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).

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

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

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

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

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

Hand pollination

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

Traits and evolutive distance

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

were estimated by dividing the fruit 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 relevelling 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 standarized Hedges' d [(mean of mixed 50% mix - mean of cross pollination)/pooled SD]. 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.

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 assymetrical 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.

We conducted mantel test to check for correlations between heterospecific pollen effect and phylogenetic distance. Due to improvents 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.

Phylogenetic signal of traits?

Add pollen on stigma counts to methods

[Next bit to results, also divided it with]

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 aboout this in methods too.

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

Add effect sizes to analysis!!

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

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 significatively 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 statistically clear correlation for both markers (p<0.05). The correlations with ITS and RBCL markers was respectively of 0.29 and 0.25. We found a significant phylogenetic signal of traits for pollen size, stigma measurements and style length (p<0.05). Although with a lack of a significant correlation pagel's lambda values were also relatively high (>0.45) for incompatibility index, ovary length and levels of selfing. Moreover, Mantel test between heterospecific pollen effect and traits gave also a positive significant correlation with a r value of 0.4. When the effect was look trait by trait with Mantel test, stigma type and stigma measurements (length, width and area) gave a significant positive correlation with heterospecific pollen effect.
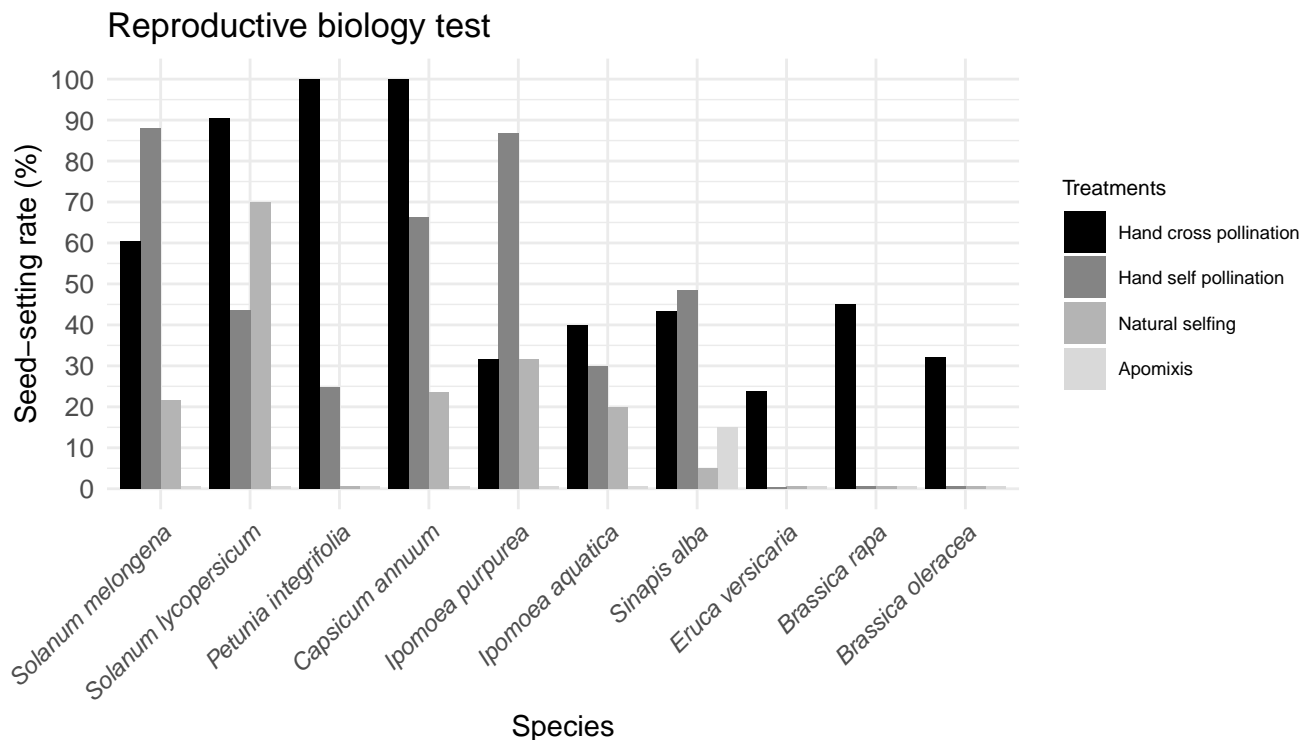
Figure 2: Barplot with the different treatments that provide information of the reproductive biology of the ten species. The y axis is the proportion of ovules converted to seed in percentage. The different treatments (N=10) which are presented in the legend are, hand cross-pollination, hand self-pollination, natural selfing and apomixis. More information about these treatments can be found in Methods and Appendices.

