

working title Compatibility system and stigma size are the main predictors of heterospecific pollen effect

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Pollinator sharing can have negative consequences for plant fitness with the arrival of foreign pollen. However, the costs of heterospecific pollen are not yet well understood. We conducted a glasshouse experiment to understand how phylogenetic relatedness and plant traits mediate the impacts of heterospecific pollen transfer. We conducted 1800 crosses by experimentally transferring pollen (50% and 100% ratio) with reciprocal crosses between 10 species belonging to three different families: Brassicaceae, Solanaceae and Convolvulaceae. Seed set was used as proxy of plant fitness. We found that for 65% of the treatments with 50% mix reduced seed set. Moreover, the reduction in seed set was dependent on the degree of relatedness and reproductive traits of the pollen recipient and not the pollen donor. Our results show that certain traits, particularly compatibility system, are critical in understanding the costs of heterospecific pollen.

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

INTRODUCTION

In most ecosystems, plant species normally coexist and share their floral visitors with other species Waser et al. (1996). From the plants' perspective, pollinator sharing can be positive for some plants Carvalheiro et al. (2014) or negative for others Pauw (2013), depending on the facilitation gradient. An increasing number of visits often correlates with higher chances of fertilization Engel and Irwin (2003). However this is not always the case, among these possible flower visitors there are also nectar robbers and pollen thieves Inouye (1980); Magrach et al. (2017). Receiving both sufficient quantity and quality deposited on the stigma is thus highly relevant to the pollination success of the plant Aizen and Harder (2007).

34 By visiting many plant species, many pollinators are responsible for conspecific pollen loss and the
35 transport of foreign pollen, both of which can have important detrimental effects on species fitness
36 Morales and Traveset (2008); Ashman and Arceo-Gómez (2013); Arceo-Gómez and Ashman (2016).
37 Foreign pollen arrival can play an important role in plant species fitness but outcomes are variable and
38 appear to be context dependent as there is not always a decrease in fitness Morales and Traveset (2008).
39 Some of this variation is likely due to the enormous variability of foreign pollen transferred across
40 systems ranging from 0 to 75 percent. However, most studies report ranges of heterospecific pollen
41 between 0 and 20 percent of the total pollen load Bartomeus et al. (2008) Montgomery and Rathcke
42 (2012); Ashman and Arceo-Gómez (2013); Fang and Huang (2013), yet even these relatively low
43 amounts of heterospecific pollen transferred can decrease fitness greatly Thomson et al. (1982). While
44 we now have some understanding of the impacts of heterospecific pollen quantity, we have less
45 understanding of other factors that could be driving the variation in impacts upon fitness. Ashman and
46 Arceo-Gómez (2013) postulated the first predictive framework that identifies a need to understand how
47 plant traits might mediate heterospecific pollen effect, whereby mating system and pollen size were
48 predicted to potentially mediate the impact of foreign pollen transfer on plant fitness. This concept is
49 supported by specific case studies, such as Tong and Huang (2016) that demonstrate an asymmetrical
50 effect in 6 species of *Pedicularis* whereby the pollen of long styled species was able to grow the full
51 length of the style on short styled species but not vice versa. While this suggests that the impacts of
52 heterospecific pollen may differ among pollen donor and recipient, few studies have been conducted to
53 ascertain whether this pattern is in fact a general trend or to identify the extent to which other plant
54 traits are critical to heterospecific pollen impacts.

55 Plant traits are crucial to understand heterospecific pollen effect but the multifactorial nature of the
56 traits that are involve in the pollen-pistil interaction make difficult to unravel what are the main traits
57 in driving the effect. These traits can be seen from a male perspective of both donor and recipient
58 where pollen size, pollen aperture number and pollen allelopathy are key components to understand the
59 outcome of foreign pollen arrival Murphy and Aarssen (1995); Ashman and Arceo-Gómez (2013). In
60 Ashman and Arceo-Gómez (2013) small pollen is predicted to cause a greater fitness decrease, although
61 this can be true there are also other possibilities to consider which can obscure a predictive framework
62 like big pollen can clogg small stigmas with fewer pollen grains, bigger stigmas are less likely to be

63 clogged by small pollen grains and bigger pollen can outcompete smaller pollen grains due to faster
64 pollen tube growth rate Williams and Rouse (1990). Moreover, to understand the different mechanical
65 or chemical effects of pollen also the female traits of the pollen recipient have to be considered, from
66 the literature these main traits are: stigma size, style length, number of ovules, incompatibility system
67 and also a structural trait such as flower morphology Montgomery and Rathcke (2012); Ashman and
68 Arceo-Gómez (2013); Tong and Huang (2016). For example, greater stigmatic area is positively
69 correlated with greater heterospecific pollen deposition Montgomery and Rathcke (2012) and therefore
70 possibly with an increase in negative effect. For species that are self-incompatible the barriers towards
71 heterospecific pollen are stronger than self-compatible species Ashman and Arceo-Gómez (2013).
72 Nonetheless, an effect of foreign pollen is a bit obscured by the variability within species, however
73 species that are strong selfers or strong outcrossers have less variability in mating systems and
74 predictions of effect could be more realistic (see figure 1 from Whitehead et al. (2018)). Although past
75 research has progress in the understanding of what traits can mediate the effect as we have shown here,
76 there are multiple traits involved and multiple possible scenarios still to be explored empirically for a
77 full understanding of the importance of heterospecific pollen effect in nature.

