

High Outcrossing in the Annual Colonizing Species *Ambrosia artemisiifolia* (Asteraceae)

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- **Background and Aims** Variation in mating patterns may be particularly evident in colonizing species because they commonly experience wide variation in plant density. Here, the role of density for the mating system of *Ambrosia artemisiifolia* (common ragweed), a wind-pollinated annual colonizing species previously reported as self-compatible, is explored.
- **Methods** The effect of population density on the proportion of self- and cross-fertilized seeds was examined using allozyme markers and experimental arrays conducted over two seasons in the field. Also the reproductive success of isolated plants located in diverse habitats was measured. The potential occurrence of a physiological mechanism preventing self-fertilization, i.e. self-incompatibility, following controlled self- and cross-pollinations in the glass-house was examined.
- **Key Results** Outcrossing rates estimated using allozyme markers were uniformly high, regardless of the spacing between plants. However, when single plants were isolated from congeners they set few seeds. Observations of pollen-tube growth and seed set following controlled pollinations demonstrated that plants of *A. artemisiifolia* possess a strong self-incompatibility mechanism, contrary to earlier reports and assumptions.
- **Conclusions** The maintenance of high outcrossing rates in colonizing populations of *A. artemisiifolia* is likely to be facilitated by the prodigious production of wind-borne pollen, high seed production and extended seed dormancy.

Key words: Self-incompatibility, outcrossing rate, density dependence, colonization, wind-pollination, *Ambrosia artemisiifolia* (ragweed), Asteraceae.

INTRODUCTION

The immobility of plants exerts an important influence on mating which is commonly ‘context dependent’, varying with local ecological and demographic conditions and the availability of compatible mating partners. Density-dependent and frequency-dependent influences on rates of self- and cross-fertilization have now been widely reported (e.g. Farris and Mitton, 1984; Kohn and Barrett, 1994; Cheptou *et al.*, 2002; Kalisz *et al.*, 2004; Eppley and Pannell, 2007). For species that grow in ephemeral habitats, and particularly those that are annual, demographic factors and mate availability can vary greatly among neighbourhoods and between years. This unpredictability can have important reproductive consequences with respect to mating and fertility and may explain the common occurrence of self-compatibility in many annual colonizing species (Baker, 1955; Mulligan and Findlay, 1970; Price and Jain, 1981; Pannell and Barrett, 1998; Cheptou, 2004; although for exceptions, see Abbott and Forbes, 1993; Sun and Ritland, 1998; Cheptou *et al.*, 2002).

Demographic factors, such as the local density of plants, are well known to influence mating and fertility. Both animal- and wind-pollinated species often show a positive correlation between plant density and outcrossing rate (e.g. Vaquero *et al.*, 1989; Murawski and Hamrick, 1991; Motten and Antonovics, 1992; van Treuren *et al.*, 1993; but see Karron *et al.*, 1995; Herlihy and Eckert, 2004;

Brunet and Sweet, 2006). For animal-pollinated plants this relationship can arise because of density-dependent changes in pollinator behaviour (reviewed in Antonovics and Levin, 1980). In contrast, because wind-pollinated plants do not rely on visits by pollinators, demographic effects on outcrossing are a feature of the interaction with local microclimatic conditions and the plants themselves. For example, Eppley and Pannell (2007) recently demonstrated that in *Mercurialis annua*, a wind-pollinated androdioecious annual, selfing rates were context-dependent depending on the local density and relative frequencies of hermaphrodites and males. In general, context-dependent mating arises from either differences in the amount of pollen captured (pollen quantity) or the type of pollen captured (pollen quality).

Here, the effect of plant density on mating in *Ambrosia artemisiifolia* (common ragweed) was investigated. This wind-pollinated monoecious annual is common in eastern North America as a weed of arable crops but has been introduced to Europe, Asia and Australia where it has become invasive (Bassett and Crompton, 1975; Bass *et al.*, 2000; Chauvel *et al.*, 2006). There have been no studies of the mating system of *A. artemisiifolia* but most workers, probably because of its annual weedy habit, have assumed the species is self-compatible and capable of selfing (e.g. Genton *et al.*, 2005). Indeed, Jones (1936) conducted genetic studies of sexual variation in *A. artemisiifolia* and reported all monoecious forms to be self-fertile. Similarly, Bassett and Crompton (1975) stated that *A. artemisiifolia*

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produces viable seed through both self- and cross-fertilization and McKone and Tonkyn (1986) and Lundholm and Aarssen (1994) both report that the species is self-fertile. The present studies were therefore motivated on the assumption that, like many annual colonizers, *A. artemisiifolia* was self-compatible.

