SHORT COMMUNICATION

Reproductive isolation and hybrid pollen disadvantage in *Ipomopsis*

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Abstract

One cause of reproductive isolation is gamete competition, in which conspecific pollen has an advantage over heterospecific pollen in siring seeds, thereby decreasing the formation of F1 hybrids. Analogous pollen interactions between hybrid pollen and conspecific pollen can contribute to post-zygotic isolation. The herbaceous plants *Ipomopsis aggregata* and *I. tenuituba* frequently hybridize in nature. Hand-pollination of *I. aggregata* with pollen from F1 or F2 hybrids produced as many seeds as hand-pollination with conspecific pollen, suggesting equal pollen viability. However, when mixed pollen loads with 50% conspecific pollen and 50% hybrid pollen were applied to *I. aggregata* stigmas, fewer than half of the seeds had hybrid sires. Such pollen mixtures are frequently received if plants of the two species and F1 and F2 hybrids are intermixed, suggesting that this advantage of conspecific over hybrid pollen reduces backcrossing and contributes to reproductive isolation.

Introduction

One mechanism of reproductive isolation involves gamete competition. In some animals, conspecific sperm has an advantage over heterospecific sperm in fertilizing eggs (Price, 1997; Howard *et al.*, 1998). Similarly, in some flowering plants, conspecific pollen has an advantage over heterospecific pollen in fertilizing ovules (Carney *et al.*, 1994; Rieseberg *et al.*, 1995; Arnold, 1997). The idea that conspecific pollen has a competitive advantage is not new (Darwin, 1859), but recent authors have particularly emphasized its importance as a major cause of reproductive isolation in nature (Arnold, 1997; Howard, 1999). In plants, such 'conspecific pollen precedence' is typically demonstrated by placing pollen from two species together on a stigma and using genetic markers to identify the sire of the seed. A lower than expected proportion of hybrid offspring indicates conspecific pollen precedence, which is a potential form of post-mating, but prezygotic, reproductive isolation that restricts the formation of F1 hybrids.

Here we illustrate how similar kinds of gametic competition could also result in post-zygotic isolation, by influencing reproductive fitness of hybrids and thereby formation of advanced generation hybrids. Well-recognized forms of post-zygotic isolation in plants include reduced survival of hybrids and hybrid sterility (Stebbins, 1958). Sterility need not be complete; hybrids that are fertile and make some pollen may have low male fertility if they exhibit reduced pollen viability or sire fewer seeds on particular kinds of mates. There is a long tradition of using pollen viability as an indicator of hybrid performance (Stebbins, 1950; Smith & Daly, 1959), and in some cases the genetic basis of reduction in viability has been characterized (Tadmor *et al.*, 1987; Rieseberg *et al.*, 1999). It is, however, also possible that hybrid pollen would perform poorly in competition when present in mixed pollen loads on the stigma, although performing similar to conspecific pollen in single donor crosses. In this scenario, there is conspecific pollen precedence, as previously described for mixtures of pollen from two species. However, in this case the pollen

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precedence would constitute post-zygotic isolation that reduces formation of backcross progeny, and could influence the extent of introgression and subsequent evolution in a hybrid zone.

