The influence of floral morph ratios and low plant density on mating and fertility in a tristylos colonizing species

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**Abstract:** Sexual reproduction in heterostylos populations may be vulnerable to demographic conditions because of the small number of mating types in populations. Here, we investigate mating and fertility under natural and experimental conditions in tristylos *Lythrum salicaria* L., an invasive species that exhibits a wide range of floral morph ratios and demographic contexts. We grew 147 open-pollinated seed families from six populations with different morph structures to estimate intermorph mating \( d \). In a field experiment, we used progeny ratios from 47 spatially isolated individuals to estimate \( d \), and measured the intensity of pollen limitation experienced by the morphs. The M- and S-morphs experienced high rates of \( d \), regardless of population size or morph ratio. Estimates for the L-morph revealed low levels of intramorph mating in three dimorphic and two trimorphic populations, but near complete intramorph mating in a monomorphic population. Despite high levels of intermorph mating in the field experiment, the morphs experienced significant pollen limitation of fruit and seed set, but this did not differ in intensity among the morphs. Our field experiment demonstrates that although plant isolation was associated with pollen limitation of seed set, “long-distance” bee-mediated pollen flow served to maintain intermorph mating. Tristyly in *L. salicaria* is remarkably robust to the demographic variation associated with colonization.

**Key words:** *Lythrum salicaria*, intermorph mating, tristyly, pollen flow, biological invasions, pollen limitation.

**Résumé :** La reproduction sexuée des populations hétérostyliques peut être vulnérable aux conditions démographiques à cause de faible nombre de types sexuels dans les populations. Les auteurs ont examiné ici le croisement et la fertilité dans des conditions naturelles et expérimentales chez la plante tristylique *Lythrum salicaria* L., une plante envahissante qui présente une grande variété de ratios de morphes floraux et de contextes démographiques. Ils ont fait pousser 147 familles de semences à pollinisation libre à partir de six populations possédant différentes structures de morphes afin d’estimer le croisement intermorphes \( d \). Lors d’une expérience en plein champ, ils ont utilisé les ratios de la descendance de 47 individus spatialement isolés afin d’estimer \( d \), et mesuré l’intensité de la limitation par le pollen que les morphes subissaient. Les morphes M et S présentaient des taux élevés de \( d \), sans égard à la taille de la population ou au ratio des morphes. Les estimations chez le morphé L révélaient de faibles niveaux de croisement intramorphes chez les trois populations dimorphiques et les deux populations trimorphiques, mais une compatibilité intramorph presque complète dans une population monomorphique. Malgré les hauts niveaux de croisements intermorphes lors de l’expérience en plein champ, les morphes subissaient une limitation par le pollen significative de la production de fruits et de semences, mais elle ne différait pas significativement en intensité selon les morphes. L’expérience en plein champ démontre que même si l’isolement des plants était associé à une limitation par le pollen de la grenaison, le flux de pollen véhiculé par les abeilles sur de longues distances servait à maintenir le croisement intermorphes. La tristylie chez *L. salicaria* est remarquablement robuste à la variation démographique associée à la colonisation. [Traduit par la Rédaction]

**Mots-clés :** *Lythrum salicaria*, croisement intermorphes, tristylie, flux pollinique, invasions biologiques, limitation en pollen.

**Introduction**

In populations of self-incompatible plants, the ecological and demographic context in which mating occurs and the availability of cross-compatible mates play key roles in determining the number and genetic quality of offspring. In animal-pollinated species, small population size and low density can limit reproductive success owing to a lack of pollen vectors resulting in pollen limita-
tion of seed set (Ågren 1996; Larson and Barrett 2000; Ashman et al. 2004). These conditions can induce Allee effects on sexual reproduction and may favour the selection of self-fertilization (Allee 1931; Lloyd 1980; Barrett 2011; Pannell 2015). Despite the immobility of plants and the potential vulnerability of their reproductive ecology to the number and identity of neighbours, we have only limited understanding of how the social context in which plants occur influences both their mating and fertility.

Mating in heterostylous populations can be particularly vulnerable to demographic influences. Unlike species with homomorphic incompatibility, in which populations usually contain numerous mating types, heterostylous populations most commonly contain only two or three cross-compatible mating types (Darwin 1877; Ganders 1979; Barrett and Shore 2008). The mating types (floral morphs) differ in sex-organ length and are usually self- and intramorph-incompatible. Negative frequency-dependent selection operating in large populations of heterostylous species should result in equal frequencies of the floral morphs (isoplethy); however, a variety of ecological and demographic factors can cause deviations from isoplethy with potential consequences for mating and fertility (Ishihama et al. 2003; Wang et al. 2005; Stehlik et al. 2006). Deviations from isoplethy are especially likely in heterostylous species with well-developed colonizing ability in which founder events and restrictions on sexual recruitment may contribute to biased morph ratios (e.g., Eichhornia crassipes, Barrett and Forno 1982; Oxalis pes-caprae, Ferrero et al. 2015). The principal goal of this study was to investigate the influence of morph ratio bias and low plant density on mating and fertility in an invasive heterostylous species. Our study system for this investigation is Lythrum salicaria L., which exhibits a wide range of population sizes and floral morph structures.

