies related to the conservation of *T. integrifolia* has been published (e.g. Widén 1987, 1991, 1993, Widén & Andersson 1993).

Our main purpose of this study was to explore the role of inbreeding depression in the decline of *T. integrifolia* by experimental studies of progeny fitness in relation to cross proximity (cf. Byers 1998). Inbreeding depression is often expressed at late life history stages (e.g. Karron 1989, Belaoussoff & Shore 1995, Nason & Ellstrand 1995). Widén (1993) found clear indications of inbreeding depression at early life history stages of our study species. The inbreeding experiments performed on material derived from the three populations Benestad, Grödby, and Råby, showed a significant effect of crossing-category for most, but not all, life-history stages; germination, survival, and flowering at Grödby, and to a certain extent at Benestad and Råby. Height differed significantly between crossing-categories for Benestad, but not for Grödby and Råby. For other morphological traits we found no significant differences between crossing-categories at any site. Several studies report a positive effect on progeny vigour with increasing crossing distances (e.g. Morán-Palma & Snow 1997, Byers 1998).

The magnitude of inbreeding depression does not explain the dramatic decrease in population sizes since 1980. Most of the experimentally induced inbreeding depression can be referred to breeding between plants with the same maternal field parent, while plants with different maternal field parents from the same patch rarely gave lower fitness than crosses between plants in different patches (cf. Oostermeijer, Altenburg, &

den Nijs 1995). Genetic depletion seems not to be a threat to *T. integrifolia*, since an earlier study showed significant quantitative genetic variation for a range of life-history and morphological traits in both the Benestad and Grödby populations (Widén & Andersson 1993). The only morphological effect of inbreeding that we found in our experiments was a decrease in plant height. If inbreeding-depression indeed played a role in the historical extinctions of populations, we would have expected plant-height to decrease before populations went extinct. The general increase in plant height from 1818 till 1960, indicated by the herbarium specimens, is rather an indication of light shortage, possibly as a consequence of pastures being overgrown – etiolation as a response to light shortage has previously been confirmed for *T. integrifolia* (Widén & Andersson 1993).

The processes behind the rapid decline of *T. integrifolia* at the local population level are not fully understood, but the experiences gained during the period 1980 to 2009 indicate that three mechanisms may be involved:

1. Grazing of flowering stems – which affects seed-dispersal.
2. Herbivory by molluscs – which affects seedling-survival.
3. Longer periods of drought – which mainly affects seedling-survival, but also flowering, seed-development, and, when extreme, the survival of adult, well-established plants.

A demographic study of the species 1980 to 1983 revealed that grazing reduced seed production, e.g. by 100 % at Benestad in 1981. The impact of grazing varied between zero and 97.5 % at other sites during 1980–1982 (Widén 1987). Grazing by livestock has been economically supported since the 1980s, and has been an important management practice (the NOLA-project). However, grazing is generally not restricted to any specific time of the year, but starts early (late

April–early May) and goes on throughout the season. Consequently most flowering stems were probably grazed before seed dispersal during the 1980s (few estimations of the impact of grazing were made after 1982). The decline in population sizes in the 1990s may partly be a result of the intense and unrestricted grazing promoted by the NOLA project during the 1980s. Although *T. integrifolia*, being a poor competitor, seems dependent on grazing to prevail, grazing before midsummer could seriously hamper seed-dispersal, since flowering stems are readily eaten by the two main grazers, rabbit (*Oryctolagus cuniculus*) and cattle (*Bos taurus*). The action plan focused on the grazing regime (Widén & Wetterin 1999), and there have been changes since 2000; grazing is restricted before midsummer, which makes seed-dispersal possible. However, the populations have continued to decrease in size after 2000. Recruitment seems to have been very low during the last decades contrary to the situation during 1980–1983 (Widén 1987).

The extreme decrease in the formerly large Tosteberga population could partly be attributed to rabbit grazing. An exclusion experiment in 2004 at Tosteberga revealed that rabbits were the main herbivores at that site when cattle were excluded (Widén, unpublished). Hunting of rabbits in this area was extensive until the early 1980s, after which time it almost ceased, giving rise to a rapid increase in the population size of rabbits (Widén, personal observations). Few estimations of the proportion of reproductively successful flowering stems at Tosteberga were made after 1982, but grazing by rabbits as well as by cattle has probably inhibited most of the seed production at this site since the early 1980s. Grazing was intense even before 1980; Nilsson & Gustafsson (1977) reported only about 50 flowering stems at Tosteberga in 1976. The rabbit was successfully introduced for hunting in Skåne in the early 20th century (Göransson, Frylestam, & Berg 1983), thus

grazing by rabbits cannot be responsible for the pronounced decline in number of populations of *T. integrifolia* in the late 19th century. Further, rabbit grazing cannot explain the decline in population sizes after 1980 at other sites than Tosteberga, because very little grazing on *T. integrifolia* by rabbits have been observed elsewhere (Widén, personal observations). Cattle grazing could have contributed to the decline in population size at Benestad, Grödby S, Benestad, Lövhall, and Åby. At Kverrestad, on the other hand, grazing by cattle had little effect on seed production, but tall vegetation hampered seedling establishment (Widén 1987).

Generally, the seedling stage represents the most vulnerable part of the life cycle in plants (Harper 1977, Fenner & Thompson 2005) and herbivory, e.g. mollusc grazing, is one of the major causes of seedling deaths (Cook 1979, Mole & Westoby 2004). Many plants eaten by molluscs in the autumn were recorded in the demographic study of *T. integrifolia* 1980–82 (Widén 1987). After the wet summers of 1980 and 1981 a high proportion of the plants had clear signs of damage caused by molluscs in the autumn. July 1982 was dry and less favourable for snails and slugs. Consequently, the damage caused by molluscs in the autumn 1982 was only half or even less of that recorded in the two previous years (Widén 1987). The same trend with a high frequency of plants damaged by molluscs after a wet summer and lower frequency of mollusc damage on leaves of *T. integrifolia* after dry periods has been observed in the artificial populations at Benestad and Kverrestad in 2006–2008 (Widén, unpublished). Several molluscs have been observed on or close to leaves of *T. integrifolia* (*Arion lusitanicus*, *Cepaea hortensis*, *Deroceras reticulatum*, and *Helix pomatia*), although actual eating has not been observed (Widén, unpublished). *A. lusitanicus*, e.g. is known to be a threat to rare plants (Bruelheide & Scheidel 1999).

In an experimental cultivation study 2008 M. Magnusson (unpublished) found that the slug *A. lusitanicus* preferred both adult and seedling plants of *T. integrifolia* to three other grassland species (*Pulsatilla vulgaris*, *Helianthemum nummularium*, and *Leontodon hispidus*). Experimental sowing of seeds of *T. integrifolia* at Benestad during the last five years has not led to any recruitment (Widén, unpublished). The seedlings, if ever observed, were often recorded as eaten by molluscs, probably by the invasive species *A. lusitanicus*, a species common at the site, especially in the wet year 2007.

A number of serious droughts during the 1990s and later made seedling establishment practically impossible, and also killed many adult plants (Widén, unpublished). Drought is one other major cause of seedling mortality in plants (Cook 1979, Mole & Westoby 2004). The demographic study 1980–83 of *T. integrifolia* revealed a high recruitment rate, if seed production was not prohibited by grazing of flowering stems or if seed germination was not repressed by a dense grass sward (Widén 1987). However, even the short period of low precipitation in the summer of 1982 killed young plants in the permanent plots (Widén 1987). Mortality was clearly age-dependent except at the Benestad site, which has a different hydrology than the other sites (Regnéll 1976), leading to a lower mortality during the 1982 drought in Skåne. A severe drought occurred again in July–August 1983 (Widén 1987), but unfortunately no data was recorded in the permanent plots after July 1983.

The rapidly decreasing viability of seeds confirms that *T. integrifolia* populations cannot rely on a seed-bank for their persistence. However, while viability decreased more in the inbred seeds, they also took longer to germinate (cf. Widén 1993). Therefore, though we see no serious signs of inbreeding depression as a result of within-patch crosses, we can draw the conclusion that further inbreeding would make the species more vulnerable to changes in precipitation

during the germination phase.

The evidence so far indicates little or no recruitment in the extant populations. The populations seem to consist of old individuals flowering with an interval of several years. Less than 50 % of the plants that flowered during 1980 to 1983 did so more than once (Widén 1987), and a high proportion of the plants present in permanent plots in 1980 and still alive in 1983 (seedlings excluded) did not flower at all during this period (69 % at Benestad, 72 % at Kverrestad, 43 % at Tosteberga, and 47 % at Grödby). Variation in number of flowering stems of *T. integrifolia* among years is regulated by a complex interaction of wet and dry summers. Drought is a major factor regulating the flowering frequency. The number of flowering stems decreases dramatically after one or several successive years with periods of drought. One case in point is the decrease in number of flowering stems in 2003, the year after a severe drought in the late summer of 2002. The most dramatic decrease in number of flowering stems occurred at Tosteberga in the early 1990s. In 1992, drought even killed many adult plants (Widén, personal observations).

The decline in population size is also manifested in areas occupied by the plant. Many patches with *T. integrifolia* became extinct during the 1980s and 1990s, both at Benestad and at Grödby (Fig. 1). Patches that were small and isolated at the beginning of the study are probably remains of larger continuous areas covered by the plant. The larger patches occupied by *T. integrifolia* at Grödby are at present undergoing a fragmentation process.

We know the Benestad and the Grödby populations both to have decreased in size since the 1850s, but in different ways and for different reasons. At Benestad scattered plants found down-hill in the 1980s, close to the present occupied areas, as well as the numerous herbarium specimens collected during the 19ᵗʰ century and till the species was protected by law in 1935, indicate that the population has covered a much larger area, but we don't know it's exact distribution. After the quarrying activities ceased at Benestad towards the second half of the 19ᵗʰ century (Regnéll 1976), the patchy structure has probably been rather stable, since the habitat between the patches is unsuitable for *T. integrifolia*. And because of the relative isolation of the plants, it can be expected that the population would have lost some genetic variance through genetic drift (cf. Leimu et al. 2006). The fragmentation and population size decrease at Grödby seems to have been much more drastic, after a large part of the area was planted with pine (*Pinus sylvestris*) around 1960 (Nilsson & Gustafsson 1977). However, as the isolation of the present-living plants has only gone on since the 1960s, one might expect that less genetic variation would have been lost than at Benestad. However, considering the shorter generation time at Grödby (according to Widén 1987), the loss of genetic variation might be much more rapid there than at Benestad. The genetic distribution at Råby seems confusing, since between-patch crosses sometimes gave lower fitness than within-patch crosses. Since the between-patch crossing distances are short compared to the distances of the same crossing category for the other populations, and since the Råby patches are relatively large and close to each other, it is hard to imagine our results being a case of optimal crossing-distance (sensu Waser & Price 1989). Possibly, an extreme stirring and moving of soil as a consequence of ploughing disrupted any kind of structure in the Råby population.

Summary
The main reason for the pronounced historic decline of *T. integrifolia* in Sweden is habitat loss. The recent extinction and decline of population sizes are probably caused by a combination of reduced seed production due to grazing, periods of drought and mollusc herbivory on seedlings. Today the extant populations seem

to consist of old individuals that flower with an interval of several years. In this study, we found no clear signs of inbreeding depression that could explain the rapid decline in population sizes and population number. Without proper management actions it is most likely that the Swedish populations of *T. integrifolia* will go extinct in a near future, and though inbreeding depression might speed up the process, it does not play the leading part.

Acknowledgments

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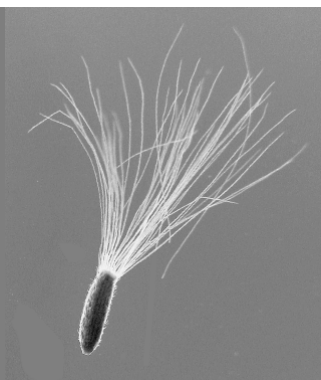
II

Self-incompatibility does not explain declining population sizes of *Tephroseris integrifolia* (Asteraceae)

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Abstract

Small populations face problems of inbreeding depression and extinctions due to stochastic events. One of the problems that a small plant population might be challenged with is a reduced seed-set. This is often attributed to pollination failure, but could, in self-incompatible species, be an effect of loss of S-alleles through drift. In the rare and self-incompatible *Tephroseris integrifolia* (L.) Holub, seed-set has been found to be density-dependent in cultivation, and positively correlated with population size in wild populations. In this study of three populations in different stages of fragmentation I investigate the connection between self-incompatibility and reduced seed-set in fragmented populations, and whether this could be one of the causes of the severe decline of *T. integrifolia* in Sweden during the past decades. The main conclusions are: 1. Self-incompatibility does not prevent the plants from reproducing except in extremely small population fragments (with 1–3 plants). 2. There probably haven't been many generation shifts since the populations were fragmented, since otherwise, a substantial number of S-alleles would have been lost through drift, and seed-set would have been much lower. 3. Considering how rapidly the population sizes are decreasing, these populations are likely to be extinct before SI would cause any reproduction difficulties.

Keywords: Sporophytic self-incompatibility, small populations, fragmentation, extinction, relict populations, threatened species.

Introduction

Self-incompatibility (SI) prevents plants from fertilising themselves and reduces the risk of being fertilised by close relatives (De Nettancourt 2001), which protects them from inbreeding depression (Castric & Vekemans 2004). About 70 % of all angiosperm species have some form of SI system, and the evolution of SI is thought to be part of the reason why angiosperms have been so successful (Franklin-Tong & Franklin 2003).

