The effect of polyploidy and mating system on floral size and the pollination niche in Brassicaceae

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Abstract

Premise of research. Polyploidy, a major evolutionary process in flowering plants, is expected to impact floral traits which can have cascading effects on pollination interactions, but this may depend on selfing propensity. In a novel use of herbarium specimens, we assessed the effects of polyploidy and mating system on floral traits and the pollination niche of 40 Brassicaceae species.

Methodology. We combined data on mating system (self-compatible or self-incompatible) with inferred ploidy level (polyploid or diploid) and use phylogenetically controlled analyses to investigate their influence on floral traits (size and shape) and the degree of pollination generalism based on the frequency and the richness of heterospecific pollen morphospecies captured by stigmas.

Pivotal Results. Flower size (but not shape) depended on the interaction between ploidy and mating system. Self-incompatible polyploid species had larger flowers than self-incompatible diploids but there was no difference for self-compatible species. The breadth of pollination niche (degree of generalism) was not affected by ploidy but rather strongly by mating system only. Self-incompatible species had more stigmas with heterospecific pollen and higher heterospecific pollen morphospecies richness per stigma than self-compatible species, regardless of their ploidy.

Conclusions. Our results demonstrate that mating system moderated the influence of ploidy on morphological features associated with pollination generalism but that response in terms of heterospecific pollen captured as a proxy of pollination generalism was more variable.

Introduction

Polyploidy, or whole genome duplication involving one (autopolyploidy) or more (allopolyploidy) parental species, is a major evolutionary process in plants (Soltis et al., 2009; Van der Peer et al., 2017; Baduel et al., 2018) with 35% of extant flowering plant species being of recent polyploid origin (Wood et al., 2009). Polyploidy provides new genetic material for ecological diversification and rapid niche differentiation, and is identified as a major driver of global patterns of polyploid persistence (Levin, 1983; Ramsey and Schemske, 2002; Martin and Husband, 2009; Van der Peer et al., 2017; Baniaga et al., 2020). While knowledge of how polyploidy affects the abiotic niche is vast (e.g., Brittingham et al., 2018; Baduel et al., 2018; Wei et al., 2019; Rice et al., 2019; Glennon et al., 2014; Baniaga et al., 2020), much less is known about the effect of polyploidy on the biotic niche; in particular, on species interactions, such as pollination, that promote and sustain polyploid populations (e.g., Segraves and Anneberg, 2016; Casazza et al., 2017; Segraves, 2017; Gaynor et al., 2018).

Polyploidy is likely to alter plant-pollinator interactions because shifts in ploidy can have significant effects on morphological and physiological aspects of flowers (reviewed in Segraves and Anneberg; 2016). For example, the most common phenotypic effect attributed to whole genome duplication is the increased size of flowers, petals, and inflorescences (a 'gigas' effect) (Vamosi et al., 2007; Porturas et al., 2019). Moreover, shifts in ploidy can modify pollinator access (e.g., floral tube length; McCarthy et al., 2019), alter self-pollination rate (e.g., stigma-anther distance, Casazza et al., 2017; or result in loss of self-incompatibility (Novikova et al., 2023). Because floral advertisement and mechanical fit are important in filtering pollinators (Armbruster, 2017), these phenotypic changes may affect a plant's pollination niche (Phillips et al., 2020). Indeed, changes that increase the mating probability of a new polyploid are expected

63 to play a key role in its establishment, as they contribute to overcoming the inherent minority
64 cytotype disadvantage by differentiating the pollination niche (Levin, 1975; Fowler and Levin,
65 1984; Rodriguez, 1996; Theodoridis et al., 2013; Spoelhof, 2020). For instance, if polyploidy
66 leads to larger flowers or shorter floral tubes then it can lead to greater accessibility of flowers
67 (e.g., McCarthy et al., 2019) and the recruitment of new pollinators could broaden a polyploid's
68 pollination niche (i.e., increased generalism via increased diversity or changes in the composition
69 of flower visitors) relative to its diploid progenitors (Segraves and Anneberg, 2016; Casazza et
70 al., 2017). Alternatively, phenotypic shifts that restrict some pollinators or reduce reliance on
71 pollinators could lead to the narrowing of the pollination niche (i.e., reducing the diversity of
72 flower visitors; increasing self-pollination) relative to its diploid progenitors (e.g., Vamosi et al.,
73 2007; Thompson and Merg, 2008). Thus, changes in the pollination niche are expected to
74 accompany polyploidy, but whether there are universal patterns of change (Casazza et al., 2017;
75 Segraves, 2017; Rezende et al., 2020) or whether patterns vary with age of polyploids (as seen in
76 McCarthy et al., 2019) is still unknown.
77 Mating system could interact with ploidy, however, to modify the flower traits and
78 pollination niche. Self-compatibility lessens reliance of pollinator service and selfing can ease a
79 new polyploid's establishment (Novikova et al., 2023). In contrast, self-incompatible polyploids
80 and diploids (in the absence of any other reproductive assurance mechanisms) both rely on
81 pollinators to affect reproduction so a new polyploid might be under greater selection to recruit
82 new (or a wider range) to pollinators to ensure adequate pollination and reduce competition with
83 its diploid parent(s) (e.g., Seagraves and Thomson 1999). Thus, we predict that self-compatible
84 diploids and polyploids may have similarly small flower sizes and narrow pollinator niches,
85 whereas self-incompatible diploids and polyploids may have greater differences in flower traits

