Limited mate availability decreases reproductive success of fragmented populations of *Linnaea borealis*, a rare, clonal self-incompatible plant

A. R. Scobie and C. C. Wilcock*

*School of Biological Sciences, University of Aberdeen, Cruickshank Building, St Machar Drive, Aberdeen AB24 3UU, Scotland, UK*

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- **Background and Aims** Small populations of rare plant species are increasingly reported to have high levels of reproductive failure. The objective of this study was to understand the principal constraints on sexual reproduction in small fragmented populations of a rare clonal self-incompatible plant.

- **Methods** The pollinator spectrum, diversity of flower colour, natural pollination and fruit-set levels of *L. borealis* were examined in Scotland. Artificially crossed seed production was compared within and between different flower colour types and patches.

- **Key Results** *Linnaea borealis* was pollinated by a diverse spectrum of insect species and the principal pollinators were muscid, syrphid and empid flies which mostly moved only small distances (<0.25 m) between flowers when foraging. Natural pollination levels were high, indicating high pollinator effectiveness, but fruit set was very low in most patches. Flower colour diversity was low in most patches and only those with a diversity of flower colour types had high fruiting success. Pollination experiments showed *L. borealis* to be highly self-incompatible and artificial crosses within and between patches and flower colour types confirmed that low fruit success was the result of a lack of compatible mates and limited pollen movement between them. Evidence of isolation from pollen exchange was apparent at as little as 6 m and severe at 30 m and beyond.

- **Conclusions** Limited mate availability and isolation from pollen exchange compromise the reproductive success of fragmented populations of *L. borealis* in Scotland. A diversity of compatible mates situated within close proximity (<6 m) is the key requirement to ensure high natural fruiting success. This study emphasizes that an understanding of the breeding system, pollinator spectrum and potential for interconnectivity via pollinator movement are fundamental to identify isolation distances and to establish when conservation intervention is necessary for rare species.

**Key words:** *Linnaea borealis*, clonal, self-incompatible, reproductive failure, fragmented populations, isolation, pollination.

**INTRODUCTION**

There are increasing reports of sexual reproductive failure in small populations of rare plant species (Les et al., 1991; Demauro, 1993; Kéry et al., 2000; Luijten et al., 2000; Warburton et al., 2000; Wolf and Harrison, 2001; Fischer et al., 2003; Aigner, 2004). Due to habitat loss and fragmentation many plants have become confined to small, highly isolated populations with an increased risk of extinction (Saunders et al., 1991; Schemske et al., 1994). One of the major consequences of fragmentation is loss of genetic diversity as a result of random genetic drift, increased inbreeding, and reduced gene flow between populations (Barrett and Kohn, 1991; van Treuren et al., 1991; Ellstrand and Elam, 1993; Young et al., 1996; Young and Brown, 1999) and consequently loss of offspring and population fitness (Oostermeijer et al., 1995; Fischer and Matthies, 1998; Luijten et al., 2000; Fischer et al., 2003; Willi et al., 2005) and ability to adapt to environmental change (Willi et al., 2006). Gene flow can counter the effects of genetic drift (Ellstrand and Elam, 1993) but the likelihood of pollen exchange and migration by seed is reduced with increasing distances between populations (Schaal and Leverich, 1996). Opportunities for sexual recruitment within populations and seed migration between them are further reduced by increased risk of both pollen and pollination limitation (Wilcock and Neiland, 2002).

Species with self-incompatible breeding systems are particularly vulnerable to the effects of isolation on reproductive success. For successful fertilization, self-incompatible plants require cross-pollination with a compatible mate, a plant that differs by at least one allele at the S-locus (de Nettancourt, 1977). The erosion of S-allele diversity in small populations leads to a shortage of compatible mates and a reduction in seed set (Byers and Meagher, 1992; Byers, 1995; Vekemans et al., 1998). In extreme cases, where all remaining plants have the same S-alleles, seed set may be reduced to zero (Demauro, 1993). In clonal self-incompatible plants extensive vegetative spread increases the risks of geitonogamy and the deposition of incompatible pollen on stigmas (Charpentiern, 2002; Araki et al., 2007). In isolated monoclonal patches geitonogamy is inevitable and will result in total reproductive failure (Wilcock and Jennings, 1999).

In populations with low mate availability, pollen exchange between neighbouring populations can provide compatible mates and maintain seed set (Eriksson and Bremer, 1993).
The extent of connectivity via pollen flow between neighbouring populations varies within and between species and with years, population size, distance and type of vegetation between populations, pollinator spectrum, and the foraging range of the pollinators (Powell and Powell, 1987; Ellstrand, 1992; Westerbergh and Saura, 1994; Kwak et al., 1998; Somanathan et al., 2004). However, long-distance dispersal events do occur (Ellstrand and Marshall, 1985; Goodell et al., 1997; Young and Brown, 1999) but the frequency of these events and their contribution to total seed production per generation is unclear.

For the successful conservation of rare plant species it is essential to understand the potential for small, isolated populations to recover and spread following restoration of suitable habitat. Identifying the threshold distance above which populations of self-incompatible plants with limited mate availability are too isolated for pollen exchange to maintain levels of seed set sufficient for recruitment and migration is a critical step in determining whether intervention is necessary. This study examines the impact of isolation on reproductive success in the rare, clonal dwarf shrub *Linnaea borealis* (twinflower), a UK Biodiversity Action Plan and Scottish Biodiversity List species.

The principal aim of this study was to experimentally investigate the constraints on sexual reproduction in *L. borealis*, and specifically to (a) identify the main pollinators of *L. borealis* and their foraging behaviour, (b) determine levels of pollination in natural populations, (c) compare natural and artificially crossed seed production within and between populations, and (d) assess the affect of isolation on fruiting success.