The present study had three objectives concerning the reproductive biology of *A. artemisiifolia*. First, using allozyme markers, the influence of plant density on mating patterns in field experiments conducted over 2 years was investigated. Secondly, the reproductive success of isolated *A. artemisiifolia* plants in the field was examined to assess to what extent low mate availability might reduce seed fertility. Finally, because the results from these two experiments suggested that *A. artemisiifolia* does not have the capacity for significant self-fertilization, the compatibility status of plants was investigated using controlled self- and cross-pollinations in the glasshouse.

METHODS AND MATERIALS

Study system

Ambrosia artemisiifolia (Asteraceae) grows abundantly in disturbed habitats on a variety of soil types, including cultivated fields, roadsides, waste places and gardens. It is a successful pioneer in early successional ecosystems (Bazzaz, 1974). In Ontario, Canada, where the present studies were conducted, plants flower in August–September with female flowers produced in the axils of bracts or upper leaves and male flowers in terminal racemes (Payne, 1963). There is no vegetative propagation, so that all reproduction occurs through seed (Bassett and Crompton, 1975).

Density arrays

During summer 2005, 750 ragweed seedlings were transplanted from ten different sites at the Koffler Scientific Reserve (KSR) at Jokers Hill in Southern Ontario (44°03'N, 79°29'W). Seedlings that were uniform in size with between six and eight leaves were selected. The seedlings were then grown in a common environment in a glasshouse at the University of Toronto in 4-inch pots until developing buds were visible. In early August, just before flowers opened, the plants were transported back to KSR and they were planted in three freshly tilled fields each separated by approx. 500 m. Plants were randomly assigned to one of three density arrays (plants 30 cm, 90 cm, 150 cm apart). Each array was represented twice in each field for a total of six replicates per density array. In each density treatment, a total of 37 plants was used, with 18 focal plants with neighbours on all sides. Plants were positioned in hexagonal arrays so that all inter-plant distances were equal. In 2006 the experiments were repeated using the same general protocols but with two modifications. First, density arrays of 90 cm, 450 cm and 900 cm were used, and only one array per field. Secondly, plants were germinated from seed and the arrays set up 3 weeks earlier in the season so that flowering in the experiment did not

overlap with flowering of local ragweed populations. Both of these modifications were undertaken in an effort to reduce the potential influence of background levels of pollen on outcrossing rates.

For the 2005 experiment, stigmas were collected to assess stigmatic pollen loads from one of the three density treatments in each field once a week for 4 weeks. At the end of the season, 20 seeds were collected from each maternal plant to assess mating patterns in each array. Seeds were collected at two time intervals 2 weeks apart, to span the range of maturation (and stigma exposure) times. Stigmatic pollen loads were analysed with repeated-measures, general linear models (Neter *et al.*, 1996: mixed procedure of SAS, release 9-1, SAS Inst. Inc., 2002). The dependent variable was log-transformed to assure normally distributed residuals.

Allozyme markers and gel electrophoresis were used to determine the outcrossing rate of each density array (2005: 18 arrays, six of each density treatment; 2006: six arrays, two of each density treatment). For each density treatment, 20 seeds from each of the 18 focal plants were used to estimate outcrossing rates for that array. Variation at four isozyme loci was resolved using horizontal starch gel electrophoresis. Seeds from each maternal plant were separately ground in three drops of 0.1 M Tris–HCl extraction buffer (Soltis *et al.*, 1983), and the extract absorbed onto 3-mm chromatography paper wicks and placed directly onto 11–12% starch gels. Using a lithium-borate buffer system (pH 8.3), four variable loci were resolved from three enzyme systems: alcohol dehydrogenase (*Adh*), glutamate oxaloacetate transaminase (*Got*) and phosphoglucosyltransferase (*Pgm*). Gels were stained for enzyme activity following recipes in Wendel and Weeden (1991). Four alleles were detected at an *Adh* locus, three alleles at *Got-1*, four alleles at *Got-2* and three alleles at *Pgm*.