and pollination niches. Moreover, because reduced reliance on pollinators is also correlated with reduced allocation to floral display (reviewed in Goodwillie et al., 2010), we predict that self-compatible species overall will have smaller flowers and narrower pollination niches than self-incompatible ones, regardless of ploidy.

While reviews of polyploidy-driven phenotypic changes point towards effects on pollination (e.g., Vamosi et al., 2007), and direct comparisons between diploid and polyploid for pollinator changes are accumulating, few studies quantitatively compare pollination niche breadth (reviewed in Rezende et al., 2020). Roccaforte et al. (2015) found that tetraploid *Erythronium* were visited by more pollinator taxa (including several unique insect taxa) than diploids, leading to a broader pollinator niche that was also differentiated from that of the diploids. Likewise, Thompson and Merg (2008) observed that tetraploid *Heuchera grossulariifolia* had a more diverse pollinator assemblage than diploids. However, insect pollination assemblages were similarly diverse among the ploidal cytotypes of *Chamerion angustifolium* (Kennedy et al., 2006). While this handful of studies that directly characterized pollinator taxa provides mixed evidence of shifts in pollinator niche, it does suggest that a broader test across multiple species might reveal ploidy-mediated differences in the pollination niche.

While broad comparative tests may seem out of reach due to the infeasibility of direct pollinator observation across many taxa, diversity of heterospecific pollen on stigmas is a proven proxy for pollination generalism and offers a novel avenue for broad comparison. Because sharing pollinators leads to interspecific transfer of pollen among plants (Morales and Traveset, 2008; Ashman and Arceo-Gómez, 2013; Wei et al., 2021) stigmas capture the pollination history of a plant. Thus, the amount and diversity of heterospecific pollen (HP) on stigmas reflects the

level of pollinator sharing, and consequently, plant generalism, i.e., the breadth of the pollination niche (Fang and Huang, 2013; Arceo-Gómez et al., 2016; Wei et al., 2021; Ashman and Wei, submitted). The power of these data for comparative studies of species interactions has been demonstrated from stigmas collected from live plants within and among communities at global scales (e.g., Arceo-Gómez et al., 2016; Arceo-Gómez et al., 2019; Wei et al. 2021), as well as from dried plants on herbarium specimens (Johnson et al., 2019; Rakosy et al., 2023). Because morphospecies identification is adequate to capture species-level differences in HP richness that are related to pollination generalism (see Arceo-Gómez et al. 2016) this approach can be leveraged to address the question of how polyploidy and mating system affect the pollination niche at a broad scale.

Here we investigated the influence of polyploidy and mating system on floral phenotype and pollination niche breadth in 40 species of Brassicaceae. Brassicaceae is a family of c. 4000 species with worldwide distribution (Appel and Al-Shehbaz, 2003; Al-Shehbaz, 2012; BrassiBase 2023) with characteristic four-merous cross-shaped flowers (Appel and Al-Shehbaz, 2003; Nikolov, 2019). Although species show conserved general floral architecture, there is great diversity in floral size and shape that reflect distinct pollination niches among taxa (Gómez et al., 2016). Hermaphroditic species are self-compatible or have sporophytic self-incompatibility (Hiscock and Tabah, 2003; Hiscock and McInnis, 2003). Polyploidy has been a major mode of speciation in Brassicaceae with nearly half of the species estimated to be of recent polyploid origin (Appel and Al-Shehbaz, 2003; Román-Palacios et al., 2020). Moreover, the sporophytic self-incompatibility system in Brassicaceae is not disabled by whole genome duplication the way gametophytic systems can be (Barringer, 2007; Miller et al.; 2008) thus, the direct effect of polyploidy is separated from that of the mating system. We collected data from herbarium

specimens on floral traits and on the breadth of the pollination niche (determined as the incidence and richness of HP on stigmas; Johnson et al., 2019; Rakosy et al., 2023). Specifically, we asked 1) Do polyploids have larger flowers than diploids, or does this depend on mating system or its interaction with ploidy? 2) Do polyploids have broader pollination niches than diploids? Does the breadth of the pollination niche depend on mating system, ploidy, or their interaction?

