

working title Compatibility system and stigma size of pollen recipient as main predictors of heterospecific pollen effect

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Pollinator sharing can have negative consequences for species fitness with the arrival of foreign pollen. However, the costs of heterospecific pollen are not yet well understood. For this reason, we have conducted a glasshouse experiment where we try to understand how phylogenetic relatedness and the different traits of these species are involved in this process. We experimentally crossed 10 species belonging to three different families: Brassicaceae, Solanaceae and Convolvulaceae. Overall, more than 4000 crosses were done and seed set and pollen tubes were considered as proxy of effect. We found that for all species foreign pollen (50% or less) reduced seed set. Moreover, the seed set reduction is not dependent on the degree of relatedness of the pollen donor. However, the effect is governed by the degree of relatedness and the traits of the species recipient. Our results show that the outcome of heterospecific pollen deposition is determined in greater degree by the traits of the pollen recipient than the pollen donor and that certain traits such as compatibility system are crucial to understand the costs of heterospecific pollen.

Keywords: heterospecific pollen, plant reproduction, fitness, interspecific competition, phylogenetic distance.

INTRODUCTION

Paragraph 1 General idea to our concept

In natural systems plant species normally coexist and share their floral visitors with other species Bascompte et al. (2003). This pollinator sharing from the plant perspective at the pre-pollination stage can be negative due to competition Pauw (2013) or positive due to facilitation Carvalheiro et al. (2014). Once the floral visitor has arrived to the flower, pollen deposition on the stigma can take place and hence ovule fertilization. An increasing number of visits generally correlates with higher chances of

35 fertilization Engel and Irwin (2003). However this is not always the case, among these possible flower
36 visitors we find also nectar robbers and pollen thieves Inouye (1980) and the quality of pollen that is
37 deposit on the stigma is also highly relevant to the pollination succes Aizen and Harder (2007).
38 Moreover, other less study issues in the pollination process are conspecific pollen loss and the arrival of
39 foreign pollen which can have important detrimental effects on species fitness Morales and Traveset
40 (2008) Ashman and Arceo-Gómez (2013).

41 **Paragraph 2** Introducing topic and knowledge gap

42 Recent studies have advanced in the ecological understanding of heterospecific pollen effect Morales and
43 Traveset (2008) Ashman and Arceo-Gómez (2013) Arceo-Gómez and Ashman (2016). A general
44 overview of foreign pollen arrival is that it can play an important role on species fitness but seems to be
45 context dependent and not always produce a decrease in fitness Morales and Traveset (2008). Part of
46 this unpredictability is due to the enormous variability of foreing pollen transferred in nature, where
47 levels between 0 and 75 percent are seen, but most commonly values ranges between 0 and 20 percent
48 of the total pollen load Bartomeus et al. (2008) Montgomery and Rathcke (2012) Ashman and
49 Arceo-Gómez (2013) Fang and Huang (2013), being the generalist species the ones that receive greater
50 loads of heterospecific pollen Fang and Huang (2013). Surprisingly, this low ranges of heterospecific
51 pollen have been shown to decrease fitness greatly Thomson et al. (1982). Although heterospecific
52 pollen quantity is fundamental to understand the outcome of the interaction so is the different traits of
53 both pollen donor and recipient. Ashman and Arceo-Gómez (2013) postulated the first predictive
54 framework for traits of heterospecific pollen effect, where different traits such as compatibility system
55 and pollen size among others seems to be crucial to understand foreing pollen effect. Moreover, in Tong
56 and Huang (2016) an assymetric effect was shown in a crossing experiment between 6 species of the
57 genus *Pedicularis* where the pollen of long styled species was able to grow the full length of the style on
58 short styled species but not viceversa. Despite these recent caveats, we still lack empirical evidence to
59 affirm what are the main traits that drive heterospecific pollen effect for both pollen donor and recipient
60 at seed production level. Interestingly, to comprehend how these traits interact is also crucial to look at
61 the phylogenetic relatedness of the species. There is a considerable amount of literature of crosses
62 between close related species Brown and Mitchell (2001) Arceo-Gómez et al. (2016) Tong and Huang
63 (2016) but few works focused on heterospecific pollen of far related species Thomson et al. (1982)

64 Galen and Gregory (1989) Neiland and Wilcock (1999) which also show a noteworthy fitness decrease.
65 Although the effect of close related species is predicted to be greater Ashman and Arceo-Gómez (2013)
66 the presence of pollen of non related species on multiple species Arceo-Gómez and Ashman (2016) and
67 the higher chances to coexist with a species that has less niche overlap (Ref) make foreign pollen from
68 far related species also an important subject of study in order to understand the importance of
69 heterospecific pollen in natural systems. Notwithstanding, the effect of heterospecific pollen of far and
70 close related species at community level remains to be explored beyond single pairwise interactions.

