

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 (**Figure 1**) 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).

113 **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
Solanaceae	Solanum	Solanum lycopersicum
Solanaceae	Solanum	Solanum melongena



Figure 1: Phylogenetic tree of the ten species used in the experiment from three different families from bottom to top: Solanaceae, Convolvulaceae and Brassicaceae.

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 in order to see if foreign pollen
117 can trigger fruit production by itself or even seeds through ovule usurpation. Therefore, we performed
118 180 different heterospecific treatments (N=10). Seed set was the proxy of effect for all our treatments.
119 Moreover, hand cross-pollination (between individuals of the same species), hand self-pollination,
120 apomixis (bagged emasculated flowers) and natural selfing were tested for each species (N=10). For the
121 treatments with foreign pollen and hand cross-pollination, flowers were emasculated the day prior
122 anthesis and hand pollinated next day with a toothpick. Hand-pollination was conducted with 3-4
123 gentle touches on the stigma surface. For each species 20 anthers were collected and their pollen
124 counted with a hemocytometer, each anther was counted 4 times and then an average of these counts
125 was performed. Once, the average number of pollen grains per anther was known, the proportion of
126 anthers per mix was calculated in order to achieve a 50-50% mix. To confirm that the treatments
127 applied were the desire proportions, the total stigmatic load of pollen was counted and the proportions
128 calculated between the two species of the mix. Because pollen from the same family was difficult to
129 distinguish, and we expected similar properties in mixing, we counted pollen from just one randomly
130 selected species within each donor family different to the focal's family (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 just Solanaceae species, we counted for all the species the
140 number of seeds produced per average number of ovules. Levels of self-incompatibility were estimated
141 by 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 releveling 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 improvents 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 aboout
171 this in methods too.

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

173 Add effect sizes to analysis!!

174 Mention that SI index is calculated (Schoen & Lloyd 1984)

175 RESULTS

176 Results of hand cross-pollination, hand self-pollination, natural selfing and apomixis are presented in
177 **Figure 1** (see appendix 1 for table with values). Heterospecific pollen reduced seed set significantly
178 with the 50-50% heterospecific pollen treatments for 65% of the pairwise interactions $p < 0.05$. Moreover,
179 average effect sizes differed across species and across families, see **Figure 2**. Despite some variability in
180 the effect of the different pollen donors per species, in general terms the effect of heterospecific pollen
181 from the distinct nine treatments per species was homogeneous (see **Figure 3**), just for four species out
182 of ten, just one donor did not overlap the confidence intervals with other donors. Therefore, none of the
183 donors had a clear stronger or weaker effect across species. When the donors were groped by family not
184 big differences were seen, just for *S. lycopersicum* the confidence intervals of Brassicaceae and
185 Convolvulaceae did not overlap. In addition,

186 Now talk about the 100%, Make plot!!!!

187 The 100% foreign pollen treatments barely produced seeds or fruits and just for *Sinapis alba* we did not
188 find significant differences between the hand cross-pollination and one treatment with pollen from a
189 confamilial- IB Unclear. Solanaceae species with berry fruit type developed small fruits or even normal
190 fruits in some cases under which treatment. *S. lycopersicum* seems to produced small fruits (35% of the
191 treatments) independently of pollen and pollen donor due to also apomictic treatments did, never
192 normal size. *C. annuum* produced some fruits (9%) of both small and normal size and finally *S.*
193 *melongena* produced seedless normal fruits with just confamilial pollen (3%), for both species seems

194 that fruit formation was induced by pollen on the stigma because of lack of fruit production with
195 treatments that tested for apomixis. clarify this descriptive statistics part- Also a figure with a summary
196 of the treatments effect would be cool, or at least in the appendices