Materials and Methods

We identified 40 hermaphroditic species within 22 genera of Brassicaceae that varied in ploidy and mating system. Given our desire to test the combined effects mating system and polyploidy while confronted with an uneven distribution of the factors within genera, we sampled broadly within the family to identify species that could complete a fully crossed design of mating system and ploidy. To do so, we first selected species with known mating system using the data in Grossenbacher et al. (2017). We used self-compatibility (self-compatible (SC) or self-incompatible (SI)) as a surrogate of mating system as it correlates with dependence on pollinators and realized mating system - although imperfectly, particularly for self-compatible species (Raduski et al*.,* 2012). From this initial list, we verified availability of samples in herbaria using the iDigBio portal (occurrence data extracted using the package ridigbio; Michonneau and Collins, 2022) and identified species with ≥ 20 specimens available. This list was then cross referenced with the chromosome count data available in The Chromosome Counts Database (Rice et al., 2015). The ploidy level (polyploid or diploid) was inferred for each species following the methods detailed in Rice et al. (2019). Accordingly, polyploids are considered as those taxa that had undergone a polyploidization event since divergence from the

most recent common ancestor of their genus, including those lineages that have potentially diploidized since then. Specifically, the number of polyploidization transitions and single chromosome changes (dysploidy) that have occurred on each branch of the phylogeny were estimated based on the likelihood models implemented in the ChromEvol (v2.0) software (Glick and Mayrose, 2014). After accounting for phylogenetic uncertainties and filtering species whose ploidy inference reliability was low (Glick and Mayrose, 2014; Rice et al., 2019), we retained only species with no, or very limited, intraspecific variation in chromosome numbers (based on the frequency of the most abundant ploidal type > 80% from data available in The Chromosome Counts Database (Rice et al., 2015). Thus, while species with mixed ploidy were excluded, the polyploids selected may include allo or autopolyploids, and represent tetraploids or higher. Nevertheless, polyploid age was also inferred for each species by applying the PloiDB inference pipeline (Halabi et al. 2023) on the time-calibrated Brassicaceae phylogeny (see appendix). Specifically, for each polyploid species, we defined its age as the age of the most recent inferred polyploidization event leading to it. Ploidy age for those species characterized as diploids was set to correspond to the time of an ancient WGD that was inferred prior to the divergence of the Brassicaceae family by Kagale et al. (2014). We thus analyzed the effect of ploidy both from a categorical and continuous perspective.

The filtered list contained 104 taxa of which 11 were self-incompatible polyploids. Thus, we selected the 9 self-incompatible polyploids with sufficient herbarium material in the visited herbaria (see below) and 31 other species to create a balanced design of roughly 10 species per ploidy-mating system combination (9 polyploid-SI, 11 polyploid-SC, 10 diploid-SI, 10 diploid-SC). The species coded by their ploidy-mating system types are shown on the phylogeny in Figure 1.

For each species we identified at least 10 herbaria sheets for floral traits and 10 for stigma sampling. Floral traits and stigmas for pollination analysis were collected from different plants because these are better represented in different stages of flowers (peak flower expansion for floral traits versus spent flowers or very young fruits for stigma analysis; see below). Floral traits were assessed on digital images, whereas stigmas were sampled in person at four herbaria (CM, NY, US and FLAS; Thiers, 2023). On a given herbarium sheet, sampled plants were selected at random with the criteria that they have flowers of the appropriate stage. To mitigate the impacts of destructive sampling on historical materials, we collected only one stigma per sheet on sheets containing several flowers or fruits. Few exceptions were made for rare species in the visited herbaria, for which we may have sampled two stigmas from different plants within a sheet. For species with numerous sheets, we sampled broadly across the available temporal and geographical range. We followed the same criteria for floral traits.

Floral trait measures

For each identified herbarium sheet (Table A3), we obtained high-resolution digital images and used imageJ (Schneider et al., 2012) to measure traits related to floral attraction or that limit pollinator access to floral rewards. Some species of Brassicaceae have flowers with petals differentiated into a claw (narrower portion of the petal's base) and a blade (expanded portion that forms the limb; Nikolov, 2019). In these flowers, the blade is the visually attractive portion to pollinators and the claw functionally forms a tube that limits the accessibility to the nectaries located at the flower base (Nikolov, 2019). Other species have flowers with undifferentiated petals; thus, there are no physical barriers to accessing nectar. We measured the length and maximum width (in mm) of the attractive portion of the petal. This was the blade for

petals differentiated into two portions or total petal for flowers with undifferenced petals. In flowers with no petals or rudimentary ones, we measured the structure that would be functionally acting as an attracting unit (i.e., sepals). As a third trait, we measured the length of the flower 'tube' that is formed by the junction of petals' claws in flowers with differentiated petals. Those with undifferentiated petals the tube was scored as zero. On average, we measured 24 (range =10-53) flowers per species. We then performed principal component analysis (PCA) on the three measures standardized to zero mean and unit variance using package vegan (Oksanen et al., 2022), extracted first and second principal component (PC1 and PC2) scores that together explained 97.1% of the variance and used these values in our floral trait analyses.

