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 4XXXX crosses by experimentally transferring pollen (50% and 100% ratio) with reciporcal 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 here it is important to notice that these traits can be seen from a donor perpective or a recipient one. In first place, when the donor is considered pollen size, pollen allelopathy and the relatedness could be the two main predictors of effect. How pollen size and allepothaty are involved in heterospecific pollen effect remains unclear yet. Herrera xxxx observed that small pollen is more likely to clogg the stigma, here we argue that although this is possible, the quantities of heterospecific pollen in nature are generally low as mentioned above and bigger pollen is generally related with higher pollen tube growth rate, which add another layer of complexity to the understanding the overall importance of this trait. When the pollen recipeint is taking into account style length, stigmatic area, number of ovules and incompatibility system seems to be the main predictors of effect.

Example of flower morphology and the importance of stigmatic area Montgomery and rathcke 2012

Species that are self-incompatible have stronger barriers to heterospecific pollen 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)).

End this with pollen quantity stuff!!!

Incompatibility system is another plant trait that appears to play an important role in foreign pollen effect. Species that are self incompatible have stronger barriers to heterospecific pollen than self-compatible species Ashman and Arceo-Gómez (2013). However, note the large variability in mating systems across populations Whitehead et al. (2018) . . . . The type of incompatibility, (i.e. whether sporophytic or gametophytic) is related to the location of pollen recognition; sporophytic incompatibility relates to signaling at the stigma surface while gametophytic occurs within the style Barrett (1988). This later acting pollen recognition mechanism is associated with greater negative effect than sporophytic recognistion Ashman and Arceo-Gómez (2013). if you introduce this,I eexpect you will use it in your analysis, remove (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 69 Whitehead et al. 2018)- IB this is a side problem for you, mention just once, and maybe in discusion. 70 Now it has a lot of weigth.)(). Moreover, other traits such as number of pollen grains per flower and 71 number of ovules have been traditionally associated with the type of incompatibility system where 72 species with higher pollen ovule ratios are predicted to be xenogamous and species with low pollen 73 ovule ratios autogamous (REF) . Selfer species are known to have a reduction of herkogamy (REF) and 74 less pollen production per ovule (REF) which can be interpretated as a reduction of pollen exported 75 into the community so, pollen and ovules are important by themselfs, or only because correlated? Unclear what your point is. Other morphological traits, like stigma size can be determinant for the total pollen quatity that a stigma can receive and therefore related to do that pollen size would also play an important role. Example with pollen here.

. . .

Moreover, other traits such as number of pollen grains per flower and number of ovules have been traditionally associated with the type of incompatibility system where species with higher pollen ovule ratios are predicted to be xenogamous and species with low pollen ovule ratios autogamous (REF) . Selfer species are known to have a reduction of herkogamy (REF) and less pollen production per ovule (REF) which can be interpretated as a reduction of pollen exported into the community so, pollen and ovules are important by themselfs, or only because correlated? Unclear what your point is. Other morphological traits, like stigma size can be determinant for the total pollen quatity that a stigma can receive and therefore related to do that pollen size would also play an important role. Example with pollen here.

Paragraph 4 Maybe connect with paragrph above?

Comments on it

Species with similar traits are more closely related XXXXXXXXX I would say no. species closely related usually have similar traits, specally if those are phylogenetially conserved. (Refs? Brown and Mitchell (2001) Arceo-Gómez et al. (2016) Tong and Huang (2016)). Several studies predict that the impact of HP transfer is likely to be greater for closely related species (Ashman and Arceo-Gómez (2013)). Few studies however, have focused on the impacts of heterospecific pollen of distantly related species Thomson et al. (1982) Galen and Gregory (1989) Neiland and Wilcock (1999). 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) ; also cite studies from pollen transfer networks here such as. . .).

here you change topic, new paragraph? Further, a majority of plant species are generalist and thus receive visits from multiple different pollinators. Given these are generally the ones that receive greater loads of heterospecific pollen Fang and Huang (2013) and unrelated species are more likely to coexist with other species due to less niche overlap (Ref), understanding the role of foreign pollen from distantly related species thus deserves greater attention in understanding coexistence blah blahXXXXX refs.. Notwithstanding, the effect of heterospecific pollen of far and close related species at community level remains to be explored beyond single pairwise interactions.

Paragraph 5 Introducing our experiment

In this study we investigated how floral reproductive traits and relatedness mediate the impact of HP transfer by asking the following research questions : To what extent do (i) floral reproductive traits and (ii) relatedness, mediate the impacts of heterospecific pollen on seed set. We do this by creating an artificial co-flowering community with 10 species belonging to three different families with different traits.

