

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).

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

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

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

77 For the understanding at what level or intensity the interference of pollen can occur is important to
78 consider the relatedness of the interacting species. Closely related species are more likely to have similar
79 traits Letten and Cornwell (2015). The similarity in traits between closely related species can lead to
80 higher chances of ovule usurpation/abortion Arceo-Gómez and Ashman (2011) and therefore studies
81 predict and show a greater negative effect of closely related species Ashman and Arceo-Gómez (2013);
82 Arceo-Gómez and Ashman (2016). Few studies however, have focused on the impacts of heterospecific
83 pollen on fitness of distantly related species Galen and Gregory (1989); Neiland and Wilcock (1999).
84 Despite the fact that far related species are also able to decrease species fitness (REFS). Yet, most
85 insects and most stigmas have been found to carry multiple species of foreign pollen with little attention
86 to degree of relatedness (Arceo-Gómez and Ashman (2016); Fang and Huang (2013). Understanding
87 the role of foreign pollen from distantly related species thus deserves greater attention. The relatedness
88 of foreign pollen gives a first snapshot of where the pollen competition can occur and therefore could
89 be a proxy of effect. With that said, Arceo-Gómez and Ashman (2016) is the only work until our
90 knowledge 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 heterogeneous reproductive 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: Brassicaceae, Convolvulaceae and Solanaceae (**Table 1**). The species of the study had different reproductive traits and different degree of relatedness (see phylogenetic tree, **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) and the rooms of the glasshouse were temperature controlled with temperature oscillations between day and night.

115 **Table 1** Species list with family and genus.

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 top to bottom: Brassicaceae, Convolvulaceae and Solanaceae

116 **Hand pollination**

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

133 **Traits and evolutive distance**

134 The traits measured for each species were pollen per anther, pollen size, number of ovules and stigma,
135 style, ovary width and length. For the stigma, the stigmatic area was also measured and moreover the
136 stigmas were divided in wet/dry type with the help of the stereomicroscope. All the morphometrical
137 measurements were performed with a stereophotomicroscope with the exception of pollen size that was
138 carried out with a light microscope. Pollen was counted for 20 anthers of each species with 4 replicates
139 per sample with an hemocytometer. Previously, anthers were squashed on a known solution with the
140 pippete tip and homogeneize with a vortex for 30 seconds. Ovule number was counted with the help of
141 a stereomicroscope and a small grid over a petri dish from 15 randomly selected flowers. Fruits per
142 number of flowers treated were counted for just Solanaceae species with fleshy fruit. For all the species
143 we counted the number of seeds produced per average number of ovules. Levels of self-incompatibility

were estimated by dividing the seed set of hand self-pollination by hand cross-pollination Lloyd and Schoen (1992).

Analysis

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

To compare the magnitude of effect of heterospecific pollen across species we conducted standardized Hedges' d [(mean of mixed 50% mix - mean of cross pollination)/pooled SD] with **effsize** package. We did in three different ways: effect sizes of each donor per focal species; effect sizes per family of the different donors per focal species; effect sizes of all the donors grouped per focal species.

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

In order to test the relative effect of traits on seed production with foreign pollen we performed Mantel test in R (**vegan** package, Euclidean distance) between the asymmetrical matrix of heterospecific pollen effect (10 by 10 matrix) with the different distance matrices of traits. Heterospecific pollen effect was obtained through the subtraction of seed production by hand cross-pollination minus seed production of the different heterospecific pollen treatments. To find a model with the best explanatory traits we used the function **bioenv** from R. We also conducted Mantel test between the matrix of heterospecific pollen effect and the distance matrix from all the traits. Moreover, we explored also the correlation between traits and heterospecific pollen effect through generalized mixed models where the response

variable was heterospecific pollen effect and the explanatory variable the different traits. In addition, we tested the correlation between the total amount of pollen deposited on the stigma with heterospecific pollinations and the stigma size through Pearson's correlation.