Genotypes were inferred based on segregation patterns characteristic of either dimeric or monomeric codominant enzymes. Single and multi-locus outcrossing rates (t and t_m , respectively), and pollen (p) and ovule (u) allele frequencies were jointly estimated using the program MLTR (vers. 0.9; Ritland, 1986). This program uses maximum-likelihood to infer the genotypes of the maternal parents, allele frequencies in the pollen pool, and the proportion of progeny that are the result of outcrossing versus selfing. Standard errors of the estimates were obtained from the standard deviation of 1000 bootstrap values, using the seed family as the unit of re-sampling. Within each field, separate outcrossing rates were estimated for the density arrays (2005: six arrays per field; 2006: one array per field) using a common pollen allele frequency pool for each field.

To determine whether estimates of the multi-locus outcrossing rates were significantly different from 1.0, the distribution of 1000 bootstrap values were examined following methods outlined in Eckert and Barrett (1994). Using this method, parameter estimates for each array were considered to be significantly <1.0 if $100[1 - (\alpha_{PC}/2)]\%$ of the bootstrap values were all <1.0 (where α_{PC} represents the Type 1 error rate per contrast). Similarly, for each density array, whether the difference between the multi-locus and

single-locus estimators of mating was significantly different from zero was tested. Overall differences in outcrossing rate between the density treatments were tested using ANOVA in SAS. The outcrossing rates for each density treatment were weighted by the inverse of the bootstrap variance estimates, to account for the sampling error in each estimate (see Barrett *et al.*, 1994).

Isolation experiment

To determine the reproductive success of isolated plants in the field, solitary individuals were planted at KSR during the summer of 2006. The seed for this experiment was collected in October 2005 from open-pollinated plants within the vicinity of KSR. Following stratification and germination procedures suggested in Willemsen (1975), seeds were grown from 40 maternal families in June 2005 in a glasshouse at the University of Toronto. Before buds were visible, 30 plants were transplanted into isolated habitats at KSR. The individuals were planted in old-field habitat, in a small patch (approx. 0.5 × 0.5 m), which was cleared of surrounding vegetation. Plants were watered upon transplanting, again 3 d later, and 1 week later to ensure successful establishment. The 30 sites were at least 300 m from known *A. artemisiifolia* populations, and in almost all cases were separated by forest and other woody vegetation. Plants were visited throughout flowering to ensure both female and male flowers were present. At the end of the flowering season all seed was harvested from each plant to estimate seed production.

Self-incompatibility experiment

Self-incompatibility in *A. artemisiifolia* was tested by comparing self- and cross-pollinated flowers for both pollen-tube growth and seed set. During autumn 2006, an additional 50 plants were grown using the same batch of seeds and germination conditions as described above. Plants were grown in a glasshouse using an artificial light regime to mimic the natural conditions during flowering in Southern Ontario (14 h light/10 h dark). Just before flowering, plants were randomly assigned to one of three treatments: cross-pollen donors (30 plants), cross-pollen recipients (10 plants) and self-pollen recipients (10 plants). For each plant, five flowers were brushed with either self-pollen or a mixture of pollen from five donor plants. It was necessary to use separate plants for the self- and cross-pollen treatments, to prevent the possibility of cross contamination between pollination treatments. Pollen donors were maintained in one glasshouse, cross-pollen recipients in a separate house, and each self-pollen recipient plant was isolated in its own glasshouse to prevent cross contamination. Cross-pollen recipients were emasculated continuously throughout flowering to avoid any self-pollen deposition. For the self-pollen recipients, covers created out of three layers of spun-fibre material were placed over female flowers to control the time of self-pollination. Twenty-four hours after pollination, styles were fixed in alcohol and examined for pollen tube growth. The aniline blue staining method and fluorescence microscopy