METHODS

The study was conducted in a glasshouse at University of New England (Armidale, Australia) from November 2017 to March 2018. Rooms were temperature controlled depending on the requirements of the species with day and night temperature differences. The species selected (**Table 1**) belonged to three different families, Solanaceae, Brassicaceae and Convolvulaceae. The criteria of species/family selection was based on close/distant related species (see phylogenetic tree for relatedness fig 1)I would explain more the bauty of our nested dessign to ensure close and far distance simultaneously, heterogeneous traits, low structural flower complexity and fast life cycle. 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).

Table 1

Family	Genus	Species
Brassicaceae	Brassica	Brassica rapa
Brassicaceae	Brassica	Brassica oleracea
Brassicaceae	Eruca	Eruca versicaria
Brassicaceae	Sinapis	Sinapis alba

Family	Genus	Species
Convolvulaceae	Ipomoea	Ipomoea aquatica
Convolvulaceae	Ipomoea	Ipomoea purpurea
Solanaceae	Capsicum	Capsicum annuum
Solanaceae	Petunia	Petunia integrifolia
Solanaceae	Solanum	Solanum lycopersicum
Solanaceae	Solanum	Solanum melongena

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 (N=10) this second I don't get, maybe explain it's ultility.. Therefore, 180 different combinations were performed with N=10 per combination. 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. The mixes of pollen were realized on an eppendorf based on the pollen counts made with Neubaeur chamber (each anther was counted 4 times for 20 different anthers per species)-IB explain better and give a bit more of detail. In order to confirm that the treatments applied were 50-50 percent pollen, for each focal species the total stigmatic load of pollen was counted from one donor of each family (N=3).

Traits and evolutive distance

The traits measured for each species were pollen per anther, number of ovules, stigma width and length and stigmatic area, style width and length, ovary width and length. Moreover stigma type explain was tested. 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 an stereomicroscope and a small grid over a petri dish from 15 randomly selected flowers. The different morphometrical traits were measured with a digital stereomicrospe. Levels of self incompatibility were estimated by dividing the fruit set of hand self pollination by hand cross pollination Lloyd and Schoen (1992).

Analysis

We used the statistical language `R` (R Core Team 2018) for all our analyses. Differences of seed set between treatments and hand cross pollination for each species was tested through mixed linear models. For the following analysis we scaled the values of seed production for all the species with mean 0 and sd of 1. To test the effect of heterospecific pollen, we substracted to the seed set of hand cross pollination the seed set of heterospecific pollen treatments. In order to see correlations between heterospecific pollen effect and traits we performed Mantel test between the matrix of heterospecific pollen effect and the distance matrix of each trait (euclidean distances). Moreover, Mantel test was also conducted between heterospecific pollen effect and the square root of the matrix of phylogenetic distance due to improvement in the statistical power (Letten & Cornwell 2014). all is here, but I would break it by questions and give a bit more detail, to avoid overwhelm the reader We explored also the relations between traits and heterospecific pollen effect through generalized mixed models where the response variable was heterospecific pollen effect, the independent variable the different traits and the random effects the different treatments per species [Here I think you should think if this controls for the non independency of donors and recipients. I think not. Maybe look onto matrix regresions?). Moreover, pairwise evolutive distances were calculated with MEGA7 for two kinds of markers: 1) Internal transcribed spacer (ITS) and 2) ribulose-bisphosphate carboxylase (RBCL). The sequences of interest were downloaded from NCBI GenBank and the phylogenetic tree constructed by maximum likelihood with MEGA7.Make a section on how you contrsucted phylogeny.

I would explain three test. 0) treatment effects with GLM's, 1) Mantels: relative effects, 2) GLM's or matrix models: Absolute effects and explain them in three independent paragraphs including a rationale of why

Phylogenetic signal of traits?