There is a high risk of inbreeding depression in small populations (Gag-giotti 2003), and an SI system is one efficient mechanism to prevent inbreeding. However, when the supply of suitable mates is scarce, self-fertilisation or fertilisation of close relatives could be the only option of sexual reproduction, and therefore an SI system would be a burden (Demauro 1993).

Low seed-set is frequently observed in small plant populations, and is usually attributed to low levels of pollen deposition, as a result of infrequent pollinator visits (Widén 1993, Aizen & Feinsinger 1994, Byers 1995, Ågren 1996), or to lack

of compatible pollen as a result of low variation in self-incompatibility alleles (S-alleles) (Byers 1995, Wagenius, Lonsdorf, & Neuhauser 2007). Some attempts have been made to estimate the effects of SI on the persistence of small populations by means of computer simulations (e.g. Byers & Meagher 1992, Wagenius, Lonsdorf, & Neuhauser 2007, Goodwillie 2008, Levin, Kelley, & Starkar 2009). The simulations take into account different aspects of SI, such as: incompatibility at the gametophytic or sporophytic stage, the number and frequencies of different S-alleles in the populations at the start of the simulation, and whether there are any dominance-relationships between the S-alleles. The simulations also take into account different aspects of population dynamics, such as production and dispersal abilities of pollen and seeds, generation overlaps, inbreeding depression, and mutation rates (both in S-alleles and in other alleles). Some of the questions asked are: how long will the population persist? and at what rate will S-alleles be lost? Byers & Meagher (1992) simulated populations of different sizes and with different frequencies of S-alleles that had strict hierarchic dominance relationships, and found that at population sizes of under 50 individuals, S-alleles will be lost relatively quickly (approximately one allele every three generations). Levin, Kelley, & Starkar (2009) simulated populations with either gametophytic or sporophytic SI, as well as mendelian populations, and varying population sizes, initial numbers of S-alleles, and dispersal abilities of pollen and seeds, while assuming complete codominance between alleles, and no inbreeding depression. These simulations showed that although small populations of self-incompatible plants will quickly move towards extinction, particularly if SI is sporophytic, S-alleles will not be lost till extinction is very close at hand. They attribute the fact that S-alleles are not lost in their model, to their assumption of equal frequencies of S-alleles at the start of the simulation. Another ex-

planation could be the lack of dominance relationships, which would increase the number of compatible crosses, and thereby the number of generations before extinction, but also speed up the loss of dominant alleles. Wagenius et al. (2007) documented a positive relationship between population size and seed-set, as well as plant-density and seed-set in the self-incompatible *Echinacea angustifolia* (Asteraceae). In the same study, they performed simulations based on the properties of the *E. angustifolia* populations they surveyed in the field. The simulations were performed both for a sporophytic SI system, with completely codominant alleles, and for a self-compatible system. They found that following fragmentation, populations of self-incompatible plants, though they suffered from less inbreeding depression than populations of self-compatible plants, decreased more quickly. They also found that the burden of SI becomes more evident with time, and that the number of S-alleles decreases through drift.

In their review on SI in natural populations, Castric & Vekemans (2004) more or less dismiss SI as a threat to small or fragmented populations. They hypothesise that fecundity selection would ensure the establishment of S-alleles that enter a population through gene-flow from other populations, or even from other species, and conclude that SI would only be a threat to a population under extreme isolation.

In small or fragmented populations of self-incompatible plants seed-set is often low (Widén 1993, Heschel & Paige 1995, Luijten et al. 2000, Brys et al. 2004), and sometimes completely absent (e.g. Demauro 1993). The self-incompatible plant species *Tephroseris integrifolia* is rare in Sweden, and has constantly decreased in both population number and size since the 19th century, and despite extensive conservation measures, it is still decreasing (Widén 1987, Widén & Wetterin 1999, Isaksson & Widén paper I). Studies of natural populations of *T. integrifolia* have shown a strong positive

relationship between seed-set and number of flowering stems in population or patch (Widén 1993, Isaksson & Widén paper I), and that isolated individuals – individuals which grow alone, or very few together, more than 10 metres away from conspecific plants – seldom set seed (Isaksson & Widén paper I). In cultivation experiments of the same species, a positive relationship has been found between plant density and seed-set (Widén 1993). Since seed-set was not related to population size after hand-pollinations, Widén (1993) suggested lack of pollinators as the most plausible explanation of low seed-set in small populations, although he didn't rule out the possibility that a lack of S-alleles could cause a decreased seed-set.

While SI promotes genetic variation, to function properly it also requires genetic variation in the S-locus, or else it will hamper reproduction. Does SI in small populations really protect them against inbreeding depression, or does it simply accelerate the rapid wipe-out of the whole population? In this study I intend to study the relationship between population size and seed-set following different levels of inbreeding, to investigate if SI could be contributing to the ongoing decrease of *T. integrifolia*.

Material and methods
Study species
Tephroseris integrifolia (L.) Holub is a self-incompatible perennial herb of the family Asteraceae. The flower heads (1–4, sometimes more) are arranged in a corymb. It has 3–10 leaves arranged in a rosette, and there are usually 1–3 small stem leaves. *T. integrifolia* flowers in May to June, and it rarely flowers in two successive years. Most seeds germinate the first year, in late summer or autumn, and there is practically no seed-bank (Widén 1987). The distribution is disjunct with the westernmost populations occurring in Britain and the easternmost in Japan (Smith 1979). In Sweden there are six extant *T. integrifolia* populations – all in Skåne, the southernmost pro-

vince (Isaksson & Widén paper I). The preferred habitat type is nutrient-poor, calcareous grassland with little competition from other plants, and in Sweden it is found mostly in heavily grazed pastures (Widén 1987).

SI in Tephroseris integrifolia
All of the SI systems that have been studied in the Asteraceae family have been found to be sporophytic (e.g. Byers 1995, De Nettancourt 2001, Hiscock & Tabah 2003, Kirchner, Robert, & Colas 2006, Ferrer et al. 2009), including species of the genus *Senecio* (Brennan et al. 2003, Hiscock & Tabah 2003), to which *Tephroseris* is closely related. It is, therefore, reasonable to assume that the SI system in *T. integrifolia* is sporophytic as well. In a sporophytic SI system, the SI-phenotype of the pollen is determined not by the haploid genotype of the pollen, but by the diploid genotype of the pollen donor. Sporophytic SI is a multi-allelic one-locus system, often with complicated hierarchical dominance relationships between alleles (De Nettancourt 2001). Evidence for complicated hierarchical dominance has also been documented in *Senecio* (Hiscock & Tabah 2003). Some unexpected compatible crosses in *Senecio* have been attributed to a rudimentary gametophytic element in the sporophytic SI mechanisms (Hiscock 2000a & b, cf. Lewis, Verma, & Zuberi 1988). As a result of dominance-relationships between alleles, reciprocal crosses can be compatible in one direction and incompatible in the other. Another characteristic of sporophytic SI systems is that they 'leak', so that even self-pollination often gives at least one seed, or so that crosses which ought to be incompatible give seeds (Lewis 1994, Hiscock 2000b). I regard cross-pollinations that give no, or extremely low (see paragraph about Incompatibility below, and Fig. 1–2), seed-set as incompatible, and all other cross-pollinations as compatible. Variation in seed-set of putatively compatible crosses I analyse separately, and in the final section I discuss possible ex-

planations of this variation, such as inbreeding depression or variations in SI mechanisms.

Populations and sampling
Plants from three of the Swedish populations were used in this investigation – Benestad, Grödby, and Råby. The description of the populations is consistent with what they looked like in 2000 and 2001, when the seeds for this investigation were collected (for a more detailed description of the populations below, see Isaksson & Widén paper I). Since I am interested in how population structure affects local genetic structure, I used the structure of the Benestad population, which had the patchiest distribution, as a model for the seed collection in the other two populations.

Benestad
The Benestad population is situated within a nature reserve with grazed pastures on calcareous soils. The topography is a mosaic of wet and dry areas. *T. integrifolia* grows in the dryer areas on a south-west facing slope (Regnell 1976, 2003), in nine rather well-defined patches, with 10 to 50 metres between each patch. The sizes of the patches are between a few and 50 m^2, and in 2000 between 1 and 33 plants flowered in the separate patches (Isaksson & Widén paper I). Seeds were collected from three patches with a maximum distance of 10 metres between plants within a patch.

Grödby
Until the end of the 1950s, when most of the habitat was destroyed to make space for a pine plantation, this was one of the largest known *T. integrifolia* populations in Sweden (Nilsson & Gustafsson 1977). Today plants grow around the pine-plantation, mostly to the south and the north. Seeds were colleted from five patches – from three artificial patches about the same size as the Benestad patches within the continuous area to the south of the plantation (patches 1–3, see Isaksson & Widén paper I), and from two patches to

the north of the plantation (patches 4 and 5). The distance between the two areas is about 400 metres.

Råby
The Råby population is situated about 300 metres north-west of the Grödby population (Isaksson & Widén paper I). Seeds were collected from three artificial patches (that is, sections demarcated within larger, continuous areas of plants) of about the same size as the Benestad patches, one from each of the three larger patches.

The distance between the Råby population and the northern parts of the Grödby population is about 300 metres – shorter than the distance between the northern and the southern part of the Grödby population. However, until recently (~1960, Nilsson & Gustafsson 1977) the Grödby population was continuous, and because I am more interested in differences in population structure than in genetic distances between the populations, I have chosen to regard Grödby and Råby as two separate populations.

Cultivation and crossing experiments
Seeds were sown in plastic pots in the green-house. Seedlings were transferred to a common garden, and the plants were kept outside until just prior to flowering in 2002 and 2003 when cross-pollinations were executed. During the time of cross-pollinations (April and May), plants were kept in an insect-free greenhouse environment with minimum risk of unwanted cross-pollinations. I performed cross-pollinations by rubbing two flower heads against each other on two different occasions at an interval of one to three days. The first cross-pollinations were carried out when ray florets and outer tubular florets were developed, and the second when all florets in the two flower heads were fully developed. Doing just the latter cross-pollination would result in a lower seed-set, since fully developed florets have usually lost some of

their pollen. The main set of cross-pollinations was performed in 2002 on plants grown from seeds collected in all three populations in 2000. Additional crosses were performed in 2003 on plants collected from the Råby population in 2000 and 2001. In total, 925 flower heads were cross-pollinated, and most of the cross-pollinations were reciprocal (some flower heads had to be removed from the investigation, for example, if they had withered). 90 of these were crosses between populations, which were used as a control, to get an estimate of maximum seed-set, assuming maximum difference in S-alleles. The number of flower heads included in the analysis was 196 for the Benestad population, 191 for the Grödby population, and 448 for the Råby population.

Crossing-types

Cross-pollinations were categorised into four crossing-types, according to the level of inbreeding:

- Between siblings – crosses between plants with the same field mother
- Within patch – crosses between plants with different field mothers but from the same patch
- Between patches – crosses between plants with field mothers from different patches in the same population
- Between populations – crosses between plants with field mothers from different populations
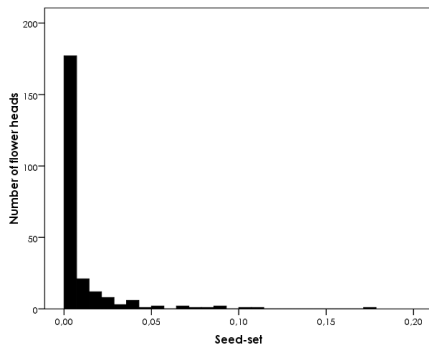
Fig 1. Seed-set in un-pollinated flower heads.

Cross-pollinations were evenly distributed between the first three categories. Since *T. integrifolia*, like other Asteraceae species, will eventually self-pollinate, if they don't receive pollen from other plants, no assistance is required for. I have noted that rubbing flowering plants with cotton-buds doesn't lead to higher seed-set than leaving the plants to pollinate themselves – which is usually no seeds at all. In the following I will call these flower heads un-pollinated.

Seed-set

From each flower head, I counted the number of well-developed achenes as well as the number of empty achenes. I measured seed-set in the flower head as the number of well developed achenes divided by the total number of achenes. 239 un-pollinated flower heads were analysed and used as a control. In the data-set from the cross-pollinations, the large number of zeroes together with a fairly normal distribution of the rest of the seed-sets made it impossible to transform this into one manageable data set, which is why it was divided into two data sets, one binomial and one continuous. However, the undivided data set is shown in diagrams.

Incompatibility

Seed-set in un-pollinated flower heads was normally 0 (in 177 cases out of 239), and seldom above 0.02 (in 32 cases) (see Fig. 1). Seed-set in cross-pollinated plants showed a peak at 0 (incompatible crosses), but there was also a high amount of crosses between 0 and 0.02 (Fig. 2). Based on this, and on the fact that sporophytic SI systems are known to 'leak' (Lewis 1994, Hiscock 2000b), I regarded cross-pollination resulting in seed-set below 0.02 as incompatible, and the rest as compatible. Incompatibility was analysed with χ^2-tests, and plants were pooled within populations. The Råby plants from the three different cross-pollination sets (plants from the 2000 field seed collection cross-pollinated 2002 and 2003, and plants from the 2001
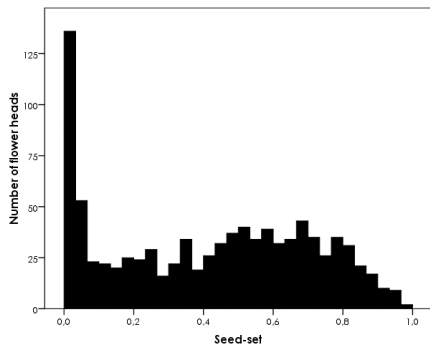
Fig 2. Seed-set in pollinated flower heads.