71 **Paragraph 3** Expanding ideas with examples

72 Interestingly, incompatibility system seems to play an important role in foreign pollen effect where
73 species that are self incompatible would have stronger barriers towards heterospecific pollen than self
74 compatible species Ashman and Arceo-Gómez (2013). The type of incompatibility, sporophytic or
75 gametophytic is related with the place of pollen recognition where the former take place at the stigma
76 level and the latter occurs within the style, this last late acting pollen recognition mechanism is
77 associated with greater negative effect Barrett (1988). Remarkably, there is a great variability in
78 mating systems across populations Whitehead et al. (2018) and therefore predict an effect of foreign
79 pollen is a bit obscured by the variability within species, however species that are strong selfers or
80 strong outcrossers have less variability in mating systems and predictions of effect could be more
81 realistic (see figure 1 from Whitehead et al. (2018)). Moreover, other traits such as number of pollen
82 grains per flower and number of ovules have been traditionally associated with the type of
83 incompatibility system where species with higher pollen ovule ratios are predicted to be xenogamous
84 and species with low pollen ovule ratios autogamous (REF). Selfer species would have a reduction of
85 herkogamy (REF) and less pollen production per ovule (REF) which can be interpreted as a
86 reduction of pollen exported into the community. Other morphological traits, like stigma size can be
87 determinant for the total pollen quantity that a stigma can receive and therefore related to do that
88 pollen size would also play an important role. Example with pollen here.

89 **Paragraph 4** Introducing our experiment

90 The great environmental variability in natural systems and complexity of floral structures make
91 heterospecific pollination studies a daunting task. Moreover, variation in sampling effort have been

shown to be determinant to characterize pollen transfer interactions Arceo-Gómez et al. (2018). Although plant-pollinator network and pollen network studies can give a first picture of the importance of foreign pollen is necessary to address how its effect is shaped with both traits and relatedness of the species. For this reason, in this study we have created an artificial co-flowering community with 10 species belonging to three different families with different traits where we try to test the following questions: 1) Does heterospecific pollen reduce seed set, if so, 2) Does heterospecific pollen effect depend on any floral trait? 3) Does heterospecific pollen effect depend on the relatedness of the species.

METHODS

The study was conducted in a glasshouse at University of New England (Armidale, Australia) from November 2017 to March 2018. Rooms were temperature controlled depending on the requirements of the species with day and night temperature differences. The species selected (Table 1) belonged to three different families, Solanaceae, Brassicaceae and Convolvulaceae. The criteria of species/family selection was based on close/distant related species (see phylogenetic tree for relatedness fig 1), heterogeneous traits, low structural flower complexity and fast life cycle. For the purpose of the experiment all the species were considered as pollen recipient and as pollen donor (see interaction matrix, fig 2). Species were watered once or twice per day and fertilized weekly (NPK 23: 3.95: 14).

Table 1

| Family | Genus | Species |
|----------------|----------|----------------------|
| Brassicaceae | Brassica | Brassica rapa |
| Brassicaceae | Brassica | Brassica oleracea |
| Brassicaceae | Eruca | Eruca versicaria |
| Brassicaceae | Sinapis | Sinapis alba |
| Convolvulaceae | Ipomoea | Ipomoea aquatica |
| Convolvulaceae | Ipomoea | Ipomoea purpurea |
| Solanaceae | Capsicum | Capsicum annuum |
| Solanaceae | Petunia | Petunia integrifolia |

| Family | Genus | Species |
|------------|---------|----------------------|
| Solanaceae | Solanum | Solanum lycopersicum |
| Solanaceae | Solanum | Solanum melongena |

109 Hand-pollination

110 Foreign pollen effect was studied through two different treatments, one with 50% conspecific pollen and
111 50% heterospecific pollen and a second one with 100% foreign pollen (N=10). Therefore, 180 different
112 combinations were performed with N=10. Seed set was the proxy of effect for all our treatments.
113 Moreover, hand cross pollination, hand self pollination, apomixis (bagged emasculated flowers) and
114 natural selfing were tested for each species (N=10). Flowers were emasculated the day prior anthesis
115 and hand pollinated next day with a toothpick. Hand-pollination was conducted with 3-4 gentle
116 touches on the stigma surface. The mixes of pollen were realized on an eppendorf based on the pollen
117 counts made with Neubauer chamber (each anther was counted 4 times for 20 different anthers per
118 species). In order to confirm that the treatments applied were 50-50 percent pollen, for each focal
119 species the total stigmatic load of pollen was counted from one donor of each family (N=3).