Stigma sampling and pollen assessment

For each species (Table A3), we sampled 10 (range = 10-14) stigmas for scoring HP receipt and a single anther to create a conspecific pollen reference. We only sampled sheets with stigmas with visual cues indicating that pollination has occurred. This was possible because stigmas usually persist attached to the rest of the gynoecium after fruit formation across Brassicaceae species (e.g., Ferrándiz et al., 1999). Thus, we sampled preferably stigmas from young fruits (e.g., gynoecium elongated with stigmas positioned beyond anthers) or, in the absence of these, stigmas from old flowers with clear signs of senescence (e.g., petals wilted). Before excising stigmas, we checked for the presence of pollen grains under stereo microscope. For species with very small flowers, we sampled young fruits to avoid inadvertently sampling unpollinated flowers. Each stigma or anther sample was stored separately in a 1.5 ml microcentrifuge tube with 70% ethanol and transported to the lab. Each sample was then acetolyzed (Kearns and Inouye, 1993;) to achieve a volume of 30 µl, and the entire contents

mounted on a microscope slide. Pollen grains were observed at ×400 magnification using a light microscope (Leica, DM500). Pollen from anthers was used as a taxon-specific reference to distinguish conspecific pollen (CP) from HP on stigmas (Fig. 2). Pollen grains that did not match CP of a given species were scored as HP, morphotyped (based on size, shape, exine patterning and texture, and aperture numbers) and enumerated (as in Johnson et al., 2019). It is important to note, however, that exact species identification is not required to characterize pollen diversity, rather morphospecies identification is adequate to capture species-level differences in HP richness of a given stigma (see Arceo-Gomez et al. 2016) since the main distinction is identifying pollen that is distinct from CP and other HP on a given stigma. Brassicaceae pollen grains are easy to distinguish (Appel and Al-Shehbaz, 2003), but we note that HP from congeners within this family on a given stigma may have been under-scored. For each stigma, we scored HP richness as the number of HP morphospecies per stigma. For each species, we calculated the frequency of pollinated stigmas with HP present. To determine whether our sample size was adequate to capture the morph richness of HP per species, we performed rarefaction analysis of heterospecific pollen morphs per stigma for each species using the package iNEXT (Chao et al., 2014; Hsieh et al., 2022). In rare cases (20/429) stigmas had no pollen of any type (conspecific or heterospecific), and these were excluded from the data set as they do not represent pollinated stigmas.

Phylogeny

To account for nonindependence of traits among the focal Brassicaceae in our analyses due to shared evolutionary history (Garland et al., 2005), we first generated a phylogenetic tree hypothesis for the 40 focal species (Fig. 1) using the megatree 'GBOTB.extended' (implemented

247 in the V.PhyloMaker package, Jin and Qian, 2019) as the backbone of our phylogeny. To control
248 for the effects of phylogenetic relatedness within species, we grafted each unique individual (i.e.,
249 stigma or flower) as a tip descending from its corresponding taxon in the phylogeny using the
250 phytools package (Revell, 2012). We assigned a branch length of zero to each such grafted
251 individual, meaning that no phylogenetic relationships were assumed between individual plants
252 within a species (Burns et al., 2019; Cullen et al., 2021).
253
254 *Data analysis*
255 To test for the effects of ploidy and mating system on floral phenotype and pollination
256 niche breadth we fitted phylogenetic generalized least squares models using the package nlme
257 (function *gls*, Pinheiro et al., 2021). The strength of the phylogenetic correlation between species
258 in our model was based on Pagel's λ, whose value was optimized to the data, using the package
259 ape (function *corPagel*, Paradis and Schliep, 2019). All models had the same prediction structure
260 with ploidy level, mating system and the interaction between them. To determine whether
261 predictor variables affect the floral phenotype and pollination niche breadth we built three
262 models using size (PC1) and shape (PC2) of each flower and HP richness of each stigma within
263 species as response variables, respectively. A fourth model was built to determine whether
264 ploidy and mating system were associated with the frequency of stigmas receiving HP as the
265 response variable. Model assumptions were visually inspected and multicollinearity between the
266 predictors in each model was accessed via the Variation Inflation Factor (VIF). To assess the
267 impact of treating ploidy as binary rather than continuous we repeated each of these models with
268 ploidy age rather than ploidy level. That is, we fitted phylogenetic generalized least squares
269 models for the response variables of interest (flower size and shape, frequency of stigmas with