In this study, we examine two forms of post-zygotic isolation between plants of *Ipomopsis aggregata* (Pursh) V. Grant and *I. tenuituba* (Rydb.) V. Grant: the traditional mechanism of reduced pollen fertility of hybrids, and the novel mechanism of conspecific pollen precedence over hybrid pollen. These two species hybridize in many areas in the western US (Grant & Wilken, 1988), including Poverty Gulch in Gunnison County, CO. That contact site has been the focus of several studies of reproductive isolation. Conspecific pollen shows no precedence when mixed with pollen from the other species (Alarcón & Campbell, 2000). However, following application of three-way mixtures of pollen to stigmas, pollen from natural hybrids of unknown ancestry sired fewer seeds than pollen from either *I. aggregata* or *I. tenuituba* (Melendez-Ackerman & Campbell, 1998). This result suggests hybrid pollen disadvantage. To establish its significance in nature, however, we must first quantify how often pollen loads of various mixtures land on stigmas, and secondly examine performance of the pollen in those kinds of mixtures that are commonly received. Ideally, this is done using plants of known genetic background (Rieseberg & Carney, 1998). Although the first kind of information is generally unknown, we do have data on pollen mixtures for this system. When plants of the two species and hybrids were intermingled in equal proportions and exposed to pollinators, *I. aggregata* and hybrid pollen were by far the most common mixture of pollen found on stigmas (Campbell *et al.*, 2002). On flowers of *I. aggregata*, this particular mix was five times as common as any other mix, three times as common as hybrid pollen alone and more common than pure conspecific pollen of *I. aggregata*. We now embark on the second step. As pollen mixtures were overwhelmingly of one type, we examine whether conspecific pollen of *I. aggregata* has precedence when presented on the stigma in mixture with hybrid pollen of known genetic background (F1 or F2).

Materials and methods

Study organisms

Ipomopsis aggregata and *I. tenuituba* plants were potted from the vicinity of Poverty Gulch (populations L and A–C, respectively, in Campbell *et al.*, 1997) and maintained at the Rocky Mountain Biological Laboratory. The flowers are self-incompatible and protandrous. At Poverty Gulch, hummingbirds are usually the most frequent pollinators of both species of *Ipomopsis*, and they easily transfer pollen between the species (Campbell *et al.*, 1997, 1998). They prefer to visit flowers of *I. aggregata* and hybrids, and make frequent transitions between those types. Computer simulations suggest this behaviour is the reason that mixtures of *I. aggregata* and hybrid pollen are common on stigmas (Campbell *et al.*, 2002).

We generated the necessary hybrids by hand-pollinating. The F1 hybrids were products of crosses made in 1994 and 1995 between plants from populations L and C, and included cases of both species as the maternal parent. The F2 hybrids were products of crosses made in 1998. Plants were maintained in paper pots, kept during the summers in a Weatherport and sunk into the ground to overwinter.

Experimental crosses

In 1999, we performed hand-pollinations with a 50 : 50 mix of F1 and *I. aggregata* pollen onto *I. aggregata* recipient plants. Insufficient F1 with known genotypes were available to perform pollinations on F1 recipients. Pollen mixtures were prepared by weighing pollen from freshly dehisced anthers, as mass correlates well with pollen number (Alarcón & Campbell, 2000). All plants were homozygous for one of three alleles (S, M or F) at the slower migrating locus of 6-phospho-glucose dehydrogenase (assay methods in Campbell & Dooley, 1992). We tested six different pairs of pollen donor plants. Each pair consisted of one *I. aggregata* and one F1 hybrid, homozygous for different alleles, either S or M. These SS and MM genotypes do not differ in siring success (Alarcón & Campbell, 2000). Each donor pair was tested on 12–15 recipient plants. We used two different sets of plants for the donors and the recipients to avoid self-pollination. On each recipient, we performed two replicate mixed pollinations on pistillate-phase flowers for each of the six donor pairs, and, as controls, two replicate single-donor pollinations for each donor plant, unless there were insufficient flowers. This procedure generated six cross groups, each with a mixed pollination treatment, a hybrid pollen treatment, and an *I. aggregata* pollen treatment. Seeds were collected and counted from all pollinations. For fruits resulting from mixed pollinations, we genotyped each seed at the 6-PGD locus.

In 2000, we performed an analogous experiment using 50 : 50 mixtures of F2 and *I. aggregata* pollen. We tested eight pollen donor pairs, each consisting of one F2 and one *I. aggregata* plant. All pairs had plants with different genotypes at the 6-PGD locus that allowed unambiguous assignment of paternity (e.g. MM and FS donors on SS recipient). Each of the eight types of mixed pollinations was performed on three to 10 recipient plants. As in 1999, we also performed single donor control pollinations using each of the 16 donors, to yield eight cross groups, each with three cross types. We distributed replicates of each of the 24 crosses across pollination dates, to avoid possible bias introduced by performing crosses at particular times of the blooming

season. In total we used 12 recipient *I. aggregata* plants, all of which were SS or MM homozygotes potted from the field.