120 Traits and evolutive distance

121 The traits measured for each species were pollen per anther, number of ovules, stigma width and length
122 and stigmatic area, style width and length, ovary width and length. Moreover stigma type was tested.
123 Pollen was counted for 20 anthers of each species with 4 replicates per sample with an hemocytometer.
124 Previously anthers were squashed on a known solution with the pipette tip and homogenized with a
125 vortex for 30 seconds. Ovule number was counted with the help of an stereomicroscope and a small
126 grid over a petri dish from 15 randomly selected flowers. The different morphometrical traits were
127 measured with a digital stereomicroscope. Levels of self incompatibility were estimated by dividing the
128 the fruit set of hand self pollination by hand cross pollination Lloyd and Schoen (1992).

129 Analysis

130 We used the statistical language R (R Core Team 2018) for all our analyses. To test the effect of

131 heterospecific pollen, we subtracted to the seed set of hand cross pollination the seed set of
132 heterospecific pollen treatments. Therefore, small values mean low effect and viceversa. To be able to
133 compare among species, seed set was previously scaled with mean 0 and standard deviation of 1. In
134 order to see correlations between heterospecific pollen effect and traits we performed Mantel test
135 between the matrix of effect and the distance matrix of each trait (euclidean distances). Moreover,
136 Mantel test was also conducted between heterospecific pollen effect and the square root of the matrix of
137 phylogenetic distance due to improvement in the statistical power (Letten & Cornwell 2014). We
138 explored also the the relations between traits and heterospecific pollen effect through generalized mixed
139 models where the response variable was heterospecific pollen effect, the independent variable the
140 different traits and the random effects the different treatments per species. Moreover, pairwise evolutive
141 distances were calculated with MEGA7 for two kinds of markers: 1) Internal transcribed spacer (ITS)
142 and 2) ribulose-bisphosphate carboxylase (RBCL). The sequences of interest were downloaded from
143 NCBI GenBank and the phylogenetic tree constructed by maximum likelihood with MEGA7.

144 Phylogenetic signal of traits?

145 RESULTS

146 Results of hand cross pollination, self hand pollination, natural selfing and apomixis are presented in
 147 table 2. Heterospecific pollen reduced seed set significantly with the 50-50% heterospecific pollen
 148 treatments for 65% of the pairwise interactions $p < 0.05$. Across families we found a very similar effect
 149 but when species were looked at species level they responded differently even within the same family, for
 150 instance for two species of the Brassicaceae family *Brassica oleracea* and *Eruca versicaria* we found
 151 very contrasting effects of foreign pollen where for the first one, all donors reduce seed set significantly
 152 and for the second, just two species did out of nine. The 100% foreign pollen treatments barely
 153 produced seeds or fruits and just for *Sinapis alba* we did not find significant differences between the
 154 hand cross pollination and one treatment with pollen from a confamilial. Solanaceae species with berry
 155 fruit type developed small fruits or even normal fruits in some cases. *S. lycopersicum* seems to
 156 produced small fruits (35% of the treatments) independently of pollen and pollen donor due to also
 157 apomictic treatments did, never normal size. *C. annuum* produced some fruits (9%) of both small and
 158 normal size and finally *S. melongena* produced seedless normal fruits with just confamilial pollen (3%),
 159 for both species seems that fruit formation was induced by pollen on the stigma because of no
 160 production of fruit in apomictic treatments.