Phylogenetic signal of traits?

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

Also the plots of ratios to appendices.

NMDS to appendix?

RESULTS

Results of hand cross-pollination, hand self-pollination, natural selfing and apomixis are presented in **Figure 1** (see appendix 1 for table with values). Heterospecific pollen reduced seed set significantly with the 50-50% heterospecific pollen treatments for 65% of the pairwise interactions $p < 0.05$. Moreover, average effect sizes differed across species and across families, see **Figure 2**. Despite some variability in the effect of the different pollen donors per species, in general terms the effect of heterospecific pollen from the distinct nine treatments per species was homogeneous (see **Figure 3**), just for four species out of ten, just one donor did not overlap the confidence intervals with other donors. Therefore, none of the donors had a clear stronger or weaker effect across species. When the donors were groped by family not big differences were seen, just for *S. lycopersicum* the confidence intervals of Brassicaceae and Convolvulaceae did not overlap (see **Figure 4**). In addition, for the 100% hetrospecific pollen treatments we did not find almost seed production. However, for just one species (*S. alba*) the control pollination and the heterospecific pollination with pollen from a confamilial had similar seed production. For two Solanaceae species *S. melongena* and *C. annuum* 100% pollen treatments produced few seedles fruits (3% and 9% respectively) and they did not for the apomictic treatments.

Results of Mantel test between heterospecific pollen effect and phylogenetic distance gave a positive

196 statistically clear correlation for both markers ($p < 0.05$). The correlations with ITS and RBCL markers
197 was respectively of 0.29 and 0.25. We found a significant phylogenetic signal of traits for pollen size,
198 stigma measurements and style length ($p < 0.05$). Although with a lack of a significant correlation
199 pagel's lambda values were also relatively high (> 0.45) for incompatibility index, ovary length and
200 levels of selfing. Moreover, Mantel test between heterospecific pollen effect and traits gave also a
201 positive significant correlation with a r value of 0.4. When the effect was look trait by trait with Mantel
202 test, stigma type and stigma measurements (length, width and area) gave a significant positive
203 correlation with heterospecific pollen effect.

204 [explain ratios and total pollen](#) add table with morphometrical traits to appendix