**MATERIALS AND METHODS**

**Study species**

*Linnaea borealis* L. has a circumpolar distribution and is associated with the forests of the northern boreal zone. Seed set failure in the species has been reported from North America, Britain and Scandinavia (Wilcock and Jennings, 1999; Wilcock, 2002). In Britain the species has undergone a considerable decline and is now almost entirely confined to the north-east of Scotland. The loss and fragmentation of native Scots pine (*Pinus sylvestris*) woodland, unrestricted grazing of the pinewood habitat by deer and other livestock, mechanical timber harvesting and ground preparation techniques, and the shading out of patches by the encouragement of dense tree regeneration are the likely causes of decline (UK Biodiversity Group, 1999). The remaining populations are typically highly isolated, often with several kilometres between them. *Linnaea borealis* is capable of extensive vegetative spread by stolons and can form large clonal patches which may be hundreds of years old (Antos and Zobel, 1984). Flowering occurs from early June to late July when a profusion of flowers may be produced in good light conditions. In heavy shade, flowering is often poor or completely absent. Each inflorescence consists of a pair (occasionally three or four) of small white/pink campanulate, sweetly scented flowers. Following successful fertilization, the ovary ripens to produce a single seeded fruit with two partially enclosing bracts with viscid hairs adapted for animal dispersal (Lusby, 1994). The species is highly self-incompatible with a gametophytic self-incompatibility system (Wilcock and Jennings, 1999). A genetic survey of 33 Scottish *L. borealis* populations by Kohn and Ennos (2000) revealed low within-population genetic diversity, with 97% of patches containing no more than four genotypes and 37% of patches consisting of just a single clone.

**Study sites**

Patches were studied in three different forest areas within the Cairngorms National Park in the north-east of Scotland. Although this area is the UK stronghold for *L. borealis*, in addition to conservation and access constraints, many patches flower poorly, further limiting the number available for the study of pollination and fruit set. Twelve patches were selected for study on the basis of accessibility and regular annual flowering. The study sites were as follows:

**Abernethy Forest (Speyside).** A semi-natural Scots pine woodland, one of the largest remnants of Caledonian pine forest in Scotland. Abernethy forest has >30 *L. borealis* patches, many of which are under Scots pine stands with trees that are 150–200+ years old. Patches are separated by distances ranging from 0.25 to 3 km. Five patches were used in this study, referred to as Abernethy patches 1–5.

**Balmoral Estate (Deeside).** A mixed Scots pine and Douglas fir plantation with Scots pine trees that are 150–200 years old. There are eight *L. borealis* patches in the woodland and distances between them range from 30 m to 1 km. Three patches were used in this study, referred to as Balmoral patches 1–3.

**Curr Wood (Speyside).** A Scots pine plantation situated to the north of Abernethy forest across the river Spey. Curr Wood has >15 *L. borealis* patches with distances between patches ranging from 60 m to 1 km. Four patches were used in this study, referred to as Curr Wood patches 1–4.

**Observation and sampling of insect pollinators**

In June and July of 2007 sampling and observations of insect visitors to the flowers of *L. borealis* were carried out from 1000 h to 1700 h, over a series of visits to patches in Abernethy, Balmoral and Curr Wood (3 d at each forest site in total). Insects were trapped while they visited a flower, swiftly killed using ethyl acetate and stored in small vials for identification. All insects were identified to family level following Chinery (1993). Hoverflies were identified to species level following Stubbs and Falk (2002) and bumblebees following Edwards and Jenner (2005). Muscidae, Anthomyiidae and Empididae flies were sent to Del Smith, a dipterist, for identification to species level. Insects were examined for the presence of *L. borealis* pollen grains using a high-power dissecting microscope and any pollen grains counted and their position on the insect noted. *Linnaea borealis* pollen is large (42 μm × 34 μm), triporate with a distinctive exine, and is readily distinguishable from other pinewood species which may be present on the pollinators. The abundance of each insect species was subjectively divided into...
three categories based on the approximate number of flower visits per hour to the \(3 \times 3 \text{ m}\) observation area: high (>100 visits h\(^{-1}\)), medium (10–100 visits h\(^{-1}\)) and low (<10 visits h\(^{-1}\)). Insect species ‘not recorded’ from some sites but present in others during the observation period does not necessarily mean that they were absent from the site. The distance that insects moved between flowers at Abernethy and Curr Wood patches was studied over 2 d in early July 2008 from 1000 h to 1700 h. Insects were followed whilst foraging in the patch, a small stick was inserted beside each flower visited and the distance between the sticks measured sequentially at the end of each foraging bout. At least 50 movements were recorded for each of the main insect taxa.

**Natural pollination levels**

To investigate natural levels of pollination, in 2007 a sample of 20 tagged *L. borealis* flowers (one per twin inflorescence), open for 7 d during peak flowering, was taken from patches in Abernethy, Balmoral and Curr Wood (12 patches in total) and preserved in FAA (formalin–acetic acid–alcohol). Stigmas were rinsed in water, stained with lactophenol cotton blue and mounted on a slide. The total number of *L. borealis* pollen grains on each stigma was recorded.

**Natural fruit-set levels**

Natural fruit-set levels were recorded in ten patches from Abernethy, Balmoral and Curr Wood in 2006 and again in 2007 with an additional two patches in Curr Wood. Depending on the size of the patch, between five and ten 1-m\(^2\) quadrats were placed randomly in areas of flowering, and each flower on every inflorescence was examined for the presence of a fruit or failed ovary.