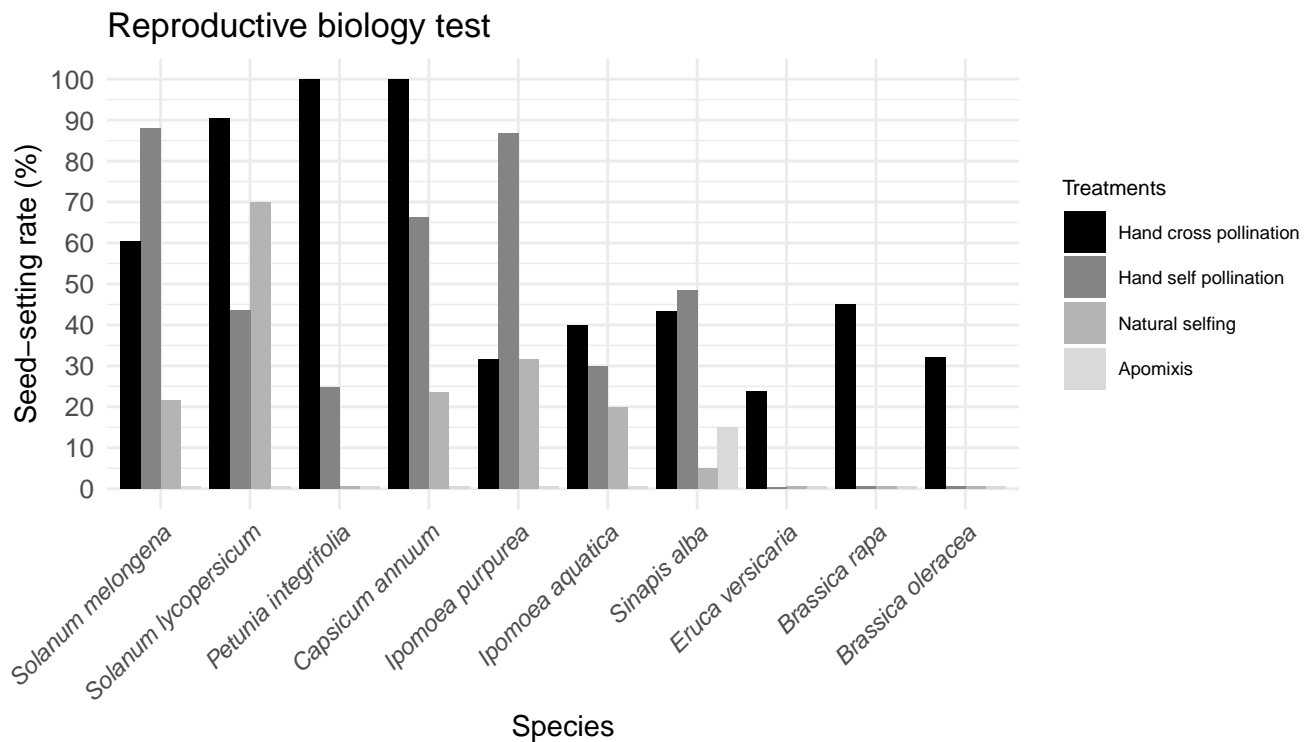


Figure 2: Barplot with the different treatments that provide information of the reproductive biology of the ten species. The y axis is the proportion of ovules converted to seed in percentage. The different treatments (N=10) which are presented in the legend are, hand cross-pollination, hand self-pollination, natural selfing and apomixis. More information about these treatments can be found in Methods and Appendices.

197 Maybe for this plot I have to do hedges'g instead of cohean because of different sample sizes when I
 198 group the treatments per family. Check



Figure 3: Average effect size and confidence intervals (95%) of the 9 heterospecific treatments of 50% pollen to the different 10 focal species coloured by family.

