American Journal of Botany

RESEARCH ARTICL

Whole genome duplication does not promote common modes of reproductive isolation in *Trifolium pratense*

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Manuscript received 20 October 2019; revision accepted 10 February 2020.

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Citation: Porturas, L. D. and K. A. Segraves. 2020. Whole genome duplication does not promote common modes of reproductive isolation in *Trifolium pratense*. *American Journal of Botany* 107(5): 833–841.

doi:10.1002/ajb2.1466

PREMISE: Although polyploidy has been studied since the early 1900s, fundamental aspects of polyploid ecology and evolution remain unexplored. In particular, surprisingly little is known about how newly formed polyploids (neopolyploids) become demographically established. Models predict that most polyploids should go extinct within the first few generations as a result of reproductive disadvantages associated with being the minority in a primarily diploid population (i.e., the minority cytotype principle), yet polyploidy is extremely common. Therefore, a key goal in the study of polyploidy is to determine the mechanisms that promote polyploid establishment in nature. Because premating isolation is critical in order for neopolylpoids to avoid minority cytotype exclusion and thus facilitate establishment, we examined floral morphology and three common premating barriers to determine their importance in generating reproductive isolation of neopolyploids from diploids.

METHODS: We induced neopolyploidy in *Trifolium pratense* and compared their floral traits to the diploid progenitors. In addition to shifts in floral morphology, we examined three premating barriers: isolation by self-fertilization, flowering-time asynchrony, and pollinator-mediated isolation.

RESULTS: We found significant differences in the morphology of diploid and neopolyploid flowers, but these changes did not facilitate premating barriers that would generate reproductive isolation of neopolyploids from diploids. There was no difference in flowering phenology, pollinator visitation, or selfing between the cytotypes.

CONCLUSIONS: Our results indicate that barriers other than the ones tested in this study—such as geographic isolation, vegetative reproduction, and pistil–stigma incompatibilities—may be more important in facilitating isolation and establishment of neopolyploid *T. pratense*.

KEY WORDS floral morphology; Fabaceae; flowering phenology; legume; neopolyploidy; pollination; premating isolation; prezygotic barriers; reproductive isolation; whole genome duplication.

Understanding the factors that drive reproductive isolation and speciation is a major focus in evolutionary ecology. One common mode of speciation in plants is polyploidy, or the duplication of an entire set of chromosomes (Reisberg and Willis, 2007; Wood et al., 2009). Although polyploidy has been studied since the early 1900s, most attention has been focused on the molecular and genomic effects of whole genome duplication (Soltis et al., 2010). Consequently, fundamental aspects of polyploid ecology and evolution remain unexplored. In particular, surprisingly little is known about how newly formed polyploid species (hereafter "neopolyploids") become established in nature. Models predict that under many conditions, polyploids should be relatively ephemeral and go extinct within a few generations as a result of reproductive disadvantages associated with being the minority in a primarily diploid population (Levin, 1975, 2019; Fowler and Levin, 1984, 2016; Felber, 1991; Rodriguez, 1996; Baack, 2005; Rausch and Morgan, 2005). However, polyploidy is extremely common in nature (24% in extant vascular plant species; Barker et al., 2016); thus, a key goal in the study of polyploidy is to determine the mechanisms that promote neopolyploid establishment in populations.

For neopolyploids to establish and persist in a predominantly diploid population, they must be at least partially reproductively isolated from their diploid progenitor. If no reproductive isolation exists between neopolyploids and their diploid progenitors, and the two cytotypes mated freely, we would expect one of two outcomes. First, if the two cytotypes were capable of producing offspring, the offspring would be triploid individuals, which are often only semi-fertile because of meiotic irregularities and a high production of gametes with an abnormal number of chromosomes (Ramsey and Schemske, 1998). The second outcome would be that no offspring could be generated, known as "triploid block" (Marks, 1966). Both of these scenarios would lead to reduced fecundity of neopolyploids. Because neopolyploids will be rare compared to their diploid counterparts, the frequency-dependent reproductive disadvantage of the neopolyploids should prevent their successful establishment in the population. This is known as the "minority cytotype exclusion principle" (Levin, 1975). However, minority cytotype exclusion can be mitigated through reproductive isolation. Instantaneous postmating isolation between neopolyploids and diploids is often a product of whole genome duplication via processes such as gametic incompatibility, hybrid inviability, or hybrid sterility. However, premating barriers must also exist to circumvent the minority cytotype's exclusion, because these barriers promote assortative mating and aid in the avoidance of ineffective pollinations that result in wasted gametes and proportionally fewer offspring (e.g., Levin, 1975; Husband and Sabara, 2003; Husband et al., 2016).