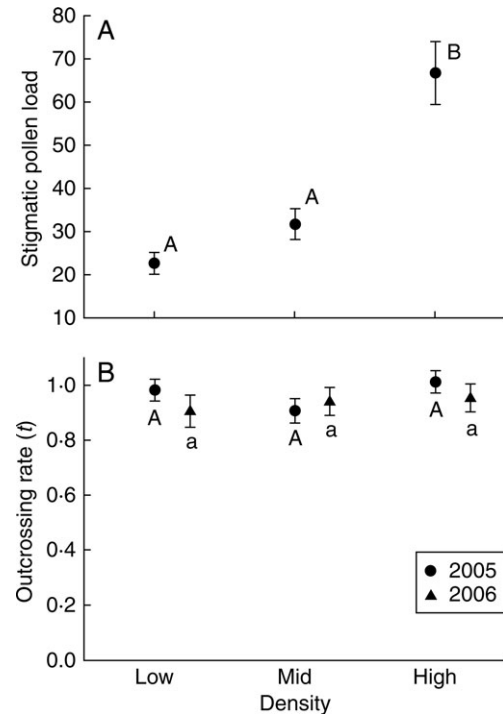


FIG. 1. Influences of plant density (low, mid, high) on (A) mean (\pm s.e.) number of pollen grains on stigmas and (B) multi-locus outcrossing rate ($t \pm$ s.e.) for 2005 and 2006 in experimental field arrays of *Ambrosia artemisiifolia*. Letters indicate the outcomes of Dunn-Šidák multiple comparisons. See text for statistical details.

were used to examine and count pollen germination and pollen-tube penetration. The remaining flowers were allowed to develop seed. Seed set was compared between plants in each treatment and the resulting data analysed with generalized linear models (Genmod procedure of SAS, release 9.1; SAS Inst. Inc., 2002; Allison, 1999) with logit transformations to accommodate the binomial distribution of data.

RESULTS

Density arrays

In 2005 pollen receipt varied significantly between plants in the three density arrays ($F_{2,62.2} = 22.60$, $P < 0.0001$; Fig. 1A). Stigmas on plants in low and mid-density arrays captured equivalent amounts of pollen ($t_{54.3} = 2.03$, $P > 0.05$). In contrast, stigmas on plants in high density arrays captured significantly more pollen than those in either low or mid-densities (high vs. low $t_{63.1} = 6.54$, $P < 0.001$; high vs. mid $t_{71.2} = 4.52$, $P < 0.001$; Fig. 1A). There was no significant difference between the same density arrays across the three different fields ($F_{6,60.9} = 1.17$, $P > 0.3$).

Multi-locus outcrossing rates for all arrays were close to 1.0 in both 2005 and 2006 (Fig. 1B). Based on the distribution of bootstrap values, one of the 26 arrays had an outcrossing rate that differed significantly from 1.0 (2005, one mid density array). For all arrays, the differences between

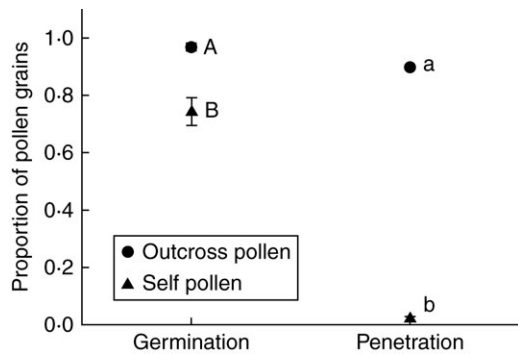


FIG. 2. Relationship of proportion of pollen grains (\pm s.e.) that fall into each subclass (germination, penetration of the style) for *Ambrosia artemisiifolia* plants that received outcross pollen and self pollen. Letters indicate the outcomes of Dunn–Šidák multiple comparisons within each subclass between the two treatments, with capital letters for germination, and lower-case letters for penetration. See text for statistical details.

the multi-locus and single-locus estimators of outcrossing rate were not significantly different from zero.