**Hand-pollination experiments**

Pollination experiments were carried out to investigate the effects of controlled intra-patch and inter-patch cross-pollination on fruiting success of *L. borealis* in Abernethy, Balmoral and Curr Wood in 2007. Two replicate patches were treated for each cross. Intra-patch crossing – stigmas of flowers on one side of a patch were pollinated with pollen from flowers furthest away on the other side of the patch; (b) inter-patch crossing – stigmas were pollinated with pollen from a different flowering patch within the same forest area that was >50 m away; (c) tagged open pollination – inflorescences were tagged and fruit set following natural pollination was recorded. A self-pollination treatment was carried out in a single patch in each of the three forest areas to confirm the breeding system – stigmas in bagged flowers were pollinated with pollen from anthers of the same flower. Five weeks after all the pollinations had been carried out, bags were removed and each flower was examined for the presence of a fruit or failed ovary.

**Flower colour diversity and intra- and inter-flower colour type crosses**

The diversity of flower colour types was examined in a selection of *L. borealis* patches at Abernethy, Balmoral and Curr Wood in 2007. A transect was taken through each patch and a flower (when present) was sampled and photographed every 1-5 m. As this involved destructive sampling for photography it was not possible to survey all patches, and those with low flowering densities were excluded. Photographs were visually examined to assess flower colour variation and provide an estimate of clonal diversity within and between patches.

Controlled hand pollinations were carried out between flowers with the same flower colour type and between flowers with different flower types following the same procedure as for the hand-pollination experiments. Each of the intra- and inter-flower colour type crosses were carried out at four different patches with 16 inflorescences (32 flowers) treated for each cross.

**Detailed mapping of a diverse patch at Balmoral**

Due to the high fruitering success reported by Wilcock and Jennings (1999) of an *L. borealis* patch at Balmoral, and similar observations made by this study in 2006, it was decided to investigate Balmoral patch 2 in detail in 2007. Flower colour diversity was examined (as above) and the area covered by each flower colour type and the distance between distinct flower colour types were measured and mapped. Fruit-set levels were recorded in ten 1-m\(^2\) quadrats positioned throughout the patch and the distance from each quadrant to the nearest different flower colour type was measured.

**Data analysis**

**Natural pollination levels.** To investigate whether stigmatic pollen loads varied between the 12 patches examined in 2007, a linear mixed model was used with patch as a fixed effect and forest area as a random effect. Pollen count data were square root transformed prior to analysis. A linear mixed model was also used to test whether there was any effect of patch flowering density on stigmatic pollen load. In the model, flowering density (as a covariate) was a fixed effect and patch nested within forest area was a random effect. A generalized linear mixed model (GLMM) with a binomial distribution and logit-link function was used to investigate whether the probability of a flower being pollinated varied between patches. Patch was a fixed effect in the model and forest area a random effect.

**Natural fruit-set levels.** A GLMM (as above) was used to investigate whether the probability of a flower producing a fruit
varied between patches and years. Due to the large variation in flowering density, for this analysis a random sub-sample of 50 inflorescences (100 flowers) was taken from each patch where density was high and all inflorescences were sampled from patches where there were <50. Both flowers on the twin inflorescence were included as individual observations. Patch, year and their interaction were fixed effects in the model, forest area, and inflorescence nested within patch by year were random effects. To test whether there was any effect of flowering density of the patch on the probability of a flower setting fruit in 2007, a GLMM (as above) was used with flowering density (covariate) as a fixed effect and inflorescence nested within patch as random effects. To test for differences in the probability of a flower producing a fruit following intra- and inter-flower colour type crosses, a GLMM (as above) was used with treatment as a fixed effect, and patch and inflorescence nested within patch as random effects. For all models, variance components were estimated using restricted maximum likelihood (REML) and tests for fixed effects were carried out using F-tests (balanced data) and Wald $\chi^2$-tests (unbalanced data).

Fruit set and distance to the nearest different flower colour type at Balmoral was investigated using Pearson's correlation. Fruit set data from the ten 1-m² quadrats was arcsine transformed for correlation. All statistical analyses were performed using GenStat 11.

RESULTS

Pollinator species and their behaviour at patches of L. borealis

Insect pollinator species visiting the flowers of L. borealis, their abundance at Abernethy, Balmoral and Curr Wood, their pollen load and position of pollen on the insect are shown in Table 1. Twenty insect species from seven families were recorded visiting the flowers, the majority of which belonged to the order Diptera and the others to Hymenoptera. The most abundant pollinators at L. borealis patches were the small muscid flies Trichops cunctans and T. sémicinérus. Three hoverflies, Melanostoma scalare, Platycheirus albimanus and Rhingia campestris, and the empid fly Empis tesselata were also common visitors to flowers at L. borealis patches. Of the three bee species, the small halictid Lasiglossum fratellum was the only abundant visitor. The two bumblebees, Bombus lucorum and B. pratorum, were only very occasional visitors to the patches examined. Most pollen carried by pollinators was of L. borealis. Pollen loads of L. borealis on the majority