Fig 3. Seed-set in all three populations together, unpollinated flower heads as well as between population crossed flower heads included.

field seed collection cross-pollinated 2003) were analysed both together and separately.

Reciprocity
I checked for reciprocity in 273 reciprocal crosses i. e. two flower heads for each cross-pollination – if one cross had to be excluded, e. g. because the flower head had withered before seeds could develop, the reciprocal flower head was excluded from the analysis as well. I divided seed-set into three categories; seed-set = 0, 0 < seed-set < 0.02, and seed-set > 0.02, and checked if the seed-set in each reciprocal pair fell into the same category.

Seed-set of compatible crosses
Only compatible crosses (seed-set above 0.02) were included in the analysis. To achieve approximate normality, seed-set was transformed with the logit function:
$$logit(seedset)=ln(seedset/(1-seedset))$$
Seed-set was analysed for all three populations together, and for the populations separately, using a general linear model. I tested for the effect of crossing-type, population, and paternal field grandmother (nested within population). I also tested for the interaction between crossing-type and population, and the interaction between crossing-type and paternal field mother.

All tests were performed in SPSS 16.0 (SPSS Inc., Chicago, IL).

Results
Seed-set in 835 cross-pollinated flower heads (between populations crosses excluded) varied between 0 and 0.98 (Fig. 2). In 62 of these no seeds were developed at all, and in 43 flower heads seed-set was higher than 0 but lower than 0.02. When comparing all cross-pollinations, regardless of population origin (Fig. 3), sib-crosses had the lowest seed-set among the cross-pollinated plants. Within-patch crosses had a higher seed-set than sib-crosses but lower than between-patch crosses. Crosses between populations gave a seed-set similar to that of between-patch crosses. When populations are treated separately, the pattern is similar to that seen when origin is not taken into account (Fig. 4). However, the effect seems to be stronger in the most fragmented population, Benestad, and weaker in the least fragmented population, Råby.

Incompatibility
The incompatibility analysis revealed that the proportion of incompatible crosses differed significantly between crossing categories in the Benestad ($p = 0.00073$) and Grödby ($p = 0.0038$) populations, but not in the Råby population ($p = 0.279$) – regardless of whether the Råby plants were analysed all together or divided into groups according to when they were collected or pollinated (Fig. 5).
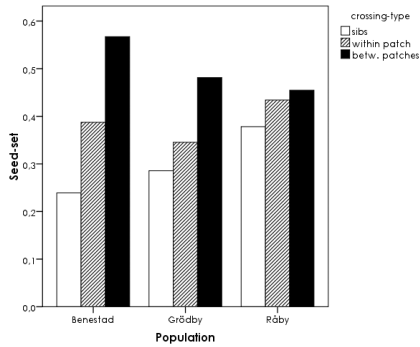
Fig 4. Seed-set in different crossing-types. Crosses which gave 0 seed-set included.

Fig 5. Portion of compatible crosses (seed-set > 0.02). Number of compatible crosses and total number of crosses above bars.

Of the 90 between-populations crosses all except one (which produced only one seed) were compatible (not shown).

Reciprocity

From the sample of 273 reciprocal crosses that were checked for reciprocity, of the 49 crosses that had a seed-set of 0 (exactly) in at least one direction, 11 had a seed-set of 0 (exactly) in both directions, and 22 had a seed-set greater than 0.02 in the other direction. Two reciprocal crosses gave seed-set between 0 and 0.02 in both directions.

Seed-set of compatible crosses

The effect of crossing-category in compatible crosses (671 investigated flower heads) was the strongest in the Benestad population, where all three categories differed significantly from each other ($p < 0.001$, Fig. 6). The effect was the same in the Grödby population ($p = 0.003$, Fig. 6), but only sib-crosses and between-patch crosses differed significantly from each other. For the Råby population, though the tendency seemed to be the same, there was no significant effect of crossing-type on seed-set ($p = 0.158$, Fig. 6). Between populations crosses produced a lower seed-set than within population crosses from Benestad ($p = 0.032$), but it was not significantly different from the seed-set of within population crosses in Grödby ($p = 0.341$) (not shown). Råby plants were not used as fathers in the between-populations crosses.

Discussion

Seed-set has been found to be related to population size in self-incompatible plants (Widén 1993, Heschel & Paige 1995, Luijten et al. 2000, Brys et al. 2004). Widén (1993) also demonstrated that seed-set is density-dependent in *T. integrifolia*. He concluded that the reason for the low seed-set in small populations is lack of pollen. In the field, *T. integrifolia* plants growing more than 10 metres away from other plants rarely set seed (Isaksson & Widén paper I). In an earlier study which includes the field parents of the plants in this study (Isaksson & Widén paper I), seed-set in the field was positively correlated with the number of flowering plants within a
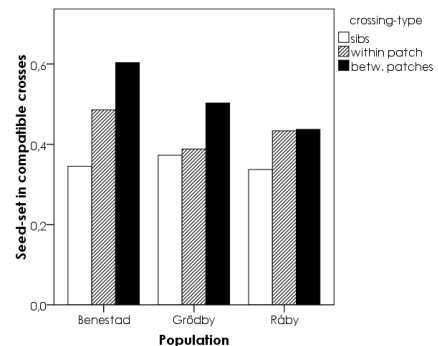
Fig 6. Seed-set in compatible crosses (seed-set < 0.02 excluded).

patch (where seed-set was measured both as number of incompatible crosses compared with number of compatible crosses (assuming that plants with a seed-set lower than 0.02 had not received any compatible pollen) and as average seed-set).

I conclude that the SI system of *T. integrifolia* is sporophytic, based on two facts: 1. The reciprocal differences seen in the cross-pollinations, which indicate a dominance relationship between S-alleles (cf. Hiscock & Tabah 2003). 2. The fact that some flower heads which had been left to self-pollinate set a few seeds, which indicates a 'leak' in the SI system not found in gametophytic systems (De Nettancourt 2001). This is also consistent with several studies of Asteraceae species in which SI was found to be sporophytic (e.g. Demauro 1993, Byers 1995, Kirchner, Robert, & Colas 2006, Abbott et al. 2009, Ferrer et al. 2009). A dominance relationship between S-alleles increases the amount of compatible crosses (Bateman 1952), but it also increases the risk of S-alleles being lost, particularly since the most dominant alleles occur in lower frequencies than the rest. Similarly, transient SI (an SI system where plants become self-compatible with age) also leads to a higher amount of compatible crosses, as well as a more rapid loss of S-alleles (Goodwillie 2008).

The variations of seed-set of compatible crosses could perhaps be attributed to the 'irregularities' of sporophytic self-incompatibility (De Nettancourt 2001, Hiscock & Tabah 2003). Another possible explanation of low seed-set in some compatible crosses could be inbreeding depression – cross-pollination of genetically similar individuals (though different at the S-locus) leading to homozygosity for deleterious recessive alleles (Wiens et al. 1987, Charlesworth 1989, Allphin, Wiens, & Harper 2002).

Of the three populations investigated in the present study, it is Benestad which had the smallest and most isolated patches, and which had the lowest seed-set in the field (Isaksson & Widén paper I). That this might be related to a lack of S-alleles is confirmed by the greater effect of crossing-type on seed-set in the Benestad plants. However, in the crossing experiments, seed-set of compatible crosses was higher for within and between patch crosses for the Benestad plants than for the others. It is, therefore, possible that the lower seed-set in the Benestad population is caused by factors other than SI, such as less frequent pollinator visits, perhaps as a consequence of the general scarcity of *T. integrifolia* at this site. The between populations crosses (Benestad–Grödby), gave a higher seed-set when the Benestad plants acted as mothers, than when the Grödby plants acted as mothers. This might be an example of purging of genetic load, since the Benestad patches have been small and isolated for a long time. However, there is also a possibility that the Benestad plants were best suited for the green-house conditions – in consistence with Crnokrak & Barrett (2002), who suggest that cases of increased fitness after generations of inbreeding might be the result of selection for laboratory conditions. Nevertheless, though seed-set for the natural populations is lower in Benestad than in Grödby and Råby, seed-set in the Benestad patches was on average higher than in similar-sized patches in Grödby and Råby (Isaksson & Widén paper I), which speaks in favour of the purging hypothesis. It is interesting that in Widéns study from 1993, though Grödby was found to have a lower seed-set than Benestad both after sib-crosses and after within population crosses (it is not stated whether within population crosses were performed within or between patches) the difference in seed-set between these two crossing-categories is greater for Benestad than for Grödby!

SI protects plants against inbreeding, and it decreases the chances of seed-set following cross-pollination between two closely related plants. Since population fragmentation leads to a general de-

crease in genetic variation as a result of increased genetic drift, we might also expect a decrease in variation in S-alleles. Willi et al. (2005) in a study of populations of the self-incompatible *Ranunculus reptans*, found that inbreeding depression was high in small populations, and concluded, therefore, that SI might not protect small populations from inbreeding. The suggestions of Castric and Vekemans (2004), that populations might be rescued from lack of S-alleles through gene-flow from other populations, or through occasional hybridisations with closely related species, hardly applies to *T. integrifolia* in Skåne, since the present populations grow very far from each other, and the only species of the same genus, *T. palustris* is even more uncommon, and grows in completely different habitat types (Wigermo & Håkansson 2005).

It is difficult to estimate the consequences, either negative or positive, of being self-incompatible, through the study of real populations, or using computer simulations. In simulations, it is difficult to take all necessary factors into account (inbreeding depression, number of S-alleles, gene flow between populations, population sizes, habitat fragment sizes, generation times, and dispersal distances for seeds and pollen) and to give them realistic values. In the study of real populations, you get only a momentary image of a process, which would have to continue for perhaps a hundred years before you might see any change in SI variation as a result of a decrease in population size. The empirical-experimental-simulatory study of Wagenius et al. (2007) shows the importance of the age of a habitat-fragment. Their simulation of a population of self-incompatible *Echinacea angustifolia* (a species similar to *T. integrifolia* in longevity and flowering-frequency) did not reach extinction until several hundred years after fragmentation (Wagenius, Lonsdorf, & Neuhauser 2007), and even in the smallest habitat fragments (100 m^2) it took 100 years for the effective number of

S-alleles to reach zero. When comparing these results of Wagenius et al. (2007) with the results of my crossing-experiments – where I found a high portion of compatible crosses within fragments (even between siblings, about 60 % of the crosses were compatible), it is reasonable to assume that the number of generations since fragmentation is small. This means that the *T. integrifolia* populations in Skåne are probably relicts of larger populations. If the populations remain small, but the plants manage to reproduce successfully, it is likely that S-alleles will be lost through drift, and that seed-set will decrease. However, if the populations continue to decrease in size with the same speed as they have done during the past three decades, they will probably have disappeared before drift could alter any gene frequencies.

Acknowledgements

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III

No connection between population size and local genetic variation in AFLP-markers in a threatened plant species (*Tephroseris integrifolia*)

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Abstract

Genetic population structure is the consequence of past dispersal and survival. Small-scale genetic variation in plant populations is essential for sexual reproduction, since plants to a great extent reproduce with their neighbours. As a consequence of extensive changes in the European cultural landscape, many plant populations have disappeared or become smaller or fragmented – processes which are likely to lead to genetic depletion. However, in nine Estonian and two Swedish populations of a rare plant species, *Tephroseris integrifolia* (L.) Holub (Asteraceae), we found no relation between population size and local (within ~10 m^2) genetic variation in AFLP markers. One possible explanation for the lack of difference in local genetic variation is that the processes leading to a decrease in population sizes and numbers also have lead to a halted recruitment, thus we might now have relict populations which do not reproduce at all, and accordingly, what we see now is a more or less random sample of the populations as they were before land management changed. Analyses of genetic distances between populations revealed that the Swedish populations were significantly different from the Estonian populations, but also that one of the Estonian populations, from a relatively isolated island, was significantly different from the rest, and also seemed to harbour less genetic variation.

Key words: Local genetic variation, AFLP, rare species, small populations.

Introduction

The genetic structure within a plant population is created through a process of dispersal, establishment, and survival. This process is dependent on several factors. Since sexual reproduction in plants often takes place between individuals which are relatively close to each other, changes in pollinator behaviour, seed dispersal mechanisms, or plant survival may change the chances of plant reproduction. Insect pollinated, out-crossing plants are, for their sexual repro- duction, dependent on what conspecific plants are within flying-distance of the pollinating insects (Kunin 1993, Wilcock & Neiland 2002). Habitat fragmentation can decrease access to suitable pollen-donors, which could lead to reduced seed-set through pollination failure (Wilcock & Neiland 2002, Tomimatsu & Ohara 2006), as well as to inbreeding depression (Van Geert, Van Rossum, & Triest 2008). The genetic composition of a plant population may give us some clues to past seed-dispersal mechanisms, as well

Table 1. Descriptions and geographic coordinates of populations for AFLP analysis. Gray indicates that individuals were not included in the analysis of genetic variation within population. For descriptions of primer combinations (B13, G20, & Y10), see Table 2.