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

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

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.

DISCUSSION

Open with the main results. . .

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 related species can affect negatively fitness but the effect from close related is species can also have important detrimental effects. Moreover, the effect of close related species can be masked by the possibility of hibridization as it occure between two of our species of Brassica. Moreover, although different effect between distinct donors can occur we want to note the importance on 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 have been very studied, the recipient. . .

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

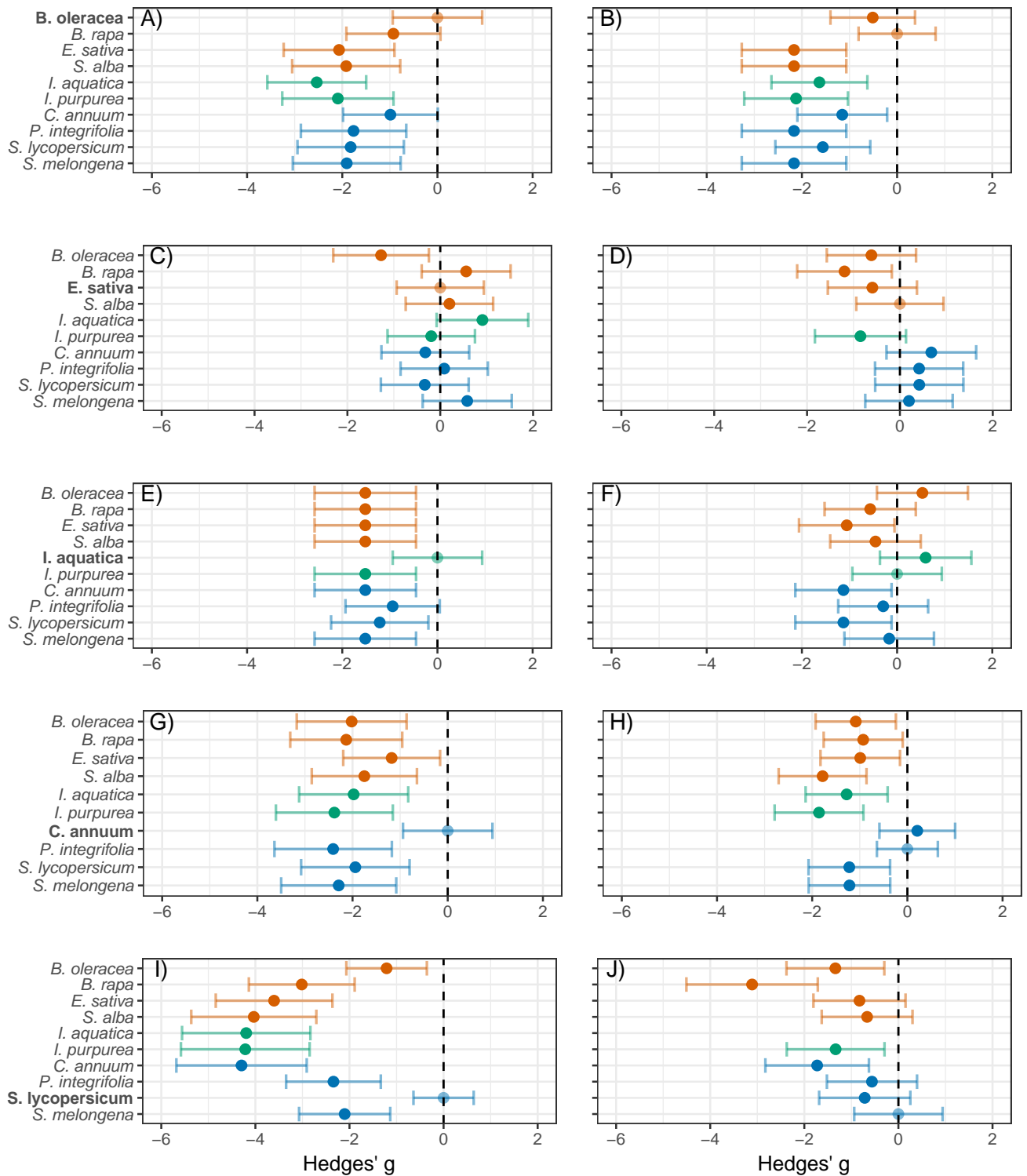What are the implications of the findings?