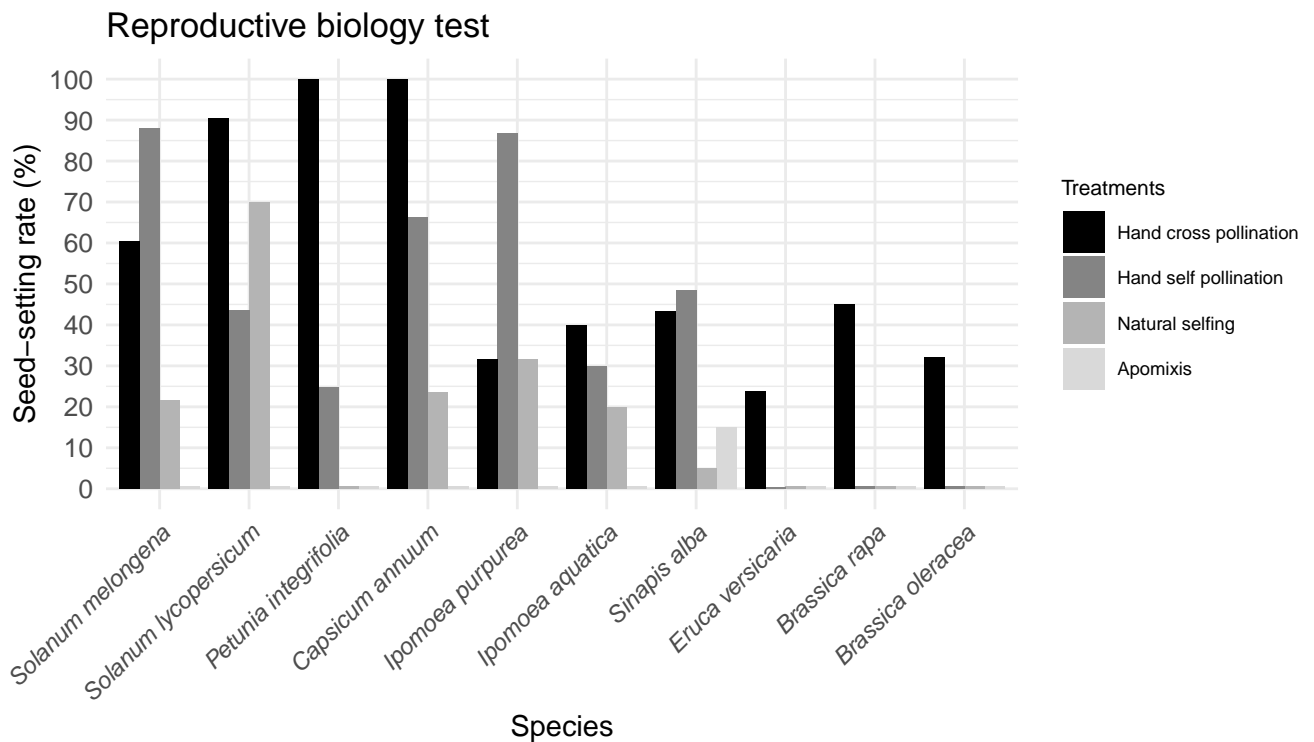


Figure 2: Barplot with the different treatments that provide information of the reproductive biology of the ten species. The y axis is the proportion of ovules converted to seed in percentage. The different treatments (N=10) which are presented in the legend are, hand cross-pollination, hand self-pollination,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

DISCUSSION

Interestingly the effect of the different donors per species were very homogeneous which lead us to think that the female reproductive traits of the pollen recipient are the main ones in explaining heterospecific pollen effect. Our main predictive trait of effect is stigma size, and because we found a correlation between the pollen quantity deposited on the stigma and stigma size, we argue that that the total load of pollen deposited per treatment can obscured what are the main traits in driving heterospecific pollen effect.

Curiously between the species with greater effect sizes, we found completely self incompatible species, species with the smallest stigma and the species with shortes style. Develop more...

Although we have found a positive correlation between phylogenetic distance and heterospecific pollen effect, this results have to be treated carefully. From our results we want to highlight that also far



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.

related species can affect negatively fitness but the effect from close related species can also have important detrimental effects. Moreover, the effect of close related species can be masked by the possibility of hybridization as it occurs between two of our species of Brassica. Moreover, although different effects between distinct donors can occur we want to note the importance of the traits of the recipient that determine an homogeneous effect between donors as we have shown in figure xxx. These different traits will define how it will be the effect across the different species independently of the nature of the donor and also differently even between species of . Although traditionally the nature of the pollen donor has been very studied, the recipient...

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

What are the implications of the findings?