<table>
<thead>
<tr>
<th>Pollinator species</th>
<th>Order</th>
<th>Family</th>
<th>Abernethy</th>
<th>Balmeral</th>
<th>Curr Wood</th>
<th>L. borealis pollen load (grains)</th>
<th>Position of pollen on insect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alliopsis silvestris</td>
<td>Diptera</td>
<td>Anthomyiidae</td>
<td>n.r.</td>
<td>n.r.</td>
<td>med.</td>
<td>&lt;20</td>
<td>l, t, a</td>
</tr>
<tr>
<td>Lasiomma latipenne</td>
<td>Diptera</td>
<td>Anthomyiidae</td>
<td>n.r.</td>
<td>n.r.</td>
<td>low</td>
<td>&lt;20</td>
<td>h, l, t</td>
</tr>
<tr>
<td>Empis grisea</td>
<td>Diptera</td>
<td>Empididae</td>
<td>n.r.</td>
<td>med.</td>
<td>n.r.</td>
<td>&lt;20</td>
<td>h, l, t, a</td>
</tr>
<tr>
<td>Empis lucidus</td>
<td>Diptera</td>
<td>Empididae</td>
<td>low</td>
<td>n.r.</td>
<td>low</td>
<td>&lt;20</td>
<td>l, t</td>
</tr>
<tr>
<td>Empis tesselata</td>
<td>Diptera</td>
<td>Empididae</td>
<td>med.</td>
<td>med.</td>
<td>med.</td>
<td>20–100</td>
<td>h, l, t, a</td>
</tr>
<tr>
<td>Phaonia angelicae</td>
<td>Diptera</td>
<td>Muscidae</td>
<td>low</td>
<td>n.r.</td>
<td>n.r.</td>
<td>&lt;20</td>
<td>h, l, t</td>
</tr>
<tr>
<td>Thricops cunctans</td>
<td>Diptera</td>
<td>Muscidae</td>
<td>high</td>
<td>high</td>
<td>high</td>
<td>&lt;20</td>
<td>h, l, t, a</td>
</tr>
<tr>
<td>Thricops semicincterus</td>
<td>Diptera</td>
<td>Muscidae</td>
<td>high</td>
<td>n.r.</td>
<td>high</td>
<td>&lt;20</td>
<td>h, l, t, a</td>
</tr>
<tr>
<td>Scathophaga sternoraria</td>
<td>Diptera</td>
<td>Scathophagidae</td>
<td>low</td>
<td>n.r.</td>
<td>low</td>
<td>&lt;20</td>
<td>l, t</td>
</tr>
<tr>
<td>Chrysotoxum arcuatum</td>
<td>Diptera</td>
<td>Syrphidae</td>
<td>n.r.</td>
<td>n.r.</td>
<td>low</td>
<td>&lt;20</td>
<td>l, t, a</td>
</tr>
<tr>
<td>Melanostoma scalare</td>
<td>Diptera</td>
<td>Syrphidae</td>
<td>med.</td>
<td>med.</td>
<td>med.</td>
<td>&lt;20</td>
<td>h, l, t</td>
</tr>
<tr>
<td>Platycheirus albimanus</td>
<td>Diptera</td>
<td>Syrphidae</td>
<td>med.</td>
<td>med.</td>
<td>med.</td>
<td>&lt;20</td>
<td>h, l, t</td>
</tr>
<tr>
<td>Platycheirus nielseni</td>
<td>Diptera</td>
<td>Syrphidae</td>
<td>n.r.</td>
<td>low</td>
<td>n.r.</td>
<td>&lt;20</td>
<td>h, t</td>
</tr>
<tr>
<td>Rhingia campestris</td>
<td>Diptera</td>
<td>Syrphidae</td>
<td>med.</td>
<td>n.r.</td>
<td>med.</td>
<td>&gt;100</td>
<td>h, l, t</td>
</tr>
<tr>
<td>Sericomyia silentis</td>
<td>Diptera</td>
<td>Syrphidae</td>
<td>low</td>
<td>n.r.</td>
<td>n.r.</td>
<td>20–100</td>
<td>h, l, t, a</td>
</tr>
<tr>
<td>Sphegina clunipes</td>
<td>Diptera</td>
<td>Syrphidae</td>
<td>n.r.</td>
<td>low</td>
<td>n.r.</td>
<td>&lt;20</td>
<td>t</td>
</tr>
<tr>
<td>Volucella pellucens</td>
<td>Diptera</td>
<td>Syrphidae</td>
<td>n.r.</td>
<td>low</td>
<td>n.r.</td>
<td>&lt;20</td>
<td>l, t</td>
</tr>
<tr>
<td>Bombus lucorum</td>
<td>Hymenoptera</td>
<td>Apidae</td>
<td>low</td>
<td>low</td>
<td>low</td>
<td>&gt;100</td>
<td>h, l, t, a</td>
</tr>
<tr>
<td>Bombus pratorum</td>
<td>Hymenoptera</td>
<td>Apidae</td>
<td>low</td>
<td>n.r.</td>
<td>low</td>
<td>&gt;100</td>
<td>h, l, t, a</td>
</tr>
<tr>
<td>Lasiglossum fratellum</td>
<td>Hymenoptera</td>
<td>Halictidae</td>
<td>n.r.</td>
<td>high</td>
<td>n.r.</td>
<td>&gt;100</td>
<td>h, l, t, a</td>
</tr>
</tbody>
</table>

* Pollinator abundance: high, medium (med.), low or not recorded (n.r.).
† Position of pollen on insect: head, h; legs, l; thorax, t; abdomen, a.
of the insect species sampled were low (<20 grains), but *E. tesselata* and the hoverfly *Sericomyia silentis* carried intermediate pollen loads (20–100 grains). The hoverfly *R. campestris* and the three bee species carried the highest pollen loads (>100 grains).