There were no significant differences among the outcrossing rates of the three density arrays in either year (2005: $F_{2,10} = 3.29$, $P > 0.05$; 2006: $F_{2,7} = 0.44$, $P > 0.6$; Fig. 1B). In 2005 there was no significant difference in outcrossing rate between the same density arrays among the three fields ($F_{3,10} = 1.28$, $P > 0.3$).

Seed production of isolated plants

Plants in isolated locations at KSR set between 0 and 254 seeds (mean $58.04 \pm$ s.e. 15.11), well below the maximum potential seed set since the present observations indicated that the plants produced hundreds of female flowers.

Testing for self-incompatibility

A significant difference in successful pollen tube penetration of the stigmatic tissue was found between plants that received cross- versus self-pollen ($F_{1,11.1} = 1721.53$, $P < 0.0001$; Fig. 2). The two classes of unsuccessful penetration (no germination, and germination but no penetration) also yielded significant differences between the two treatments (no germination: self vs. cross $F_{1,10.4} = 23.00$, $P < 0.001$; germination: self vs. cross $F_{1,9.82} = 157.73$, $P < 0.0001$; Fig. 2). Qualitative differences in the growth of pollen tubes between cross- versus self-pollen can be clearly seen in Fig. 3, where cross-pollen tubes grow straight and long and penetrate the stigma (Fig. 3A), whereas self-pollen tubes are unable to penetrate the stigma and there is formation of callose (Fig. 3B) typical of a self-incompatibility reaction (de Nettancourt, 1977).

There was a significant difference in the proportion of seed set between plants that received cross- versus self-pollen ($\chi^2 = 79.55$, $P < 0.0001$). Plants that received cross-pollen set 0.56 ± 0.03 (mean \pm s.e.) proportion seed; whereas plants that received self-pollen set 0.04 ± 0.02 (mean \pm s.e.).

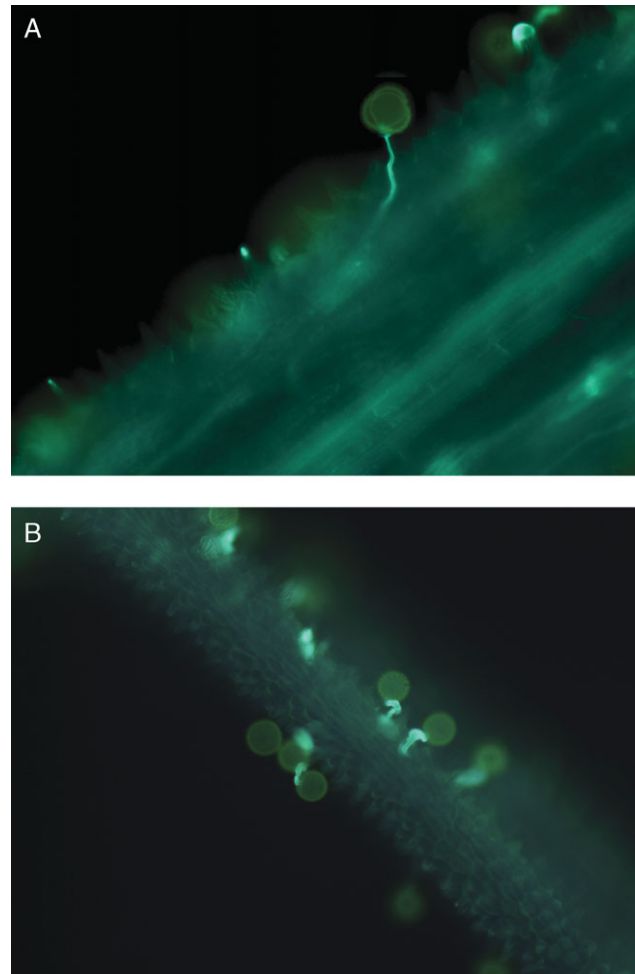


FIG. 3. Pollen grains and tubes of *Ambrosia artemisiifolia* under a fluorescence microscope showing pollen tube growth for (A) cross-pollen and (B) self-pollen at $\times 200$ magnification. Self-pollen tubes do not penetrate the style due to the formation of callose, while cross-pollen tubes penetrate the style, growing long and straight towards the ovule.