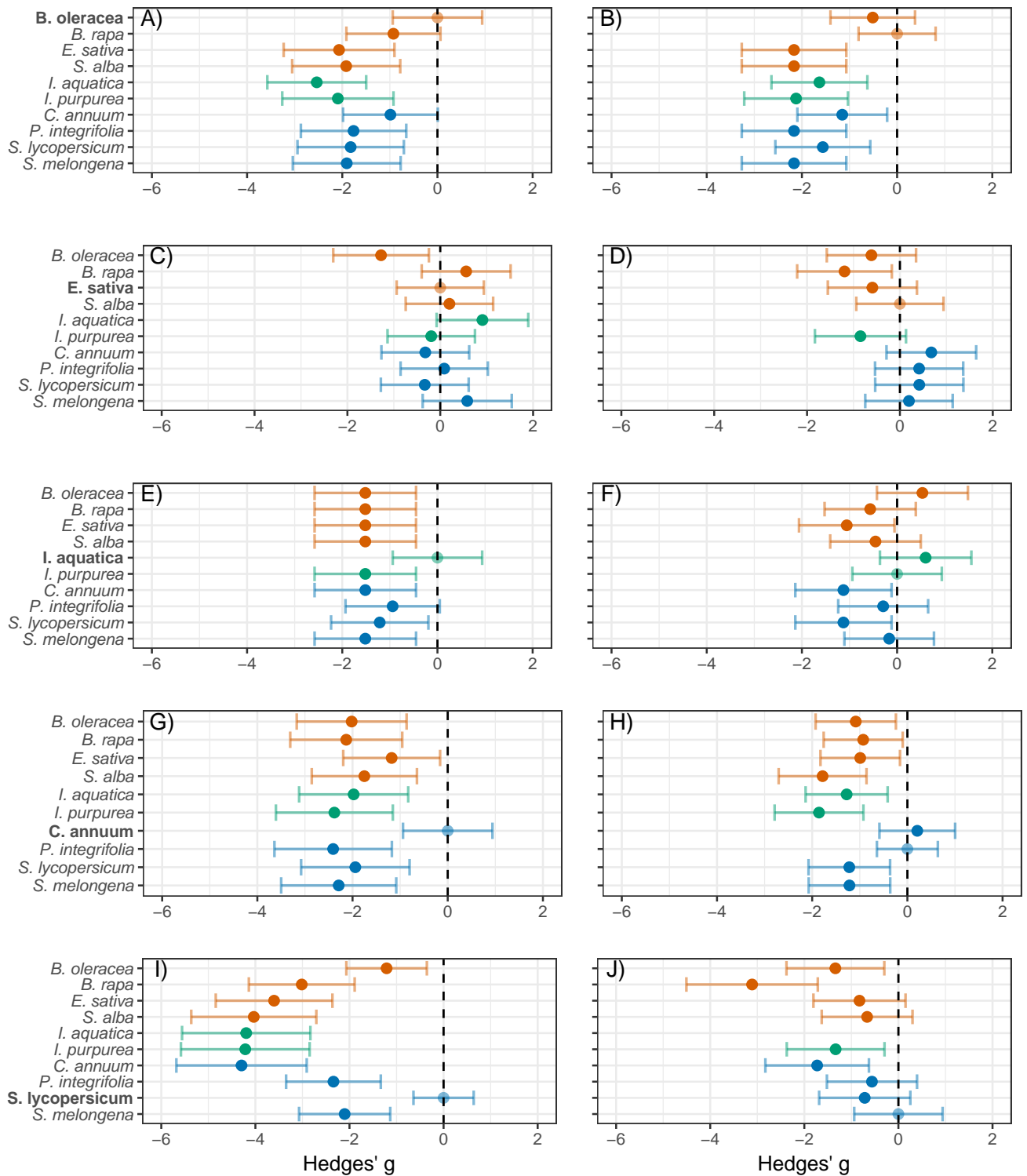


Figure 4: Effect sizes for the 10 different species. The different families appear with different colours, when a species was focal was coloured differently from its family.