HP, and HP richness) including the interaction between mating system and ploidy age as predictors for each model. In all models, predictors had VIF < 4, thus no multicollinearity was detected (Zuur et al., 2010). Type III sum of squares of each fitted model was calculated using package car (Fox and Weisberg, 2019). When a significant interaction was found, post-hoc contrasts were performed with the estimated marginal means of the phylogenetically-controlled models using package emmeans (Lenth, 2022). For these, effect size estimates, standard errors, Satterwhite degrees of freedom *t*-test and *P*-values are reported. All analyses were conducted in R (R version 4.0.4; R Core Team, 2021).

Results

The effect of ploidy and mating system on floral traits

Across the 40 species and 653 flowers, the attractive portion of the petal ranged from 0.47 to 11.80 mm (mean = 3.67) in length and from 0.23 to 8.52 mm (mean = 2.47) in width. Flower tube length varied from 0 to 16.73 mm (mean = 4.46). Principal component analysis on these variables (Fig. 3) indicated that the PC1 explained the vast majority (91%) of the variance, whereas PC2 and PC3 much less (6.1% and 2.8%). PC1 was associated with overall size, as all three measured variables (length and width of the attractive portion, and length of the flower tube) were positively associated with it (variable loadings on PC1: 0.58, 0.57 and 0.56, respectively). In contrast, PC2 represented flower shape as it was positively associated with flower tube length (0.79), but negatively with the length (-0.19) and width (-0.58) of the attractive portion of the petal. So larger values of PC1 are associated with increases in all dimensions, whereas larger values of PC2 reflect longer but narrower flowers.

Linear models showed that variation in the size of flowers (PC1) was not explained by ploidy level (β= -0.044± 0.048; χ^2= 0.84; df= 1; *P*= 0.36) or mating system alone (β= 0.080±0.049; χ^2= 2.60; df= 1; *P*= 0.11) but it was associated with the interaction between them (β= 0.21±0.059; χ^2= 12.44; df= 1; *P*<0.001; Fig. 4A). SI polyploids had larger flowers than SI diploids (effect size ± SE= -0.1666±0.0534, df= 42.2; *t*= -3.122; *P*= 0.0032) but flowers of SC polyploids did not differ from SC diploids (0.0449±0.0509, df= 36.6; *t*= 0.881; *P*= 0.38; Fig. 4A). Moreover, SI polyploids had larger flowers than SC polyploids (-0.2909±0.0326, df= 65.7; *t*= -8928; *P*= 0.0001), but there was no difference in floral size between SI and SC diploids (-0.0794±0.0520, df= 38.8; *t*= -1.529; *P*= 0.13; Fig. 4A).

Although there was a weak indication of an interaction between ploidy and mating system on flower shape (PC2) (β= -0.184±0.106; χ^2= 2.99; df= 1; *P*= 0.08, Fig. 4B) there were non-significant main effects of ploidy (β= 0.112± 0.085; χ^2= 1.75; df= 1; *P*= 0.19) and mating system (β= -0.069± 0.087; χ^2= 0.62; df= 1; *P*= 0.43).

The effect of ploidy and mating system on the pollination niche

Across the 40 species, 36% of the 409 stigmas had receipt of HP and the HP richness per stigma ranged from 0 to 8 (mean = 0.7). Within a given species, the proportion of stigmas with HP ranged from 0 to 0.81 (mean = 0.35; Fig. 1). Rarefaction analyses demonstrated that stigma sampling effort of 10 per species sufficiently sampled the HP richness within each species and also captured the variation among species (Fig. 5).

The proportion of stigmas with HP per species was not affected by ploidy (β= -0.064±0.076; χ^2= 0.71; df= 1; *P*= 0.40) but was strongly impacted by mating system (β= 0.186±0.082; χ^2= 5.09; df= 1; *P*= 0.02; Fig. 4C). There was no interaction between mating

system and ploidy (β= -0.115±0.076; χ^2= 1.13; df= 1; *P*= 0.29). Over both diploids and polyploids, SI species had 12% more stigmas with HP than SC species (Fig. 4C).

Heterospecific pollen richness on stigmas was also not associated with ploidy (β= -0.014±0.217; χ^2= 0.004; df= 1; *P*= 0.95) nor its interaction with mating system (β= -0.153±0.326; χ^2= 0.22; df= 1; *P*= 0.64). However, HP richness was associated with mating system (β= 0.505±0.239; χ^2= 4.44; df= 1; *P*= 0.035), where SI species received 0.4 more HP morphs on average than SC species (Fig. 4D).