78 For the understanding at what level or intensity the interference of pollen can occur is important to
79 consider the relatedness of the interacting species. Closely related species are more likely to have
80 similar traits Letten and Cornwell (2015). The similarity in traits between closely related species can
81 lead to higher chances of ovule usurpation/abortion Arceo-Gómez and Ashman (2011) and therefore
82 studies predict and show a greater negative effect of closely related species Ashman and Arceo-Gómez
83 (2013); Arceo-Gómez and Ashman (2016). Few studies however, have focused on the impacts of
84 heterospecific pollen on fitness of distantly related species Galen and Gregory (1989); Neiland and
85 Wilcock (1999). Despite the fact that far related species are also able to decrease species fitness
86 (REFS). Yet, most insects and most stigmas have been found to carry multiple species of foreign pollen
87 with little attention to degree of relatedness (Arceo-Gómez and Ashman (2016); Fang and Huang
88 (2013). Understanding the role of foreign pollen from distantly related species thus deserves greater
89 attention. The relatedness of foreign pollen gives a first snapshot of where the pollen competition can
90 occur and therefore could be a proxy of effect. With that said, Arceo-Gómez and Ashman (2016) is the
91 only work which has proven a greater effect of close related species through a meta-analysis but with

low sample sizes and lack of significance. Therefore, there is a need to study the effect of heterospecific pollen of far and close related species at community level beyond single pairwise interactions.

Heterospecific pollen studies in nature have the complexity added of great environmental variability which can lead to confounding interpretations. Moreover, the great diversity of floral structures and small sizes of some of them such as Asteraceae species make tedious or almost impossible the studies of heterospecific pollen on the field. For this reason, we investigated how floral reproductive traits and relatedness mediate the impact of heterospecific pollen by creating an artificial co-flowering community in a glasshouse with 10 species belonging to three different families with different traits. Our study tries to answer the following questions: (i) Do heterospecific pollen reduce seed set? To what extent do (ii) floral reproductive traits and (iii) relatedness, mediate the impacts of heterospecific pollen on seed set.

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 experimental design had species from three different families: Solanaceae, Brassicaceae and Convolvulaceae (**Table 1**). The species of the study had different reproductive traits and different degree of relatedness where the reciprocal crosses between species allowed us to have multiple different scenarios of both traits and relatedness. Moreover, the species selected had fast life cycle and low structural flower complexity in order to perform the pollination treatments and grow the different species from seeds. 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

Family	Genus	Species
Brassicaceae	Eruca	Eruca versicaria
Brassicaceae	Sinapis	Sinapis alba
Convolvulaceae	Ipomoea	Ipomoea aquatica
Convolvulaceae	Ipomoea	Ipomoea purpurea
Solanaceae	Capsicum	Capsicum annuum
Solanaceae	Petunia	Petunia integrifolia
Solanaceae	Solanum	Solanum lycopersicum
Solanaceae	Solanum	Solanum melongena

114 Hand-pollination

115 Foreign pollen effect was studied through two different treatments, one with 50% conspecific pollen and
116 50% heterospecific pollen and a second one with 100% foreign pollen (N=10) in order to see if foreign
117 pollen can trigger fruit production by itself or even seeds through ovule usurpation. Therefore, 180
118 different combinations were performed with N=10 per combination. Seed set was the proxy of effect for
119 all our treatments. Moreover, hand cross pollination (between individuals of the same species), hand
120 self pollination, apomixis (bagged emasculated flowers) and natural selfing were tested for each species
121 (N=10). For the treatments with foreign pollen and hand cross pollination, flowers were emasculated
122 the day prior anthesis and hand pollinated next day with a toothpick. Hand-pollination was conducted
123 with 3-4 gentle touches on the stigma surface. For each species 20 anthers were collected and their
124 pollen counted with a hemocytometer, each anther was counted 4 times and then an average of these
125 counts was performed. Once, the average number of pollen grains per anther was known, the
126 proportion of anthers per mix was calculated in order to achieve a 50-50% mix. In order to confirm
127 that the treatments applied were the desire proportions, the total stigmatic load of pollen was counted
128 and the proportions calculated between the two species of the mix. Therefore, 20 different
129 heterospecific crosses 50-50% were counted for all the focal species with crosses of just one species of
130 the other two families (N=3).

131 Traits and evolutive distance

132 The traits measured for each species were pollen per anther, number of ovules and stigma, style, ovary
133 width and length. For the stigma, the stigmatic area was also measured and moreover the stigmas were
134 divided in wet/dry type. All the morphometrical measurements were performed with a
135 stereophotomicroscope. Pollen was counted for 20 anthers of each species with 4 replicates per sample
136 with an hemocytometer. Previously, anthers were squashed on a known solution with the pippete tip
137 and homogeneize with a vortex for 30 seconds. Ovule number was counted with the help of a
138 stereomicroscope and a small grid over a petri dish from 15 randomly selected flowers. Fruits per
139 number of flowers treated were counted for Solanaceae species and for the rest, the number of seeds
140 produced per average number of ovules of each species. Levels of self incompatibility were estimated by
141 dividing the fruit set of hand self pollination by hand cross pollination Lloyd and Schoen (1992).

142 **Analysis**

143 To evaluate heterospecific pollen effect on seed production we performed linear mixed models. The
144 distinct heterospecific pollination treatments were compared through relevelling each variable with the
145 cross pollination treatment which was our control for optimum seed production for all the species. The
146 different replicates of each treatment were considered as random effects. Seed production was scaled for
147 all the species with mean 0 and standard deviation of 1 prior to the analysis. All the analysis were
148 conducted with the statistical language R (R Core Team 2018).