Figure 5: A

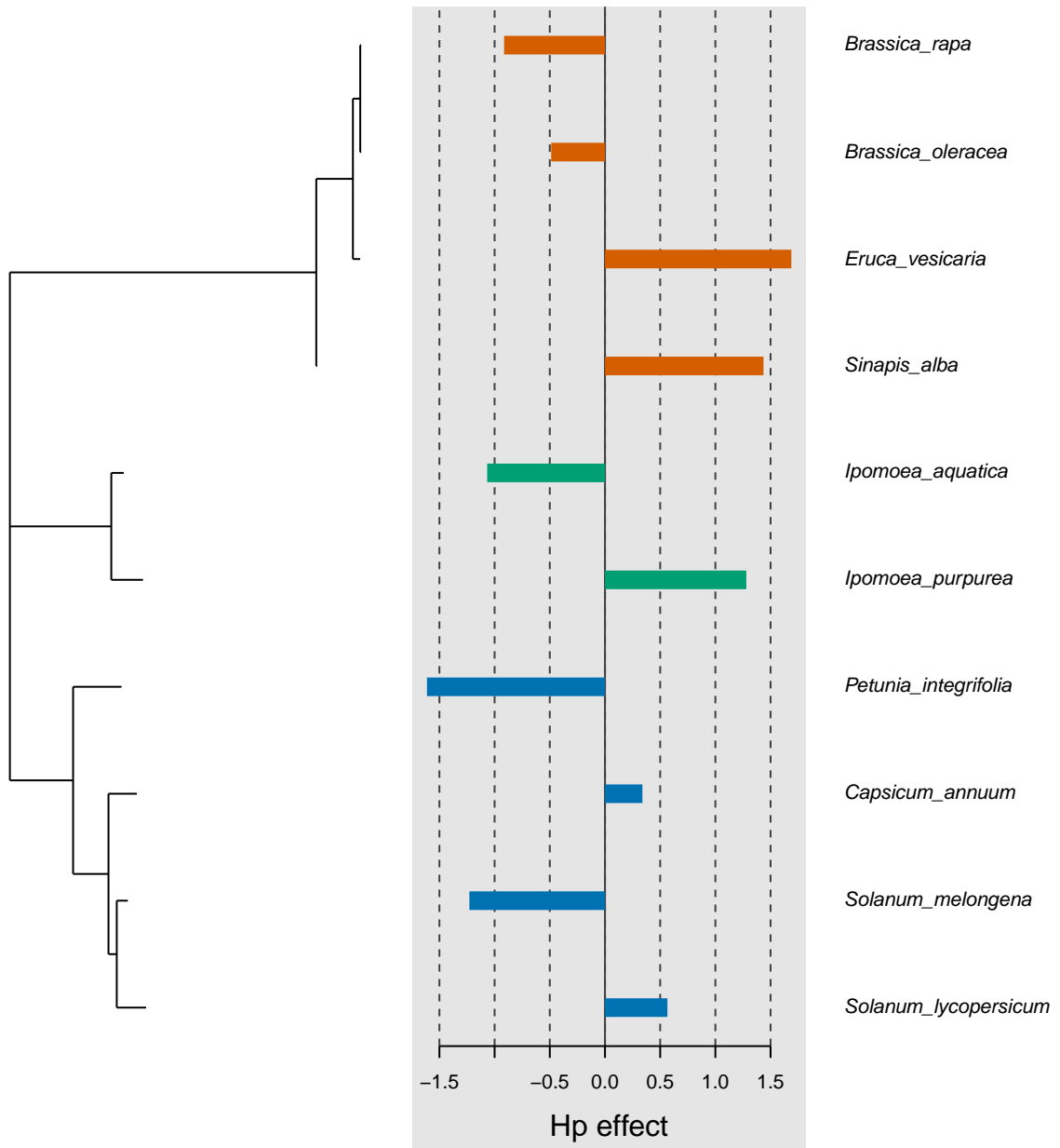


Figure 6: Phylogenetic signal of the average heterospecific pollen effect size per species

227 CONCLUSIONS

228 ACKNOWLEDGEMENTS

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APPENDIX

1.