Mating patterns in heterostylous populations are rarely investigated because it is generally assumed that the floral morphs are self-compatible and all mating is between the floral morphs (intermorph or disassortative mating). However, in several heterostylous species, incompatibility is only partially expressed, and in some cases can be absent altogether or unlinked to floral morph type (reviewed in Barrett and Cruzan 1994). Mating system estimates in heterostylous species that do not possess conventional heteromorphic incompatibility have revealed a wide range of mating patterns including variable selfing rates and significant levels of intramorph (assortative) mating (e.g., Amsonia, Ganders 1975; Eichhornia paniculata, Barrett and Husband 1990; Decodon verticillatus, Eckert and Barrett 1994; Narcissus triandrus, Hodgins and Barrett 2006, 2008; Oxalis alpina, Weber et al. 2013; Luculia pinceana, Zhou et al. 2015). In such cases, deviations from symmetrical disassortative mating can result in biased morph ratios (reviewed in Barrett and Hodgins 2006). Although most estimates of mating in heterostylous populations have used allozymes or microsatellite markers, a few studies have used the style-length loci themselves as genetic markers to estimate intermorph and intramorph mating by progeny testing open-pollinated seed families (e.g., Ganders 1975; Barrett 1979; Barrett et al. 1987). We used this approach here because L. salicaria exhibits tetrasomic inheritance, complicating the use of allozymes or microsatellite markers.

A neglected aspect of the population biology of colonizing species concerns the consequences of spatial isolation for mating and fertility. Most studies in reproductive ecology focus on populations of moderate to large size. The ecology and genetics of “plant outliers”, plants that occur outside population boundaries, are therefore not well characterized (reviewed in Levin 1995). In particular, the extent to which spatial isolation may be buffered by “long distance” pollen flow, either from larger populations or from other isolated individuals, is rarely investigated, aside from studies investigating gene flow using genetic markers in tropical forest trees where conspecific individuals are usually spatially isolated and at low density (Nason et al. 1998; White et al. 2002; Ashley 2010). If cross-pollination is compromised by low density through unreliable pollinator service, increased rates of self-pollination may be likely. However, the extent to which pollen limitation influences mating and fertility will depend on the compatibility status of individuals in populations (Lloyd and Schoen 1992), and comparative evidence indicates that self-incompatible species are more vulnerable to pollen limitation than self-compatible species (Larson and Barrett 2000). One of the objectives of this study is to investigate whether morph-specific differences in the expression of incompatibility may differentially affect the reproductive performance of L. salicaria plants occurring at low density.

Here, we investigate mating patterns and fertility in tristylos L. salicaria, an invasive herb that exhibits a wide range of population sizes and floral morph ratios in its adventive range of Ontario, Canada (Eckert and Barrett 1992; Balogh and Barrett 2016). We examined two demographic contexts, natural population variation in morph ratios and a field experiment involving plant isolation, to examine the following specific questions: (i) to what extent are levels of intermorph (disassortative) mating affected by the floral morph structure of populations? Specifically, we contrasted progeny morph ratios sampled from parents in trimorphic and dimorphic populations with expectations for intermorph and intramorph mating to assess whether floral morph loss from populations is associated with deviations from intermorph mating. In addition, we also progeny tested a small monomorphic population composed of the L-morph in which we observed seed set to determine whether mating was predominantly intramorph and (or) whether long-distance pollen flow from other populations could account for seed production, and (ii) what is the influ-
ence of low plant density on mating and fertility? We performed an experiment with spatially isolated plants of the three floral morphs in two old fields separated by forest and ponds at a field station to determine whether fruit and seed set was pollen limited and, if so, whether pollen limitation varied among the morphs. Using progeny tests of the isolated plants, we also assessed the extent to which intermorph gene flow may alleviate any pollen limitation.

**Materials and methods**

**Study system**

*Lythrum salicaria* (Lythraceae) is a showy-flowered, herbaceous, amphibious perennial native to Eurasia. It has been introduced to North America where it colonizes a wide range of wetland habitats including freshwater marshes, river edges, low-lying pastures and roadside ditches. Flowers of *L. salicaria* are visited by diverse pollinators, particularly *Bombus* spp. and *Apis mellifera*, but also small pollen-collecting bees, wasps, butterflies, and occasionally, hummingbirds (Thompson et al. 1987; Brown et al. 2002; King and Sargent 2012). Populations of *L. salicaria* are commonly tristylos and the species possesses a trimorphic incompatibility system that generally prohibits self- and intramorph fertilization. However, populations commonly exhibit partial self-incompatibility, with the M-morph displaying the weakest incompatibility and the S-morph the strongest, based on controlled self- and cross-pollinations (Darwin 1877; Colautti et al. 2010; Balogh and Barrett 2018). At present, it is unclear whether variation in partial self-incompatibility influences mating patterns in populations of *L. salicaria*. Despite extensive literature on the reproductive ecology of *L. salicaria*, there have been no studies on the mating system of the species.