To date, studies investigating the role of prezygotic barriers in reproductive isolation of polyploids have primarily compared systems of established polyploids and their diploid sister groups (Husband and Sabara, 2003; Jersáková et al., 2010; Roccaforte et al., 2015; Pegoraro et al., 2016; Barringer and Galloway, 2017). For example, Husband and Sabara (2003) estimated mechanisms of reproductive isolation in natural populations of *Chamerion angustifolium* (Onagraceae) and determined that the majority of isolation between cytotypes was due to prezygotic isolation, caused specifically by pollinator fidelity and the spatial distribution of cytotypes within populations. Similarly, Roccaforte et al. (2015) quantified the contribution of isolating barriers between diploid *Erythronium mesochoreum* (Liliaceae) and its tetraploid sister species *E. albidum* (Liliaceae). They found that geographic isolation was the primary barrier driving reproductive isolation in this polyploid complex, followed by pollinator-mediated isolation and floral phenology, with postzygotic barriers contributing the least to total reproductive isolation. Whole genome duplication is also known to break down reproductive self-incompatibility mechanisms, correlate with changes in mating systems, and alter the rate of self-fertilization (Ramsey and Schemske, 1998; Glick et al., 2016). There is evidence from phylogenetic comparative studies that polyploids generally tend to self-fertilize at higher rates than diploids, and this propensity toward selfing may help neopolyploids overcome minority cytotype exclusion (Barringer, 2007; Robertson et al., 2011). Together, these studies suggest that established polyploids and diploids are often isolated through at least one, but often a combination of prezygotic barriers, particularly when living in sympatry. Although this previous work investigating the mechanisms that maintain reproductive isolation and promote the persistence of established polyploids has been instrumental in the study of polyploid reproductive ecology, there remains a gap in our understanding of how polyploids establish given their reproductive disadvantages. Specifically, we have yet to determine which prezygotic mechanisms promote isolation and facilitate establishment in the generations immediately following polyploid speciation (Husband et al., 2016).

To the best of our knowledge, only one study to date has quantified the relative importance of various prezygotic isolating mechanisms of neopolyploids from their diploid progenitors. Husband et al. (2016) found that in *C. angustifolium*, neopolyploids had some phenotypic traits that were more similar to diploids than to established polyploids, and other traits that more closely resembled established polyploids and differed from diploids. Additionally, they found differences in the degree to which the several reproductive barriers contributed to the reproductive isolation of neopolyploids and established polyploids from diploids. This work by Husband et al. (2016) provides direct evidence that the mechanisms and degree of reproductive isolation experienced by established polyploids may not be the same for neopolyploids, especially during the critical generations immediately following whole genome duplication. These results highlight how the phenotypes of neopolyploids can be significantly different from older-generation polyploids (Butterfass, 1987; Oswald and Nuismer, 2011) and suggest that to truly understand the pervasiveness of polyploidy, we require more studies investigating the mechanisms of premating isolation of neopolyploids.

To address this deficit and build upon the foundational work of Husband et al. (2016), we induced neopolyploidy in red clover and observed changes in floral morphology and three common premating barriers to determine their importance in generating reproductive isolation from diploids. By studying premating isolation of neopolyploids under common garden conditions, we add to the limited number of studies that examine neopolyploids under more natural conditions. The premating barriers that we examined were temporal isolation via flowering phenology, the breakdown of self-incompatibility, and pollinator-mediated isolation via differences in flower visitor communities and flower visitor behavior.

MATERIALS AND METHODS

Study organism

To investigate whether premating isolation occurs in neopolyploids in relation to their diploid parents, we used the herb *Trifolium pratense* L. (Fabaceae), or common red clover. Red clover is frequently planted as fodder, and although it has origins in Europe, *T. pratense* is now globally naturalized (GBIF Secretariat, 2017). Red clover is an excellent species to use for studies of reproductive isolation in neopolyploids for a number of reasons: there are published methods for inducing polyploidy in this species (Taylor et al., 1976), diploid red clover naturally produces unreduced gametes at low frequencies (Parrott and Smith, 1986), tetraploid populations have been identified in nature (Elçi, 1982; Pinar et al., 2001; Buyukkartal, 2008, 2013), the diploid species is strongly self-incompatible, and, lastly, it reaches reproductive maturity relatively quickly (3–4 mo).

Generating neopolyploids

Neopolyploid red clover seeds were generated following the methods described by Taylor et al. (1976). In brief, diploid plants were grown from seed (organic medium red clover; Dirt Works, New Haven, Vermont) and cross pollinated by hand. Twenty-four

hours after pollination, we clipped the inflorescences and placed the stalks in 2% w/v sucrose. These were then incubated in a pressure chamber filled with nitrous oxide (N_2O) at 90 psi for either 24 or 36 h, and seeds were then allowed to develop with a constant supply of sucrose solution until the inflorescence tissue was dried.

Cytological analysis

We identified the cytotype of plantlets grown from N_2O-treated seeds by evaluating nuclear DNA content using flow cytometry (Kron et al., 2007). Flow cytometric methods followed the protocols of Godsoe et al. (2013). In brief, plant nuclei were isolated from leaf tissues by chopping leaves in magnesium sulfate buffer ([10 mM $MgSO_4$-$7H_2O$, 50 mM KCl, 5 mM Hepes, adjusted to pH 8], 6.8 mM dithiothreitol, Triton × 100 at 1 mg/mL, and 1 mM PVP-40). The resulting supernatant was filtered through a 30 μm nylon filter, and samples were centrifuged and the supernatant was discarded. We then stained the nuclei with propidium iodide solution containing a rainbow trout red blood cell standard (rainbow trout blood diluted with 1:11 Alsever's solution, 5 mg/mL propidium iodide, and magnesium sulfate buffer). Our propidium iodide solution differed from Godsoe et al.'s (2013) recipe by omitting RNase from the solution. Samples were processed on a BDAccuri C6 flow cytometer at the Syracuse University Flow Core facility (Syracuse, New York, USA), and cytotype was determined by analyzing the data using Flowing version 2.5.1 (Perttu Terho, Turku Centre for Biotechnology, Turku, Finland; http://www.flowingsoftware.com).