Popluation – sample	Population description
Estonian populations	
Allika	Small population along gravel road surrounded by overgrown pastures
Allika 1	
Allika 2	
Järve	Small population within grazed pastures (number of flowering stems might be underestimated due to recent grazing)
Kadakalaid	Very small island in Estonian archipelago with dense stands of *Juniperus communis*.
Kadakalaid 1	
Kadakalaid 2	
Keila-Joa	Close to the north coast, some plants on the verge of chalk cliffs eroding into the see
Muuksi linnamägi	Large population within pastures on the site of an iron age fortress.
Muuksi linnamägi 1	
Muuksi linnamägi 2	
Muuksi linnamägi 3	
Pakri neem	Fairly large population in grassland which showed no signs of recent grazing
Sooääre	Very small population within a pasture which showed no signs of having been grazed that year
Türisalu	Small population within a pasture which showed no signs of having been grazed that year
Vohilaid	Fairly large population on an island to the east of Hiumaa. Most of the plants growing on chalk gravel a few metres from the see.
Vohilaid 1	
Vohilaid 2	
Vohilaid 3	
Vohilaid 4	
Swedish populations	
Benestad	Small, patchy population within a nature reserve with yearly grazing
Grödby S	Small population within grazed pastures.

Coordinates	Flowering Stems 2005	Population Surface	Individuals analysed			Total
			B13	G20	Y10	
	21	110 m²				15
59°23'31N 024°23'24E			1	1	1	1
59°23'31N 024°23'24E			14	13	12	14
59°23'34N 024°22'58E	30	130 m²	9	11	9	11
	200	0.2 km²				15
58°59'00N 023°00'13E			8	8	8	8
58°59'16N 023°00'12E			7	7	7	7
59°24'06N 024°19'00E	50	1150 m²	10	8	9	10
	5000 000	0.5 km²				18
59°30'44N 025°31'26E			4	3	3	5
59°30'43N 025°31'28E			3	3	3	4
59°30'41N 25°31'32E			6	6	7	9
59°23'23N 024°02'37E	1260	~1000 m²	11	11	12	12
59°22'35N 024°21'32E	12	15 m²	9	7	7	9
59°24'55N 024°18'52E	12	16.5 m²	7	4	6	8
	3500	2000 m²				20
58°55'30N 023°01'39E			0	0	2	2
58°55'29N 023°01'41E			11	11	10	11
58°55'29N 023°01'42E			4	4	3	5
58°55'29N 023°01'42E			2	2	1	2
55°31'36N 013°54'14E	37	50 m²	9	9	9	11
56°05'26N 014°30'51E	230	~1000 m²	11	10	11	11
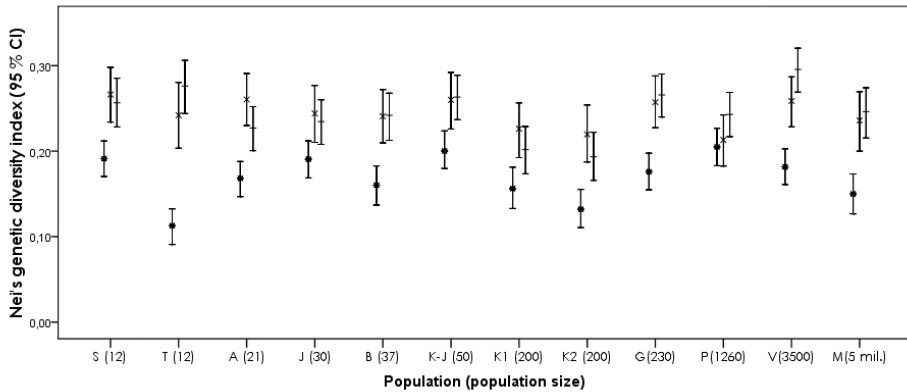
Fig. 1. Genetic diversity for three primer combinations in Swedish and Estonian *T. integrifolia* populations ranked by sizes. (Number of flowering stems in brackets.) Stars: B13, crosses: G20, and bars: Y10 (see Table 1).

as to the future chances of sexual reproduction for individual plants within the population (Ouborg, Piquot, & Van Groenendael 1999, Van Geert, Van Rossum, & Triest 2008).

The genetic variance within a population is predicted to decrease when population size decreases, and has been confirmed e.g. in a meta-study by Leimu et al. (2006) on 46 studies covering 41 species, but also in studies of single species (e.g. Lammi, Siikamäki, & Mustajärvi 1999, Landergott et al. 2001, Kang, Jiang, & Huang 2005, Coppi, Mengoni, & Selvi 2008). There are mechanisms which are believed to hamper the detrimental effects of decreasing population sizes, such as a non-random mating system, which lessens the risk of inbreeding, and strong selection, which helps preserve healthy gene-combinations. When unexpectedly high levels of within population variation have been found in small populations, this is usually referred to recent decreases in population size (e.g. Luijten et al. 2000, Mhemmed, Kamel, & Chedly 2008, Coppi, Mengoni, & Sevi 2008, Kang, Jiang, & Huang 2005), while low levels of genetic variation within populations is usually associated with historical bottlenecks or long periods of small population sizes (e.g. Landergott et al. 2001, Coppi, Mengoni, & Selvi 2008).

Landscape fragmentation and changes in land management may have extensive effects on the ecology and genetics of natural populations (Honnay & Jacquemyn 2007, Aguilar et al. 2008). The changes in European cultural landscape during the 20th century have entailed intensification and landscape simplification (Stoate et al. 2001). In Skåne, southern Sweden, a marked decrease in pastureland and hay-fields occurred already during the 19th century, when there was a five-fold increase in cultivated surface (Emanuelsson et al. 2002). This process continued during the 20th century, when many pastures were also abandoned to be overgrown with bushes, while at the same time many remaining pastures have been subject to an increased use of artificial manure (Emanuelsson et al. 2002).

After 1950, much of the agricultural land in Estonia was abandoned, and many pastures were overgrown (Kana, Kull, & Otsus 2008). The total grassland area decreased with only about 3.5% from 1950 to 2002, but only 11% of the grasslands in 2002 were grasslands also in 1950, which has to a great extent decreased genetic diversity of Estonian grasslands (Kana, Kull, & Otsus 2008). During the Soviet period, although Estonian agriculture, as was the case in all of Europe, was characterized by large-

Table 2. Primer combinations with numbers and sizes of bands, Fst, Gst, and mantel tests.

	B13	G20	Y10	average
MseI primer	GATGAGTCCTGAGTAA**CTA**	GATGAGTCCTGAGTAA**CAT**	GATGAGTCCTGAGTAA**CAC**	—
EcoRI primer	GACTGCGTACCAATTC**ACT**	GACTGCGTACCAATTC**AGG**	GACTGCGTACCAATTC**ACC**	—
Identified bands	292	186	229	—
Polymorphic bands	178	145	160	—
Min. band length	42.5 bp	46 bp	47 bp	—
Max. band length	499 bp	297 bp	384 bp	—
Fst	0.063	0.075	0.092	**0.07677**
Gst	0.0883	0.1106	0.1236	**0.1075**
Mantel test	r=0.59 (p=0.011)	r=0.64 (p=0.007)	r=0.60 (p=0.002)	**r=0.61**

scale farming, small-scale farming was also carried on in private plots of about 0.6 ha (or more) per person, utilized by farmers otherwise working at collective farms (Abrahams 1994), something which might have favoured species that were typical of the traditional cultural landscape in Europe. The Estonian part of this study is concentrated to the two counties Harjumaa and Hiiumaa, where 40–50 % of the arable land was abandoned during the first years of the 1990s (Peterson & Aunap 1998).

The focus of this study is the genetic variation within pollination-distance of a plant species, *Tephroseris integrifolia*, in populations of different sizes. *T. integrifolia* is a very rare species in Sweden, and it has decreased rapidly and drastically during the past 140 years – c. 85 % of the known Swedish populations have disappeared, and the remaining six populations are continually growing smaller (Isaksson & Widén paper I). Since *T. integrifolia* has continued to decline in Sweden during the past decades, despite farreaching conservation measures taken to save the remaining populations (Widén & Wetterin 1999) we suspect that there might be other causes for the ongoing decrease in population sizes – e.g. inbreeding depression as a consequence of genetic drift, following previous population decreases. In Estonia, on the other hand, though *T. integrifolia* is not exceedingly common, it is not considered

threatened (Ingelög, Andersson, & Thernborg 1993) and the populations are both larger (Isaksson pers. obs.) and more numerous (Kukk 2004) than the Swedish populations.

We have chosen to compare eleven populations of varying sizes – two Swedish and nine Estonian – to see if there is a difference in genetic variation between Swedish and Estonian populations, and between populations of different sizes. Specifically, we want to answer the following questions:

1. Is there a relationship between population size and small-scale genetic variation?
2. Is there a difference between Swedish and Estonian populations in small-scale genetic variation?
3. Is there a relationship between genetic and geographic distances between the populations?

Materials and methods
Study species
The Field fleawort (*Tephroseris integrifolia*) is a self-incompatible, composite plant which has a disjunct distribution throughout temperate regions of Eurasia, from Britain to Japan. It grows on calcareous, nutrient-poor grasslands (Smith 1979) – a threatened habitat type in most of Europe. In Sweden *T. integrifolia* is associated with intense grazing (Widén 1987). Both Swedish and Estonian populations of *T. integrifolia* are

edge-populations compared with the main distribution area. Seed dispersal mechanisms have not been studied for this species, but the fruit being an achene with a pappus it is, like many other Asteraceae species, likely to be classified as wind-dispersed (Benvenuti 2007). Relatively closely related species (of the genus *Senecio* and other Asteraceae species) have been shown to disperse about 2 metres at wind speeds of 4.5 metres per second (Sheldon & Burrows 1973), and since the achenes of *T. integrifolia* are similar to *Senecio* achenes in shape and size, as well as in length and width of the pappi, it is likely that wind does not disperse their seeds much farther than a few metres. Pollinators that have often been observed on *T. integrifolia* are syrphids (hoverflies) and muscids (flies) (Widén pers. obs.) and plants more than 10 metres away from other plants rarely set seeds (Isaksson & Widén paper I, Widén 1993). This species therefore seems to have limited dispersal abilities, both for seeds and for pollen.

Populations and sampling
Seeds were collected in June and July from two Swedish and nine Estonian populations (see Table 1 for details). Seven populations were from the Estonian mainland and two from small islands to the east of the island Hiiumaa – Vohilaid and Kadakalaid – of which Kadakalaid was the most isolated. In each population, seeds from ~10 plants were collected within a 2×5 m² surface. In some cases, seeds were also collected from other patches within the same population. We sowed the seeds and collected young, fresh leaves for DNA-extraction. Only one offspring from each field mother was included in the analyses.

DNA extraction and molecular methods
DNA was extracted from young, fresh leaves according to Lodhi et al. (1994).

Amplified fragment length polymorphism (AFLP) analysis (Vos et al. 1995)

was carried out using the AFLP kit for normal-sized plant genomes from Gibco BRL (Life Technologies, Täby, Sweden). For restriction, ligation and pre-amplification we followed the protocol from the manufacturer, but reduced amounts of reagents were used.

For selective amplification we used three primer combinations (Table 2). The reaction volume (5 µL) of the selective PCR reactions contained 3.9 µL ddH$_2$O, 0.5 µL 10x reaction buffer (100 mM Tris-HCl pH8.3, 500 mM KCl, 15 mM MgCl$_2$), 0.023 µL AmpliTaq Polymerase (5 units/µL; Applied Biosystems Stockholm, Sweden), and 0.4 µL template DNA (14 ng/µL). Size-variable fragments were amplified by a touch-down procedure with an initial 14 cycles of denaturing for 60 s at 94 °C, annealing for 60 s. at 65 °C in the first cycle, and then dropping 0.7 °C every cycle until the annealing temperature reached 56 °C, and extension for 90 s. at 72 °C; followed by 23 cycles of denaturing for 30 s. at 94 °C, annealing for 30 s. at 56 °C, and extension for 90 s. at 72 °C; and ended by a final extension for 10 min at 72 °C.

AFLP-band analysis
In each selective primer pair, the EcoRI primer was Cy5-labelled, to make the amplified fragments visible on the sequencer. The PCR product from each reaction was diluted in 20 mL formamide and mixed with home-made internal size standards (PCR fragments of which the size had been determined through comparison with commercially available size standards from Amersham Bioscience) to enable size determination of the fragments. For each 40 well gel that was run on the sequencer, two wells were reserved for a Cy5-labelled 50–500 bp external standard (Amersham Bioscience).

Fragments were separated with ALF Express II DNA analyzer (GE Healthcare). The banding patterns were analysed manually with ALFwin Fragment Analyser 1.03.01 software (GE Healthcare). We scanned the gels for band

lengths above 40 (the double size of the primers) to about 500 bp. Maximum band length for each primer was chosen according to the maximum band length of the majority of the samples (Table 2). Only clear bands above 0.1% of full detection with a symmetrical shape on the curve-view screen were selected for analysis. Samples with less than 20 clear bands were excluded from the analysis. The population origins of the samples were kept unknown during this process to avoid subjective estimations. Two individuals were present on every gel to serve as additional control.