Statistical analysis

In evaluating the performance of hybrid pollen in competitive mixtures, we treated each pollen donor pair as one replicate. This procedure avoided pseudoreplication caused by correlations between performance of the same donor in multiple pollinations or on multiple recipients. For each donor–recipient combination we first calculated the proportion of the seeds that were sired by the hybrid donor. We then averaged those values across the multiple recipient plants to obtain a single proportion for each of the 14 donor pairs tested (six F1 and eight F2). We combined results from the two experiments as proportions sired by the hybrid donor were very similar for the 1999 F1 and 2000 F2 experiments (see Results). The 14 values were then compared with the null expectation of 0.5 in the absence of conspecific pollen precedence, using a one-sample *t*-test. As some hybrid plants employed as pollen donors were related, we supplemented this analysis with a conservative one-sample *t*-test, using the mean proportion averaged across the two pollen donors in those cases where two donors were full sibs.

We also compared the mean seed production across the three types of crosses represented in each cross group: *I. aggregata* pollen alone, hybrid pollen alone and the 50 : 50 mixture. For the single donor crosses, higher seed production from *I. aggregata* pollen than from hybrid pollen would indicate a loss of male fertility unrelated to pollen competition. We analysed both mean seeds per flower (i.e. including cases of abortion leading to zero seeds) and mean seeds per fruit (not including cases of zero seeds). For each of the six cross groups in 1999 and eight cross groups in 2000, we first determined the mean seeds per fruit (or flower) for each of the three types of crosses on each recipient plant. We then averaged these values across the recipients to yield mean values for the 14 cross groups × three cross types. This design resembles a randomized block design in that the three cross types are blocked by cross groups. However, the cross groups are of two types, involving F1 pollen in 1999 and F2 pollen in 2000. We employed Proc Mixed in SAS (Ver. 6.12) to implement a model with fixed effects of cross type (*I. aggregata*, hybrid or mixed) and year of the experiment, and a random effect of cross group nested within year. We also specified an *a priori* contrast between the two kinds of single donor pollinations.

Results

Of the 544 seeds resulting from mixed pollinations with F1 and *I. aggregata* pollen, 231 (or 42%) had F1 sires. Of the 450 seeds from mixed pollinations with F2 and *I. aggregata* pollen, 173 (or 38%) had F2 sires. Both types of hybrid donors usually sired fewer than their share of seeds in mixed pollinations with conspecific *I. aggregata* pollen (Fig. 1). A one-way ANOVA detected no difference between the mean proportion sired by the six F1 pollen donors (0.44) and the eight F2 pollen donors (mean = 0.40, $F_{1,12} = 0.64$, n.s.). Overall, the mean proportion sired by hybrids was 0.414, which differed significantly from the null expectation of 0.5 ($t = 3.155$, d.f. = 13, $P < 0.01$) with a 95% confidence interval of 0.35–0.47. A conservative test allowing for possible correlations between the performance of full sib hybrid pollen donors still detected significantly lower than 0.5 hybrid siring success ($t = 2.825$, d.f. = 7, $P < 0.05$).