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

CONCLUSIONS

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

March 13, 2019

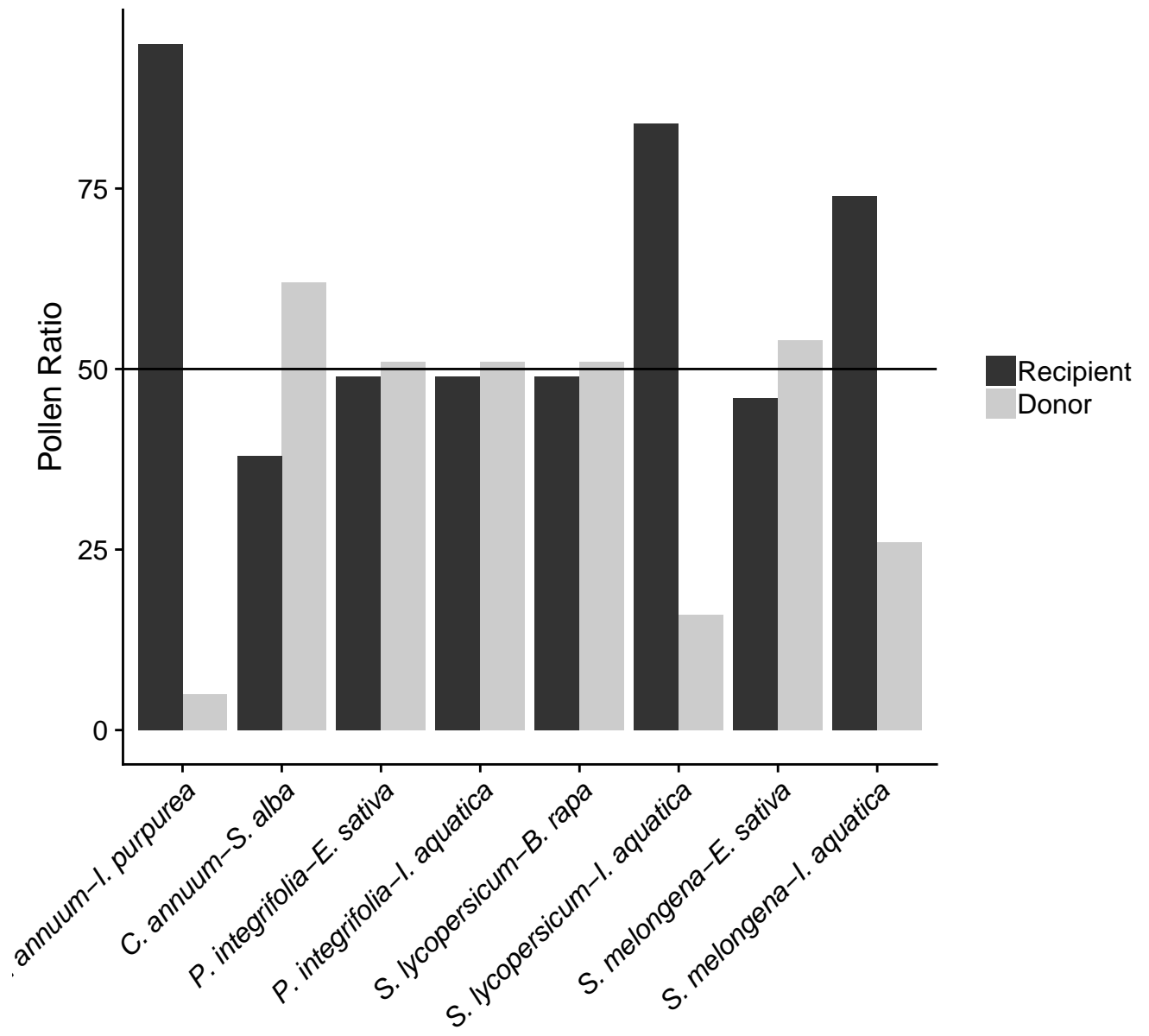| Species	Cross	Self	Natural_selfing	Apomixis
Solanum melongena	60.47525	87.9702970	21.56436	0