Analyses of genetic variation
Since the number of bands and band resolution differed between primer combinations, they were analyzed separately. We analysed within population diversity with AFLPdat (Ehrich 2006) in R version 2.7.0 (R Development Core Team 2008), which calculates Nei's gene diversity with the formula

$$D = n / (n-1) \times [1-freq(1)2+freq(0)2)]$$

(assuming that the band frequency corresponds to allele frequency); and estimates a confidence interval for the diversities from bootstrapping over markers (we used 1000 bootstrap replicates). Between-populations diversity tests and Mantel tests were performed in Arlequin 2.000 (Schneider, Roessli, & Excoffier 2000). A PCO analysis was applied to the corrected average pairwise differences from the Arelquin analysis, using NTSYSpc2.2 (Rohlf 2005). The Mantel test was performed in Arlequin, comparing an Fst distance matrix for the populations with a geographic distance matrix done in AFLPdat based on the geographic coordinates of the populations. Gst was calculated using POPGENE (Yeh, Yang, & Boyle 1999). For the within population variation, only plants collected within the 10 m² plots were included. For the Kadakalaid population, two such plots were analysed. For the between-populations analysis, and the Mantel test, plants collected outside the 10 m² plots were included as well. Arle-

quin input files and POPGENE32 input files were created with AFLPdat in R.

Results
The three primer combinations gave each between 186 and 292 bands (see Table 2). B13 gave the highest number of bands, 292. Y10 gave 229 bands. G20 gave 186 bands. We have chosen to analyse the results of the three primer-combinations separately. Primer combinations Y10 and G20 gave relatively similar results for genetic diversity, while primer combination B13 differed somewhat from the others.

The analyses of genetic variation within populations showed no relationship between population size and amount of local genetic variation for either of the primer combinations, and there was no difference between the Swedish and the Estonian populations in amount of local genetic variation. The Kadakalaid population, which is the most isolated, had least genetic variation of all the populations (see Fig. 1).

Most of the genetic variation was within the populations – the average (between the three primer combinations) Fst for all populations was 0.077. Average Gst was slightly higher: 0.11. The isolated Estonian island population, Kadakalaid, was significantly different from all of the other populations for each of the three primer combinations. The differences between the mainland Estonian populations, as well as the Vohilaid population, were sometimes significant and sometimes not. In all of the three PCOs based on corrected average pairwise differences (calculated with Arlequin), the Benestad and Grödby populations were grouped together, the Kadakalaid population was isolated, and the rest of the Estonian populations were more or less grouped together (Fig. 2a–c). The two Swedish populations, Benestad and Grödby, were never significantly different from each other, and both were significantly different from the other populations for the Y10 primer combination. For the G20 primer combination,
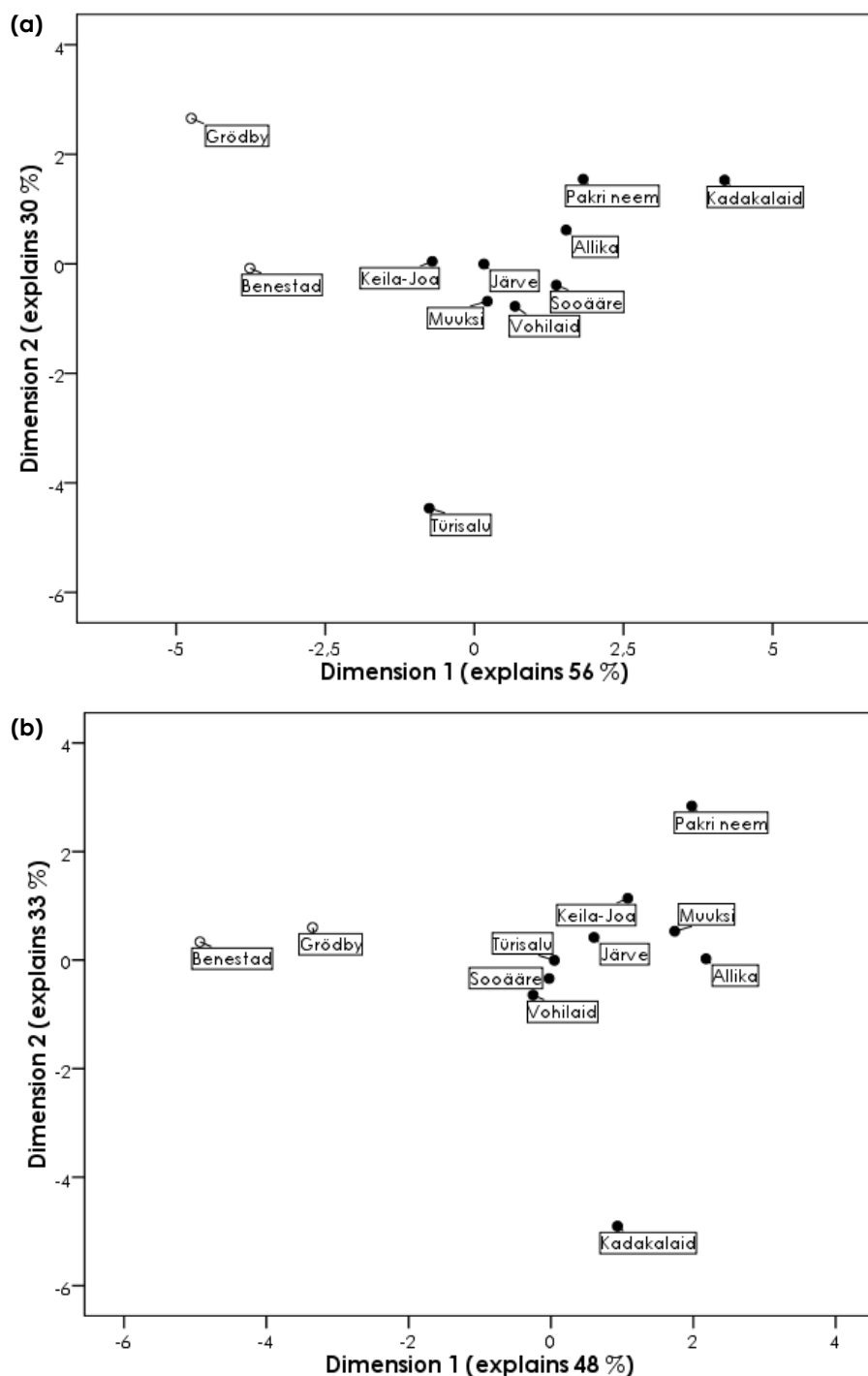
Fig. 2. PCOs applied to genetic average pairwise distances for (a) primercombination B13 (b) primercombination G20, and (c) primercombination Y10. Filled symbols for Estonian populations, open symbols for Swedish populations.

(c)
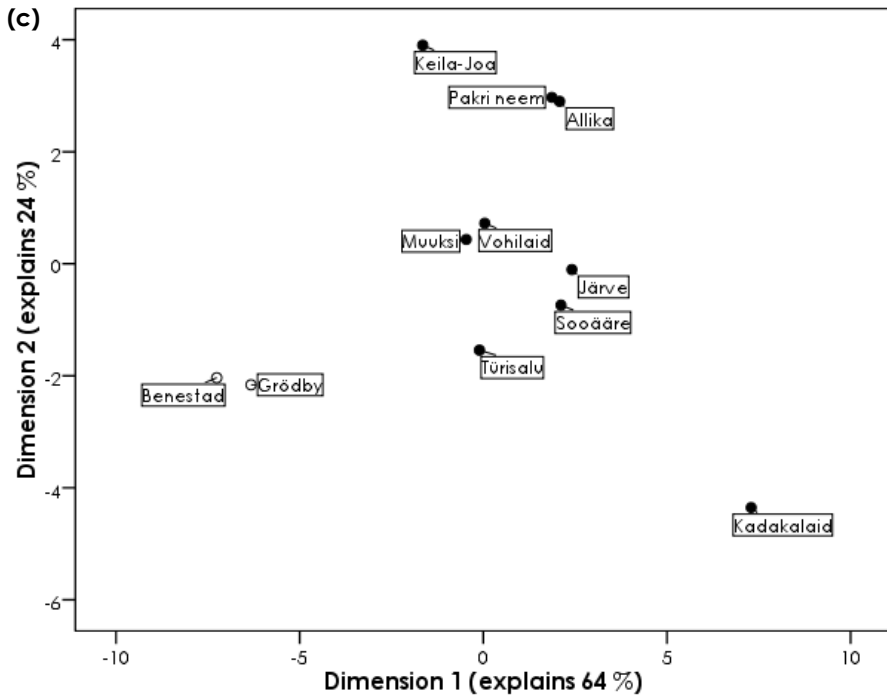
Grödby was not significantly different from Vohilaid. For the B13 primer combination, Benestad was not significantly different from Keila-Joa. The Mantel test showed a significant relationship between genetic and geographic distances when all populations were included, but not when only the Estonian populations were included. Due to the small number of populations, no separate Mantel test was performed for the Swedish populations.

Discussion
The most striking result of this study is that there is no relationship between local genetic variation and population size in the eleven investigated *T. integrifolia* populations. It is also worth mentioning that there is no difference in local genetic variation between Estonian and Swedish populations, although *T. integrifolia* is much more abundant in Estonia.

A positive relationship between population size and genetic variation is ex-

pected (Barrett & Kohn 1991, Ellstrand & Elam 1993), and has been confirmed in other studies, e.g. in a meta-study based on 46 studies of 41 plant species by Leimu et al. (2006), where self-incompatibility seemed to enhance this relationship, but they found no difference between common and rare species. However, there are a few studies which report unexpectedly high genetic variation in small populations. *Globularia bisnagarica* is one example. It is a long-lived perennial which is probably dispersed through epizoochory (Honnay et al. 2007). *Arnica montana* is another example. It is a long-lived, self-incompatible perennial with no seed-bank which has declined despite of habitat restorations (Luijten et al. 2000). The same phenomenon is, perhaps not surprisingly, found in tree-species (Soejima, Maik, & Ueda 1998, De Almeida Vieira & De Carvalho 2008 Dzialuk et al. 2009), where forest-fragments contain the same genetic variation as they did 50 years ago, when they were parts of large for-

ests, where the remaining individuals have more or less ceased to reproduce – i.e. they are relict populations.

The expected population turnover time for Swedish *T. integrifolia* populations has been estimated by Widén (1987) to 23 years in the Grödby population and to 78.6 years in the Benestad population. This would mean that several generations of plants would have succeeded each other in the Grödby population since the 1950s, while many of the present Benestad plants could have been there since that time. However, there are problems making this kind of estimations, since plants that are not flowering can be very inconspicuous, and therefore assumed to be dead. Adult plants appearing in a patch several years after they are believed to have gone extinct in that spot (Widén pers. obs.) is an indication that the length of life might earlier have been underestimated.

There are several possible explanations for the lack of relationship between population size and genetic variation in this study. Similar patterns have been found on species-richness levels – e.g. Helm, Hanski, & Pärtel (2006) found no connection between plant species richness and present habitat-size in 35 alvars in Estonia – however, they did find a relationship between present species richness and landscape connectivity 70 years ago. Charlesworth (2009) makes a review of factors affecting effective population size – among them fluctuations of population size, age structure, and spatial structure (e.g. migration processes). All of these could explain the unexpected evenness in variation between populations regardless of population sizes – population sizes might have been very different a hundred years ago. If there has not been much recruitment in the smaller *T. integrifolia* populations during the last decades, there has been little possibility for genetic drift to have any effect on genetic variation, and, in addition, the dispersal-mechanism is unknown. For example long-distance dispersal by animals would give rise to a

very different genetic structure compared with wind-dispersal.

Dispersal
It is complicated both to measure and to calculate effective dispersal distances. Asteraceae species with pappi are often believed to be wind-dispersed. Sheldon & Burrows (1973) have calculated maximum primary dispersal distances for Asteraceae species, among them some relatively closely related to *T. integrifolia* (*Senecio squalidus*, *S. viscosus*, & *S. vulgaris*) to between 1.8 and 2.9 metres at wind speeds of 4.5 metres per second, taking into account among other things the height of the plants. Dispersal distances are also affected by humidity (when the pappus is wet, the seed is likely to land closer to the mother plant), and the heat of the surface together with surrounding vegetation, both factors which can affect the vertical component of wind direction (Sheldon & Burrows 1973). It is possible that the denser vegetation followed by eutrophication might hamper the dispersability of wind-dispersed seeds. However, seeds that are traditionally thought to be wind-dispersed, are often prone to stick to animal fur (Couvreur et al. 2004, Römermann, Tackenberg, & Poschlod 2005, De Pablos & Peco 2007), and have been shown to be dispersed long distances by sheep (Fischer, Poschlod, & Beinlich 1996, Mouissie, Lengkeek, & Diggelen 2005, Wessels et al. 2008), cattle (Couvreur et al. 2004, Mouissie, Lengkeek, & Diggelen 2005), and other large herbivores such as donkeys, bison and horses (Rosas et al. 2008, Couvreur et al. 2004, Couvreur et al. 2008). A study of seed dispersal by *Bison bison* in a tallgrass prairie, showed that only 17.5% of the seeds stuck to bison fur had obvious attachment mechanisms, and that many of the species that are abundant in the fur are usually suspected to be wind-dispersed – e.g. 32.2% of the seeds were from the Asteraceae family. In their study of diaspore-dispersal by cows, donkeys and horses, Couvreur et al. (2004) found that

24 % of the seeds and 42 % of the seed-lings belonged to the Asteraceae family. An over-representation of red-listed (Wessels et al. 2008) or declining (Ozinga et al. 2009) species has been found among epizoochorous plants, and Ozinga et al. (2009) draw the conclusion that 'colonization deficit' is an important factor in loss of species diversity.

If *T. integrifolia* were indeed wind-dispersed, seeds would be likely to spread about a couple of metres each generation. Since this distance is even shorter than normal pollination distances, we would expect extreme genetic structuring within populations, particularly in fragmented habitats. Small, fragmented populations would quickly loose genetic variation, and we would probably see a difference in genetic variation depending on population size, even if this relationship might be blurred by recent historic changes in population size. That we see no relationship at all between population size and genetic variation is a hint either that the now small populations have until recently been very large and all the same size, or that dispersal patterns have been very dynamic.