Insect activity was highest at patches of *L. borealis* during warm, sunny intervals but was limited when weather conditions were cool, windy and/or overcast. Only muscid flies remained active in poor weather conditions. Muscid flies, hoverflies, empid flies and halictid bees were observed moving from flower to flower, often spending a long time in each flower, and remained within the patches for long periods. However, the bumblebees *B. lucorum* and *B. pratorum* arrived at the patches, quickly visited a succession of flowers, and then moved on from the patch, often flying a considerable distance (>50 m) away. Pollinator movements between flowers (studied in 2008) followed a leptokurtic distribution (Fig. 1A). For all species combined, 72.5 % of the 501 between-flower movements were <0·25 m, 94·4 % were <1 m, and 98·6 % were <2 m (Fig. 1B). Although present in 2007, no empid flies were recorded at either site on the two study days in 2008.

**Natural pollination levels**

Mean *L. borealis* pollen loads varied from five to 44 grains per stigma (Table 2). The highest load (88 grains) was recorded on a stigma sampled from Curr Wood 1. There were significant differences in stigmatic pollen loads between patches (Table 2). As *L. borealis* produces a single seed per flower, only one viable compatible pollen grain per stigma is required for seed set and the percentage of stigmas with one or more *L. borealis* pollen grain was very high, ranging from 75 to 100 % between patches, with flowers in the majority of patches having 100 % of their stigmas bearing at least one pollen grain (Table 2). There were no significant differences between patches in levels of pollinated stigmas (Table 2). Levels were consistently high, with 75 to 100 % between patches, with flowers in the majority of patches having 100 % of their stigmas bearing at least one pollen grain (Table 2).

**Natural fruit-set levels**

Fruit-set levels were very low (<8·5 % in eight out of the ten patches examined in 2006) and, again, in 2007 (<6 % in ten out of the 12 patches examined; Table 3). Fruit set was higher in Balmoral patches 2 and 3 than all other patches examined in 2006 and 2007, with fruit-set levels of 38·6 % and 33·8 % in 2006 and 35·1 % and 25·7 % in 2007, respectively. Fruit set varied significantly between patches (χ² = 50·89, d.f. = 11, *P* < 0·001) but not between years (χ² = 0·84, d.f. = 1, *P* = 0·359), and there was no significant patch × year interaction (χ² = 2·37, d.f. = 9, *P* = 0·984). Levels were consistently high, with 75 to 100 % between patches, with flowers in the majority of patches having 100 % of their stigmas bearing at least one pollen grain (Table 2).

**Table 2.** The presence of *Linnaea borealis* pollen on open-pollinated stigmas sampled from 12 patches at three different forest sites

<table>
<thead>
<tr>
<th>Forest area</th>
<th>Patch</th>
<th>No. of stigmas examined</th>
<th>Mean <em>L. borealis</em> pollen load per stigma [range]</th>
<th>% of stigmas with <em>L. borealis</em> pollen ± 95 % CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abernethy</td>
<td>1</td>
<td>20</td>
<td>23 [3–45]</td>
<td>100 ± 0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>17</td>
<td>7 [0–18]</td>
<td>94 ± 11</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>20</td>
<td>12 [0–30]</td>
<td>90 ± 13</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>12</td>
<td>7 [0–23]</td>
<td>75 ± 25</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>20</td>
<td>16 [5–35]</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>Balmoral</td>
<td>1</td>
<td>20</td>
<td>14 [1–34]</td>
<td>100 ± 0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>20</td>
<td>25 [6–55]</td>
<td>100 ± 0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>20</td>
<td>15 [1–38]</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>Curr Wood</td>
<td>1</td>
<td>20</td>
<td>44 [4–88]</td>
<td>100 ± 0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>20</td>
<td>26 [5–55]</td>
<td>100 ± 0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>20</td>
<td>31 [8–55]</td>
<td>100 ± 0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>20</td>
<td>5 [0–18]</td>
<td>85 ± 16</td>
</tr>
</tbody>
</table>

95 % CI = 95 % confidence interval.

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**Fig. 1.** Foraging behaviour of Muscidae, Syrphidae, Halictidae and Apidae pollinators in patches of *Linnaea borealis* at Abernethy and Curr Wood: (A) separately for each taxon; (B) combined data. Number of movements recorded: Muscidae = 237; Syrphidae = 123; Halictidae (*Lasioglossum fratellum*) = 89; Apidae (*Bombus* spp.) = 52; total = 501.
low at Abernethy 1–5, Balmoral 1 and Curr Wood 2 and 3, and consistently high at Balmoral 2 and 3 (Table 3).

**Impact of flowering density on natural pollination and fruit set**

The relationships between patch flowering density and mean pollen load per stigma and fruit set are shown in Fig. 2. There was a highly significant effect of flowering density of the patch on stigmatic pollen load ($F = 27.99$, d.f. = 1, $P < 0.001$) but no effect of flowering density of the patch on fruit set ($F = 0.07$, d.f. = 1, $P = 0.791$).

**Experimental selfing, and intra- and inter-patch cross-pollination**

Fruit set following self-pollination was very low, 0–3.3% (Table 4), and did not differ significantly between forest areas ($\chi^2 = 0.01$, d.f. = 2, $P = 0.99$). Levels of fruit set following open pollination and intra-patch crossing at Abernethy and Curr Wood were also very low (3.1–6.5%) but inter-patch crossing produced much higher fruit-set levels (46.2% and 38.3%, respectively; Fig. 3). At Balmoral fruit set following open pollination and intra- and inter-patch crossing and were all very high (46.8, 66.1 and 78.3%, respectively; Fig. 3). Fruit set was significantly affected by experimental pollination treatment ($\chi^2 = 13.28$, d.f. = 2, $P < 0.01$) and forest area ($\chi^2 = 30.48$, d.f. = 2, $P < 0.001$) and there was a significant treatment × forest area interaction ($\chi^2 = 24.61$, d.f. = 4, $P < 0.001$).