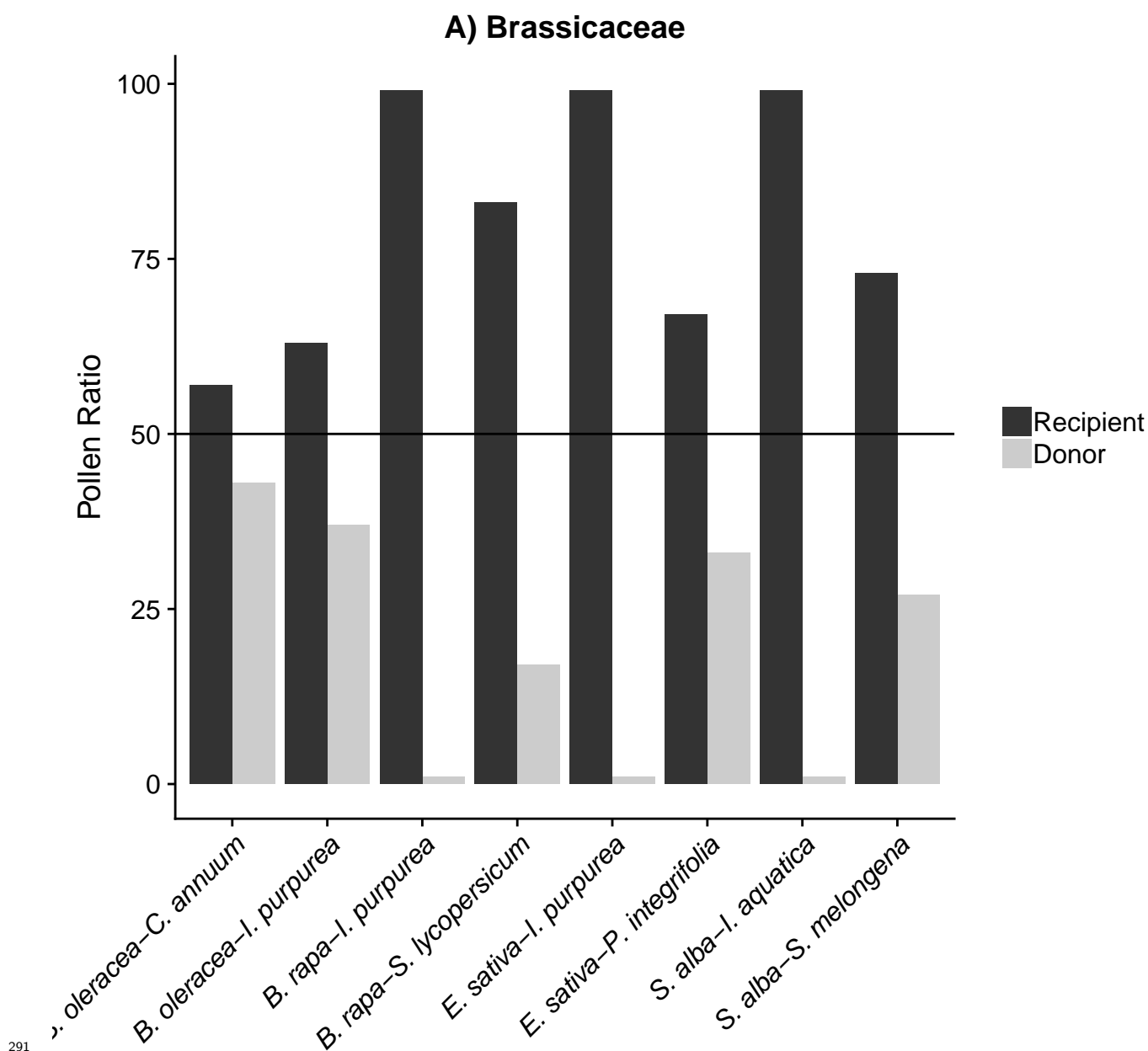
Table S1. Perecentage of seeds produced per ovule for the ten species used in the experiment. The treatments presented are hand cross-pollination, hand self-pollination, natural selfing and apomixis (emasculated flowers).