RESULTS

Results of hand cross pollination, self hand pollination, natural selfing and apomixis are presented in **Table 2**. Heterospecific pollen reduced seet set signifcatively with the 50-50% heterospecific pollen treatments for 65% of the pairwise interactions $p<0.05$. Across families we found a very similar effect but when species where look at species level they respond differently even within the same family rephrase and maybe test statistically?, for instance for two species of the Brassicaceae family *Brassica oleracea* and *Eruca versicaria* we found very contrasting effects of foreign pollen where for the first one, all donors reduce seed set significatively and for the second, just two species did out of nine. The 100% foreign pollen treatments barely produced seeds or fruits and just for *Sinapis alba* we did not find significant differences between the hand cross pollination and one treatment with pollen from a confamilial- IB Unclear. Solanaceae species with berry fruit type developed small fruits or even normal fruits in some cases under which treatment. *S. lycopersicum* seems to produced small fruits (35% of the treatments) independently of pollen and pollen donor due to also apomictic treatments did, never normal size. *C. annuum* produced some fruits (9%) of both small and normal size and finally *S. melongena* produced seedless normal fruits with just confamilial pollen (3%), for both species seems that fruit formation was induced by pollen on the stigma because of lack of fruit production with treatments that tested for apomixis.clarify this descriptive statistics part- Also a figure with a summary of the treatments effect would be cool, or at least in the appendices

Table 2. 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). turn into a figure somehow?

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

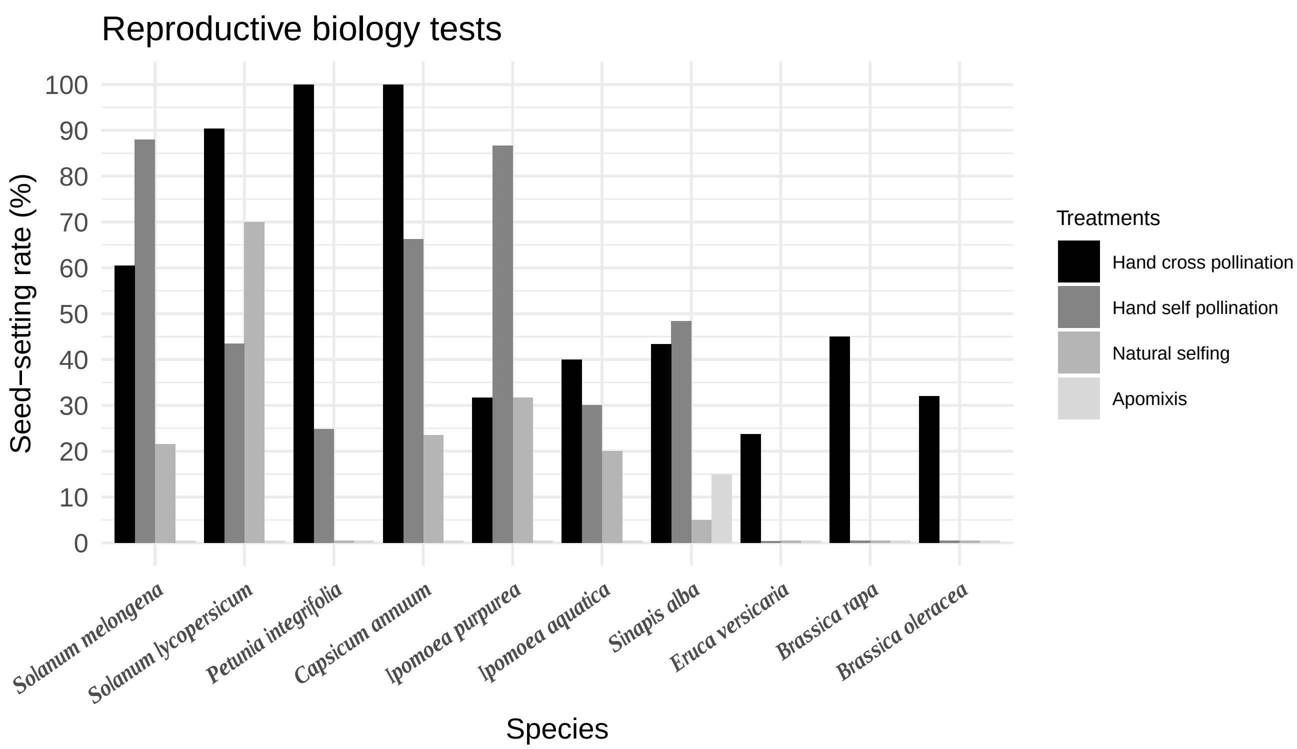

Figure 1: Barplot with the different treatments that provide information of the reproductive biology of the ten species. The y axis is the proportion of ovules converted to seed in percentage. The different treatments (N=10) which are presented in the legend are, hand cross pollination, hand self pollination,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