**Flower colour diversity within and between patches and its impact on fruiting success**

Figure 4A–C shows the diversity of flower colour obtained from flowers sampled every 1.5-m in transects through *L. borealis* patches 1–3 at Curr Wood. There was very little variation within any of the three patches and flowers showed striking uniformity from one side of the patch to the other. A similar situation was observed for Abernethy patches 1

### Table 3. Natural fruit-set levels in 12 patches of Linnaea borealis in 2006 and 2007 at three different forest sites

<table>
<thead>
<tr>
<th>Forest area</th>
<th>Patch</th>
<th>No. of flowers sampled</th>
<th>No. of fruits recorded</th>
<th>% fruit set ± 95% CI</th>
<th>Flower colour diversity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abernethy</td>
<td>1</td>
<td>944</td>
<td>76</td>
<td>8.1 ± 1.7</td>
<td>Uniform</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>83</td>
<td>1</td>
<td>1.2 ± 2.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>132</td>
<td>1</td>
<td>0.8 ± 1.5</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>35</td>
<td>0</td>
<td>0.0 ± 0.0</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>248</td>
<td>8</td>
<td>3.2 ± 2.2</td>
<td>n.d.</td>
</tr>
<tr>
<td>Balmoral</td>
<td>1</td>
<td>545</td>
<td>38</td>
<td>6.9 ± 2.1</td>
<td>Uniform</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>241</td>
<td>93</td>
<td>38.6 ± 6.1</td>
<td>Uniform</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>326</td>
<td>6</td>
<td>1.8 ± 1.5</td>
<td>Uniform</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>587</td>
<td>18</td>
<td>3.1 ± 1.4</td>
<td>Uniform</td>
</tr>
</tbody>
</table>

n.d. = no data, for fruit set patch was not examined in 2006, for flower colour diversity flowering density was too low for transects to be taken.

95% CI = 95% confidence interval.

FIG. 2. Effect of patch flowering density on levels of pollination and fruit set of *Linnaea borealis*: (A) pollen load per stigma and flowering density of the patch (trend line: linear); (B) fruit set and flowering density of the patch. Infl./m² = inflorescences m⁻².
The findings of this study have clearly shown that reproductive failure is widespread in Scottish populations of *Linnaea borealis*. Failure is not the result of a lack of insect visitation, or pollination, but limited mate availability within patches and isolation from pollen exchange between patches. Limited mate availability has been increasingly identified as the cause of reproductive failure in small populations of clonal, self-incompatible plants, e.g. *Hymenoxys acaulis* var. *glabra* (Demauro, 1993), *Rubus saxatilis* (Eriksson and Bremer, 1993), *Aster furcatus* (Les et al., 1991), *Scirpus maritimus* (Charpentier et al., 2000), *Calystegia collina* (Wolf and Harrison, 2001), *Arnica montana* (Luijten et al., 2002), *Dithyrea maritima* (Aigner, 2004), *Ramunculus reptans* (Willi et al., 2005), *Maianthemum bifolium* (Honray et al., 2006), and it is likely to be more widespread than is currently recognized.

Due to the limited pollen-dispersal capabilities of flies, which dominate the pollinator spectrum of *L. borealis*, even small distances provide a barrier to pollen exchange between mates. A degree of isolation from pollen exchange was evident when flower colour types were separated by distances as small as 6 m and increased rapidly beyond this distance. As artificial cross-pollination between isolated patches can restore their reproductive success, a diversity of compatible mates situated within close proximity is therefore the key requirement to ensure high natural fruiting success of *L. borealis*.

**Pollinator species diversity and pollen movement**

This is the first time that a detailed examination has been made of the pollinator spectrum of *L. borealis* in Scotland and has revealed that the species has a wide diversity of insect visitors. The species has a generalist pollination strategy and the principal visitors were flies (Diptera). This study demonstrates that there is not a lack of pollinators for *L. borealis* in Scotland. A similarly diverse pollinator spectrum was recorded by Barrett and Helenurm (1987), studying *L. borealis* in central New Brunswick, who listed 21 species of visitors to the flowers, including members of the Syrphidae, Muscidae, Apidae and Halictidae.

Insect pollinators when foraging in patches of *L. borealis* moved very limited distances between flowers. Pollinator movements followed a leptokurtic distribution as previously reported by numerous other studies (e.g. Price and Waser, 1979; Pyke, 1979; Zimmerman, 1979; Schaal, 1980; Schmitt, 1980; Waddington, 1981; Richards, 1997; Nuortila et al., 2002). The high proportion of short distance movements by pollinators between flowers of *L. borealis* shows that pollen dispersal is limited. However, pollen carry-over can result in pollen being transported beyond the first flower visited (Schaal, 1980; Waddington, 1981; Geber, 1985; Robertson, 1992; Morris et al., 1994; Richards, 1997; Escaravage and Wagner, 2004),

### TABLE 4. Test for self-incompatibility in Abernethy, Balmoral and Curr Wood Linnaea borealis patches

<table>
<thead>
<tr>
<th>Forest area</th>
<th>No. of flowers studied</th>
<th>No. of fruits recorded</th>
<th>% fruit set ± 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abernethy</td>
<td>32</td>
<td>1</td>
<td>3±1 ± 6±0</td>
</tr>
<tr>
<td>Balmoral</td>
<td>32</td>
<td>0</td>
<td>00 ± 00</td>
</tr>
<tr>
<td>Curr Wood</td>
<td>30</td>
<td>1</td>
<td>3±3 ± 6±4</td>
</tr>
</tbody>
</table>

95 % CI = 95 % confidence interval.