149 In order to test the relative effect of traits on seed production with foreign pollen we performed Mantel
150 test between the assymetrical matrix of heterospecific pollen effect (10 by 10 matrix) with the different
151 distance matrices of traits (Euclidean distances). Heterospecific pollen effect was obtained through the
152 subtraction of seed production by hand cross pollination minus seed production of the different
153 heterospecific pollen treatments. To find a model with the best explanatory traits we used the function
154 **bioenv** from R. We also conducted Mantel test between the matrix of heterospecific pollen effect and
155 the distance matrix from all the traits. Moreover, we explored also the correlation between traits and
156 heterospecific pollen effect through generalized mixed models where the response variable was
157 heterospecific pollen effect and the explanatory variable the different traits. In addition, we tested the
158 correlation between the total amount of pollen deposited on the stigma with heterospecific pollinations
159 and the stigma size through Pearson's correlation.

160 We conducted mantel test to check for correlations between heterospecific pollen effect and phylogenetic
161 distance. Due to improves in statistical power we used the square root of the phylogenetic distance
162 (Letten & Cornwell 2014). Two different phylogenetic distances were used from two kinds of markers: 1)
163 Internal transcribed spacer (ITS) and 2) ribulose-bisphosphate carboxylase (RBCL). The sequences
164 were obtained from GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>, accessed 20 Oct. 2018). The
165 sequences were aligned with clustal omega and the pairwise evolutive distances calculated with MEGA7.

166 [Phylogenetic signal of traits?](#)

167 [Add pollen on stigma counts to methods](#)

168 [Next bit to results, also divided it with]

169 Total pollen deposited on stigmas was significantly correlated with the stigmatic area, pearsons
170 correlation=0.57 and p-value=0.008. Proportions of pollen talk about it to 50-50 mix? Talk about
171 this in methods too.

172 Add also plot with fruit production and put it in appendix. Also the plots of ratios to appendices.

173 RESULTS

174 Results of hand cross-pollination, self hand-pollination, natural selfing and apomixis are presented in
175 **Figure 1** (see appendix 1 for table with values). Heterospecific pollen reduced seed set significantly
176 with the 50-50% heterospecific pollen treatments for 65% of the pairwise interactions $p < 0.05$.

177 Across families we found a very similar effect but when species were looked at species level they respond
178 differently even within the same family rephrase and maybe test statistically?, for instance for two
179 species of the Brassicaceae family *Brassica oleracea* and *Eruca versicaria* we found very contrasting
180 effects of foreign pollen where for the first one, all donors reduce seed set significantly and for the
181 second, just two species did out of nine. The 100% foreign pollen treatments barely produced seeds or
182 fruits and just for *Sinapis alba* we did not find significant differences between the hand cross pollination
183 and one treatment with pollen from a confamilial- IB Unclear. Solanaceae species with berry fruit type

184 developed small fruits or even normal fruits in some cases under which treatment. *S. lycopersicum*
185 seems to produced small fruits (35% of the treatments) independently of pollen and pollen donor due to
186 also apomictic treatments did, never normal size. *C. annuum* produced some fruits (9%) of both small
187 and normal size and finally *S. melongena* produced seedless normal fruits with just confamilial pollen
188 (3%), for both species seems that fruit formation was induced by pollen on the stigma because of lack
189 of fruit production with treatments that tested for apomixis. clarify this descriptive statistics part- Also
190 a figure with a summary of the treatments effect would be cool, or at least in the appendices