Plants of *L. salicaria* produce very large numbers of small, easily dispersed seeds (thousands to 2.5 million per plant) resulting in prolific colonizing ability (Thompson et al. 1987). The species is perennial, with some individuals living over 12 years, but reproducing exclusively by seed (Yakimowski et al. 2005). As a consequence, the species displays a wide range of population sizes from isolated individuals and very small colonies to large, monospecific, high density stands containing many thousands of plants (Eckert and Barrett 1992). Variation in population size in *L. salicaria* is associated with pollen limitation of seed set with smaller populations experiencing more intense pollen limitation than larger populations (Ågren 1996). Extensive surveys of style-morph ratios in populations of *L. salicaria* in Europe and eastern North America indicate significant differences between the two continents. In France and the Iberian Peninsula, ~95% of *L. salicaria* populations (*n* = 198 populations) are trimorphic (Eckert et al. 1996a; Costa et al. 2016). In contrast, surveys of populations in eastern North America (*n* = 216) indicate that ~25% of populations are dimorphic with the vast majority of dimorphic populations composed of the L- and M-morphs (Eckert and Barrett 1992; Balogh and Barrett 2016). Occasional populations containing a single floral morph are also reported from Ontario (Balogh and Barrett 2016), raising the question of whether these populations are self-sustaining through sexual reproduction and to what degree intramorph incompatibility limits mating and fertility.

The inheritance of alleles determining style morph in *L. salicaria* was first enumerated by Fisher and Mather (1943), based on controlled crosses among the floral morphs. They demonstrated that two unlinked loci (*S* and *M*), with the *S*-locus epistatic to the *M*-locus, govern inheritance, and that because *L. salicaria* is an autotetraploid, the loci exhibit tetrasomic inheritance. Fisher (1941) calculated the gamete frequencies produced by autotetraploid individuals of different genotypes and predicted the genotype frequencies one would expect to find in a population at isoplethic equilibrium. Fisher and Mather (1943) extended this work to include the phenomenon of double reduction, in which sister chromatids are inherited by the same gamete at various rates (Darlington 1929; Bever and Felber 1992). The frequencies of double reduction at the *M*- and *S*-loci of *L. salicaria* have been estimated to be approximately 10% and 2.5%, respectively (Fisher 1949; Fyfe 1953). Furthermore, rather than possessing homozygous/heterozygous allelic states as in diploid organisms, autotetraploid *L. salicaria* can possess between zero to four copies of a dominant or recessive allele, resulting in a total of 25 possible genotypes at the *S* and *M* loci (Fisher 1941; Supplementary data, Table S1). At equilibrium, by far the most common genotypes are nulliplex or simplex (i.e., possessing zero or one dominant allele at a locus), whereas only seven percent of the individuals possess duplex genotypes (which possess two dominant alleles at a locus) or higher (Heuch and Lie 1985). As outlined below, the occurrence of tetrasomic inheritance and double reduction in *L. salicaria* needs to be taken into account when inferring maternal genotypes for progeny testing.

**Progeny testing of maternal seed families from natural populations**

In summer 2015, we sampled an average of 24.5 (range 4–34) maternal seed families from six *L. salicaria* populations in southern and central Ontario (Fig. 1). The populations were originally located in summer 2013 as part of an extensive survey of floral morph ratios in *L. salicaria* populations in Ontario (Balogh and Barrett 2016). In each population, we estimated census number (population size) and floral morph ratios using methods previously

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The populations were chosen to include the complete range of population morph structures (trimorphic, two populations; dimorphic, three populations; monomorphic, one population). At peak flowering in each population (July–August), we used aluminum forestry tags with unique identifiers to label the floral morphs of plants to be progeny tested. In late October–early November 2015, we revisited the six populations and harvested six mature fruit from each tagged plant. The fruits were sampled from a single inflorescence with a special effort made to include fruits from throughout the inflorescence (bottom to top) to maximize the range of “pollination environments” experienced by each plant. We allowed fruits to dry in open Eppendorf tubes at 6 °C until April 2016 to break seed dormancy.

In April 2016, we sowed the seed from each fruit into Promix BX soil (Promix Company, Rivière-du-Loup, Quebec, Canada) and in mid-May transplanted seedlings into SC7 sized Ray Leach “Cone-tainers” that are 3.81 cm wide and 13.97 cm deep (Stuewe and Sons, Inc., Tangent Oregon, USA) filled with Promix BX soil. The mean number of seedlings grown to flowering for each maternal family was 17 (range: 2–37) and depended on germination success, which was generally high for most families. The sample sizes for families and plants per floral morph for each population are presented in Table 1. We added a 2–20–20 fertilizer with micronutrients on a biweekly basis following the manufacturer’s instructions. We recorded the date of flowering and floral morph of each plant.

Estimation of intermorph mating in natural populations

We estimated morph-specific rates of intermorph mating (= disassortative mating or d) in each population using the morph ratios of open-pollinated seed families weighted by the number of offspring produced by each of the genotypes within each morph (Supplementary data, Table S2). We estimated d in our study (and its inverse, 1–d, intramorph mating = assortative mating) rather than outcrossing (and selfing) because the genetics of tristyly make it difficult or impossible (depending on maternal genotype) to distinguish assortative mating from selfing. We adapted our methods from those detailed in Barrett et al. (1987), where only the diploid case with linkage between the M- and S-loci was considered, and we extended these methods for the tetrasomic case with unlinked M- and S-loci, as found in L. salicaria (Fisher and Mather 1943). We represented genotype frequencies as elements in a data vector in R where each element contained the frequency of that genotype in the population or progeny. We assigned which element defined each genotype using the genotype numbers in the Supplementary data, Table S1. We defined j as the paternal genotype, i as the maternal genotype, crossfreq as a 25 × 25 matrix initially containing zeros which expresses the probability of cross-fertilization between genotype i and genotype j, and Fj or Fi as the frequency in a population of a given genotype i or j. We set the variable mat.morph to represent all genotypes (x), which define the floral morph expressed by genotype x; these are genotype 1 for L-morph parents, genotypes 2–5 for the M-morph, and genotypes 6–25 for S-morph; when i equals one of the genotypes in each of these categories, then mat.morph will represent i and the other genotypes in that morph. We then used the equation:

$$\text{crossfreq}_{ij} = \frac{F_j}{1 - \sum_{x=\text{mat.morph}} F_i} \times \frac{1}{\sum_{x=\text{mat.morph}} F_i}$$

to calculate the mating frequency between each compatible genotype in the population. Equation 1 only defines intermorph mating and therefore the frequencies of intramorph mating equal zero on the crossfreq matrix. We
calculated the frequency with which each genotype produced a given gamete following Fisher and Mather (1943) using the estimated double reduction rates of $\alpha = 0.10$ at the $M$-locus and $\alpha = 0.025$ for the $S$-locus (Fisher 1949; Fyfe 1953). We summed these values following the procedures detailed in Fisher and Mather (1943) to establish a $25 \times 25$ array ($\text{progfreq}$) in which dimensions one and two represent paternal and maternal parents and the third dimension represents the probability that the parents produced a given offspring genotype. We used $\text{progfreq}$ and $\text{crossfreq}$ to determine the expected number of offspring from each genotype after intermorph mating. We created a third matrix, $\text{progeny}$, which is a $25 \times 25$ matrix, to represent the output of each progeny genotype $k$ from each maternal genotype $i$ crossed with all paternal parents of genotype $j$. We filled this matrix using the equation:

$$
\text{progeny}[i, k] = \sum_{j} \text{crossfreq}[i, j] \times \text{progfreq}[j, i, k]
$$

using the expected predicted genotypes, we then calculated the expected number of each floral morph in the progeny. To calculate the progeny ratio expected after intramorph mating, we repeated the steps above, except we defined the genotypes $x$ of $\text{mat.morph}$ to be all genotypes of the same morph as $i$.

We inferred the genotypes of parental plants using a combination of floral morph identity and progeny ratios. By definition, L-morph plants are nulliplex at the $M$- and $S$-loci and could therefore be assigned to genotype 1. For each family of the $M$- and $S$-morph, we performed a $\chi^2$ test comparing the progeny morph ratios expected for “simple” $M$- and $S$-morph genotypes, which consist of $M$-morph plants simplex at the $M$-locus (genotype 2) or $S$-morph plants nulliplex at the $M$-locus (genotype 6). We also compared the observed progeny morph ratios in $M$- and $S$-morph plants to more “complex” expectations, which consist of $M$-morph plants duplex at the $M$-locus (genotype 3) or $S$-morph plants simplex at the $M$-locus (genotype 7). We chose these genotypes for our comparisons because of prior studies (Fisher 1941; Fisher and Mather 1943; Heuch and Lie 1985) indicating that genotypes 2, 3, 6, and 7 represent $\approx 95\%$ of the $M$- and $S$-morph plants in autotetraploid, tristylos populations at isoplethic equilibrium. Our simulations of dimorphic and non-equilibrium populations demonstrated that triplex and higher dominant genotypes were rare, with complete intermorph mating, and none of the observed progeny morph ratios suggested triplex maternal genotypes. We assigned a genotype to each plant based upon which prediction was the best fit to the data according to the $\chi^2$ test (Supplementary data, Table S31). In total, 109 plants had genotypes clearly assigned; 64, 29, and 16 of the L-, M-, and S-morph, respectively. We estimated genotype frequencies in the populations using these genotypes, and then using the identities assigned from the first run of this test, we repeated the $\chi^2$ comparisons to ensure that the progeny ratios did not deviate from the predicted genotypes to any substantial degree.

We pooled the progeny of families of each floral morph in each population to estimate the frequency of intermorph mating (Table 2). We obtained the predicted frequency of $L$, $M$, and $S$-morph progeny from each genotype in the $L$- and $M$-morphs after intermorph or intramorph mating ($X_b$ and $S_b$, respectively, where $b$ represents the progeny floral morphs $L$, $M$, and $S$-morph). We then entered these values along with the observed progeny morph counts ($\text{obs}_b$) into the following equation, which outputs the $\chi^2$ fit based on $d$ values:

$$
\chi^2_d = \sum_{\text{for } b \text{ where } \text{obs}_b > 0} \frac{[(X_b + (X_b - S_b) \times d) - \text{obs}_b]^2}{\text{obs}_b}
$$

We used a modified version of this equation for families of the $S$-morph, with $n$ as the total number of progeny produced by the $S$-morph maternal parents and subscripts $L$, $M$, and $S$ representing the $L$-, $M$-, and $S$-morphs, respectively:

$$
\chi^2_d = \frac{[1/4 \times (n \times (1 + d) - (\text{obs}_L + \text{obs}_M)]^2}{(\text{obs}_L + \text{obs}_M)} + \frac{[n \times (3 - d)/4 - \text{obs}_S]^2}{\text{obs}_S}
$$
Table 2. Patterns of mating in six populations of *Lythrum salicaria* based on progeny testing of open-pollinated seed families.