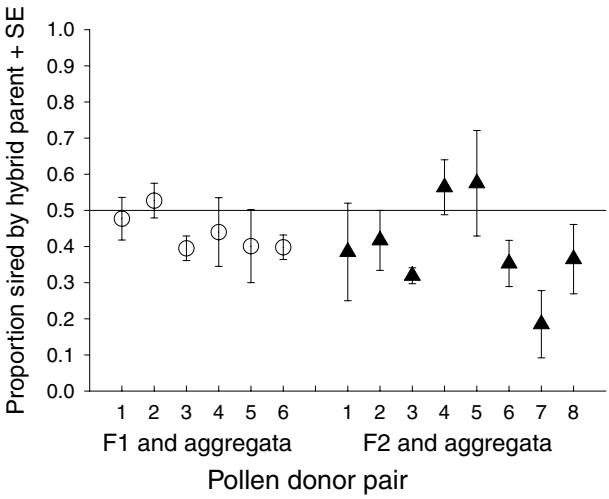


Fig. 1 Proportion of seeds sired by the hybrid parent following pollination with a mixed load. For each of six pairs of F1 and *I. aggregata* pollen donors and eight pairs of F2 and *Ipomopsis aggregata* pollen donors, we plotted the mean and SE across all recipient plants that set seeds. The number of recipients ranged from eight to 11 for the pollinations involving F1 and from two to five for the pollinations involving F2 hybrids. These numbers are lower than the number of recipient plants pollinated (three to 15) because of fruits that failed to set seed. The horizontal line at 0.5 depicts the expectation under the null hypothesis that hybrid and conspecific pollen are equally likely to sire a seed.

In contrast, when hybrid pollen was applied alone it sired as many seeds as did conspecific *I. aggregata* pollen by itself (Fig. 2a and b). Combining all of the results in a mixed model ANOVA, there was no detectable effect of cross type on mean seeds per fruit ($F_{2,24} = 0.50$, n.s.) or mean seeds per flower ($F_{2,24} = 0.75$, n.s.). Nor did *a priori* contrasts detect any difference between the *I. aggregata* and hybrid pollen treatments ($P = 0.3394$ for mean seeds per fruit and $P = 0.5696$ for mean seeds per flower). If anything, the trend was for higher seeds per flower for the hybrid pollen treatment than for the conspecific pollen treatment (Fig. 2b). Analysis of the two experi-

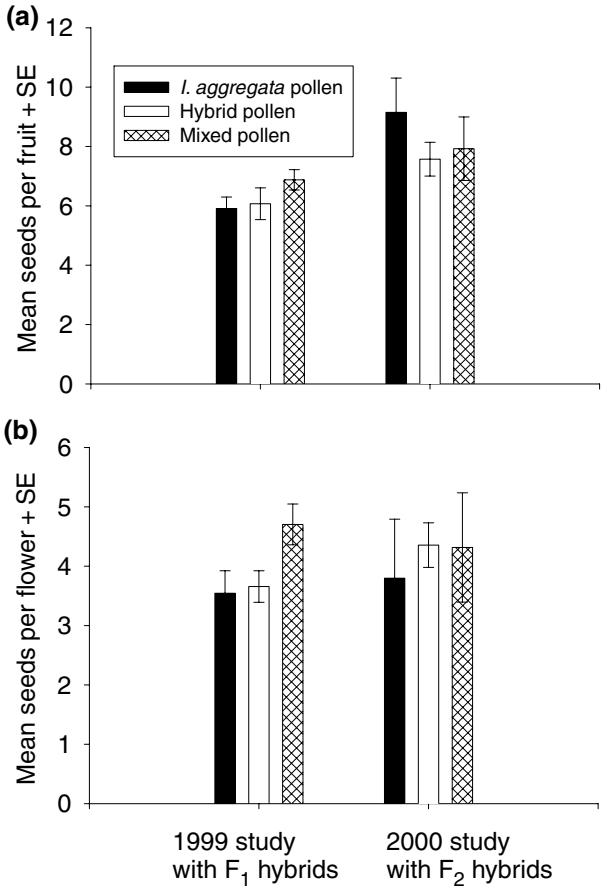


Fig. 2 Seed production from crosses with *Ipomopsis aggregata* pollen, hybrid pollen and a 50 : 50 mix of pollen. For each of the two variables, mean and SE are taken across the six cross groups in 1999 and eight cross groups in 2000. (a) Mean seeds per fruit. (b) Mean seeds per flower including pollinations that generated zero seeds.

ments separately, using randomized block ANOVA, also detected no overall effect of cross type for either mean seeds per fruit or seeds per flower (all $P > 0.05$). Seeds per fruit were, however, significantly higher in the 2000 than in the 1999 crosses (Fig. 2a; $F_{1,12} = 8.04$, $P < 0.05$).