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

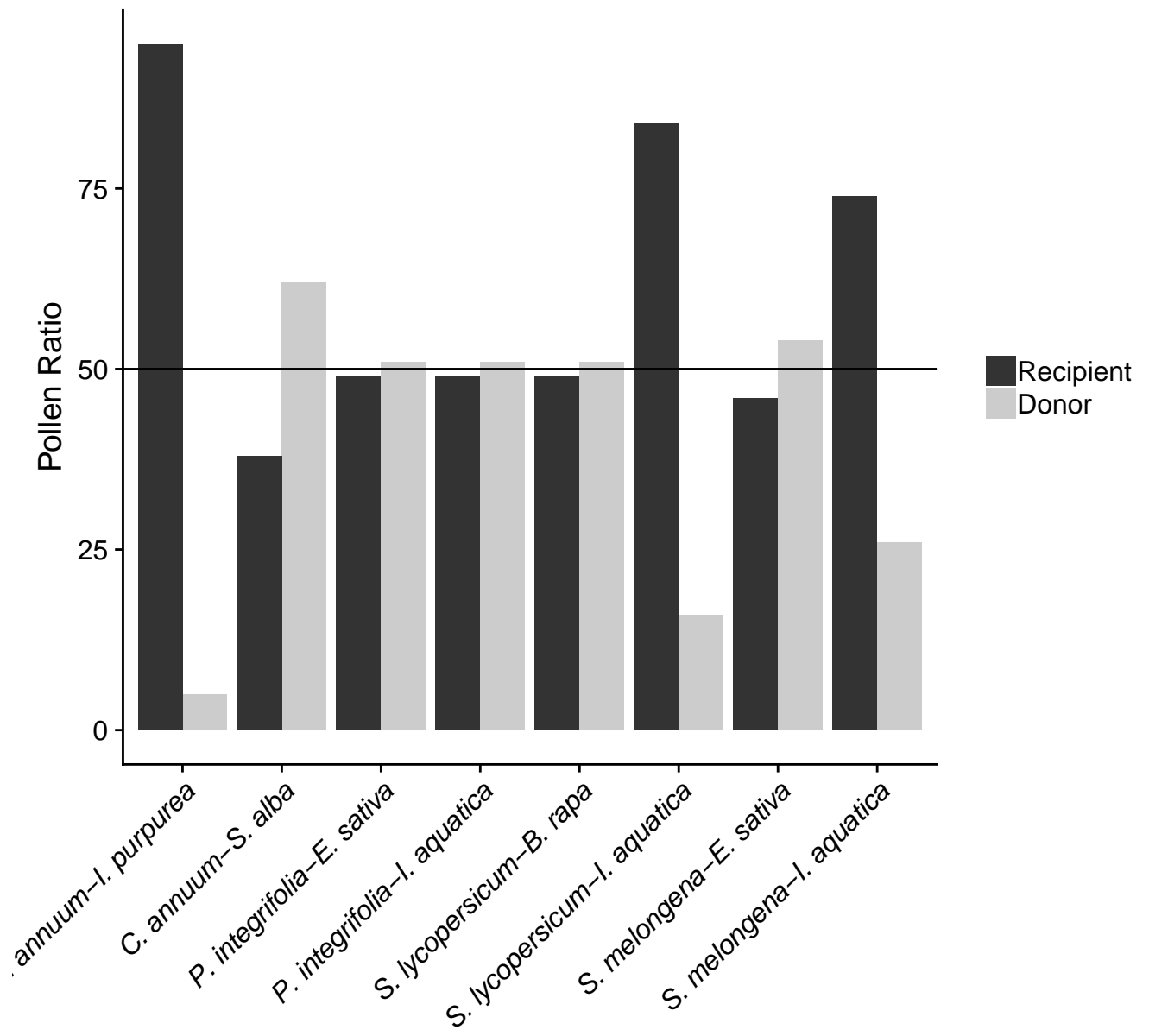
Species	Cross	Self	Natural_selfing	Apomixis
Solanum melongena	60.47525	87.9702970	21.56436	0

287 2.