161 **Table 2.** Percentage of seeds produced per ovule for the ten species used in the experiment. The
 162 treatments presented are hand cross pollination, hand self pollination, natural selfing and apomixis
 163 (emasculated flowers).

| Species | Cross | Self | Natural_selfing | Apomixis |
|-------------------|-----------|------------|-----------------|----------|
| Brassica oleracea | 32.06897 | 0.0000000 | 0.00000 | 0 |
| Brassica rapa | 44.97041 | 0.0000000 | 0.00000 | 0 |
| Eruca versicaria | 23.75000 | 0.4166667 | 0.00000 | 0 |
| Sinapis alba | 43.33333 | 48.3333333 | 5.00000 | 15 |
| Ipomoea aquatica | 40.00000 | 30.0000000 | 20.00000 | 0 |
| Ipomoea purpurea | 31.66667 | 86.6666667 | 31.66667 | 0 |
| Capsicum annuum | 100.00000 | 66.2240664 | 23.48548 | 0 |

| Species | Cross | Self | Natural_selfing | Apomixis |
|----------------------|-----------|------------|-----------------|----------|
| Petunia integrifolia | 100.00000 | 24.7727273 | 0.00000 | 0 |
| Solanum lycopersicum | 90.38043 | 43.4782609 | 70.00000 | 0 |
| Solanum melongena | 60.47525 | 87.9702970 | 21.56436 | 0 |

164 Mantel test indicates that a possible correlation exist between heterospecific pollen effect and the
165 evolutive distances, for ITS and RBCL markers we had r coefficients of 0.29 and 0.25 respectively
166 $p < 0.05$. Moreover, Mantel test indicates that also a possible correlation between stigma width and
167 stigma type exist. Trait correlations were also explored with ... and we found that...

168 Fix mantel test selfing rates and change it for compatibility index...

169 Fix this to GLMM? Yep I have to...

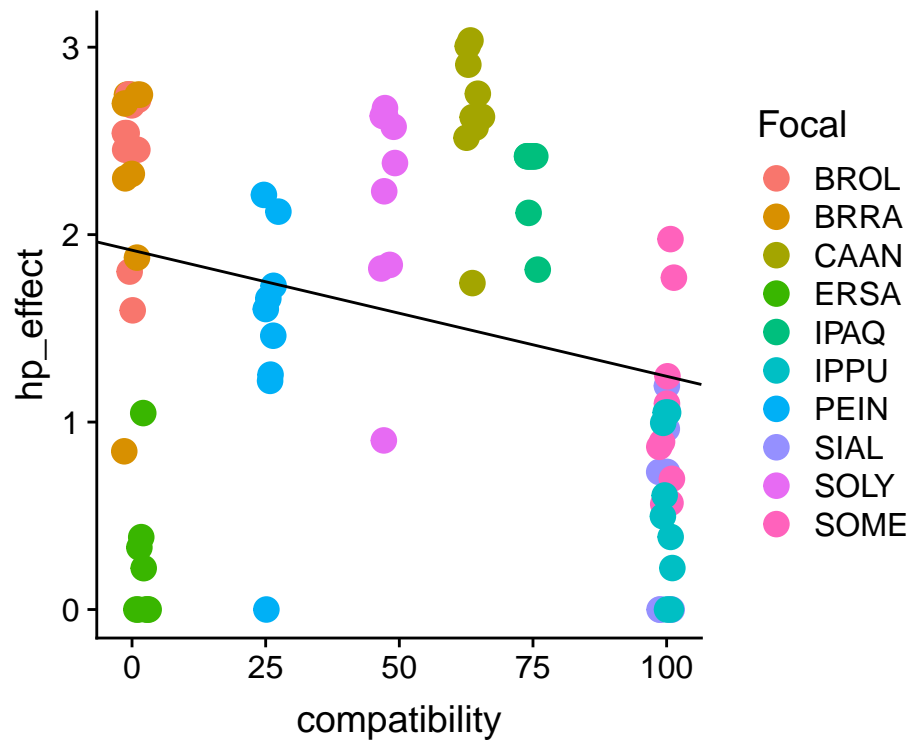


Figure 1: The effect of heterospecific pollen (scaled see set) is represented in function of the compatibility system (self/cross*100) for the the different species. Each coulored dot represents the interaction of a focal species with a different pollen donor.

DISCUSSION

Discussion

1. What are the implications of the findings?

173 CONCLUSIONS

174 ACKNOWLEDGEMENTS

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229 **List of Figures**

| | | | |
|-----|---|--|---|
| 230 | 1 | The effect of heterospecific pollen (scaled see set) is represented in function of the | |
| 231 | | compatibility system (self/cross*100) for the the different species. Each coulored dot | |
| 232 | | represents the interaction of a focal species with a different pollen donor. | 9 |