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.
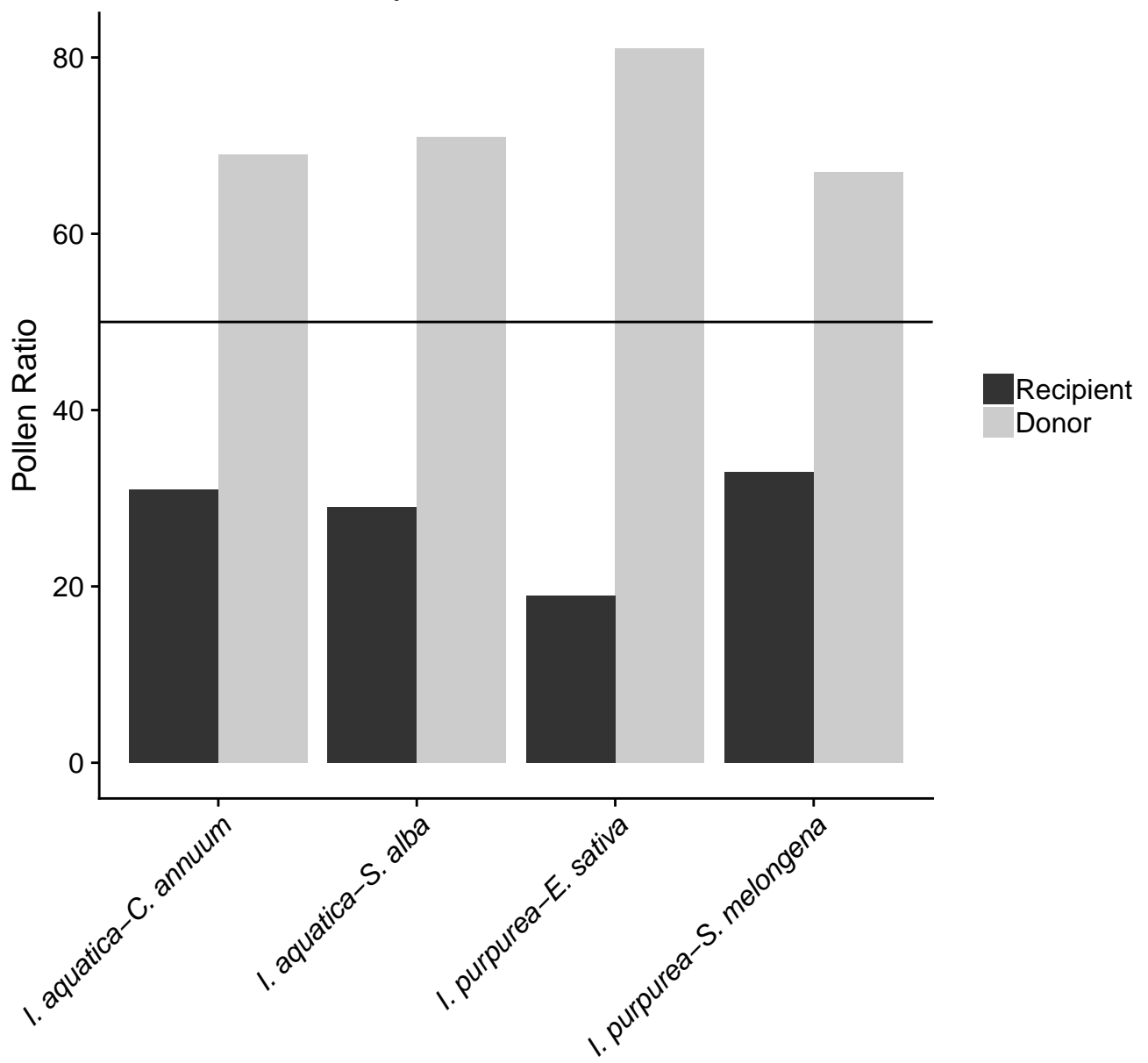

A) Brassicaceae

B) Solanaceae

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C) Convolvulaceae

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