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

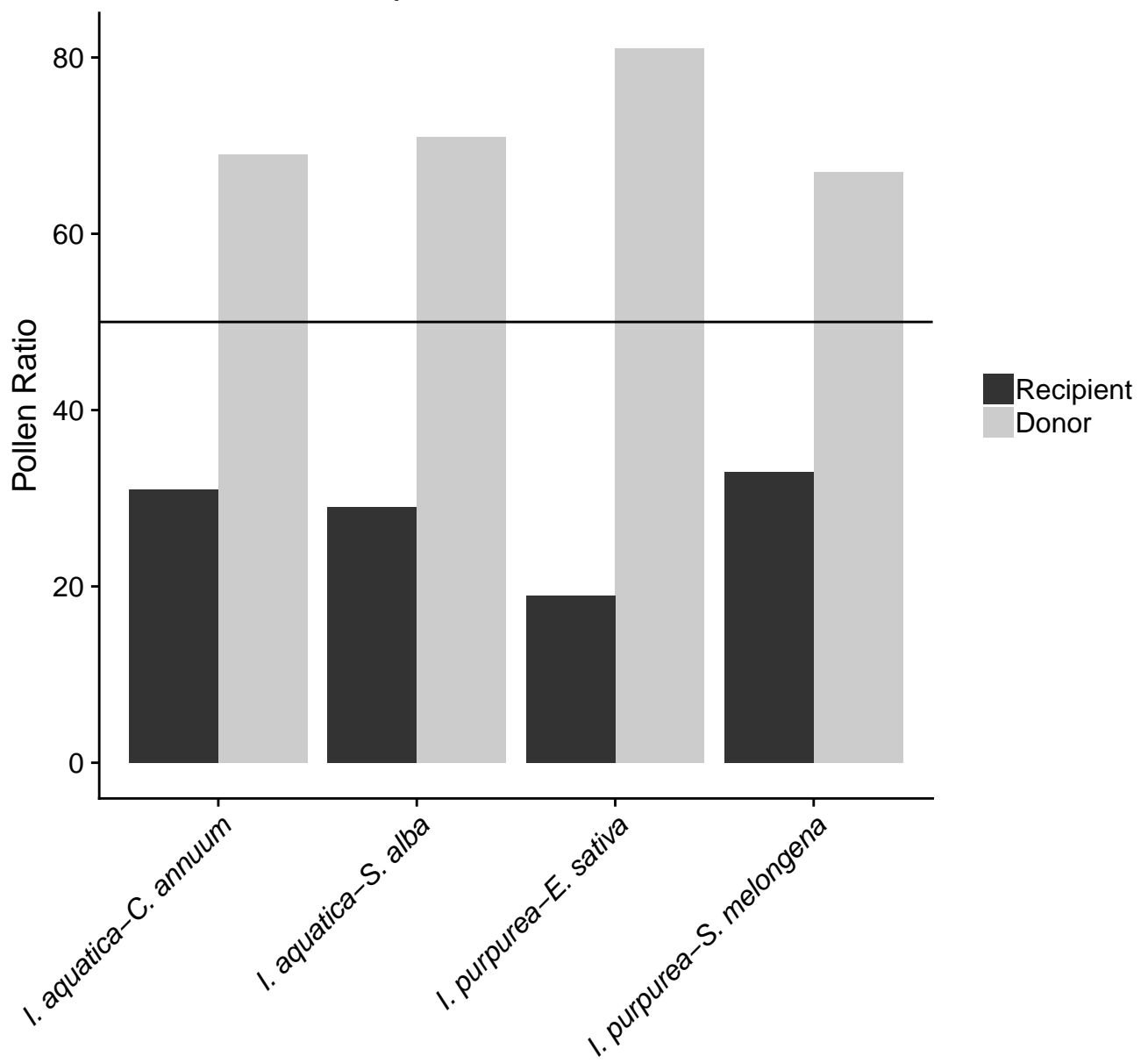


B) Solanaceae



292

C) Convolvulaceae



293

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