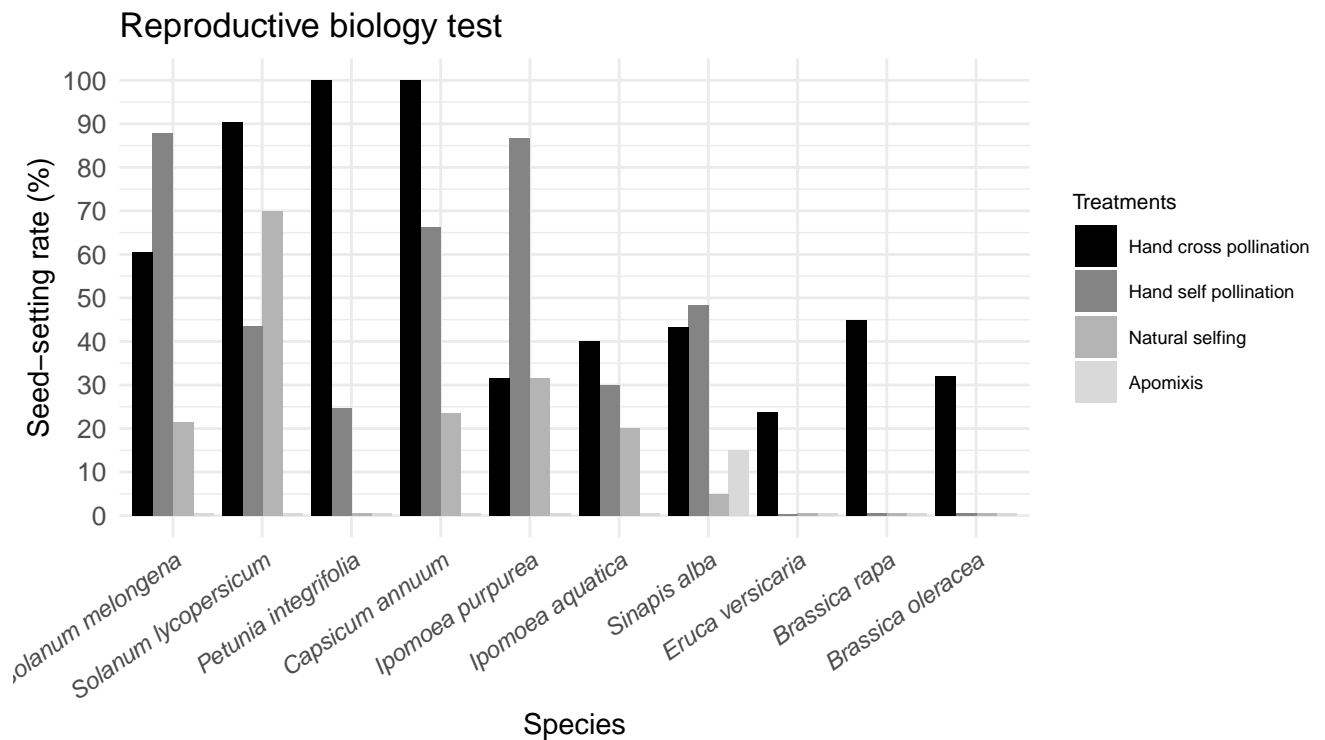


Figure 1: Barplot with the different treatments that provide information of the reproductive biology of the ten species. The y axis is the proportion of ovules converted to seed in percentage. The different treatments (N=10) which are presented in the legend are, hand cross pollination, hand self pollination, natural selfing and apomixis. More information about these treatments can be found in Methods and Appendices.

191 Mantel test indicates that a possible?? It exists! correlation exist between heterospecific pollen effect
 192 and the evolutive relative distances, for ITS and RBCL markers we had r coefficients of 0.29 and 0.25
 193 respectively $p < 0.05$ think on a figure - maybe using NMDS. Moreover, Mantel test indicates that also a
 194 possible?? correlation between stigma width and stigma type exist (stats??). Trait correlations were
 195 also explored with GLMM

196 I have done it at the moment just for Compatibility system Also I have to fix from mixed linear model
 197 to GLMM, just realize that