<table>
<thead>
<tr>
<th>Population (n)</th>
<th>Structure</th>
<th>Morph</th>
<th>Intramorph mating expectation</th>
<th>Intermorph mating expectation</th>
<th>Observed progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td>153</td>
<td>L</td>
<td>L</td>
<td>50</td>
<td>0</td>
<td>48</td>
</tr>
<tr>
<td>68</td>
<td>L, M</td>
<td>M</td>
<td>105</td>
<td>293</td>
<td>202</td>
</tr>
<tr>
<td>100</td>
<td>L, M</td>
<td>M</td>
<td>277</td>
<td>145</td>
<td>123</td>
</tr>
<tr>
<td>84</td>
<td>L, S</td>
<td>S</td>
<td>119</td>
<td>0</td>
<td>55</td>
</tr>
<tr>
<td>92</td>
<td>L, M, S</td>
<td>L</td>
<td>103</td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td>92</td>
<td>L, M, S</td>
<td>M</td>
<td>23</td>
<td>90</td>
<td>24</td>
</tr>
<tr>
<td>92</td>
<td>L, M, S</td>
<td>S</td>
<td>27</td>
<td>34</td>
<td>45</td>
</tr>
<tr>
<td>135</td>
<td>L, M, S</td>
<td>S</td>
<td>188</td>
<td>0</td>
<td>95</td>
</tr>
<tr>
<td>135</td>
<td>L, M, S</td>
<td>M</td>
<td>6</td>
<td>14</td>
<td>8</td>
</tr>
<tr>
<td>135</td>
<td>L, M, S</td>
<td>S</td>
<td>43</td>
<td>124</td>
<td>81</td>
</tr>
</tbody>
</table>

Note: The expected morph ratios produced by the floral morphs in each population after intramorph and intermorph mating as well as the observed progeny morph ratios. All predicted morph ratios were estimated using the assumption that double reduction equaled 0.10 at the M-locus and 0.025 at the S-locus and were weighted by the progeny produced per genotype in each floral morph.

We translated these equations into a script for the “mle” function in the R package “stat4” in R version 3.3.2 (R Core Team 2016), which uses the log-likelihood values of the χ² test to predict the mean and 95% confidence intervals of d based on the observed and predicted morph ratios. We present the predicted offspring, which we used for the mle function in Table 2.

Mating and fertility of isolated plants

In the summer of 2014 we selected 19 plants of each floral morph for use in a field experiment at the Koffler Scientific Reserve (KSR) near Newmarket, Ontario, Canada (44.0309N, 79.5389W). The plants were previously screened for partial self-incompatibility in the glasshouse and exhibited the typical pattern in which plants of the M-morph set variable numbers of fruit after self-pollination, and the L- and S-morphs set few if any fruit (Barlozh and Barrett 2018). We grew these individuals in the glasshouse in pots 21.6 cm diameter by 21.6 cm height (8.5 × 8.5 inches) containing Promix BX soil with Osmocote slow-release fertilizer (Scotts Miracle-Gro Company, Marysville, Ohio, USA) added as per the manufacturer’s instructions, until they were transferred to the field.

We moved the 57 plants to KSR in early July 2014 and placed them individually 50–85 m apart along mowed paths in two large old fields. We transferred plants in these pots into pots 35.5 cm diameter × 26.3 cm height (14” × 10.4” inches) that were sunk into the soil for stability, and we surrounded the outer pots with clear plastic to maintain moisture levels. We evenly assigned plants of the L-, M-, and S-morph among the 57 locations distributed across the two fields and randomly assigned individuals of the appropriate morph to each location (Supplementary data, Fig. S1). Before the experiment commenced, we removed any inflorescences that had developed prior to the plants being placed in the field.

On each plant we tagged two inflorescences and marked ~20 flowers on each with yellow or red paint at their base depending on treatment. A subset of flowers on each inflorescence were marked with yellow paint and allowed to be naturally pollinated (mean per inflorescence, 20.4 flowers; range, 5–45). On one inflorescence of half of the plants we additionally conducted hand-pollinations using a mixed compatible pollen donor (hand-pollination: mean per inflorescence, 14.2 flowers; range, 6–20) and marked these flowers with red paint. In late August, we removed all of the flowers from the plants before moving them back to a glasshouse at the University of Toronto. Ten plants did not survive the summer and are not considered further. Once fruit were mature we recorded the number of flowers in each treatment that had produced fruit and these were then harvested. Overall, we harvested 1216 open-pollinated fruit and 280 hand-pollinated fruit; a mean of 25.9 open-pollinated fruit per plant on 47 plants (15 L-, 16 M-, 16 S-morph) and 12.2 hand-pollinated fruit per plant on 23 plants (7 L-, 8 M-, and 8 S-morph).

We measured the number of seeds produced in each fruit. Based on the marked open- and hand-pollinated flowers on inflorescences which we hand-pollinated, we calculated the proportion fruit set, the mean number of seeds set per fruit, and multiplicative fertility calculated by proportion fruit set times mean seeds set per fruit after open- and hand-pollination. We then calculated an index of pollen limitation (PL) following Larson and Barrett (2000):

\[
(5) \quad PL = 1 - \frac{p_o}{p_s}
\]