Plants identified as tetraploids via flow cytometry analysis were then subject to chromosome counts from root tip cells. We sampled fine roots and soaked them in Farmer's fixative (3:1 absolute ethanol to glacial acetic acid) for ~24 h, followed by treatment with 10% HCl at 60°C for 5 min, and last stained the roots with aceto-carmine at 60°C for ~1.5 h. Four plants identified as tetraploids via flow cytometry were confirmed as tetraploids with direct counts of chromosomes. Two other tetraploids identified via flow cytometry had approximately double the number of chromosomes as determined by chromosome squashes, but small overlapping chromosomes made it difficult to provide definitive confirmation. However, these two plants displayed similar phenotypes to the chromosome-squash-verified tetraploids and did not display characteristics of the aneuploids, such as stunted growth and bumps over the leaf and stem surfaces.

Seed stocks for experiments

To obtain enough tetraploid plants to do a comparative study between neopolyploids and diploids, and to ensure that our neopolyploid and diploid plants were treated identically, both N_2O-treated red clover and untreated diploids were grown to flowering together in a greenhouse at 14–16°C day and 11–13°C night temperature cycles and 15 h daylight conditions. Crosses were done opportunistically when an inflorescence on a plant was in full bloom. Flowers were cross pollinated with flowers from another individual of the same cytotype that had an inflorescence in full bloom. If multiple inflorescences were in bloom simultaneously on multiple plants, the number of crosses between individuals was maximized. We used 14 diploids to generate a stock of diploid seeds, and six N_2O-treated tetraploids to generate a stock of neopolyploid seeds. Once N_2O-treated plants were confirmed as tetraploids via flow cytometry or both flow cytometry and chromosome counts, we cross pollinated these six neopolyploids to generate a stock of neopolyploid seeds.

Plant care

Diploid and neopolyploid seeds were grown in the Syracuse University greenhouse. These seeds were germinated in Miracle-Gro potting mix (Scotts Miracle-Gro, Marysville, Ohio, USA) and sown in individual cells of propagation trays. We set the greenhouse room conditions at 20–22°C day and 17–19°C night temperature cycles with light conditions that mimicked natural sunrise and sunset conditions of Syracuse, New York. Four weeks after planting, the seedlings that germinated were transplanted to 1.89 L pots. Both diploid and neopolyploid seeds had low germination success. Therefore, in an attempt to increase germination rates, we cold treated the remaining seeds that had not yet germinated. Cold treatment lasted for 2 wk at 6–8°C in a reach-in growth chamber. Following the cold treatment, seeds were returned to the greenhouse and grown under standard growing conditions as before. Approximately 4 wk after being returned to the greenhouse, this second group of plants was transplanted into 1.89 L pots. For the remainder of the experiment, both groups were grown in the same greenhouse conditions, then moved to the Syracuse University experimental gardens by group once they began bolting. Once transferred to the common garden, plants remained there through the end of the experiment. In total, 85 non-cold-treated seeds germinated (hereafter "group 1": diploids, $n = 31$; neopolyploids, $n = 54$) and 89 cold-treated seeds germinated (hereafter "group 2": diploids, $n = 39$; neopolyploids, $n = 50$). Once neopolyploid seedlings had at least three trifoliate leaves, they were screened via flow cytometry against the rainbow trout red blood cell standard and a diploid red clover individual to confirm cytotype.

Flower morphology

Three flowers from the top, middle, and base of an inflorescence were collected from each flowering plant. Flowers were placed on ice and transported to the lab to photograph. Flowers were photographed individually and pictures were taken using a Camedia c 7070 wide-zoom 7.1 MP camera (Olympus, Center Valley, Pennsylvania, USA), with an S8 APO dissecting microscope (Leica, Buffalo Grove, Illinois, USA), including a 0.5 cm Minitool Micro-Scale ruler (Bioquip, Compton, California, USA). Total length (TL), length of banner petal (LB), distance between tips of wing petals (WD), width of banner petal (BW), width of tube (WT), stigma–anther separation (SA), wing length (WL), and angle of banner (AB) were measured (Appendix S1). If individual flowers were not imaged clearly enough to provide a reliable measurement or if parts of flowers were damaged during handling, some of these measurements were not taken. All morphological traits were measured using ImageJ version 1.50i (Schneider et al., 2012). Total length was measured using the curved-line tool to follow the shape of the flower on the ventral side of the tube and banner petal. The angle of the banner petal was measured using the angle tool, and the rest of the traits were measured using the straight-line tool.

Floral phenology

For each plant, the date of germination and the date that the first inflorescence was in bloom were recorded. We tracked flowering

phenology throughout the season by counting the total number of inflorescences in bloom per plant at regular census periods. Inflorescences involved in the self-fertilization treatments were not excluded from phenology tracking. Inflorescences were scored as in bloom if more than half of the flowers on the inflorescence were open. This was used as the cutoff because (1) it is easy to observe and score quickly and (2) previous observations of local bees foraging on red clover were attracted to inflorescences with the majority of flowers in bloom. Because red clover is an outcrossing plant, if bees are not visiting the inflorescence when only a few flowers are open, then it is effectively not reproductively active.

Self-fertilization

Each diploid and neopolyploid plant was assigned to one of two self-fertilization treatments prior to flowering: hand-pollination and autonomous self-pollination. The hand-pollination treatment was designed to determine the frequency of self-pollinating individuals while simulating the presence of pollinators, and the autonomous self-pollination treatment determined the frequency of self-pollinating individuals regardless of pollinator presence. We used both self-fertilization treatments in the event that genome duplication alters flower morphology in a way that reduces the ability of pollen to autonomously reach the stigma in neopolyploids, therefore requiring the presence of pollinators for effective self-pollination. For both treatments, a single inflorescence on the plant was covered with a small mesh bag before flowering to ensure that no pollinators would be able to visit. For the hand-pollination treatment, we temporarily removed the mesh bag and hand pollinated flowers on the selected inflorescence with pollen originating from the same inflorescence. For the autonomous self-pollination treatment, the mesh bag remained in place throughout flowering to test if floral morphology allowed for self-pollination in the absence of pollinators. Four weeks after self-fertilization treatments, the inflorescences were removed from the plant, bagged, and brought back to the lab to assess presence or absence of seeds.