The effect of ploidy age and mating system on floral traits and the pollination niche

Ploidy age had a binary distribution (Fig. A1) since most diploids are older than most polyploids in our data set. Results of all analyses were, thus, similar to those when ploidy was treated as a categorical variable (Tables A1 and A2; Fig. A2). All major patterns held, with the main difference being that there was a significant interaction between ploidy age and mating system for flower shape (PC2) (β= 0.006±0.002; χ^2= 6.028; df= 1; *P*= 0.014; Fig. A2B). This ploidy-mating system interaction was non-significant when ploidy was treated as binary (*P*= 0.08; Fig 4B). Here, with the increase of ploidy age, PC2 values declined for SC species (β= -0.002±0.002) but increased for SI species (β= 0.004±0.002; Table A2; Fig. A2B). Because larger values of PC2 reflect longer and narrower flowers, these results show that SC species' flowers got shorter and wider (less restrictive) whereas SI species flowers got longer and narrower (more restrictive) with increasing ploidal age.

Discussion

Our investigation of 40 species in 22 genera of Brassicaceae demonstrated that polyploidy and mating system have distinct effects on floral phenotype but more nuanced ones on pollination niche breadth. Moreover, our novel approach of using herbarium samples for assessing HP receipt by stigmas and thus generalism (Fig. 5) --along with floral traits-- was validated. Our results showed that the effect of polyploidy on flower size was dependent on the mating system, with SI polyploids having larger flowers than SI diploids and SC polyploids, but SC polyploids and SC diploids were similar in flower size. Ploidy effects on the pollination niche, however, were smaller and statistically non-significant in comparison the pronounced effects of mating system. Regardless of ploidy, SI species had broader pollination niches (higher frequency of HP receipt and higher HP richness) than SC species. We discuss these results below, as well as why ploidal effects on floral traits were not reflected in the pollination niche.

The effect of polyploidy on flower size observed here is consistent with predictions of 'gigas' effects on polyploid organs and previous empirical data (Vamosi et al., 2007; Porturas et al., 2019; Rezende et al., 2020; Oliveira et al., 2022), at least for SI polyploids. This mating system-dependent effect of polyploidy represents an important advance. It seems reasonable that pathways of floral divergence from diploids to polyploids may depend on degree of pollinator dependence and here we show that to be the case across 40 related wild Brassicaceae species. Specifically, while SC polyploids can rely on self-pollination as a form of reproductive assurance, SI polyploids are pollinator dependent and thus may be subject to stronger selection pressure for larger flowers to increase pollinator differentiation from diploids. On the other hand, SC polyploids may avoid mating with diploid progenitors via autonomous selfing (Vamosi et al., 2007; Brys et al., 2015), driving selection for smaller flower size (working against the gigas effect) and reducing floral morphological divergence. Subtle differences in floral shape were not

361 statistically significant but hint at SI polyploids having shorter, wider flowers, while SC
362 polyploids tended to longer, narrower flowers. This intriguing trend could suggest divergence in
363 floral restrictiveness, with SI polyploids tending toward less restrictive flowers, but this
364 conjecture would need deeper investigation. Interestingly, when ploidal age was analyzed,
365 restrictive shape (PC2) declined with age in SC species but increased with age in SI species:
366 older SI polyploids (mostly diploids) were more restrictive than younger polyploids and older SC
367 polyploids (mostly diploids) were less restrictive than younger polyploids. Similarly, McCarthy
368 et al. (2019) found, in the genus *Nicotiana*, that younger polyploids have shorter and wider
369 corolla tubes than older polyploids, suggesting that the former experience more generalist
370 pollination, with pollinator-mediated selection altering corolla traits over time.
371 We predicted that the pollination niche of SC taxa would be narrower than that of SI
372 species. Furthermore, given the floral size change associated with polyploidy, we expected that
373 SI polyploid species would have broader pollination niches than SI diploids (and similarly for SC
374 polyploids compared to SC diploids). While our HP proxies for niche breadth confirmed a
375 pronounced mating system effect on pollination niche breadth, they did not reveal a direct
376 polyploidy effect nor an interaction with mating system. In fact, albeit non-significant, the
377 observed trend was the opposite than the expected one with SI diploids having larger pollination
378 niches than SI polyploids. This could be due to an interaction between flower size and pollinator
379 behavior. For instance, increased flower size can promote pollinator foraging constancy which
380 can reduce HP receipt (Totland, 2001; Vamosi et al. 2007; Brosi, 2016) so the signature of
381 increased pollinator attraction to larger flowers in SI polyploids on stigmas could be decreased
382 by increased pollinator constancy. Alternatively, other unmeasured floral traits that mediate
383 degree of pollinator generalism (color, phenology, fragrance; Rezende et al., 2020; Wei et al.,

2021) could modify the effect of polyploid flower size. For instance, if polyploids flowered earlier or later than diploids they could encounter different pollinator assemblages or plant community compositions, maintaining similar pollination niche breadth as diploids despite the shifts in flower size. Future studies should include these types of traits, but keep in mind that several are altered or lost (fragrance, color) after collection or are subject to sampling biases (flowering phenology and display size; Daru et al., 2018); thus, caution should be exercised when attempting to collect these from herbarium specimens.