where \(p_o\) and \(p_s\) are the measure of fruit or mean seeds set per fruit in marked open-pollinated and hand-
pollinated flowers for each plant and PL is an index ranging from 0–1 representing no pollen limitation (where there was no difference in the measure of fertility between open-pollinated and hand-pollinated flowers) to complete pollen limitation (where open-pollinated flowers set no seed) in the response variable. We set all of the negative values from this index to 0 because these results most likely resulted from experimental or statistical error (see Young and Young 1992; Burd 1994). We compared these pollen limitation indices for each output variable between the three floral morphs using ANOVA. Finally, because we did not hand cross-pollinate every plant (see above), we collected open-pollinated fruit and seeds set per fruit from all marked plants and inflorescences in the field (~40 marked flowers per plant). We tested the potential effects of hand pollination on resource allocation to open-pollinated fruits, as proposed by Zimmerman and Pyke (1988), by comparing the proportion of flowers setting fruit and the mean seeds per fruit between shoots on plants that received hand-pollination and those that did not (see Ågren and Ericson 1996 for a similar treatment on L. salicaria). There was no significant difference in reproductive measures between these treatments (proportion fruit set, \(P > 0.10\); mean seeds per fruit, \(P > 0.25\)) indicating that resource reallocation was not a significant factor in our experiment. For all of the plants in the study, we measured the proportion of marked open-pollinated flowers that set fruit and the mean seeds set per open-pollinated fruit in each of the floral morphs and compared these values among the morphs using ANOVA.

We inferred mating between plants in the experiment using progeny testing of L- and M-morph families. We chose these morphs because unambiguous inferences concerning minimum levels of intermorph mating can be obtained from genotypes of these morphs but not the S-morph. We germinated seeds from 9 L-morph and 9 M-morph families, obtaining an average of 18 progeny from each plant (range, 8–30). When the 18 families flowered, we recorded the floral morph of each plant. We performed heterogeneity \(\chi^2\) tests on morph ratios of families grown from each parental morph to detect if there were differences among individual plants in progeny ratios. We checked L-morph families for M- and S-morph progeny and M-morph families for S-morph progeny, which in both cases unambiguously indicate intermorph mating.

**Results**

**Intermorph mating in natural populations**

Of the 147 open-pollinated families from six natural populations of L. salicaria used to estimate intermorph mating, we unambiguously identified the parental genotypes of 109 using \(\chi^2\) tests (Supplementary data, Table S3). Our maximum likelihood estimates of intermorph mating in trimorphic and dimorphic populations in general were not substantially different from expectations predicted by complete intermorph mating and there was no significant difference between the M- and S-morph in estimated values of \(d\) (Fig. 2). However, there were several cases where deviations from intermorph mating were evident. In populations 84, 92, and 135 L-morph plants experienced a low level of selfing and (or) intramorph mating and in population 153 the L-morph reproduced almost exclusively through selfing and (or) intramorph mating.
population 100, we detected an excess of M-morph progeny in L-morph families resulting in an estimate of $d$ slightly greater than 1 ($d = 1.09, CI = 1.02–1.17$). In populations 84 and 92, the 95% CI of $d$ for the L-morph did not overlap with the 95% CI for the M- and S-morphs. In the monomorphic L-morph population (153), 48 of the 50 progeny were of the L-morph, a result consistent with a high degree of intramorph mating and (or) selfing ($d = 0.04, CI = -0.15–0.23$). In populations 68 and 100, both of which lacked the S-morph, we found a single S-morph progeny in one maternal family in each population indicating gene flow from nearby populations. Similarly, the occurrence of a single M- and S-morph progeny in population 153 also indicates gene flow from a neighbouring population.

Mating and fertility of plants at low density

Progeny tests of open-pollinated families of L- and M-morph plants provided unequivocal evidence of intermorph mating. We found that 23% and 11% of the total progeny produced by L- and M-morph plants ($N = 200$ and 127, respectively; Supplementary data, Table S41) were of the S-morph, and 24% of L-morph progeny were of the M-morph. Because genotypes of the L- and M-morph cannot produce S-morph plants, and the L-morph cannot produce the M-morph, except by intermorph mating, these offspring must have arisen from pollen dispersal among plants in the fields. We also detected significant heterogeneity in the progeny morph ratios in families from L- and M-morph parents (L-morph: $\chi^2 = 40, df = 16, P < 1 \times 10^{-3}$; M-morph: $\chi^2 = 30, df = 16, P < 0.05$) reflecting variation in the paternity of offspring produced in each plant and possibly some variation in the genotypes of M-morph parents represented in the experiment.

Isolated plants in our field experiment experienced significant pollen limitation for all three measures of fertility. The index of pollen limitation for fruit set and seeds set per fruit of open-pollinated flowers was 0.25 and 0.56, respectively, with little variation among morphs (Fig. 3). The pollen limitation index based on the multiplicative measure of seeds set was 0.59 and there were no significant differences among the floral morphs in the intensity of pollen limitation (Fig. 3). There was also no evidence of differences among the floral morphs in the proportion of open-pollinated flowers setting fruit, the mean seeds set per fruit, or the per plant multiplicative fertility (Fig. 4).