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

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

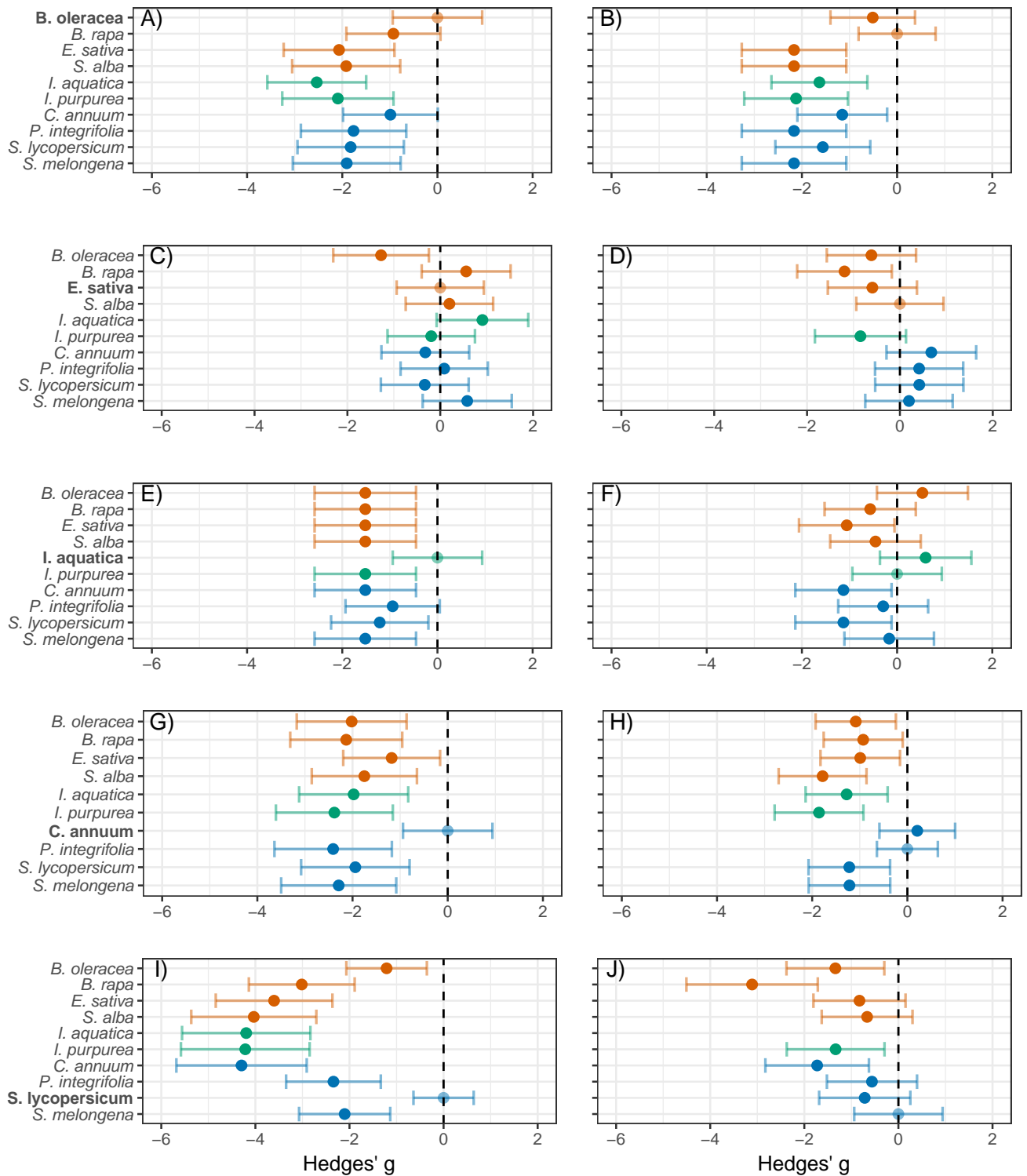


Figure 4: Effect sizes for the 10 different species. The different families appear with different colours, when a species was focal was coloured differently from its family.