Although we did not observe a universal pattern of polyploidy on the incidence or richness of heterospecific pollen on stigmas, this does not rule out the possibility of a real impact on the pollination niche. First of all, it is possible that niche breadth is different between the ploidy levels but we could not detect it with our experimental design. For instance, that the variation in pollination beyond ploidy was not fully accounted by phylogeny, or that the metric of ecological generalism had greater variability than the morphological one of flower size or shape (Williams and Conner, 2001; Fang and Huang, 2013; Arceo-Gómez et al., 2016; Gómez et al., 2016), making type I error larger for pollination than floral morphology. Second, it is possible that HP proxies do not reflect pollination generalism precisely. Despite the power of HP as a surrogate for pollination interactions (e.g., Fang and Huang, 2013; Arceo-Gómez et al., 2016; Tur et al., 2016; Wei et al., 2021), HP receipt and pollinator visitation can diverge in several ways even when pollen is collected from fresh flowers. For instance, flowers can avoid or reduce HP transfer by legitimate pollen-carrying visitors (Murcia and Feinsinger, 1996; Muchhala and Potts, 2007), and some floral visitors do not carry pollen to stigmas despite visiting (King et al., 2013; Ballantyne et al., 2015; Zhao et al., 2019; Souza et al., 2021). Finally, the use of herbarium specimens imposes some additional limitations such as the potential of

pollen detaching from stigmas during years of specimen handling in botanical collections (Rakosy et al., 2023). Nevertheless, the finding of a strong mating system effect on pollination niche (Fig. 4C,D) demonstrates that the proxy of pollen on stigmas is still a very valuable approach when observing pollination interactions across numerous widely-distributed species is not possible.

One final reason why an effect of polyploidy on pollination niche may exist but was not detected in our analysis is that our approach pooled important aspects of ploidy variation and thus obscured the signal. First, the route to polyploidy (allopolyploidy or autopolyploidy) can influence the degree of phenotypic change that occurs. For instance, current evidence suggests that autopolyploids are more likely to exhibit increased floral size compared to their diploid counterparts, whereas allopolyploids are more often intermediate or show no significant difference (Vamosi et al., 2007; Casazza et al., 2017). But how these translate into changes in pollination niche is still not clear, even though one review suggests that shifts are more frequent in allopolyploids than autopolyploids (Rezende et al., 2020). Second, combining all polyploid levels into a single category may have obscured the effect of higher ploidies on pollination niche breadth. For instance, narrower pollination niches of tetraploids could be attenuated by broader pollination niches of octoploids (or higher ploidies), or vice versa. But there is little research on the effect of various polyploid levels on pollinator visitation, and as far as we know, intraspecific tetraploids and octoploids have similar pollinator assemblages (Jersáková et al., 2010; Castro et al., 2020). However, a recent study has shown effects of intraspecific ploidal series on floral traits that could impact pollination niche (García-Muñoz et al., 2023). While the preceding discussion suggests many potential limitations in our 'pooled' approach, more importantly, it

highlights the lack of research on how any of these key features of polyploid evolution impact the pollination niche, and thus highlights the need for more investigation.

In conclusion, our study provides deeper insights into the consequences of ploidy and mating system on plant-pollinator interactions, suggesting several directions for future research. Beyond the potential factors mentioned above, future studies could also target plants with different self-incompatibility systems, owing to the different immediate impacts of genome duplication on gametophytic and sporophytic incompatibility systems (Barringer 2007, Miller et al. 2008). This would allow us to disentangle whether the correlations between selfing and polyploidy arise through whole genome duplication itself or through shared selective constraints on the self-incompatibility system. Finally, given the lack of a ploidy signal on the pollination niche across several species in our results, it would also be valuable to focus on paired ploidies within genera, on ploidy variation within species (populations with mixed ploidal cytotypes; e.g. García-Muñoz et al*.,* 2023) or on diploids and synthetically produced neopolyploids (e.g., Forester and Ashman 2018). These would reveal the direct effect of polyploidy and remove context-dependent pollination variation. Our results show that it will also be important to account for mating system to understand the reasons behind the incongruence between morphological and ecological shifts.