Discussion

The primary goal of this investigation was to elucidate the influence of floral morph ratios and low plant density on mating and fertility in invasive *L. salicaria*. Two features of this species motivated our study. First, because *L. salicaria* has prolific colonizing ability, populations display a wide range of both population size and floral morph composition (Eckert and Barrett 1992; Ågren and Ericson 1996; Eckert et al. 1996a, 1996b).}

![Fig. 3. Index of pollen limitation (PL) based on open- and hand-pollinated fruit and seed set (see the section on Materials and methods) for isolated plants of *Lythrum salicaria* in a field experiment. (A) Proportion of fruit set, (B) mean seeds per fruit, and (C) multiplicative fertility calculated as the proportion of fruit set multiplied by mean seeds set per fruit. The floral morphs did not differ in proportion of fruit set, mean seeds per fruit, or multiplicative fertility. However, all values were significantly greater than zero, indicating significant pollen limitation for all three indices of pollen limitation.](image-url)
Using progeny tests of maternal families and measure of fruit and seeds set in two demographic contexts — natural populations and a field experiment — we evaluated the sensitivity of several reproductive parameters to morph ratio bias and spatial isolation. We found that intermorph mating predominated in all five polymorphic (trimorphic, dimorphic) populations, regardless of their floral morph ratios. Also, in our field experiment on plant isolation intermorph mating was frequent because of extensive bee-mediated pollen dispersal among plants. Although individuals in the experiment suffered moderate pollen limitation (mean PL = 0.59 for multiplicative fertility), there was no evidence that its intensity varied among the floral morphs owing to morph-specific partial self-incompatibility. Earlier studies on pollen limitation in *L. salicaria* provided mixed results concerning whether morph-specific effects were evident and which morphs were susceptible (O’Neil 1992; Ågren and Ericson 1996; Waites and Ågren 2004). In the ecological contexts that we investigated, we found no strong evidence that partial self-incompatibility in the M-morph of *L. salicaria* plays a significant role in affecting mating patterns, or in alleviating pollen limitation at low density. We now consider the implications of our results for the reproductive ecology of invasive populations of *L. salicaria* and in explaining the patterns of floral morph ratios reported in native and introduced populations.

**Mating in natural populations of varying morph composition**

Using the progeny test method, we found that the vast majority of mating in trimorphic and dimorphic populations of *L. salicaria* involved intermorph cross-fertilization and, concomitantly, that selfing and intramorph mating occurred at very low levels (Fig. 2). This indicates that trimorphic incompatibility functions effectively to promote disassortative mating, regardless of the pollen loads delivered to stigmas by insect pollinators, which may contain a significant amount of intramorph pollen (Mulcahy and Caporello 1970; Waites and Ågren 2004; Costa et al. 2017). The resilience of intermorph mating, despite deviations from isoplethy (three of five polymorphic populations had anisoplethic morph ratios — populations 84, 92, and 135, see Table 1), should promote negative frequency-dependent selection, and over time populations are predicted to proceed from biased morph ratios to a phenotypic equilibrium involving isoplethy (Fisher 1941; Heuch and Lie 1985). However, both theoretical and empirical studies of the dynamics of morph ratios in populations of tristylous species (e.g., Morgan and Barrett 1988; Eckert and Barrett 1992, 1995) indicate that the time that this takes can be quite protracted, despite increases in population size and persistent frequency-dependent mating. These studies indicate that the composition of founding genotypes, population structure, features of life history, and patterns of sexual recruitment can each slow progress to the isoplethic equilibrium.

Whereas progeny tests of the M- and S-morph in polymorphic populations generally revealed high rates of intermorph mating (Fig. 2), the small excess of L-morph progeny from L-morph parents in populations 84, 92, and 135 indicated a low rate of intramorph mating and (or) selfing, which cannot be distinguished using the progeny test method used here. This result may help to explain the empirical observation that the L-morph is the most common morph in tristylous populations, based on extensive surveys of natural populations of *L. salicaria* (overall average frequencies from surveys: L-morph = 0.379, M-morph = 0.296, S-morph = 0.338; population count $N = 558$, plant count $n = 83 885$; Heuch 1979a; Anderson and Ascher 1995; Ågren and Ericson 1996; Eckert et al. 1996a; Balogh and Barrett 2016; Costa et al. 2016 and references cited in these sources).
tramorph and (or) self-mating in the L-morph produces only L-morph progeny because this morph is homozygous recessive at the S- and M-loci controlling tristyly. In contrast, all of the other tristylos genotypes segregate two or three floral morphs from intramorph mating. In a deterministic model of morph-frequency dynamics, Heuch (1979b) calculated that with rates of intramorph mating of 8.4%–11% the average observed frequency of the L-morph in European populations could be obtained. In extreme cases, such as tristylos Narcissus triandrus in which intramorph mating is permitted, it has been shown that high rates of assortative mating in the L-morph cause this morph to predominate in populations throughout the geographical range of the species (Barrett et al. 2004; Hodgins and Barrett 2008). Thus, low levels of selfing or assortative mating, as observed in our study, could explain the generally higher frequency of the L-morph in tristylos populations of L. salicaria.

Progeny tests of a small number of L-morph families in the single monomorphic population in our study revealed that all progeny except for two were of the L-morph. This result indicates that the observed seed produced in this population arises almost exclusively from selfing and (or) intramorph mating and suggests that partial self-incompatibility in the L-morph can play a role in maintaining populations in which cross-compatible floral morphs are absent. Although monomorphic populations of L. salicaria are infrequent, a survey of 114 invasive populations in Ontario revealed the occurrence of eight populations that were composed exclusively of the L-morph and one population that contained only the M-morph (Balogh and Barrett 2016). Partial self-incompatibility may function to maintain monomorphic L-morph populations whereas monomorphic M-morph populations are likely to segregate L- and M-morph progeny (Fisher and Mather 1943). However, occasional long-distance pollen flow events have the potential to convert monomorphic to polymorphic populations and the finding of single mid- and shortstyled offspring in families from population 153 provides evidence for this process. Depending on the spatial isolation of populations and the degree of long-distance seed flow, monomorphic floral morph structure may be quite transient and this may account for its general rarity in L. salicaria.