Figure 5: A

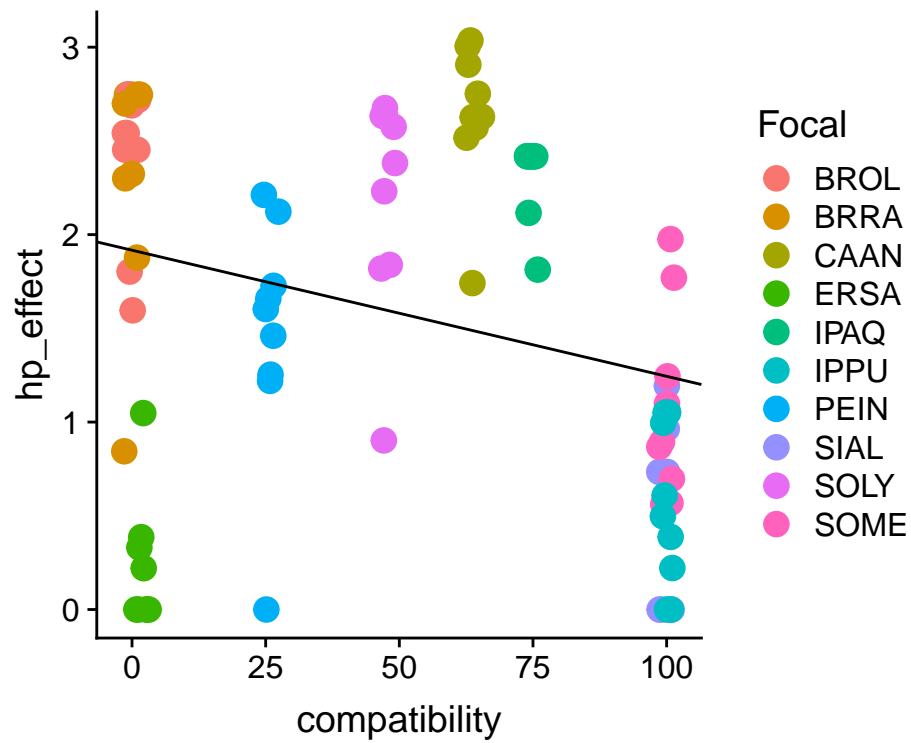


Figure 6: The effect of heterospecific pollen (scaled see set) is represented in function of the compatibility system (self/cross*100) for the the different species. Each coulored dot represents the interaction of a focal species with a different pollen donor.

206 [Compatibility index don´t multiply per 100 from Lloyd](#)

207 **DISCUSSION**

208 Discussion

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

211 What are the implications of the findings?

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

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

216 Now I try to develop each part

217 1) Donor pollen size < Recipient pollen size

218 Effect:

- 219 • Donor's pollen could clog the stigma
- 220 • Chemical inhibition

221 Traits associated with bigger pollen of the recipient:

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

226 2) Donor pollen size = Recipient pollen size

- 227 • Very relatedness dependant this point

228 • Similar probabilities of taken space on the stigma

229 3) Donor pollen size > Recipient pollen size

230 Effect:

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

232 -small pollen grains can get embedded

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

237 CONCLUSIONS

238 ACKNOWLEDGEMENTS

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APPENDIX

1.