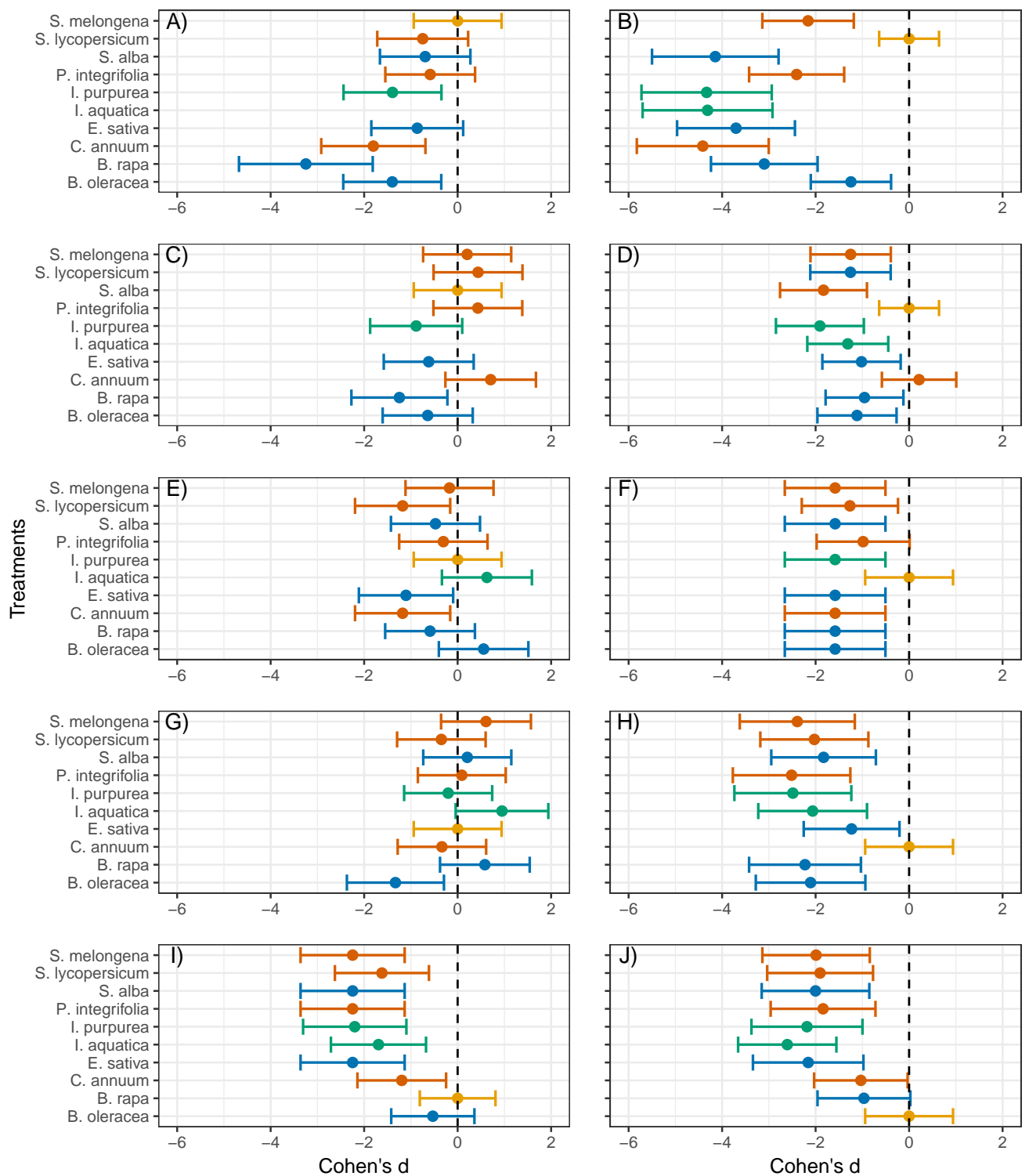


Figure 2: Effect sizes for the 10 different species. The different families appear with different colours, when a species was focal was coloured differently from its family.