Partial self-incompatibility in the M-morph was not a significant factor influencing mating patterns in the present study but may provide reproductive assurance in this morph during colonizing events. Previous studies of morph-frequency variation found that ~25% of L. salicaria populations in Ontario were dimorphic containing the L- and M-morphs (Eckert and Barrett 1992; Balogh and Barrett 2016). Selfing and (or) assortative mating of M-morph founders with partial self-incompatibility may be responsible for the origin of some of these populations, as the vast majority of genotypes of the M-morph are heterozygous and would segregate L- and M-styled plants (Heuch and Lie 1985). However, there is no evidence that the frequency of partial self-incompatibility in plants of the M-morph of L. salicaria varies across the geographical gradient of invasion in Ontario (Colautti et al. 2010), as one might predict if selfcompatibility provided reproductive assurance at low density in isolated populations at the range front of the biological invasion (Baker 1955; Barrett 2011; Pannell 2015). Nevertheless, it is too early to discount the role of partial self-incompatibility in enabling the establishment of colonies following dispersal of M-morph colonists and more studies of mating in small dimorphic populations would be valuable to address this question.

Our data suggest that following the establishment of dimorphic populations, intermorph mating may increase in frequency leading to isoplethic L- and M-morph ratios. Indeed, this may have occurred in dimorphic populations 68 and 100 in which morph ratios are close to being equal in frequency. Significantly, of the 41 dimorphic populations so far reported from surveys in Ontario, 26 possess isoplethic morph ratios (Eckert and Barrett 1992; Balogh and Barrett 2016) indicating that tristyly can still function effectively in promoting high rates of disassortative mating despite morph loss. Mating patterns in colonizing populations of L. salicaria may be quite dynamic, changing rapidly from the establishment phase to consistent intermorph mating if compatible mates arise in progeny or by gene flow. Thus, partial self-incompatibility in the M-morph may provide reproductive assurance for only a few generations during the establishment phase in dimorphic populations. In contrast, in populations composed of the L-morph, partial self-incompatibility may enable sexual reproduction to continue for many generations until migrants of the M and S-morph arrive by seed or pollen flow.

**Plant isolation and long-distance pollen flow**

The patterns of inheritance in tristylos populations provide an opportunity for the detection of long-distance pollen flow using open-pollinated families because maternal seed families of the L- and M-morph cannot produce plants of the S-morph without crossing with an S-morph paternal individual. Similarly, the L-morph cannot produce M-morph progeny after intramorph mating (Fisher and Mather 1943). Simulation models indicate that rates of gene flow of approximately $m = 0.05$ generation can maintain trimorphism in over 95% of populations with $N > 10$ individuals over the course of 100 years (Eckert et al. 1996a). In our study LM-dimorphic populations 68 and 100 experienced a minimum migration rate, based on the frequency of S-morph progeny, of $m = 0.001$ and 0.002, respectively, and the L-morph monomorphic population exhibited a minimum $m = 0.042$ based on the presence M- and S-morph progeny. These estimates of $m$ using the tristyly loci should be considered minimum estimates that likely underestimate the true level of gene flow.
flow among individuals and populations. A major future challenge given the autotetraploid status of invasive populations of *L. salicaria* will be to develop hypervariable molecular markers to estimate mating parameters and gene flow.

Most long-distance pollen flow studies that have screened for genetic markers in progeny have focused on forest trees. However, several relevant studies have been conducted in herbaceous plants (e.g., *Raphanus sativa*, *Solanum tuberosum*, *Skogsmyr 1994*; *Primula elatior*, van Rossum et al. 2011) and have provided evidence for mating events at ~151 m (*Primula elatior*) to over 1 km (*Raphanus sativa*) from paternal parents. Our results on intermorphic mating from the plant isolation experiment indicated that moderate rates of pollen dispersal at distances of 50–85 m were common and largely mediated by *Bombus* spp., which we frequently observed visiting flowers of isolated plants. In the natural populations of *L. salicaria* that we investigated, we recorded rare instances of gene flow events exceeding 1 km, based on the distance to the nearest populations to the focal populations used in progeny testing. Once again we commonly observed *Bombus* visiting flowers in these populations for nectar and pollen. Long-distance *Bombus* foraging from 200 m to 1.5 km has been reported in the literature (Osborne et al. 1999; Walther-Hellwig and Frankl 2000; Knight et al. 2005) and given that this taxon is a major visitor to *L. salicaria* populations, our results on gene flow distances are not especially surprising.

Isolated flowering individuals of *L. salicaria* that we did not observe near our focal populations may have also been the source of pollen responsible for the rare gene flow events we recorded in populations 63, 100, and 153. This possibility points to the need for detailed surveys of isolated individuals in studies of interpopulation gene flow in plants, a concern raised by Levin (1995) in emphasizing the role of plant isolates in acting as gene flow bridges between populations. In future, high resolution genetic markers and detailed mapping approaches would be useful for thoroughly investigating the frequency and landscape-level scale of gene flow in invasive populations of *L. salicaria*, particularly from isolated plant outliers that commonly occur in this species.

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