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

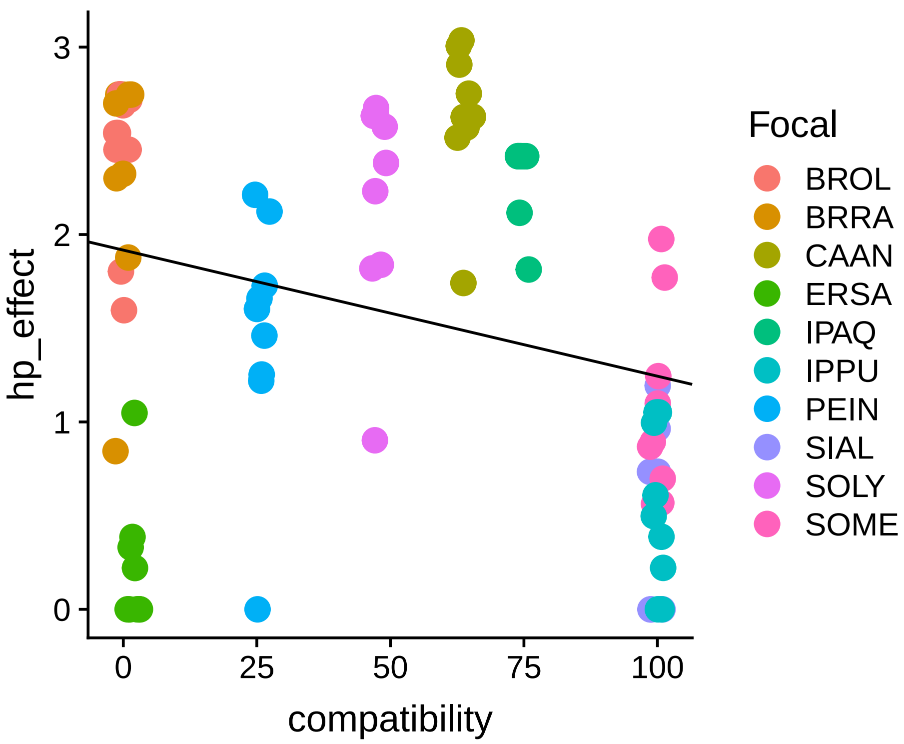

Figure 2: The effect of heterospecific pollen (scaled see set) is represented in function of the compatibility system (self/cross*100) for the the different species. Each coulored dot represents the interaction of a focal species with a different pollen donor.

Compatibility index don´t multiply per 100 from Lloyd

DISCUSSION

Discussion

What are the implications of the findings?

Other idea based on the paper of Aizen 2007:

He explains seed set in this way. O total number of ovules, d fraction of ovules that become seed set, b proportion of pollen grains that reach ovules, p number of pollen grains.

$S = dO(1 - e^b p)$

$S = dO(1 - e^b p(HpEffect))$

but this Hp effect maybe can be divided at the same time in the interaction between recipient-donor with the main traits that drive the effect. IB: maybe just a weighting factor 0-1 depending on trait matching? If effect of hetero is 0, it cancells out the term, if is maximum, it *1

Moreover, this should consider quantity of hp. lINEAR EFFECT? How to model this maybe talk with nacho. IB: In the absence of data you can try linearity, but also a sigmoidal.

IB: Cool! se also Morris et al 2010 Ecology on how to add to AIzen curve, the effects of a second curve describing the negative effect of hetero. Morris apporach is mathematically more robust if you can model the second curve of hp effects.

I also think you should use this for another paper and look into Morales -Castilla TREE paper inferring interactions. I can see a similar idea where you use a set of matrix you can multiply. 1) A matrix of plant-plant pollinator influence (a lo carvalehire 2014). This tells you which plants may get exposed to hp pollen from empirical plant pollinator networks 2) you matrix of plant plant ht effect (0-1) or a proxy based on phylogeny of trait similarity. This is also easy to quantify. 3) A vector of sensitive recipient traits. to create the probability of ht effects. I really like this ideas to link to community ecology.

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

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

Now I try to develop each part

1) Donor pollen size $<$ Recipient pollen size

Effect:

- Donor's pollen could clogg the stigma
- Chemical inhibition

Traits associated with bigger pollen of the recipient:

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

2) Donor pollen size $=$ Recipient pollen size

- Very relatedness dependant this point
- Similar probabilities of taken space on the stigma

3) Donor pollen size $>$ Recipient pollen size

Effect:

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

-small pollen grains can get embeded

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

CONCLUSIONS

ACKNOWLEDGEMENTS

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List of Tables

List of Figures

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. 10

2 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. 11