Flower visitors

Flower visitor behavior was monitored to determine whether there were immediate behavioral differences in bee responses to neopolyploid plants. Depending on the number of plants in bloom on a given day, 6–12 plants were set up ~1 m apart in a rectangular checkerboard array with alternating cytotypes. Arrays were placed in various locations within 1 km of the experimental garden. Observations of flower visitor behavior began when an insect landed on an inflorescence in the array, and the insect was followed until it left the array. The visitation pattern (whether landing on a diploid or neopolyploid inflorescence), the number of inflorescences visited, and whether the insect actively foraged or simply visited a flower were recorded. When possible, insects were collected after visitation and were brought back to the lab for identification. If we were unable to catch the insects, a size estimate was recorded. Small bees are unlikely to be effective pollinators, as previous studies have suggested that only larger bees pollinate red clover (Bender, 1999a, b). We were easily able to identify *Bombus impatiens* to species level in the field because of unique abdomen markings. Other species in the genus have variable color patterns, so field identification was unreliable. Species identified during these observations were used to generate diploid and neopolyploid bee community profiles.

Statistical analysis

To determine whether there were differences in flower morphology between diploids and neopolyploids, we performed a two-way multivariate analysis of variance (MANOVA). Our model included the eight flower morphology traits as response variables, with "cytotype" as a fixed predictor variable and "group" as an interacting fixed predictor variable. The group predictor variable allowed us to determine whether the cold treatment or difference in development time influenced the differences between cytotypes. Tukey's HSD post hoc test was then used to further evaluate differences of the morphological traits between cytotypes of the individual morphological traits. We also performed a principal component analysis (PCA) to visualize the differences and characterize the variation. Top, middle, and bottom flower measurements were averaged per plant, and experimental units were at the plant level.

To determine whether there were differences in phenology, we first calculated the days to first flower (first day of recorded flowering – first day of recorded germination) and days to peak flower (day of recorded maximum flowering – first day of recorded germination). We then used a two-way MANOVA to investigate whether there were differences in these floral phenology traits between diploids and neopolyploids. This model included the two phenology variables as response variables, with cytotype as a fixed predictor variable and group as an interacting fixed predictor variable, to determine whether the cold treatment influenced differences between cytotypes.

To determine whether neopolyploids differed from diploids in the proportion of individuals able to self-pollinate, we used a chi-square test for equality of proportions. To determine if bees played a role in premating isolation of neopolyploids by flying nonrandomly between cytotypes, we used a chi-square goodness-of-fit test to see if flights between cytotypes differed from random expectations. And lastly, to determine if bees were differentially visiting diploids and neopolyploids, we used a chi-square test of independence. All analyses were carried out using R (R Development Core Team, 2016).

RESULTS

Flower morphology

In total, 318 flowers were photographed and measured, providing 2501 individual measurements from 48 diploid and 57 neopolyploid plants. A two-way MANOVA indicated that there were significant effects of cytotype and group on floral morphology ($F_{8,94} = 8.271$, $P < 0.0001$; $F_{8,94} = 2.613$, $P = 0.013$), but the interaction term was not significant ($F_{8,94} = 0.332$, $P = 0.952$). Tukey's HSD post hoc tests indicated significant differences between cytotypes in all size traits and also the angle of the banner (Fig. 1). Although group significantly affected morphology in our MANOVA, a Tukey's HSD post hoc test showed that differences between groups were present only for one shape trait, the distance between wings ($P = 0.0006$; WD = 24% larger in group one; Appendix S2). For all size traits where diploids and neopolyploids were significantly different from one another, neopolyploids were