DISCUSSION

Our results clearly demonstrate that *Ambrosia artemisiifolia* is a highly outcrossing, self-incompatible plant. Provided that population densities are sufficient, seed set is generally high. Isolated plants in the present study experienced reduced seed set, although they still set some seed. Our glasshouse experiment demonstrated that plants exposed to only self-pollen set very few seeds relative to those that were cross-pollinated. Furthermore, fluorescence microscopy provided evidence that *A. artemisiifolia* is self-incompatible based on observations of pollen germination and pollen tube growth. These findings were unexpected and contrary to previous reports that the species is self-compatible (e.g. Jones, 1936; Bassett and Crompton, 1975).

A significant effect of plant density on stigmatic pollen loads was detected with plants in high-density patches capturing more pollen grains. However, because flowers of *A. artemisiifolia* are uni-ovulate, plants at lower densities still captured sufficient pollen for high seed set.

The measures of stigmatic pollen loads did not differentiate between self- and cross-pollen, so it is likely that a substantial portion of the pollen captured was self-pollen. In contrast to most wind-pollinated plants where pollen is held in the anthers until it is removed by the wind, studies on the release of pollen in *A. artemisiifolia* indicate that as anthers swell and dehisce, pollen first falls passively downwards to vegetation and is then swept away by the wind (Bianchi *et al.*, 1959). This process causes deposition of large quantities of self-pollen on stigmas within the same plant (geitonogamy). Opportunities for geitonogamous pollination are exacerbated by the inflorescence architecture of *A. artemisiifolia*. Male flowers are typically positioned above female flowers and despite some protandry there is considerable overlap between the sex functions. Self-incompatibility in *A. artemisiifolia* may therefore function largely to limit the deleterious consequences of inbreeding that arise from high levels of geitonogamous pollination.

Controlled pollinations in the glasshouse demonstrated that *A. artemisiifolia* has a self-incompatibility system. Seven of the ten plants set no seed following self-pollination, with three plants setting small amounts of seed. There may be some leakiness in the SI system, as is commonly observed in self-incompatible species (de Nettancourt, 1977; Levin, 1996; Stephenson *et al.*, 2000) and has been reported in other species of Asteraceae (Ferrer and Good-Avila, 2007). It is possible that previous reports of self-compatibility in *A. artemisiifolia* (Jones, 1936; Bassett and Crompton, 1975) involved plants with weak self-incompatibility. However, neither of these studies was designed to specifically test self-incompatibility, and so it is not possible to evaluate the rigour of their experimental designs or the validity of the results. Surveys of Asteraceae indicate that many species are partially self-incompatible (Ferrer and Good-Avila, 2007), and, in some cases, this may have resulted from selection for reproductive assurance in colonizing populations or other demographic factors (Hiscock, 2000, Cheptou *et al.*, 2001, 2002; but see Brennan *et al.*, 2005). Further studies of the incompatibility status of *A. artemisiifolia* populations would certainly be warranted, especially in the invasive range (see Genton *et al.*, 2005) where demographic factors associated with repeated colonizing events may possibly favour the breakdown of self-incompatibility.

The relative costs and benefits of self-compatibility versus self-incompatibility in colonizing species depend on a variety of demographic and life-history conditions including plant density, propagule number, life-span, seed dormancy and the capacity for clonal reproduction. Pannell and Barrett (1998) investigated the effects of different life-history traits on the reproductive success of self-compatible and self-incompatible phenotypes in a metapopulation context. Their results suggest that the advantage of self-compatible phenotypes through reproductive assurance is diminished if plants can survive unfavourable conditions in a dormant seed bank. Germination studies in *A. artemisiifolia* indicate that seeds can remain viable for 39 years or more when buried in the soil (Toole and

Brown, 1946). Pannell and Barrett (1998) also found that enhanced seed productivity can offset the disadvantage possessed by self-incompatible plants. According to Dickerson and Sweet (1971), a small *A. artemisiifolia* plant produces about 3000 seeds, while large plants can produce up to 62 000 seeds. Thus, although *A. artemisiifolia* is an annual species with no vegetative propagation, its prolific seed production, seed dormancy and high population densities may offset the cost of being self-incompatible allowing successful colonization.