Population size and the effect of sample area

Plants are often more likely to produce offspring with their nearest neighbours than with more distant individuals (Turner, Stephens, & Anderson 1982) while at the same time many plants have barriers against producing offspring with close relatives (Richards 1997, Barrett 2002, Hiscock & Tabah 2003). The possibilities of finding mates outside the population are limited, since plants can't move. Even in a very large population, most gene exchange will occur within very short distances (Turner, Stephens, & Andersson 1982). This adds a dimension to population-genetics not present in most animal populations.

As pointed out by Leblos, Estoup, & Streiff (2006) the effect of reduction in population size on genetic variance is strongly influenced by isolation by distance, and the sampling method is very important – you can choose either to sample from an area proportional to the total area of the population, or you can choose to sample from an area of exactly the same size in each population. Which you choose will strongly affect the results. According to their simulations (Leblos, Estoup, & Streiff 2006) where populations of different sizes suffered an instantaneous loss of individuals and of habitat area, genetic variation decreased more slowly in populations with strong isolation by distance than in panmictic populations, and this effect was stronger for a smaller sample area (Leblos, Estoup, & Streiff 2006). A lack of recruitment of new individuals might also render higher-than-expected genetic variation. Van Geert, Van Rossum, & Triest (2008) found much lower genetic variation in the seedling than in adult plants in a *Primula vulgaris* population, concluding that habitat degradation might hamper the establishment of new individuals. It might be added, that establishment of new individuals could be facilitated if seeds were dispersed over a large area, since that would decrease competition from plants of the same species, as well as from other plants (reviewed in Fenner & Thompson 2005 p. 67 ff) – a patch which was ideal for seedling establishment when the plants in the parent generation were established could be very hostile for their offspring.

Peripheral populations

Populations for preservation are often selected on a national or state basis. The choices can sometimes seem absurd, when you consider that species are for that reason frequently chosen to be protected in the periphery of the species distribution, where the genetic variation is putatively lower than in the centre of the distribution area, just because a state border happens to be close to the periphery of the species distribution (Lesica & Allendorf 1995). Lammi, Siikamäki, & Mustajärvi (1999) proved small popula-

tions of *Lychnis viscaria* to be less genetically variable, but not less fit, than larger populations, and drew the conclusion that small, isolated populations might harbour important evolutionary potential. This is consistent with the Kadakalaid population in this investigation, which had least genetic variation, but which also differed the most from the other populations.

Small-scale genetic variation – causes and consequences

The genetic variation in present-day populations is the consequence of past dispersal and extinction. The relatively high genetic variation in AFLP markers suggests that the Swedish *T. integrifolia* populations that we see today are relicts of previously large populations, and this is confirmed by the lack of inbreeding depression (Isaksson & Widén paper I) and the high seed-set both in natural populations (Isaksson & Widén paper I) and in crossing-experiments (Isaksson paper II). If the present Swedish *T. integrifolia* populations are indeed relicts of previously large populations, and if the plants that are still alive are very old, we cannot expect local genetic variation to be lower than in random samples from large and thriving populations. It seems therefore, that the supply of suitable mates is sufficient for the plants to produce high-quality seeds even in the smallest populations in this investigation (cf. Isaksson & Widén paper I, Isaksson paper II), at least during years when most plants are flowering. However, if there have been drastic changes in seed dispersal dynamics, so that produced seeds are not likely to end up in habitats favourable for seedlings, these seeds will have been produced in vain.

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IV

Negative association between population size and quantitative genetic variance in *Tephroseris integrifolia* (Asteraceae) – evidence for a recent bottleneck?

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Abstract

The Field Fleawort, *Tephroseris integrifolia* (L.) Holub, is a plant species confined to nutrient-poor, calcareous grasslands, a habitat-type which has declined considerably in many European countries during the past 150 years. As a result, *T. integrifolia* has become very rare in some of these countries. We investigated the broad sense heritability for *T. integrifolia* plants from thirteen North European populations, from Britain, Denmark, Sweden, and Estonia. Surprisingly, heritability was for most characters the largest in the Swedish populations, which are the smallest and the smallest for the Estonian populations, which are the largest. It is probable that the smaller populations are relicts of large populations, which could explain why not much variation has been lost. Increased quantitative variation could, however, be the consequence of homozygosity in rare recessive alleles, something which has been observed during the first generations of a bottleneck. It seems, therefore, that not many generations of *T. integrifolia* have passed since the populations decreased in size, something which is consistent with other studies of the same species.

Keywords: Quantitative genetics, genetic variation, small populations, fragmentation, extinction, relict populations, threatened species.

Introduction

A large number of the plant species in Europe are native to nutrient-poor grasslands (Pärtel et al. 2005). This is a habitat-type which has been dependent on human management for thousands of years (Richards 1990). During the past centuries there has been considerable change in the European cultural landscape. Approximately 30 % of the total grassland-cover in (non USSR) Europe disappeared between 1700 and 1980, most of which took place before 1850 (Richards 1990). However, the more severe changes have taken place after 1940, when the use of tractors and artificial fertilisers became more common, changing most of the remaining grasslands into a completely new habitat type (Ratcliffe 1984, Bernes 1994). Gradually, agriculture has become more efficient and also more static, through the cessation of the rotation of crops and the more rigid division of land into fields and pastures, and as a consequence the area covered with nutrient-poor grasslands has decreased in Europe (Emanuelsson 2009). Therefore, populations of plants native to these habitats have become fragmented and many are threatened

with extinction (Hodgson et al. 2005, Poschlod et al. 2005, Schleuning et al. 2009). Several studies have demonstrated that the effect of fragmentation on species richness or population size might be delayed, so that we don't yet see the full effect of changes that took place 50 or 100 (Cousins, Ohlson, & Eriksson 2007), or even 150 (Tasser et al. 2007), or 200 years ago (Gustavsson, Lennartson, & Emanuelsson 2007). The processes of change have, due to political and economical differences, varied between countries, particularly, many processes have started later in the eastern and central parts of Europe as a consequence of a more rigid feudal system (Emanuelsson 2009).

Small populations
Stochastic events – demographic or environmental catastrophes – may lead to the extinction of small populations (Turelli 1977, Vindens, Engen, & Sæter 2008). Small populations loose genetic variance due to genetic drift (Young, Brown, & Zich 1999, Van Rossum, De Sousa & Triest 2004). Genetic depletion can be observed in molecular, putatively neutral, markers, e.g. as the loss of heterozygosity, which will be $1/[2N_e]$ per generation (Wright 1931), where N_e is the effective population size – thus, the smaller the population, the larger the loss of genetic variance. Genetic depletion is also reflected in the phenotypic variance and leads to a decrease in additive genetic variance, $V_A = V_P/[V_D+V_E]$, where V_P is the phenotypic variance in the population, V_D the variance of dominance deviations and V_E the variance due to environmental effects (Futuyma 1998). If there is no epistasis, dominance or selection, the loss of additive genetic variance will also be $1/[2N_e]$ per generation. A loss of phenotypic variance leads to a reduced capacity to tackle future environmental changes as a consequence of decreased evolvability (Houle 1989, Reed & Frankham 2003). Genetic depletion also increases the risk of inbreeding depression; as the expression of recessive, possibly detrimental, alleles increases with increased homozygosity (Reed & Frankham 2003).

Reduced variation in quantitative characters as a consequence of decreased population size are well documented in experimentally inbred populations, at least for characters not strongly related to fitness – which is demonstrated in a meta-study of 22 investigations by Van Buskirk & Willi (2006) – but are weakly supported by studies of natural populations, according to a review by Willi, Van Buskirk, & Hoffmann (2006). Variation in quantitative markers is sometimes found to be negatively correlated with population size in natural populations (e.g. Waldmann 2001) and has been noted in the first generations of inbreeding in experimental populations (e.g. Van Heerwarden et al. 2008). Unexpectedly high levels of genetic variation have been found in several studies of small populations and could be explained by the populations being relicts of larger populations (e.g. Young, Brown, & Zich 1999, Luijten et al. 2000). In the meta-study by Van Buskirk & Willi (2006), it is found that variation in fitness-related morphological characters often increases during the first generations of inbreeding – a consequence, they conclude, of dominance and epistasis effects on life history traits.

While organisms chosen for bottleneck experiments usually have a relatively short generation time (e.g. Bryant & Meffert 1988, Saccheri, Nichols, & Brakefield 2006), generation times in natural populations vary and for long-lived species, a hundred years after habitat fragmentation, we might only see the effects of a few generations of inbreeding, which is when we might expect an increase in variation in some quantitative characters (Van Buskirk & Willi 2006). The period of increasing variation at the beginning of a bottleneck might be prolonged if the mechanisms behind the decline of the populations also hamper successful regeneration, in which case extant populations are relicts of larger,

historical populations (Tilman et al. 1994, Bachmann & Hensen 2007, García 2008).

The Field fleawort, *Tephroseris integrifolia*, is one of the plant species which is confined to nutrient-poor grasslands and its decline in Sweden has been studied in detail (Widén 1986, 1987, 1991, 1993, Widén & Andersson 1993, Widen & Wetterin 1999, see also Isaksson & Widén paper I, Isaksson paper II, Isaksson, Nordström, & Widén paper III). Despite a serious decline in population number and size (Isaksson & Widén paper I), the small Swedish populations don't suffer from serious inbreeding depression (Isaksson & Widén paper I), when cross-pollinated with plants from the same patch, they usually set seed, though they are self-incompatible, which indicates that they have not lost many S-alleles through drift (Isaksson paper II) and the Swedish populations seem to harbour about as much local genetic variation in molecular markers as the larger Estonian populations (Isaksson, Nordström, & Widén paper III).

While the study of variation in putatively neutral genetic markers is a good tool to find loss of genetic variation in small populations, it is also important to investigate the morphological response to genetic variation, i.e. additive genetic variance, or heritability. In an earlier study of two Swedish populations of *T. integrifolia*, a smaller population (Benestad) seemed to harbour more variation than a larger population (Grödby) (Widén & Andersson 1993). However, all Swedish populations are relatively small e.g. compared with the Estonian populations (Isaksson pers. obs.) and also have a relatively similar history. We wanted to make a broader study of populations from a greater geographical range, with a more varied history and with more varying numbers of plants.

In this study we investigate the relationship between population size and variation in morphological characters in thirteen North European populations of *T. integrifolia*, from four different countries – Britain, Denmark, Sweden, and Estonia. The decrease in the Swedish *T. integrifolia* populations is closely associated with the disappearance of pastures and the rationalisation of agriculture (Isaksson & Widén paper I). While agricultural history in Britain, Denmark, and Sweden have followed more or less the same processes as the rest of Western Europe, Estonian agrarian history is more complicated, since Estonia has been under the rule of so many different regimes (Emanuelsson 2009). According to Pärtel et al. (1999), the destruction of semi-natural grass-lands has started towards the end of the fifties – however, Estonian landscape has gone through several periods of change during the 20th century. By the time of world war one, most agricultural land in Estonia was owned by German-Baltic nobility. In the 1920s, agricultural land was sold to farmers and towards the end of the 1940s, under Soviet regime, it went through collectivisation, which led to a lot of smaller farms being abandoned (Emanuelsson 2009). However, in Estonia, the picture is further complicated by the custom of small private plots where small-scale farming was done parallel to large-scale collective farming during the Soviet period (Abrahams 1994). In the 1970s and 1980s, about 15% of Estonia was turned into nature reserves (Emanuelsson 2009). Additional semi-natural grasslands in Estonia are now threatened as a consequence of many farms being abandoned in the 1990s, after the Soviet era (Emanuelsson 2009).

We use *T. integrifolia* as a model organism to answer the following questions:

- What is the relationship between population size and variation in quantitative characters?
- Is there a difference between North European regions in local or regional genetic variation?

Materials and methods

Study species

Tephroseris integrifolia L. Holub (Asteraceae – earlier *Senecio integrifolius*) is a perennial, self-incompatible herb which grows in nutrient-poor, calcareous grasslands. It occurs throughout Eurasia, from Britain to Japan. In several European countries it has declined considerably during the past decades (e.g. Widén 1987, Krach & Krach 1991, and Preston et al. 2002 compared with Smith 1979). Each stem has 1-4 flower heads with 30-150 florets in each head. The fruit is a one-seeded achene with a pappus. In the wild, a plant rarely flowers two years in a row and the number of flowering plants in one population varies considerably between years (Isaksson & Widén paper I). The Swedish populations have been carefully monitored and investigated since the 1980s (e.g. Widén 1986, 1987, 1993, Andersson & Widén 1994) and during this period they have declined drastically in spite of extensive conservation measures. Observed pollinators are hover flies and other flies (Widén pers. obs.), which usually don't fly long distances. This explains why seed-set is exceedingly density dependent, which has been demonstrated in a common garden experiment (Widén 1993), and why plants growing more than ten metres away from others of the same species seldom set any seeds (Isaksson & Widén paper I). Widén (1987) calculated the expected half-life of plants in four Swedish *T. integrifolia* populations to between 7.2 and 39.3 years. One of these populations (Tosteberga) is included in this study, with an expected half-life of 8.7 years for individual plants.