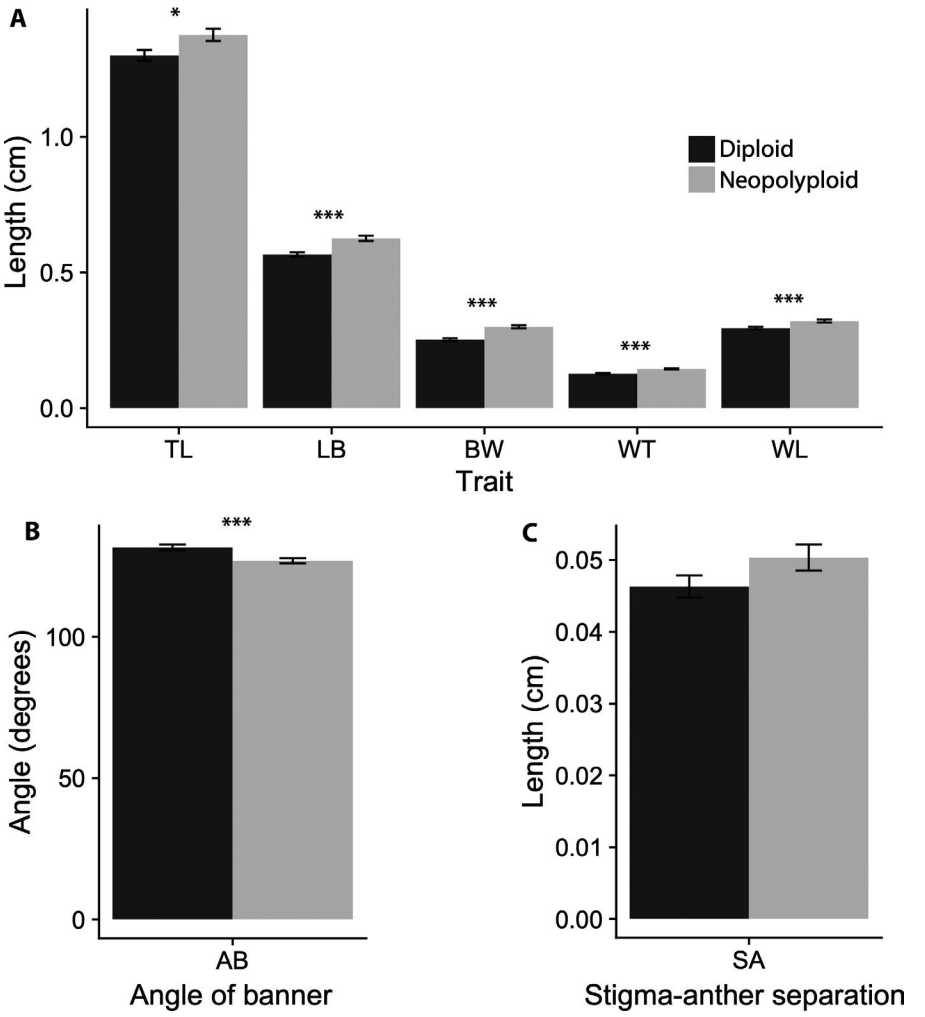

FIGURE 1. Comparisons of flower morphology between diploid (black) and neopolyploid (gray) *Trifolium pratense*. Size-related traits are shown in panel A, and shape-related traits in panels B and C. (A) TL (total length of flower), LB (length of banner petal), BW (width of banner petal), WT (width of flower tube), and WL (length of wing petal). (B) Angle of the banner petal in relation to the flower tube. (C) Distance between the tip of the stigma and the nearest anther. Tukey's HSD post hoc tests of pairwise significant differences between diploids and neopolyploids are indicated with asterisks. *P < 0.05, ***P < 0.001, ns = not significant. Error bars indicate standard errors of the mean.

larger and the angle of the banner petal was sharper (TL = 6% larger, LB = 10% larger, WD = 18% larger, BW = 18% larger, WT = 14% larger, SA = 9% larger, WL = 9% larger, AB = 5 degrees fewer). We also used PCA to explore differences in floral morphology (Fig. 2). In this analysis, we found that size-related traits were more important in driving the differences between diploids and neopolyploids because all of the size traits, along with wing distance, had larger loadings on the first principal component (PC1), which accounted for 53% of the total variation. Stigma–anther separation and angle of the banner petal had larger loadings on the second principal component (PC2), which accounted for 14% of the total variation.

Floral phenology

We tracked floral phenology on 43 diploids and 55 neopolyploids. A two-way MANOVA examining the effect of cytotype and group on floral phenology traits showed that there were significant effects of both cytotype ($F_{2,93}$ = 7.533, P < 0.001) and group ($F_{2,93}$ = 15.015, P < 0.0001) on floral phenology traits. There was, however, no interaction between group and cytotype that influenced these phenology traits ($F_{2,93}$ = 1.464, P = 0.237). Group 1 plants that did not receive cold treatment flowered earlier and reached peak flower earlier than group 2 plants. In group 1, the number of days to first flower (mean ± SE) of diploids and neopolyploids was 87.9 ± 3.0 and 90.9 ± 1.8, respectively, and the number of days to peak flower was 100.0 ± 2.4 and 100.4 ± 1.7, respectively. In group 2, the number of days to first flower of diploids and neopolyploids was 88.8 ± 3.6 and 100.5 ± 3.1, respectively, and the number of days to peak flower was 107.8 ± 2.6 and 109.7 ± 2.7, respectively. Neopolyploids' total number of days of flowering completely overlapped with that of diploids (Fig. 3). The data used to generate Figure 3 come from group 1 only, because group 2 flowers were harvested (for another experiment) before the completion of their flowering cycle.

Self-fertilization

Self-fertilization was tested in 38 diploids and 54 neopolyploids. Both the hand-pollinated and autonomous self-pollination treatments revealed a similar number of self-compatible individuals (hand-pollination: 2 diploids, 4 neopolyploids; autonomous self-pollination: 4 neopolyploids), suggesting that self-fertilization is possible in neopolyploids in the absence of pollinators. Therefore, we pooled the data from the hand-pollinated and autonomous self-pollination treatments. When examining differences in self-fertilization between diploids and neopolyploids, we found a nonsignificant increase (10%) in the proportion of self-compatible plants after whole genome duplication (χ^2 = 1.230, df = 1, P = 0.267). For neopolyploids, 14.8% of individuals were able to set seed after self-fertilization, as opposed to 5.2% of individuals for diploids.