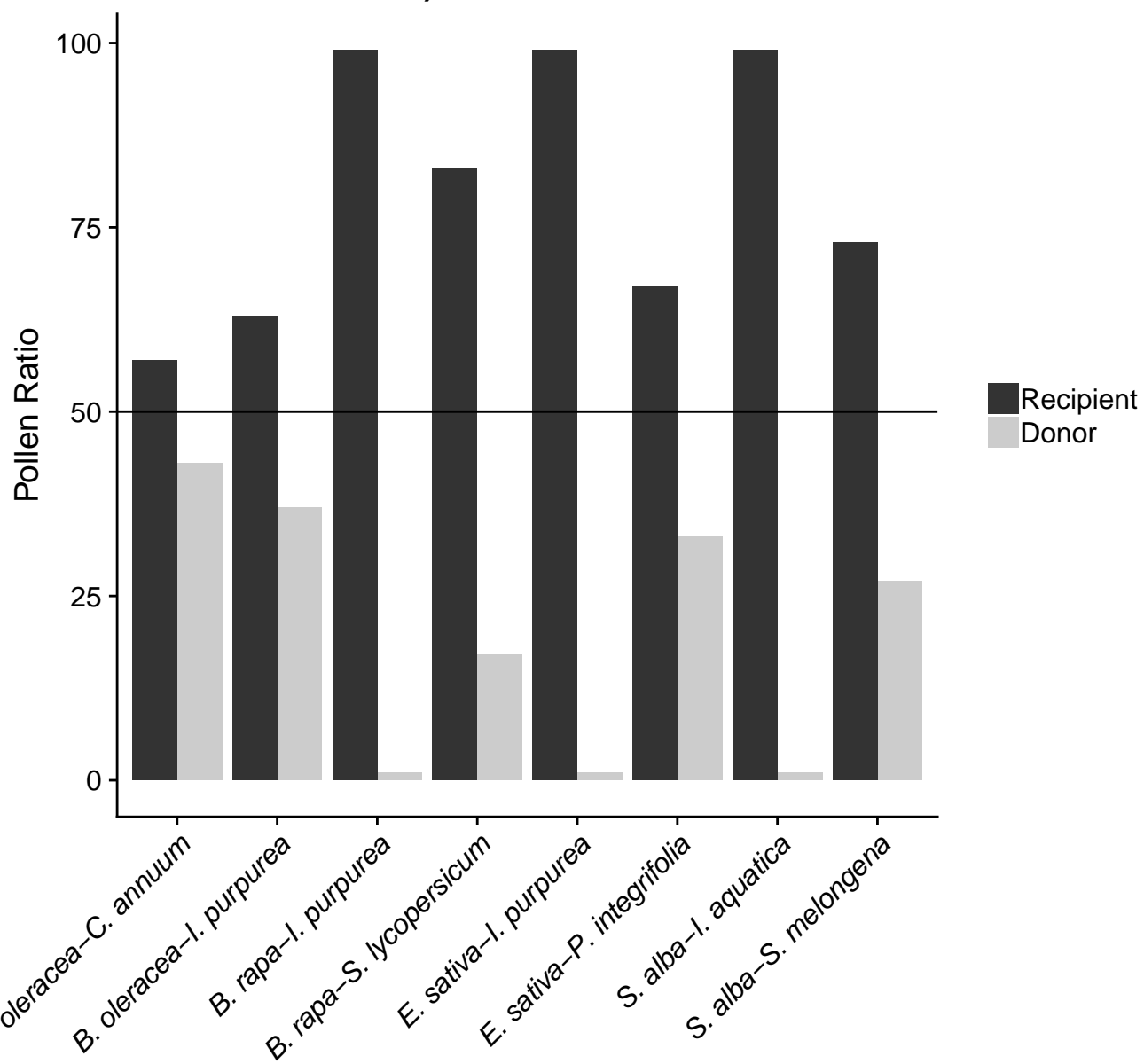
Table S1. Percentage of seeds produced per ovule for the ten species used in the experiment. The treatments presented are hand cross-pollination, hand self-pollination, natural selfing and apomixis (emasculated flowers).

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

2.