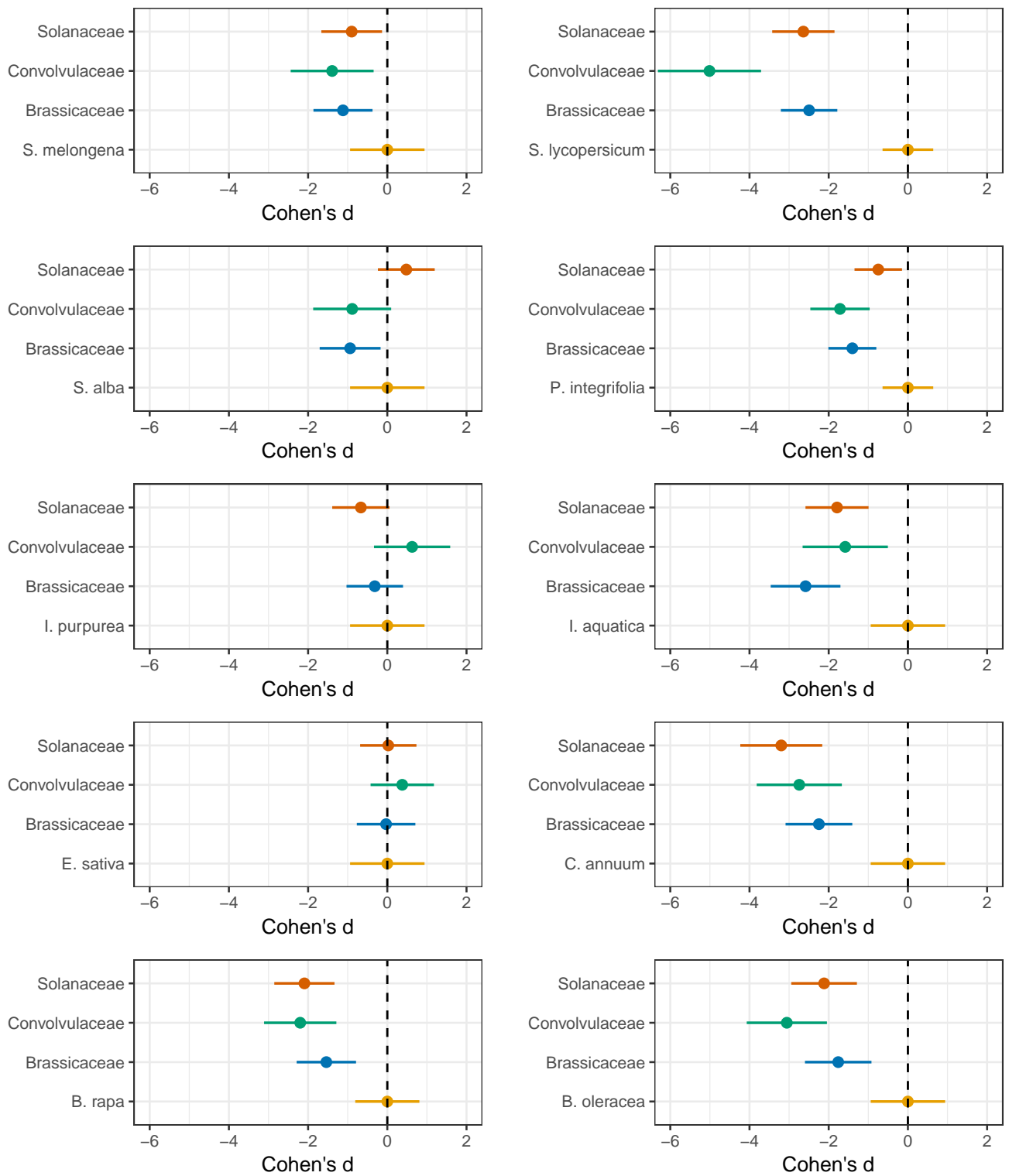


Figure 3: A

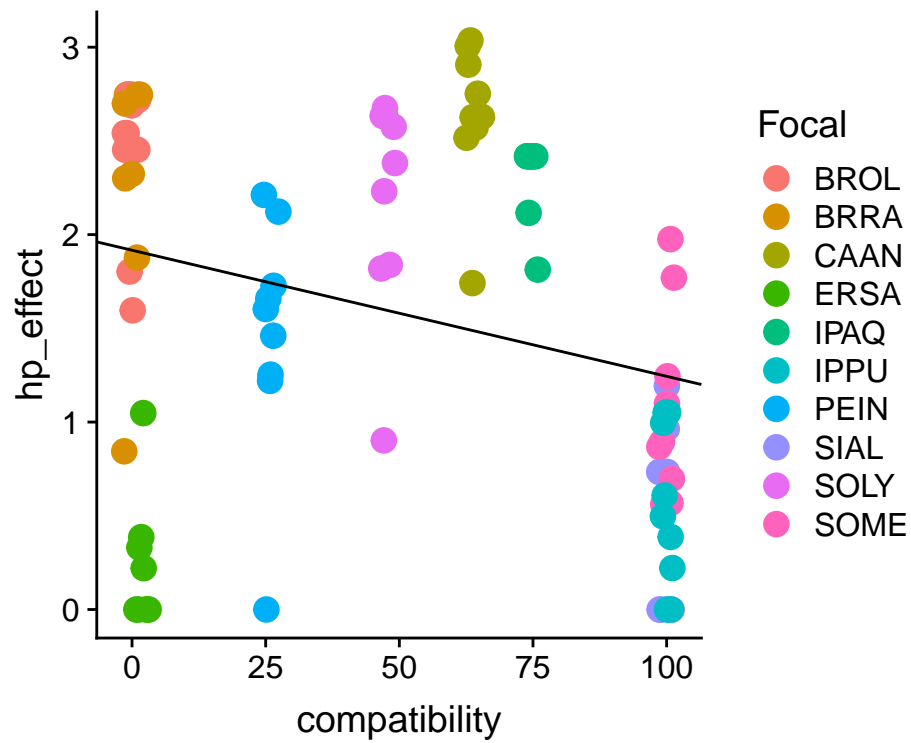


Figure 4: The effect of heterospecific pollen (scaled see set) is represented in function of the compatibility system (self/cross*100) for the the different species. Each coulored dot represents the interaction of a focal species with a different pollen donor.

199 **DISCUSSION**

200 Discussion

201 Herbs vs trees, annual vs perennial... Many flowers vs few flowered species; structural composition on
202 a system

203 What are the implications of the findings?

204 Ideas about pollen size in heterospecific pollen effect. (still have to develop it more...)

205 Let's classify pollen size in three groups in order to understand the interaction between pollen donor
206 and recipient: 1) Donor pollen size < Recipient pollen size 2) Donor pollen size = Recipient pollen size
207 3) Donor pollen size > Recipient pollen size

208 Now I try to develop each part

209 1) Donor pollen size < Recipient pollen size

210 Effect:

- 211 • Donor's pollen could clog the stigma
- 212 • Chemical inhibition

213 Traits associated with bigger pollen of the recipient:

- 214 • Recipient's pollen have faster pollen tube growth (example with my data)
- 215 • Reduction in number of ovules (Also with my species)
- 216 • Big differences in pollen size can be translated in low relatedness therefore less likely of pollen
217 germination on a far related stigma.

218 2) Donor pollen size = Recipient pollen size

- 219 • Very relatedness dependant this point

220 • Similar probabilities of taken space on the stigma

221 3) Donor pollen size > Recipient pollen size

222 Effect:

223 -In small stigmas big pollen grains can occupy great part of the stigmatic area.

224 -small pollen grains can get embedded

225 IB: Think also on using tree analysis to test if hp effect depends on complex trait combinations. Tree
226 analysis are great when two different strategies lead to the same outcome. This would never been pick
227 up by GLMs. The r package is party{} . You can see an example applied to birds is Sol et al 2010
228 Science. Ask me if you want more details or code examples.

229 CONCLUSIONS

230 ACKNOWLEDGEMENTS

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284 **APPENDIX**

285 1.