Flower visitors

We observed a total of 95 bee foraging behaviors over 18 observation periods and 35 h of observation time. Bees transitioned between 209 plants and 491 individual inflorescences. Overall, bees visited diploid and neopolyploid plants at similar frequencies; 54% of plants visited were diploids and 46% were neopolyploids. To test whether foraging behavior could lead to premating isolation of neopolyploids, we looked for evidence of

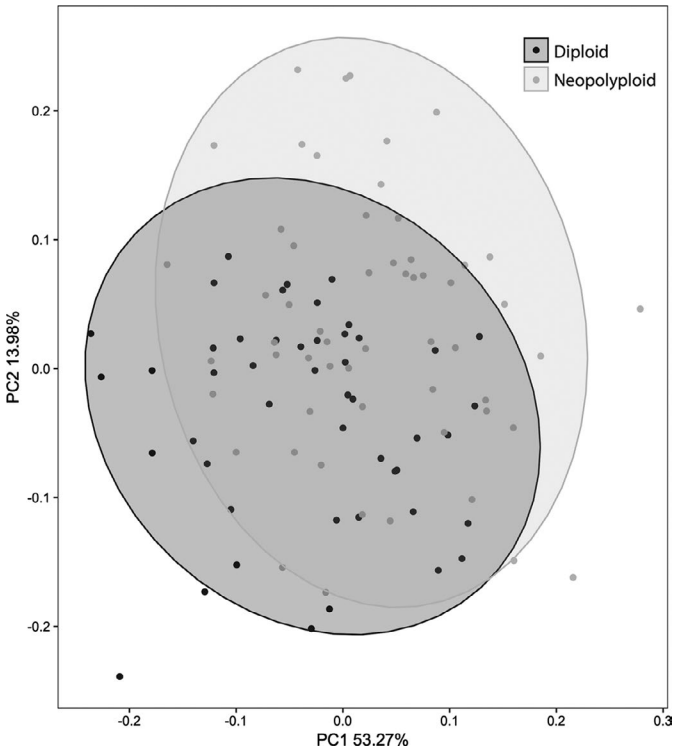

FIGURE 2. Principal component analysis of floral traits of diploid (dark gray) and neopolyploid (light gray) *Trifolium pratense*. Percentages of the total variance are indicated on the PC1 and PC2 axes, and circles represent 95% confidence estimates.

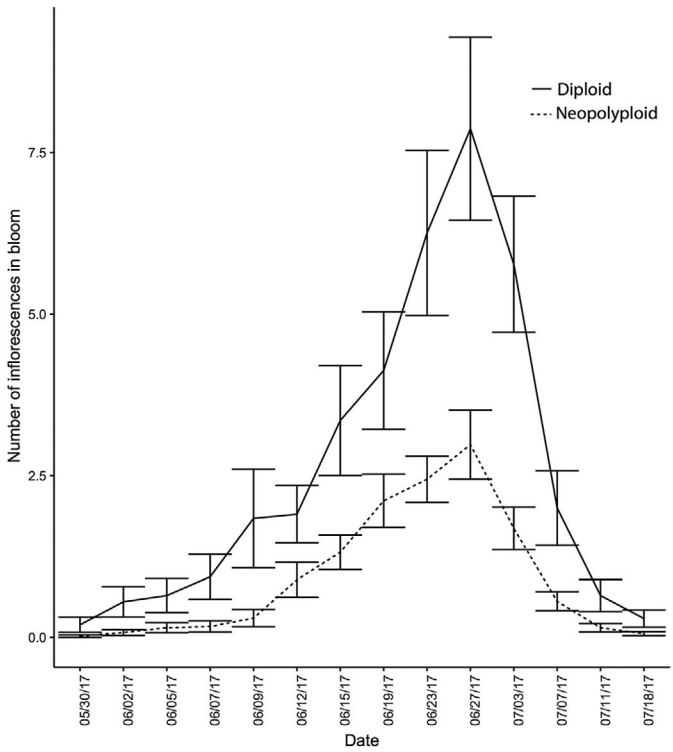

FIGURE 3. Floral phenology timeline of diploid and neopolyploid *Trifolium pratense*. Lines connect the mean and SE of the number of inflorescences in bloom of diploids (solid line) and neopolyploids (dotted line). Dates are month/date/year.

assortative mating of plants facilitated by bee behavior. Following Kennedy et al. (2006), we used a conservative measure of bee constancy (the tendency to preferentially visit either diploids or neopolyploids) to determine whether flower visitors could facilitate isolation. We used only the first transition between plants as our unit of measure to avoid complications of non-independence for the subsequent plant transitions in bee foraging bouts. We found that bee flights within (diploid to diploid; neopolyploid to neopolyploid) and between (diploid to neopolyploid; neopolyploid to diploid) cytotypes did not differ from flights that would be expected from random visitation ($\chi^2 = 6.767$, df = 3, $P = 0.080$). We also found that the bee communities visiting diploids and neopolyploids were very similar (Table 1). The most common bees in both diploid and neopolyploid communities were *Bombus* species. These bee groups did not visit one cytotype more frequently ($\chi^2 = 3.545$, df = 6, $P = 0.738$).

DISCUSSION

Polyploidy is a common mode of speciation in plants, but despite its importance in plant evolution, surprisingly little is known about how neopolyploids become established. Theory predicts that neopolyploids will be unlikely to find a suitable mate and should quickly become extinct (Levin, 1975), yet polyploid species are extremely common (Barker et al., 2016). For neopolyploids to establish and persist in a predominantly diploid population, genome duplication must induce mechanisms that promote prezygotic reproductive barriers to facilitate assortative mating and avoid ineffective pollinations that would result in wasted gametes and scant offspring (e.g., Levin, 1975; Husband and Sabara, 2003; Husband et al., 2016). To best understand how neopolyploids become established, more studies examining reproductive ecology in the generations immediately following speciation are needed. Here, we generated neopolyploid plants and compared them to diploids to determine if whole genome duplication directly altered aspects of plant reproductive biology that would lead to premating isolation from diploids. We found that genome duplication did immediately impact floral morphology of our plants, but we found no inherent changes associated with genome duplication that might facilitate premating isolation.