Discussion

Male sterility in hybrid plants is typically studied by examining pollen production and pollen viability. In these aspects of reproductive performance, hybrids between *I. aggregata* and *I. tenuituba* from Poverty Gulch, Colorado suffer no obvious disadvantage. Two previous studies examined pollen production of natural hybrids from this region. Meléndez-Ackerman & Campbell (1998) detected no differences in pollen production among *I. aggregata, I. tenuituba* and natural hybrids collected from the hybrid zone. Campbell *et al.* (1998) found similar pollen production by flowers of *I. tenuituba* and hybrids, although both types of plants produced slightly less than *I. aggregata*. Moreover, in the single donor pollinations performed here, application of F1 or F2 hybrid pollen led to as many seeds as did application of conspecific *I. aggregata* pollen alone, indicating similar pollen viability.

However, male fertility of hybrids can be impaired in more subtle ways. In this hybrid zone, reproductive isolation is generated in part by a novel mechanism in which conspecific pollen has precedence over hybrid pollen, although it has no precedence over heterospecific pollen. When plants were pollinated with a 50:50 mix of pollen from the two species, heterospecific pollen sired 51.5% of the seeds on average (Alarcón & Campbell, 2000). In contrast, following application of a three-way mix of pollen from *I. aggregata, I. tenuituba* and natural hybrids, hybrid pollen sired the fewest seeds, just 26–74% (depending on the type of recipient plant) as many seeds as expected if all three donors had equal success (Meléndez-Ackerman & Campbell, 1998). Here we showed further that hybrid pollen suffers a competitive disadvantage when present in the kind of stigmatic mixture (*I. aggregata* and F1 or F2 hybrid pollen) that is most commonly received in this system (Campbell *et al.*, 2002), indicating the phenomenon can be of importance in a natural setting. In these mixtures, F1 pollen averaged 88% and F2 pollen 80% of the null expectation (0.4 = 80% of the null value of 0.5).

The relatively poor performance of hybrid pollen in mixture could have two general consequences. First, it should impact hybrid fitness by decreasing male reproductive success of hybrids, below the level predicted by the amount of pollen donated to stigmas. Secondly, it should influence reproductive isolation and introgression by reducing the formation of backcross progeny by *I. aggregata* recipients, below the high level predicted by amounts of pollen transferred (Campbell *et al.*, 2002). As these pollen interactions appear only after initial formation of F1 hybrids, they affect only post-zygotic reproductive isolation. However, it is important to keep in mind that both reproductive isolation and hybrid fitness are potentially influenced by a large number of other mechanisms as well. We have shown elsewhere that the incomplete reproductive isolation between these species of *Ipomopsis* also involves prezygotic isolation, due primarily to visitation patterns of pollinators (Campbell *et al.*, 2002). And hybrid fitness depends also on environment-specific survival, seed production, and how much pollen is dispersed by pollinators (Campbell & Waser, 2001; Campbell *et al.*, 2002).

Establishing the importance of gamete interactions in natural hybrid zones requires two kinds of information. Although pollen and sperm competition take place post-mating, their importance depends on mating events, and both kinds of processes need to be investigated for assessing the importance of these cryptic forms of reproductive isolation. For example, in flowering plants

we need behavioural and ecological studies of pollinator movements and ideally pollen transfer to quantify the frequencies of different kinds of pollen mixtures received by stigmas. Such information is rarely available (Campbell *et al*., 2002), but in those cases where visitation to hybrids has been studied, hybrids received as many visits as at least one parental species, suggesting that mixed stigmatic loads of hybrid and conspecific pollen could be common (Wesselingh & Arnold, 2000). Information on stigmatic loads can be integrated with studies employing genetic markers to establish the success of certain kinds of pollen in siring seeds. To date, the usual emphasis has been on testing mixtures of conspecific and heterospecific pollen applied by hand to stigmas (reviewed in Arnold, 1997). Relying on this approach alone would have led to a false conclusion that pollen interactions are of no importance at this *Ipomopsis* contact site. Yet in this system, stigmas are more likely to receive mixtures of hybrid and conspecific pollen, and hybrid pollen is at a competitive disadvantage to conspecific pollen.

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