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703
704 **Appendix content**
705 **Figure A1** – Ploidy age distribution based on mating system and ploidy level.
706 **Figure A2** Influence of mating system and ploidy age on marginal means of flower size (PC1),
707 flower shape (PC2), frequency of stigmas with heterospecific pollen, and morphospecies
708 richness of heterospecific pollen per species.
709 **Table A1** Models' results for the effects of ploidy age and mating system on flower size (PC1),
710 flower shape (PC2), frequency of stigmas with heterospecific pollen, and morphospecies
711 richness of heterospecific pollen per species.
712 **Table A2** Estimates of slopes of the ploidy age trend for each level of mating system for flower
713 size (PC1) and flower shape (PC2).
714 **Table A3** Herbaria and vouchers accessed for floral trait measures and pollen on stigma.
715 **Table A4** First and second principal component extracted from the PCA performed with the
716 three floral traits measured per flower of each species.
717 **Table A5** The frequency of stigmas analyzed that had heterospecific pollen presence per species
718 and their mating system, inferred ploidy, and ploidy age.
719 **Table A6** The total number of heterospecific pollen morphotypes per stigma of each flower and
720 the mating system and inferred ploidy of each species.

Figures

Figure 1 – Phylogeny of the 40 species of Brassicaceae included in the study. Mating system (SC = self-compatible or SI = self-incompatible) and ploidy level (diploid or polyploid) of each species is noted by colored boxes at the tips. The frequency (proportion) of stigmas with heterospecific pollen (HP) per species is denoted by dark-gray bars. Flower size (PC1) of each species is denoted in light grey bars.

Figure 2 – Pollen grains of representative stigmas of four Brassicaceae species with different ploidy-mating system combinations. For each stigma, conspecific pollen (CP) of the recipient species and the morphospecies of heterospecific pollen (HP) are shown. Mating system and polyploidy of the recipient species is represented by the box colors (outer box: mating [self-incompatible =blue; self-compatible= orange]; inner box ploidy [polyploid=blue; diploid =red]). HP species richness of the sample is represented by the number of morphospecies within each box. Relative size and shape of pollen grains is retained.

Figure 3 – Bivariate plot of first two principal components (PC1 and PC2) of floral traits of the 40 species of Brassicaceae. Species scores centroids are identified by color for ploidy level and by shape for mating system (SC = self-compatible or SI = self-incompatible). Floral traits (flower tube length, length, and width of the attractive portion of the petal) are represented by arrows. Gray dots represent each flower measured (n=653).

Note: Four or five-letter codes refer to plant species: ALAL = *Alyssum alyssoides*; ALDE = *Alyssum desertorum*; ARTH = *Arabidopsis thaliana*; ARCA = *Arabis caucasica*; ARRU = *Armoracia rusticana*; ATPU = *Athysanus pusillus*; BAVE = *Barbarea verna*; BAVU = *Barbarea vulgaris*; BOST = *Boechera stricta*; BREL = *Brassica elongata*; CAAM = *Cardamine amara*; CABE = *Cardamine bellidifolia*; CAFL = *Cardamine flexuosa*; CAHI = *Cardamine hirsuta*; CAIM = *Cardamine impatiens*; CASC = *Cardamine scutata*; DIER = *Diplotaxis erucoides*; DIHA = *Diplotaxis harra*; DRCR = *Draba crassifolia*; DRNE = *Draba nemorosa*; ERVE = *Eruca vesicaria*; ERHI = *Erucaria hispanica*; ERAS = *Erysimum asperum*; ERCA = *Erysimum capitatum*; ERINC = *Erysimum inconspicuum*; ERINS = *Erysimum insulare*; EROC = *Erysimum occidentale*; LEST = *Leavenworthia stylosa*; LEUN = *Leavenworthia uniflora*; LEDE = *Lepidium densiflorum*; LELA = *Lepidium latifolium*; LEMO = *Lepidium montanum*; LEPE = *Lepidium perfoliatum*; RARA = *Raphanus raphanistrum*; RARU = *Rapistrum rugosum*; ROAU = *Rorippa austriaca*; ROPA = *Rorippa palustris*; SIAL = *Sisymbrium altissimum*; STCA = *Streptanthus carinatus*; THAR = *Thlaspi arvense*.

Figure 4 – Influence of mating system (MS) and ploidy inference on marginal means of A) flower size (PC1), B) flower shape (PC2), C) frequency of stigmas with heterospecific pollen (HP), and D) morphospecies richness of HP per species. Predictions are based on phylogenetically-corrected generalized least squares models. Error bars represent 95% confidence intervals around least square means. *$P < 0.05$, ***$P < 0.001$.

761 **Figure 5** – Rarefaction curves of heterospecific pollen (HP) richness (the number pollen
762 morphotypes per stigma) for the 38 Brassicaceae species that had HP on stigmas. The observed
763 number of stigmas (sample size) is represented by the solid portion and extrapolation by the
764 dashed portion of each species curve.