**DISCUSSION**

**Controlled cross-pollination between *L. borealis* flowers with the same flower colour type within a patch resulted in very low levels of fruit production (3-1–6.3 %), even when crossed flowers were separated by distances of up to 40 m (Table 5).** However, cross-pollination between flowers with different colour types always resulted in higher levels of fruit production (30-0–53.1 %). Fruit set following inter-flower colour type crossing was significantly higher than fruit set following intra-flower colour type crossing ($F = 24.9$, d.f. = 1, $P < 0.001$). In the two patches with a diversity of flower colour types (Balmoral 2 and 3), natural fruit-set levels were higher than all of the uniform patches (Table 3).

**Natural fruiting levels and distance to the nearest different flower colour type**

Detailed mapping of Balmoral patch 2 revealed that it consisted of a number of distinct flower colour types (Fig. 4G) situated closely together (1–12 m apart) with high levels of natural fruiting success (16.3–61.2 %). Within this patch there was a highly significant negative correlation between fruit set and distance to the nearest different flower colour type (Pearson’s correlation, $R = -0.927$, $P < 0.001$; Fig. 5A). Only different colour types situated within close proximity (<6 m) achieved the highest levels of reproductive success (>30 %). Fruit-set levels and distance to the nearest different flower colour type for combined data from Balmoral patches 1 and 2 and Curr Wood patches 1–3 showed a leptokurtic distribution (Fig. 5B). When the distance between colour types reached 30 m, fruit set decreased to <5 %, and remained low, but not reaching 0 %, as the distance between flower colour types increased to 144 m.
and examination of individual pollinator movements alone can underestimate actual pollen dispersal distances (Schaal, 1980; Ellstrand and Marshall, 1985; Ellstrand, 1992). As muscid flies, hoverflies, empid flies and halictid bees foraged in *L. borealis* patches for long periods most pollen dispersal is likely to be within patches and, since these small insects were rarely observed leaving the *L. borealis* patches, pollen dispersal beyond the patch will be limited. Westerbergh and Saura (1994) similarly concluded that the contribution to gene flow by small insect visitors (*Thricops* spp. and syrphid flies) to *Silene dioica* was likely to be limited because they were never observed leaving the patches. Only bumblebees, which have been reported to have extensive foraging ranges (Dramstad, 1996; Osborne, 1999; Walther-Hellwig and Frankl, 2000; Dramstad *et al.*, 2003; Darvill *et al.*, 2004; Kreyer *et al.*, 2004), could potentially connect isolated patches of *L. borealis* through longer distance movements but their visitation rates to patches were very low. The highly fragmented nature of Scottish *L. borealis* populations, the large distances between them (e.g. 250 m to >3 km in Abernethy), and the limited foraging range of the small insect pollinators, pose a serious barrier to between-patch pollen movement.

**Pollination levels and natural fruiting success**

The high stigmatic pollen loads show that pollinators are efficient at dispersing pollen within patches of *L. borealis*. Mean pollen loads were very high in some patches and well in excess of the single compatible pollen grain required to produce a fruit. There was a significant positive relationship between density of flowering and stigmatic pollen load, indicating that pollination levels are higher in denser-flowering patches. Many studies have shown that pollinator abundance increases with density of flowering or plants (Campbell and Motten, 1985; Geber, 1985; Sih and Baltus, 1987; Klinkhamer and de Jong, 1990; Andersson, 1991; Eckhart, 1991; Kunin, 1993; Goulson *et al.*, 1998; Totland and Matthews, 1998). Pollinators may also visit more flowers in dense flowering patches or plants (Eckhart, 1991; Andersson, 1991; Goulson *et al.*, 1998; Totland and Matthews, 1998) but this is not always the case (see Sih and Baltus, 1987; Klinkhamer and de Jong, 1990).

The level of pollinated stigmas was very high in all patches examined but, despite this, a survey of fruit-set levels revealed widespread seed-set failure. Flowering density did not influence natural fruit set, and levels were consistent over 2 consecutive years. Low fruit-set levels were reported at Abernethy patch 3 in 1996 by Neiland and Wilcock (1997), and high fruit set at Balmoral patch 2 in 1993 by Wilcock and Jennings (1999), providing long-term evidence of annual fruiting failure at Abernethy and fruiting success at Balmoral.

**Breeding system**

The pollination experiments in this study have confirmed the highly self-incompatible breeding system of *L. borealis*...
first identified by Wilcock and Jennings (1999). Artificial self-pollination at all forest sites resulted in very low levels of fruit set compared with inter-patch outcrossing. The production of some seed from carefully controlled artificial selfings shows that *L. borealis* is either capable of agamospermy or not completely self-incompatible. Wilcock and Jennings (1999) showed no evidence of agamospermy but this was based on a small sample size and the possibility cannot be completely ruled out. Leaky self-incompatibility has been observed in several other species (e.g. Les et al., 1991; Reinartz and Les, 1994; Luijten et al., 1996) and may represent a partial breakdown of self-incompatibility (Reinartz and Les, 1994).