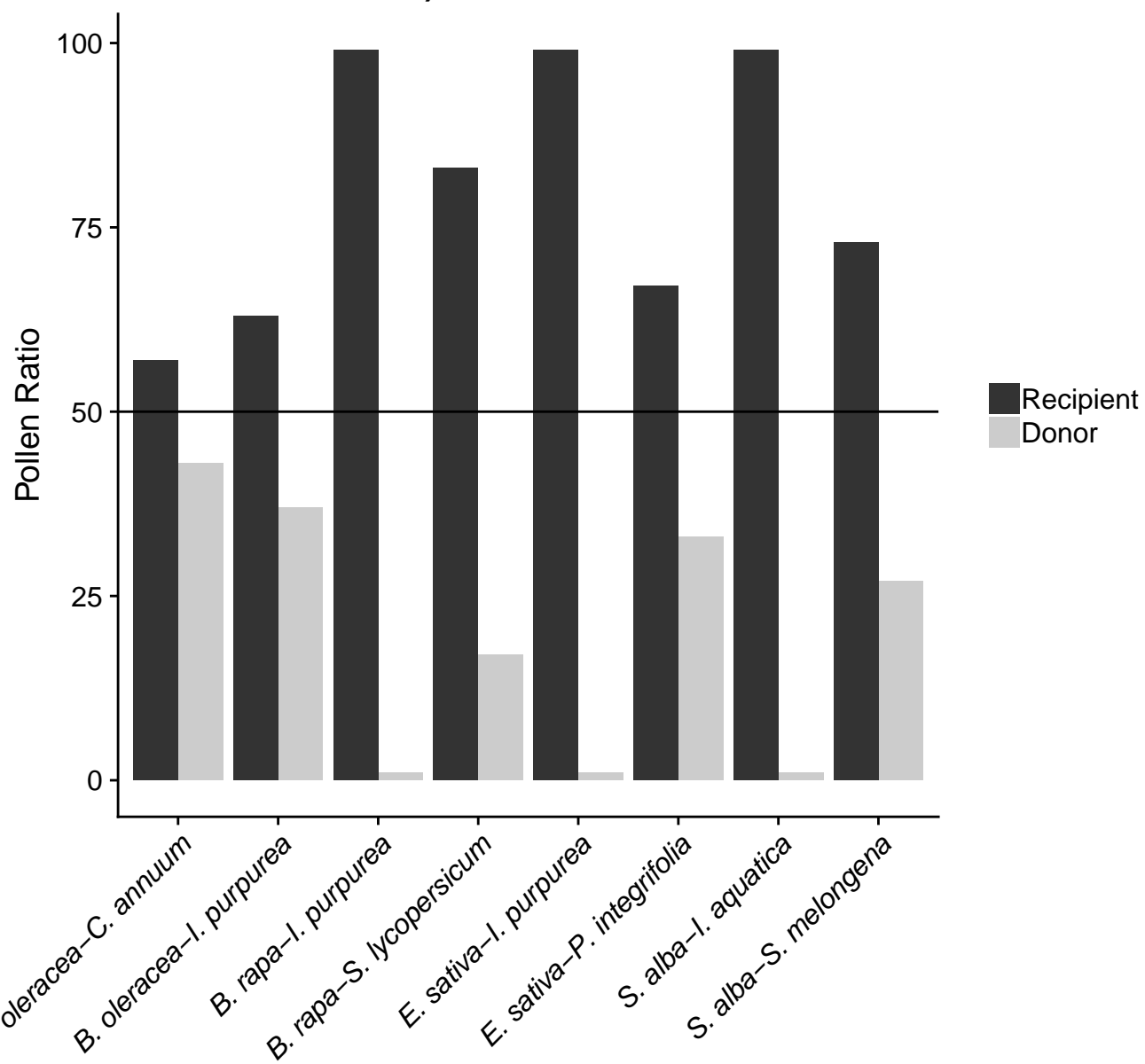
286 **Table S1.** Perecentage of seeds produced per ovule for the ten species used in the experiment. The
 287 treatments presented are hand cross pollination, hand self pollination, natural selfing and apomixis
 288 (emasculated flowers).

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
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

289 2.

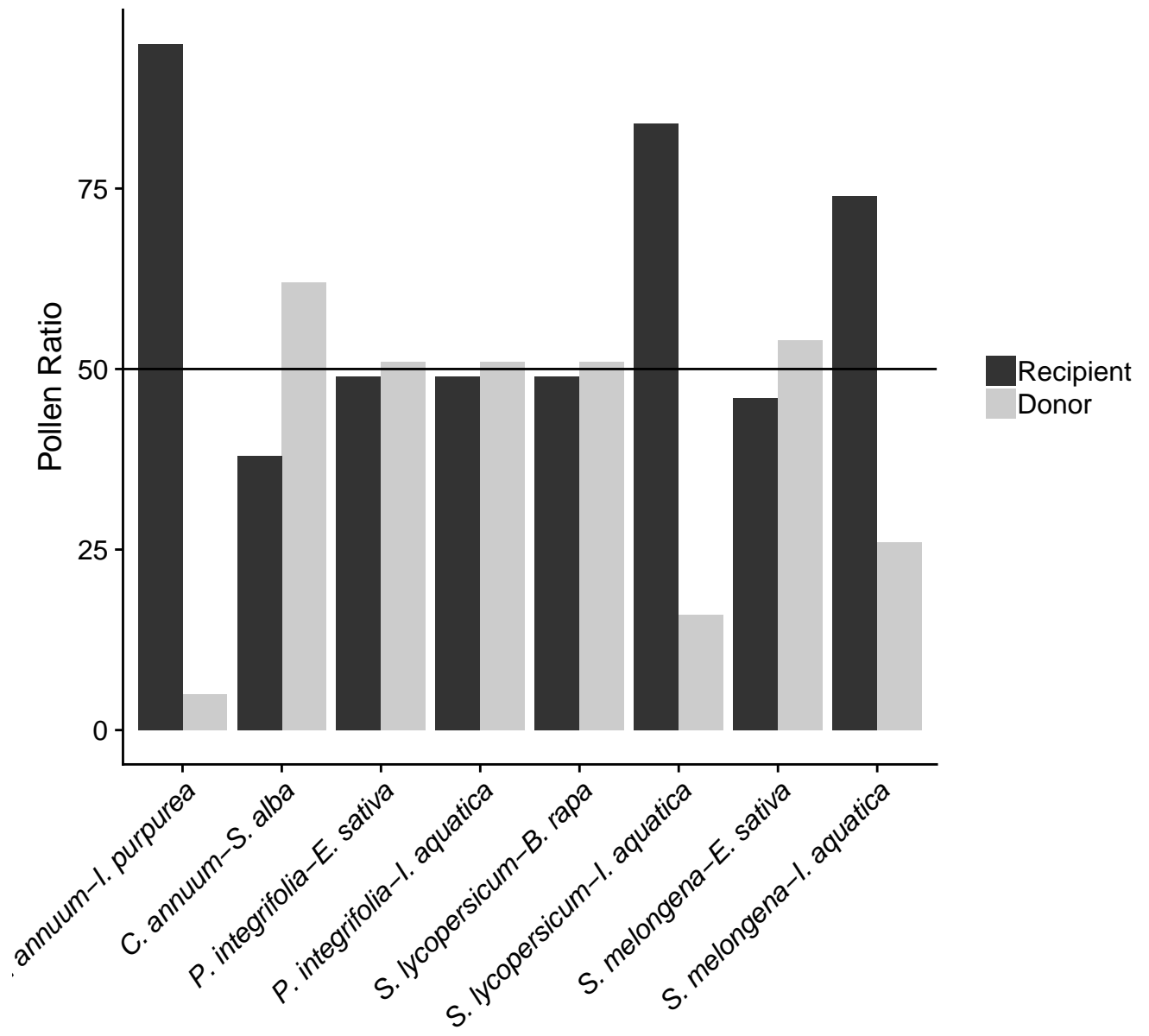
290 **Figure S1.** Pollen ratios separated by family A) Brassicaceae, B) Solanaceae, C) Convolvulaceae.
 291 Pollen was counted for crosses with species of the other two families (N=3). To prepare these ratios all
 292 the pollen grains on the stigma were counted.