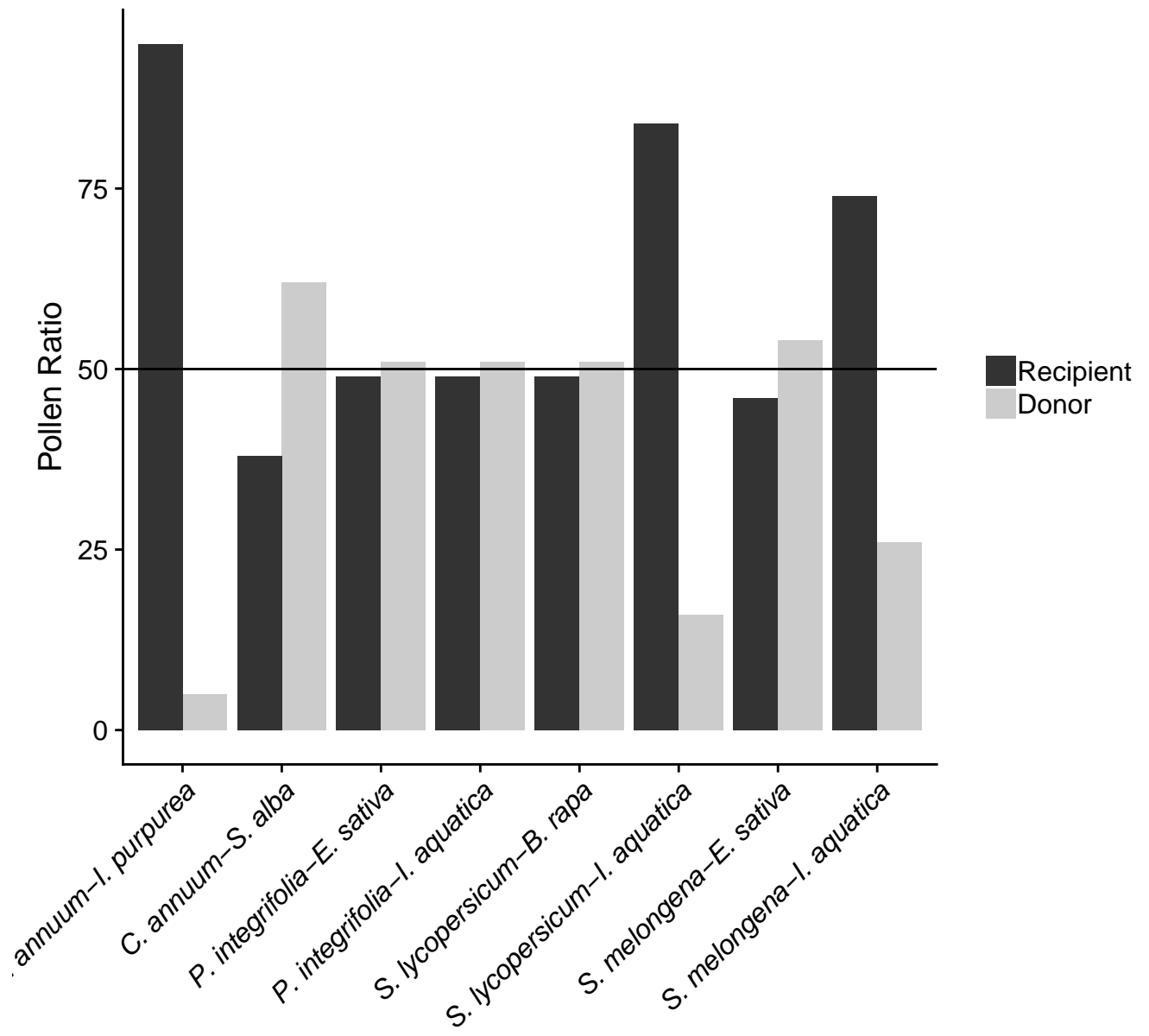
Figure S1. Pollen ratios separated by family A) Brassicaceae, B) Solanaceae, C) Convolvulaceae. Pollen was counted for crosses with species of the other two families (N=3). To prepare these ratios all the pollen grains on the stigma were counted.