### Table 5. Fruiting success of *Linnaea borealis* obtained from experimentally controlled intra- and inter-flower colour type crosses

<table>
<thead>
<tr>
<th>Type of cross</th>
<th>Distance between crossed flowers (m)</th>
<th>No. of flowers pollinated</th>
<th>No. of fruits produced</th>
<th>% fruit set ± 95 % CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-flower colour type:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>×</td>
<td>13</td>
<td>32</td>
<td>2</td>
<td>6.3 ± 8.4</td>
</tr>
<tr>
<td>×</td>
<td>14</td>
<td>30</td>
<td>1</td>
<td>3.3 ± 6.4</td>
</tr>
<tr>
<td>×</td>
<td>23</td>
<td>32</td>
<td>1</td>
<td>3.1 ± 6.0</td>
</tr>
<tr>
<td>×</td>
<td>40</td>
<td>30</td>
<td>1</td>
<td>3.3 ± 6.4</td>
</tr>
<tr>
<td>Total</td>
<td>124</td>
<td>6</td>
<td>4.0 ± 3.5</td>
<td></td>
</tr>
<tr>
<td>Inter-flower colour type:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>×</td>
<td>60</td>
<td>30</td>
<td>9</td>
<td>30.0 ± 16.4</td>
</tr>
<tr>
<td>×</td>
<td>144</td>
<td>30</td>
<td>14</td>
<td>46.7 ± 17.9</td>
</tr>
<tr>
<td>×</td>
<td>450</td>
<td>32</td>
<td>17</td>
<td>53.1 ± 17.3</td>
</tr>
<tr>
<td>×</td>
<td>2900</td>
<td>20</td>
<td>7</td>
<td>35.0 ± 20.9</td>
</tr>
<tr>
<td>Total</td>
<td>112</td>
<td>47</td>
<td>42.0 ± 9.1</td>
<td></td>
</tr>
</tbody>
</table>

95 % CI = 95 % confidence interval.

Flower colour diversity and mate availability

Studies of flower colour diversity provide some insight into potential mate availability within patches. Controlled crosses between flowers with the same colour type were highly incompatible and produced very low levels of fruit set even when separated by distances of up to 40 m. However, crosses between flowers with different colour types resulted in
significantly higher levels of fruit set. Very little variation in flower colour diversity was observed in Curr Wood patches 1–3, Abernethy 1 and 5 and Balmoral 1. The combination of low natural fruit set and flower colour uniformity at these sites shows that they have low clonal diversity and limited mate availability, and that each are possibly composed of just a single clone. In contrast to the other patches examined, Balmoral patches 2 and 3 had a diversity of flower colour types and high natural fruit set, indicating higher clonal diversity and compatible mate availability.

Implications of limited pollinator movement and low mate availability on fruiting success

At Abernethy and Curr Wood natural fruit set is limited by low mate availability within patches and isolation from pollen exchange between them. The presence of some natural fruit set in these isolated patches may be the result of agamospermy, leaky self-incompatibility and/or occasional long-distance pollen dispersal. Further investigation involving seed paternity testing is needed to distinguish between these possibilities and to assess their relative contributions to seed set. High natural fruiting success is dependent on a diversity of compatible mates situated close enough for insect pollinators to transfer pollen between them. Fruit-set levels fall below 30% when compatible mates are separated by 8 m and below 5% when separated by 30 m or more. Proximity to a compatible mate is therefore the critical factor limiting fruiting success in L. borealis.

The importance of mate proximity on fruiting success has also been found in Rubus saxatilis (Eriksson and Bremer, 1993), Calystegia collina (Wolf and Harrison, 2001) and Dithyrea maritima (Aigner, 2004). Widén and Widén (1990) showed that fruit set in female clones of the predominantly bee-pollinated gynodioecious Glechoma hederacea was dependent upon the distance to the nearest hermaphroditic clone. Fruit set was never greater than 4% at distances >10 m from the nearest pollen source compared with >50% fruit set within 2 m of the source. Isolation of 100 m reduced fruit set to 1% (Widén and Widén, 1990). Distance to the nearest potential mate may limit reproductive success in many other small populations of outcrossing species in fragmented landscapes. Foraging ranges of pollinators will influence connectivity and define the threshold of isolation for a particular species.

Future conservation and maintenance of L. borealis

Severe seed set failure in Scottish populations of L. borealis suggests that recruitment from seed is likely to be an infrequent event. During the course of this study, seedlings were very rarely observed and this problem may be widespread since Eriksson and Ehrlén (1992) described seedling recruitment as rare and seed limited in central Sweden, and Antos and Zobel (1984) observed no L. borealis seedlings in central Oregon. Without seed the species is dependent upon clonal spread, and migration will be limited, reducing the capacity for colonization of vacant habitats and acquisition of genetic diversity into existing patches. Populations will be increasingly vulnerable to random stochastic events and environmental change, and extinction is inevitable. The highly self-incompatible breeding system, combined with low within-population clonal diversity, large distances between patches and limited pollinator movement, compromise the future for L. borealis in Scotland and the species is unlikely to recover without intervention. Re-introduction of compatible mates through re-stocking of existing patches, or the creation of new ones, is required to ensure the long-term conservation and maintenance of L. borealis in Scotland.

Several authors have suggested that the introduction of new compatible mating types into genetically impoverished populations of self-incompatible plant species may be an effective technique to alleviate reproductive failure and restore population viability (Les et al., 1991; Demauro, 1993; Luijten et al., 2002; Willi and Fischer, 2005). The introduction of new compatible mating types into impoverished L. borealis populations could be achieved by artificial cross-pollination or transplantation of seed, seedlings or adult plants between carefully selected cross-compatible populations. Mates should be sourced from nearby populations to minimize the risk of out-breeding depression and the breakdown of locally adapted genotypes. Future studies should investigate the

![Fig. 5. Relationship between fruit-set levels and distance to the nearest different flower colour type in Linnaea borealis.](https://academic.oup.com/aob/article-abstract/103/6/835/129090)
most suitable protocols so that an effective conservation recovery programme can be implemented.

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