In our study, we determined that flower size increased after whole genome duplication, in accord with the gigas effect observed in many other plant species (e.g., Muntzing, 1936; Stebbins, 1971; Porturas et al., 2019). We also found differences in flower shape. These changes in floral morphology could cascade to a number of different effects important to plant reproductive ecology. For example, we know that tetraploid varietal lines of *T. pratense* can have larger flowers than diploids (Bender, 1999a, b; Vleugels et al., 2015) and that bee behavior can change depending on the cytotype (Bender, 1999a, b). Morphological changes associated with genome duplication could offer easier access to nectar or pollen rewards and cause behavioral changes in pollinators or attract different suites of pollinators altogether. However, in contrast to these expectations, our results suggest that despite the changes in flower morphology, either bees were unable to differentiate between neopolyploids and diploids or the perceived differences were unimportant in flower selection. The results showed that there was no evidence of pollinator-mediated isolation due to flower visitor behavior or through

TABLE 1. Bee visitors to diploid and neopolyploid *Trifolium pratense* plants.

Bee type	Visitors to diploids (*n*)	Proportion of community (%)	Visitors to neopolyploids (*n*)	Proportion of community (%)
Andrena spp.	12	16.7	8	13.5
Apis mellifera	5	6.9	3	5.1
Bombus impatiens	3	4.2	5	8.5
Bombus spp.	29	40.2	29	49.1
Colletes spp.	4	5.6	4	6.8
Megachile spp.	1	1.4	1	1.7
Other	18	25	9	15.3

changes in the composition of visiting bee communities. We would like to highlight the caveat that our flower-visitor-behavior arrays used unrealistic proportions of neopolyploids and diploids. In naturally derived neopolyploid populations, neopolyploids will be the minority cytotype rather than occurring in the equal proportions used in our checkerboard array. Had we used more realistic proportions and randomized placement of cytotypes within the array, we would not be able to ensure that pollinator-mediated assortative mating was due to active pollinator preference alone and not influenced by spatial aggregation of cytotypes.

We are aware of only two other studies that have compared the community and behavior of pollinators of neopolyploids and diploids. Nghiem et al. (2011) observed that both diploids (*Acacia mangium* [Fabaceae] and *A. auriculiformis* [Fabaceae]) and neopolyploids (*A. mangium* [Fabaceae]) were visited primarily by honey bees, and they showed that qualitatively, bees did not discriminate between diploid and neopolyploid *Acacia*. Another study conducted by Husband et al. (2016) also observed primarily honey bees (>90%) visiting both diploids and neopolyploids of *C. angustifolium*, and found that pollinator behavior did not contribute to reproductive isolation of neopolyploids. Although most of the bees observed in our study were not honey bees, the bees in our study are considered generalist pollinators.

Changes in flower size of neopolyploids also have the potential to influence phenological traits—if, for instance, larger flowers require longer development times and results in later flowering (Cavalier-Smith, 1978; Ramsey and Ramsey, 2014). Indeed, we did find that neopolyploidy significantly delayed the time to first flower. This result is similar to the findings of some studies that have recorded flowering times of neopolyploids, for example in *Vicia villosa* (Fabaceae; Tulay and Unal, 2010), *Heuchera grossulariifolia* (Saxifragaceae; Oswald and Nuismer, 2011), *Achillea borealis* (Asteraceae; Ramsey, 2011), *Chamerion angustifolium* (Martin and Husband, 2012), and multiple species in the genus *Miscanthus* (Poaceae; Chae et al., 2013). However, other studies have found either that genome duplication does not alter flowering timing, as in *Acacia mangium* (Nghiem et al., 2011) and *Chamerion angustifolium* (Husband et al., 2016), or that there are mixed results when neopolyploids are derived via hybridization, as in *Brassica napus* (Brassicaceae; Hansen and Earle, 1994) and *Cucumis* hybrid (Cucurbitaceae; Chen et al., 2002). Although our neopolyploids did take longer to begin flowering, this did not translate into an overall shift in flowering phenology. Both diploids and neopolyploids reached peak flowering at the same time, and neopolyploid flowering did not extend past that of diploids (Fig. 3). This suggests that for red clover, neopolyploidy does cause changes in flowering initiation, but these changes would be unlikely to lead to reproductive isolation, given that the remainder of neopolyploid flowering completely overlaps with diploids.

In addition to phenological and pollinator-based isolation, another potential isolating mechanism for newly formed polyploids is the ability to reproduce without the need for mating with other individuals. For example, asexual reproduction is one mechanism that can allow polyploids to persist without mating. Another mechanism is self-fertilization, which could also prevent ineffectual matings with nearby diploids and thereby reduce the likelihood of succumbing to minority cytotype exclusion (Levin, 1975; Rodriguez, 1996; Baack, 2005; Rausch and Morgan, 2005; Fowler and Levin, 2016; Van Drunen and Husband, 2018). The propensity for whole genome duplication to break down self-incompatibility barriers and hence increase the ability of plants to self-fertilize is well documented, particularly for plants with gametophytic self-incompatibility systems, although the mechanisms behind the breakdown are poorly understood (Ramsey and Schemske, 1998; Mable, 2004; Barringer, 2007). In our study we found that red clover, which has a gametophytic self-incompatibility mechanism (Taylor and Smith, 1979), experienced a nonsignificant increase (10%) in the proportion of self-compatible plants after whole genome duplication. Although the directionality of the change was in line with findings from previous studies, our sample sizes may have been insufficient to detect a significant change.

Together, the results of this study suggest that none of the premating mechanisms that we tested are important in facilitating reproductive isolation of neopolyploid red clover. This is surprising given our original expectation that at least one of the mechanisms shown to be important in enacting reproductive isolation in established polyploids would also be involved in reproductive isolation of neopolyploids. Although we observed shifts in floral morphology, these differences did not facilitate isolation of neopolyploids from diploids in self-pollination rates, flowering phenology, flower visitor behavior, or flower visitor communities. These observations support the conclusions of Husband et al. (2016) that although neopolyploids often show immediate changes in floral phenotype, these changes on their own do not account for the reproductive barriers observed in natural, established populations.