Regions

We sampled seeds from thirteen North European populations in Britain, Denmark, Sweden, and Estonia. In Britain (Cheffings & Farrell 2005) and in Sweden (Gärdenfors 2005) this species is regarded as endangered (EN according to IUCN criteria), while it is not present on the national red data lists for Denmark (Den danske rødliste 2004) or Estonia. However, in Red Data Book of the Baltic Region (Ingelög et al. 1993), *T. integrifolia* is listed as rare in Denmark, while in Estonia it is listed as present.

Populations

For information on size, structure, and cover-area of the populations, see Table 1! On the three British sites, the plants grew on slopes related to pre-historic constructions. The smallest population (Cissbury Ring) was infested by rabbits. All three sites were grazed. Of the three Danish sites, two (Sårup and Skørping) were grazed. In both cases the plants grew on steep slopes, which would be avoided by cattle as long as there is more easily accessible grass. At one site (Hamborg) there was no grazing – however, the slope was so steep, that vegetation was probably kept open through continuous erosion (Isaksson, pers. obs.). Of the four Estonian populations, none grew on a slope. In the westernmost population, at Vohilaid (an island to the east of the island Hiumaa) the plants grew among lime stone gravel on the shore. The plants in the population at Pakri neem grew together with high grass close to a steep slope towards the sea. We saw no signs of grazing. At Allika, all the plants grew along the edges of a gravel-road with over-grown pastures on both sides – indicating that the extant plants are a relict of a much larger population. The Muuksi linnamägi population, which was by far the largest, grew within a small area which had been grazed the previous year (which we concluded from the year-old cowpats). In 1983 the Tosteberga population was the largest Swedish population, with about 8000 flowering stems, and new seedlings were established. Ten years later the size of the population had decreased dramatically (Isaksson & Widén paper I). During the past ten years the maximum number of flowering stems in Tosteberga has been 200, in Edenryd 600, and in Råby 800 (Isaksson & Widén paper I).

Table 1. Description of *T. integrifolia* populations. Populations in Denmark, Britain, and Sweden were sampled in 2006, and Estonian populations in 2005. Covered area and population structure were estimated from the distribution of flowering plants in the area during the sampling year.

Region	Population	Coordinates	Covered area	Nr of flowering stems	Population structure	Density (stems/m²)	Habitat appearance	N. of field mothers collected from
Denmark	Hamborg	57°06'48N 8°40'21E	200 m²	40	Patchy	0.2	Slope	11
	Skørping	56°50'28N 9°54'52E	30 m²	17	Patchy	0.6	Slope	8
	Sårup	57°05'8N 8°38'59E	1500 m²	200	Mostly continuous	0.1	Slope	9
Britain	Cissbury Ring	50°51'45N 0°22'58W	140 m²	24	Patchy	0.2	Slope	10
	Mt Caburn	50°51'42N 0°22'58W	1800 m²	1800	Continuous, oval	1.0	Slope	10
	Martin Down	50°58'14N 0°03'3E	400 m²	142	Continuous, linear	0.4	Slope	10
Estonia	Allika	59°23'31N 24°23'24E	200 m²	30	Linear	5.5	Gravel roadside	12
	Muuksi linnamägi	59°30'41N 25°31'32E	0,5 km²	5 mill.	Continuous, circular	20	Pasture, flat	12
	Pakri neem	59°23'23N 24°02'37E	16000 m²	230 000	Mostly continuous	14.4	Grassland, flat	12
	Vohilaid	58°55'29N 23°01'41E	2000 m²	3500	Continuous	1.8	Chalk gravel shore	10
Sweden	Edenryd	56°04'87N 14°51'95E	800 m²	228	Continuous + some isolated patches	0.3	Flat, stony pasture	10
	Råby	56°09'69N 14°51'79E	700 m²	152	Patchy	0.2	Flat pasture	10
	Tosteberga	56°01'65N 14°46'09E	1000 m²	164	±Continuous	0.2	Flat, stony pasture	10

Sampling and field-measures

We sampled between eight and twelve plants from each population (Table 1). To make the populations comparable, we sampled from equal-sized plots (ca. 10 m²). From each plant we took one flower head with mature seeds. Height of the tallest stem for each plant was measured (except for Mt Caburn) and number of flowering stems per plant and flower heads per stem were counted.

Cultivation of offspring

All seeds were sown during two days in August 2006. From each of the ten field-mother plants from each population, we sowed 30 seeds, or all seeds, when less than 30 were available and transplanted 20 seedlings, or all seedlings, when less than 20 were available, into new pots. The seedlings were transplanted again in September. All plants were divided into (and randomized within) blocks, with members of each family evenly

divided between the blocks. In December all plants were randomized and classified according to size, 0 through 5, and we measured the longest leaf of each plant. The smallest plants, category 0, were kept in the warm green-house throughout the winter, while the rest were kept in a cold green-house from December, when the plants were randomized within blocks, till February, when the plants were again randomized within blocks and transferred back to the warm green-house.

Measures

Characters chosen for measure (Tables 3 and 4) had been found to give significant narrow sense heritabilities in an earlier study of the same species (Widén & Andersson 1993) – except the characters »number of undeveloped flower heads» and corymb length, which were not included in Widén and Andersson (1993). The flowering date of the first flower
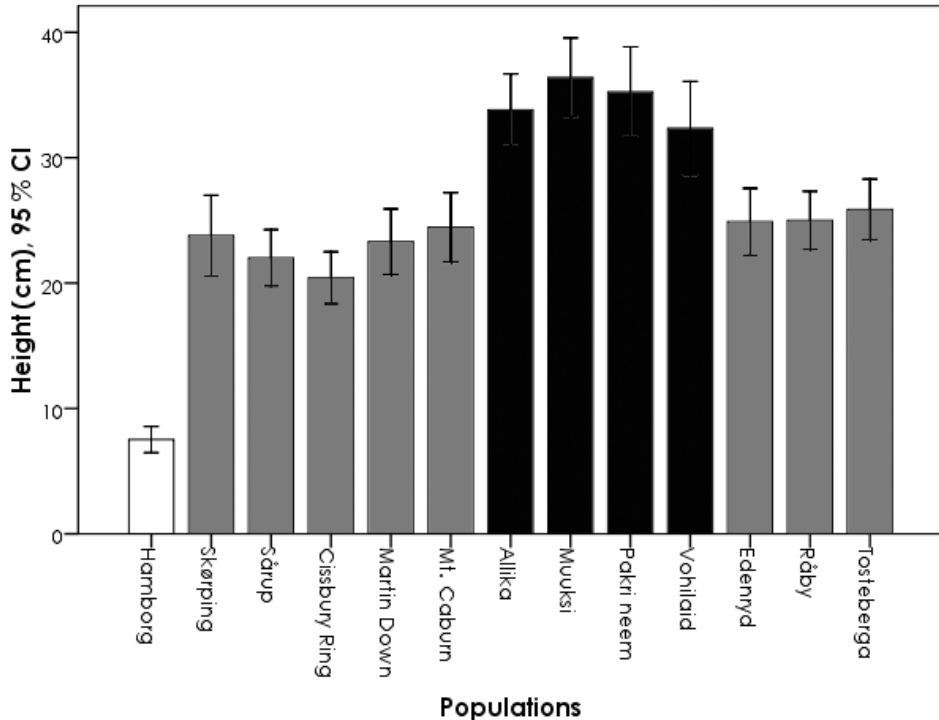
Fig. 1. Heights at flowering of *T. Integrifolia* in cultivation for different populations. Colour indicates homogeneous sub-set at p < 0.05; white = subset 1, gray = subset 2, black = subset 3.

head of each plant was recorded and this was also the date when all measures were taken (except flower- and seed-characters and height at seed-maturity). One flower head from each plant was harvested and preserved in 40 % ethanol in a cold store-room. The plants were randomly cross-pollinated within population in order to make the plants produce seeds. The date of seed-maturity was recorded and from each plant one head with seeds was harvested. Flower- and seed-measures were taken with an ocular ruler under a dissecting microscope and with a digital slide-calliper. Characters were grouped into five categories: height characters, quantity characters, flower characters, seed characters, and phenology characters (flowering time, seeding time, and length of flowering period). In total 1994 plants were cultivated, of which 896 flowered

and were measured, on average 69 from each population (Tab. 2).

Heritabilities and $Q_{ST}s$

Nested variance component analyses were done for one character and one country at the time. For flowering time and height, populations were also analysed separately. Variance components of populations $V(p)$, variance components of field mothers, nested within populations $V(m(p))$, and variance components of errors $V(e)$ were given by the computer analysis. The genetic variance was quantified as the intra-class coefficient, calculated as (Falconer & Mackay 1996):

$$V(m(p))/(V(m(p) + V(e))$$

and referred to as the broad-sense heritability, H^2. We do not know whether plants from one family are full-sibs or half-sibs which would imply multiplying the intra-class coefficient with 2 or 4 to
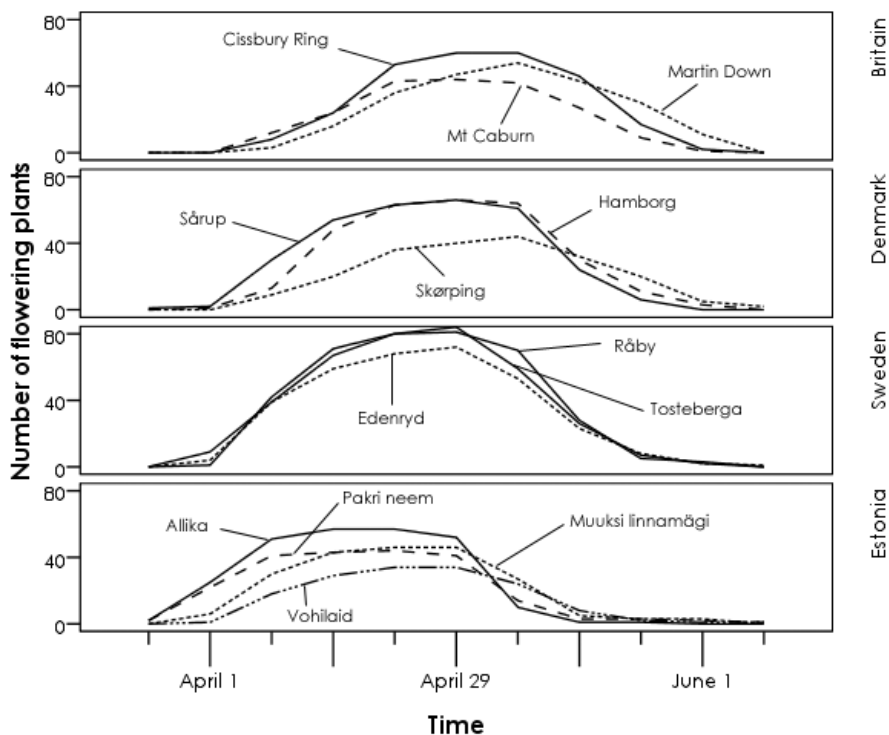
Fig. 2. Flowering period of the different populations, calculated from the first flowering date and the first seeding date of individual plants.

Table 2. Average first flowering date. Posthoc: Tukey HSD (homogeneous sub-sets with significance-values).

Country	Population	N field mothers with flowering offspring	N flowering offspring	Average first flowering	Subset (p)	Subset (p)	Subset (p)
Estonia	Allika	11	71	April 5	1 (0,837)		
Estonia	Pakri neem	11	58	April 7	1		
Sweden	Tosteberga	10	104	April 13	2		
Estonia	Vohilaid	10	47	April 14	2		
Estonia	Muuksi	10	63	April 14	2 (0,053)	3	
Sweden	Edenryd	10	86	April 17	2	3 (0,054)	4(0,597)
Sweden	Råby	9	93	April 18	2	3	4
Denmark	Sårup	9	76	April 19	3 (0,054)	4(0,597)	5(0,058)
Britain	Mt Caburn	10	49	April 19	4(0,597)	5(0,058)	
Denmark	Hamborg	9	75	April 20	4	5	
Britain	Cissbury Ring	10	66	April 23	5(0,058)	6 (0,411)	
Britain	Martin Down	10	60	April 27	6 (0,411)	7 (0,989)	
Denmark	Skørping	8	48	April 29	7 (0,989)		

get narrow-sense or broad sense heritability, respectively. We use the term broad-sense heritability, to imply that we cannot decide how much of the variation is due to additive variance or dominance variance, respectively.

Q_{ST} for each country was calculated as (cf. Spitze 1993):

$$Q_{ST} = V(p) / V(p) + 2 \times (V(m(p)) + V(e))$$

One of the Danish populations (Hamborg) consisted of very small plants, which made the Q_{ST} -values for Denmark very high, therefore, we also calculated Q_{ST} for the Swedish and Danish populations together, excluding the Hamborg population.

Table 3. Heritabilities. Significance values: * for p < 0.05, ** for p < 0.01, *** for p < 0.001 reflect significance-values of corresponding ANOVAs. Average values bold when at least one individual character within the character category is significant.