A) Brassicaceae



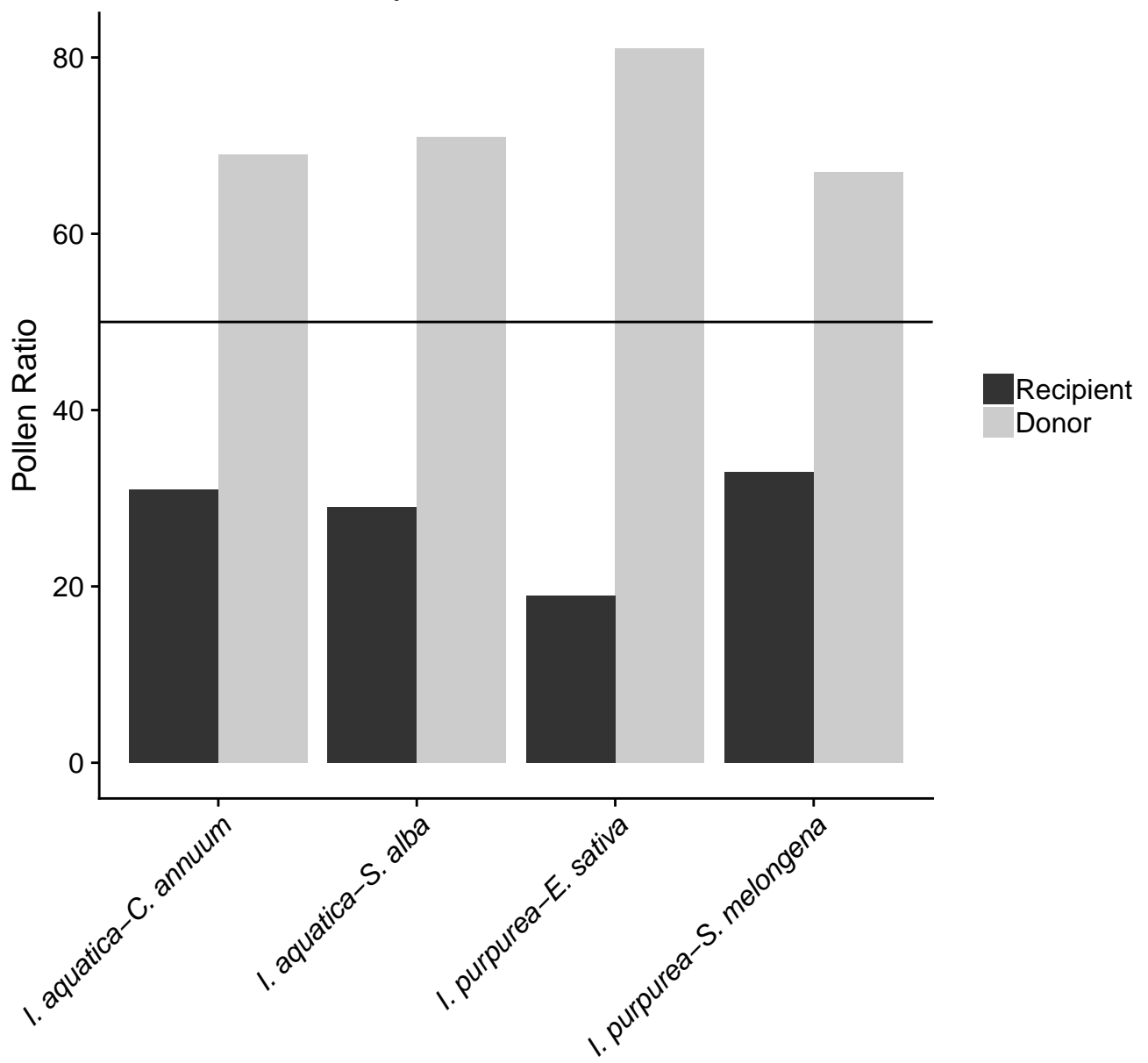
293

B) Solanaceae



294

C) Convolvulaceae



297 List of Figures

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299		of the ten species. The y axis is the proportion of ovules converted to seed in percentage.	
300		The different treatments (N=10) which are presented in the legend are, hand cross	
301		pollination, hand self pollination, natural selfing and apomixis. More information about	
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308		represents the interaction of a focal species with a different pollen donor.	12