Ambrosia artemisiifolia is an aggressive weed in North America, and invasive in Europe. Records from Europe suggest an early origin in France during the 18th century, and substantial spread since introduction as a seed contaminant in crops from North America (Chauvel *et al.*, 2006). Both herbarium records (Chauvel *et al.*, 2006) and a recent molecular study suggest multiple independent introductions (Genton *et al.*, 2005) with no loss of genetic diversity in introduced populations compared with native North American populations. This result would be unexpected in a selfing colonist because uniparental reproduction commonly leads to severe genetic bottlenecks during range expansion in invading species (reviewed in Novak and Mack, 2005; Barrett *et al.*, 2008). However, their finding of similar levels of genetic diversity in the introduced and native range of *A. artemisiifolia* is consistent with what one might expect for a highly outcrossing, self-incompatible colonist that has spread through multiple introductions. Extensive pollen dispersal through wind pollination is more likely to foster outcrossing among separate introductions and the maintenance of genetic diversity in the introduced range.

Self-incompatible plants may suffer reduced reproductive success when population density or size is low ('Allee effect'; Allee, 1951). Animal-pollinated species may be more prone to Allee effects through insufficient pollen transfer or through the transfer of different species' pollen, particularly when rare plants are surrounded by other flowering species (Kunin, 1993, 1997) or patches are quite isolated (Aizen and Feinsinger, 1994). Because *A. artemisiifolia* is wind-pollinated and plants produce copious pollen, the effects of being relatively isolated may to some extent be diminished. Long-distance transport of pollen in *A. artemisiifolia* could alleviate some of the costs associated with growing sparsely. There are records of *A. artemisiifolia* pollen appearing in air samples hundreds of kilometres from the nearest population indicating long-distance transport of ragweed pollen (e.g. Lorenzo *et al.*, 2006; Stach *et al.*, 2007), although the duration of pollen viability is unknown. Low levels of seed set were recorded in isolated plants of *A. artemisiifolia* positioned among forest patches. However, whether this seed resulted from self-pollination in plants with leaky SI or from long-distance pollen transport is not known.

Pollen limitation has been well documented and discussed in animal-pollinated plants (for reviews, see Burd, 1994; Larson and Barrett, 2000; Ashman *et al.*, 2004; Knight *et al.*, 2005), but there is much less evidence on whether pollen availability limits reproduction in wind-pollinated plants. The dispersal of wind-borne pollen

from point sources has a leptokurtic distribution, although the direction, speed and turbulence of wind and settling velocity of pollen also affects patterns of pollen deposition (Bateman, 1947; Gleaves, 1973; Dowding, 1987; Okubo and Levin, 1989; Giddings, 2000). Recent empirical work suggests that pollen capture and the proportion of fertilized ovules may decrease rapidly with increasing distance from pollen donors in wind-pollinated plants (Knapp *et al.*, 2001; Davis *et al.*, 2004; Stehlik and Barrett, 2006; Eppley and Pannell, 2007). The present density experiments indicated that pollen capture decreased with increasing distance, but this did not have an effect on outcrossing rates. Also, isolated plants set few seeds, suggesting that they may have suffered from pollen limitation. However, various features of pollen dispersal in *A. artemisiifolia* demonstrating long-distance transport (Raynor *et al.*, 1968, 1970, 1973) suggest that pollen-limited reproduction may be relatively uncommon in *A. artemisiifolia*.

The present study illustrates the importance of understanding both demographic and genetic influences on mating patterns in plant populations. Because *A. artemisiifolia* is a weedy, colonizing species, the demographic conditions in which it occurs should have important influences on mating and fertility. Although the present study suggests that *A. artemisiifolia* may be obligately outcrossing due to self-incompatibility, various features of its ecology and reproductive biology may ameliorate the costs that are normally associated with self-incompatibility in colonizing annuals. Traits that could diminish costs associated with outcrossing include producing enormous quantities of wind-borne pollen, prolific seed production and the presence of a seed bank.

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