A) Brassicaceae



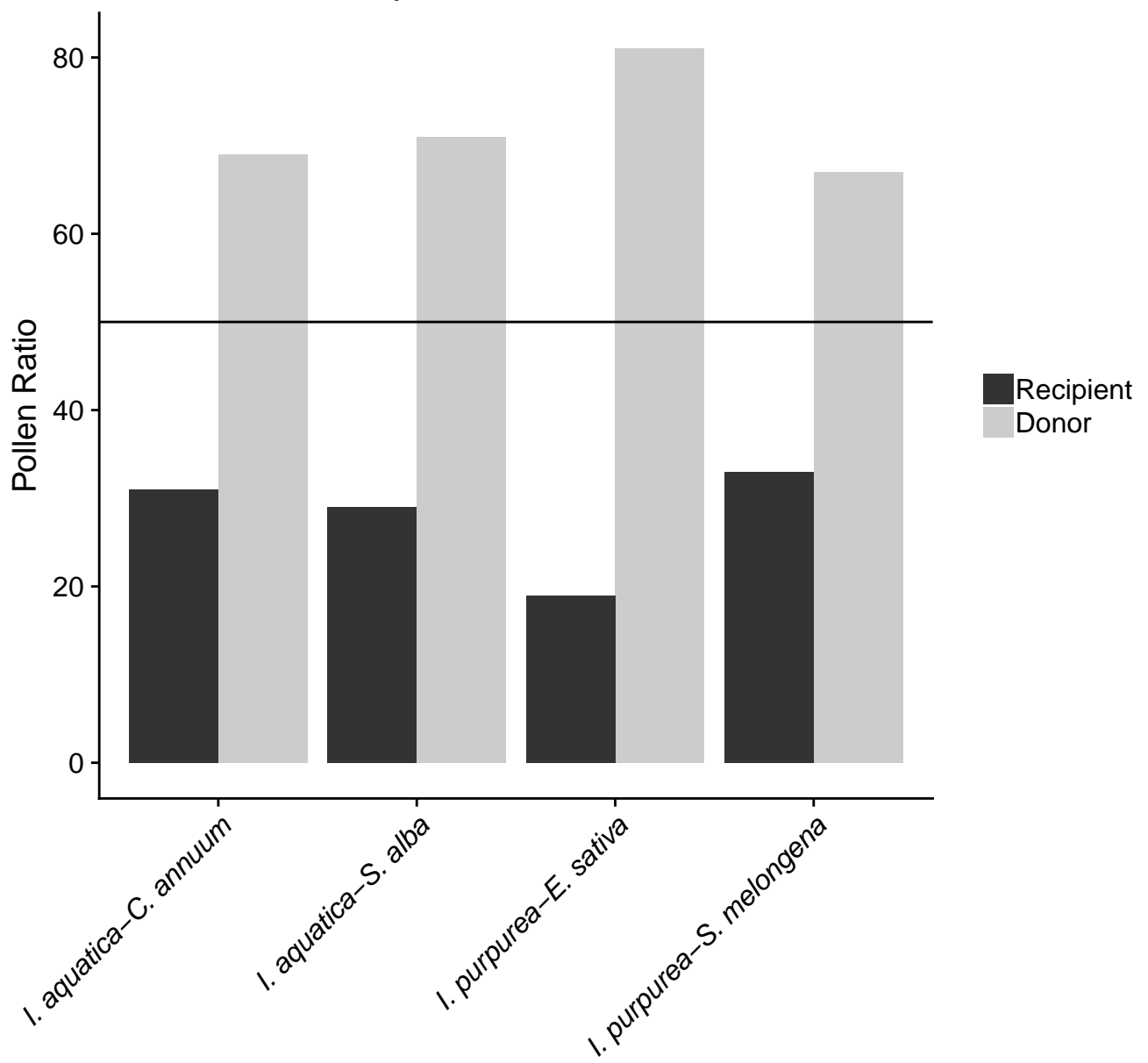
301

B) Solanaceae



302

C) Convolvulaceae



303

305 List of Figures

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