	H²-values						
	Denmark		**Britain**		**Estonia**		**Sweden**	
Height characters								
Height at flowering	0.188	***	0.000		0.027		0.104	*
Height after flowering	0.093	*	0.018		0.000		0.086	*
Length of first head stalk	0.076	*	0.187	*	0.030		0.126	*
Length of corymb	0.105	*	0.313	*	0.194	*	0.091	*
Average	**0.116**		**0.130**		**0.063**		**0.102**	
Quantity characters								
Total number of heads	0.093	*	0.000		0.050		0.113	*
Tot undeveloped heads	0.000		0.000		0.000		0.000	
Number of ray florets	0.074	*	0.083		0.000		0.307	*
Number of disc florets	0.054		0.123	*	0.024		0.222	*
Average	**0.055**		**0.051**		0.018		**0.160**	
Flower characters								
Ray floret width	0.138	*	0.269	*	0.147	*	0.199	*
Ligule length	0.099	*	0.091	*	0.121	*	0.346	*
Corolla length	0.122	*	0.096	*	0.000		0.276	*
Style length	0.144	*	0.001		0.038		0.087	*
Average	**0.126**		**0.114**		**0.077**		**0.227**	
Seed characters								
Seed length	0.152	*	0.222	*	0.059	*	0.191	*
Seed width	0.242	*	0.193	*	0.101	*	0.091	*
Pappus length	0.000		0.177		0.149	*	0.120	*
Average	**0.131**		**0.197**		**0.103**		**0.134**	
Phenology characters								
First flowering day	0.027	*	0.104	*	0.000		0.028	
First seeding day	0.011	*	0.069	*	0.033	*	0.080	
Length of flowering period	0.000		0.046		0.000		0.056	
Average	**0.013**		**0.073**		**0.011**		0.055	
Number characters with significant values	14		10		7		14	

All analyses were performed in SPSS 16.0 (SPSS Inc., Chicago, IL).

Results

P-values are given in tables and figures.

Differences between populations
The plants from the Estonian populations were taller than the rest and the plants from one of the Danish populations (Hamborg) were the shortest (Fig. 1).

Two of the Estonian populations, Allika and Pakri neem, flowered significantly earlier than the rest. The Estonian and the Swedish populations all flowered earlier than the Danish and the British populations, but the flowering was to a large extent overlapping between all of the populations (Fig. 2, Table 2).

Generally, the flowering period seemed correlated with area of origin, as plants originating from geographically close

Table 4. Q_{ST}-values. Significance values: * for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$ reflect significance-values of corresponding ANOVAs. Average values bold when at least one individual character within the character category is significant.

		Denmark		Britain		Estonia		Sweden		5 North. pop.	
					Qst-values						
Height characters											
Height at flowering		0.354	**	0		0		0		0.039	**
Height after flowering		0.435	*	0.006		0.011		0		0.027	**
Length of first head stalk		0.378	*	0		0.009		0.031	*	0.100	***
Length of corymb		0.141	*	0		0		0.006		0.038	**
	Average	**0.327**		**0.002**		0.005		**0.009**		**0.051**	
Quantity characters											
Total number of heads		0.081	*	0.047	*	0.02		0		0.00075	
Tot undeveloped heads		0.121	*	0		0.034		0		0	
Number of ray florets		0.202	*	0.026		0.017		0.027	*	0.087	***
Number of disc florets		0.092	*	0		0		0.009		0.073	***
	Average	**0.124**		**0.018**		0.018		**0.009**		**0.040**	
Flower characters											
Ray floret width		0.08		0		0.083		0		0	
Ligule length		0.139	*	0		0.068	*	0		0	
Corolla length		0.056	*	0.129	*	0		0		0.029	
Style length		0.029		0.089	*	0		0		0.037	*
	Average	**0.076**		**0.054**		**0.038**		0		**0.0165**	
Seed characters											
Seed length		0.038		0.141	*	0.1	*	0.015		0.013	
Seed width		0.019		0.052	*	0		0		0.00011	
Pappus length		0.101	*	0.158	*	0.005	*	0.072	*	0.077	
	Average	**0.053**		**0.117**		**0.035**		**0.029**		0.030	
Phenology characters											
First flowering day		0.06		0.117	*	0.106		0		0.073	***
First seeding day		0.123		0.109	*	0.06		0		0.128	***
Length of flowering period		0.006		0.009		0.012		0		0.0014	
	Average	0.063		**0.078**		0.06		0		**0.0675**	
Number of characters with significant values		**11**		**8**		**3**		**3**		**9**	

populations also flowered at the same time. The plants from the Danish populations Hamborg and Sårup, which were morphologically very different, probably due to one or a few genes causing the Hamborg plants to be very small, were much closer in flowering period than either of them were with the plants from Skørping. All three Swedish populations, which in the field grow within eleven kilometres from each other, seemed synnchronized in their flowering (Fig. 2, Table 2).

Heritability
Of the 18 characters investigated for heritability, 16 showed significant values in at least one of the four regions. Heritability was significant for 14 characters in the Danish and Swedish regions, for 10 characters in England and for 7 characters in Estonia (Table 3).

For most morphological characters, values of heritability were the largest for the Swedish populations and the smallest for the Estonian populations (Fig. 3). For flowering time, only four populations (Sårup, Cissbury Ring, Martin Down, and Tosteberga), showed any significant heritability (figures not shown).

Differences in Q_{ST} between regions
Q_{ST} values (Table 4 and Fig. 4) were higher for the Danish populations than
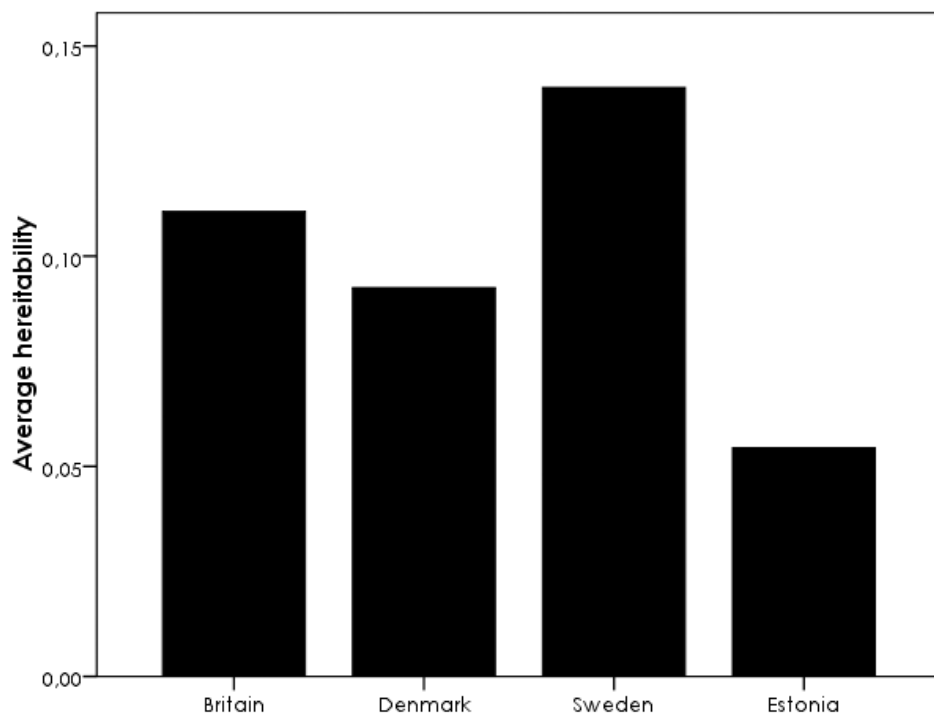
Fig. 3. Average heritability for all measured characters (calculated from the means of the different categories given in Table 2).

for the rest – particularly for characters related to height and characters which might be affected by height. When Hamborg was removed from the analysis, and the Swedish and Danish populations were analysed together, then Q_{ST} for the Nordic region was about the same as for the other regions. Thus, it is likely that the Swedish and the Danish populations are relatively closely related, although they are separated by several straits of water. For Estonia, though the Q_{ST} values were generally high compared with the other regions, only three values were significant.

Discussion
Our study demonstrates higher heritability values and significant heritability for more traits in the Swedish populations than in the Estonian populations – an unexpected result, since the Swedish populations have been small and threatened for a long time (Isaksson & Widén paper I), while the Estonian populations are still large and seem to be thriving (Isaksson pers. obs.). However, our results are consistent with an earlier study of *T. integrifolia*; Widén & Andersson (1993) found – in a paternal half-sib analysis – a small population to have both higher heritabilities and significant additive genetic variance for a larger number of traits than a large population (two Swedish populations not used in the present study). They investigated 35 characters related to morphology and life-history, and 19 of them displayed significant heritability in the small population and 14 in the large population. The magnitude of the obtained heritability values were comparable to values estimated for common and wide-spread plants, suggesting that the two investigated populations of *T. integrifolia* had not suffered from genetic erosion (Widén & Andersson 1993).

The estimated population sizes in the
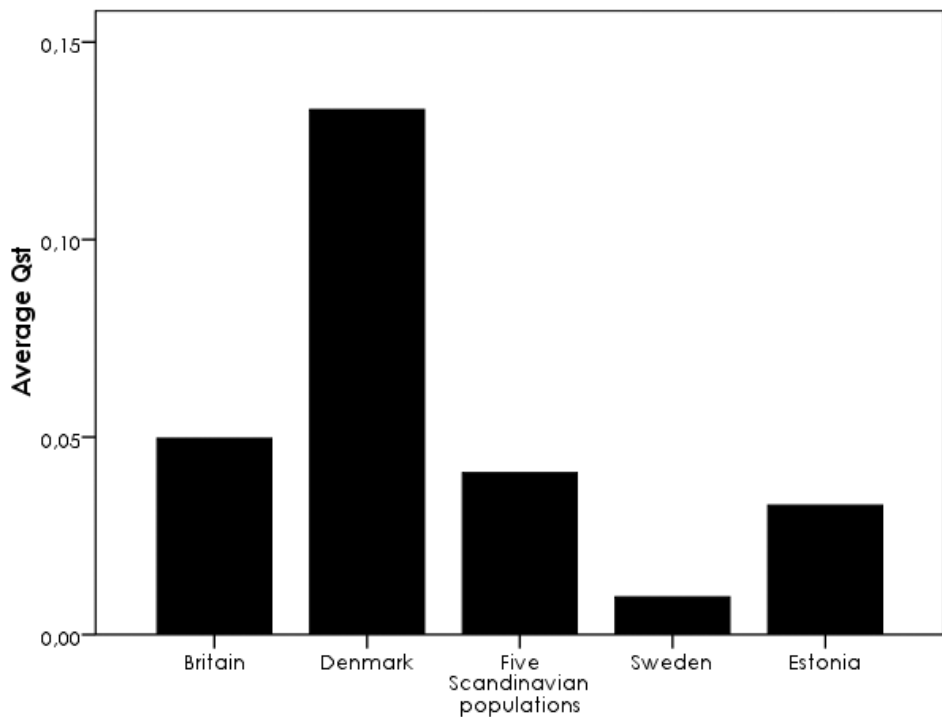
Fig. 4. Average Q_{ST} for all measured characters (calculated from the means of the different categories given in Table 2). The region 5 Nordic populations comprise the Swedish and Danish populations except the morphologically different Hamborg population.

present paper are based on the number of flowering stems during the sampling year. However, individual plants rarely flower every year (Widén 1987) and until a few years ago, the number of flowering stems were much higher in the Swedish populations than they are now (Isaksson & Widén paper I). Consequently, the number of flowering stems during a particular year is not an exact measure of population size, but would give a rough indication.

Unexpectedly high genetic variation in small populations, albeit lower than in large populations is often associated with recent decreases in population size – drift has not yet taken its toll on genetic variance – the population is then said to be a relict (e.g. Young, Brown & Zich 1999, Luijten et al. 2000). The time from habitat destruction to population extinction varies between species. Another explanation of unexpectedly high

genetic variation is a persistent seed-bank (Honnay et al. 2008). If habitat destruction hampers regeneration, while adult individuals survive, the time from habitat destruction to population extinction depends on how long-lived individuals are – if they can grow very old we might be given the false impression that the population is still viable, while it is in fact a relict population condemned to extinction, unless the habitat is restored. Obligately outcrossing populations usually maintain genetic variance for longer – unexpectedly high genetic variation is often associated with self-incompatibility (Colas et al. 1997, Young, Brown & Zich 1999, Luijten et al. 2007, Campbell et al. 2007, Honnay et al. 2007, Mathiasen et al. 2007) as well as longevity (Soejima et al. 1998, Dzialuk et al. 2009, De Almeida Vieira & De Carvalho 2008).

A short-term effect of a decrease in population size could even lead to an in-

crease in heritability as a result of increased homozygosity in previously rare recessive alleles (Willis & Orr 1993), something which, according to a meta-study by Van Buskirk & Willi (2006) has been confirmed in several empirical studies. We have not found any studies where genetic variation in molecular markers has been reported to be higher in small populations than in large populations.

Our results indicate that the small Swedish *T. integrifolia* populations are going through a bottleneck, but that they have not been small for many generations. According to papers I-III in this thesis, Swedish *T. integrifolia* populations show no obvious signs of loss of genetic variation – something which is expected several generations after fragmentation – however, the populations have decreased in size. Unlike the other three studies in this thesis, this study indicates population decrease has had an effect on genetic variation in Swedish *T. integrifolia* populations, although considering how quickly the Swedish populations have decreased in size during the 20th century, it is likely that they will disappear before these changes will have any detrimental effect on the plants.

Acknowledgements

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143. Lotten Jönsson Johansson (2008) Semi-natural grasslands: landscape, history and plant species diversity. (Joint PhD project with the Department of Physical Geography and Ecosystems Analysis.)

144. Johanna Eneström (2008) Life-history traits and population differentiation in a clonal plant: implications for establishment, persistence and weediness.

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146. Maarten Ellmer (2009) Quantitative genetic variation in declining plant populations.

147. Kerstin Isaksson (2009) Investigating genetic factors behind the decline of a threatened plant species – *Tephroseris integrifolia* (Asteraceae)