We should, however, mention that a key limitation of this work is that the results derived from studies using synthetic neopolyploids may not emulate the range of phenotypes observed in naturally derived neopolyploids. This is particularly true for neopolyploids generated by somatic doubling with mitotic inhibitors such as colchicine or N_2O. In these synthetic neopolyploids, allelic heterozygosity cannot exceed two alleles at a given locus, but natural neopolyploids generated by a union of unreduced gametes can hold up to four alleles at a given locus. The increased allelic diversity of natural neopolyploids would provide them greater adaptive potential in response to environmental pressures. Additionally, it is possible that wild polyploids may establish only from unique genotypes, and so synthetically produced neopolyploids may not recreate the genotypes and phenotypes that would facilitate establishment in nature (Ramsey, 2011; Ramsey and Ramsey, 2014). Another limitation of neopolyploids generated by use of mitotic inhibitors is that the phenotypes observed after whole genome duplication can differ depending on the pathway to polyploid formation (e.g., *Brassica napus*; Szadkowski et al., 2011). Despite these caveats, we argue that synthetic neopolyploids provide us with the opportunity to observe phenotypes that stem directly from genome duplication, without

the confounding effects of subsequent selection and drift associated with older, evolved polyploids (Ramsey and Ramsey, 2014). Additionally, synthetic neopolyploids open the door to unique studies that would be impossible or too cumbersome to achieve using natural neopolyploids. For example, one can generate both allo- and auto-neopolyploids within a single species group and compare resulting phenotypes to the diploid progenitors—this would provide a valuable lens through which to examine allopolyploidy and disentangle the effects of hybridization and genome duplication.

One trait we did not examine that has been shown to strongly influence reproductive isolation in polyploids is geographic isolation. Studies that examine reproductive isolation of established polyploids have found that geographic isolation is a primary contributor to isolation between cytotypes in, for example, *Chamerion angustifolium* (Husband and Sabara, 2003) and *Anacamptis pyramidalis* (Orchidaceae; Pegoraro et al., 2016). In our study, we excluded geographic isolation as a potential premating isolating mechanism because neopolyploids are expected to form within the distribution of their diploid progenitors. However, there is evidence that pollinators do facilitate assortative mating between cytotypes due to the spatial structure of cytotypes within populations (in *C. angustifolium*; Husband and Schemske, 2000), and models suggest that limited seed and pollen dispersal can generate "islands" within a larger, mixed-cytotype population where neopolyploids are not so greatly affected by minority exclusion (Baack, 2005). Little is known about the natural history of natural populations of established tetraploid red clover, but seeds are likely dispersed similarly to other clover species, via grazing animals, which would lend to the development of cytotype islands within a larger, mixed-cytotype population. Therefore, spatial distribution of cytotypes could potentially play an important role in pollinator-mediated isolation of neopolyploid red clover, but that was not considered in this study. Studies comparing the relative success of neopolyploids in various spatial structures, and studies comparing the relative success of polyploids with differing dispersal mechanisms, would broaden our understanding of the importance of geographic isolation as a factor contributing to neopolyploid establishment.

A major challenge in understanding the ubiquity of polyploids in nature is elucidating how they establish despite predictions that suggest they should be evolutionarily short-lived. Because polyploid establishment will occur in the generations immediately following formation, it is critical that we tackle this challenge using study systems that have not been altered through evolutionary processes such as selection and drift. Our results show that three modes of premating isolation common in established polyploids did not cause reproductive isolation of neopolyploids from diploids. More studies investigating multimodal mechanisms of prezygotic isolation are needed to draw broad conclusions about which mechanisms are most important in neopolyploid establishment. Our results indicate that other modes of isolation—such as geographic isolation, vegetative reproduction, and pistil–stigma incompatibilities—have likely been more important in facilitating isolation and establishment of natural populations of neopolyploid *Trifolium pratense*.

ACKNOWLEDGMENTS

We thank D. Althoff, T. Anneberg, A. Curé, J. Friedman, R. Rundell, and S. Wang for comments on the manuscript. We also thank the anonymous reviewers for comments and suggestions that improved the quality of this paper. We thank B. Powers and D. Toews for assistance with data collection, and J. Friedman for providing common garden space. Funding was provided by Sigma Xi and the Society for Integrative and Comparative Biology to L.D.P., and by the National Science Foundation (DEB 1556568 and 1655544) to K.A.S.

DATA AVAILABILITY

Data reported in this study are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.98sf7m0fc (Porturas and Segraves, 2020).

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

APPENDIX S1. Flower measurements of *Trifolium pratense*. (A) Stigma–anther separation (SA). (B) Distance between wing petals (WD). (C) Wing petal length (WL). (D) Angle of the banner petal (AB). (E) Length of banner petal (LB). (F) Total length of the flower (TL). (G) Width of the flower tube (WT). (H) Width of the banner petal (BW).

APPENDIX S2. Comparison of the distance between the tips of wing petals of diploid (black) and neopolyploid (gray) *Trifolium pratense* of groups 1 and 2. Significant differences between diploids in groups 1 and 2 using Tukey's HSD post hoc tests are marked with different letters. Error bars indicate standard errors of the mean.

LITERATURE CITED

Baack, E. J. 2005. To succeed globally, disperse locally: effects of local pollen and seed dispersal on tetraploid establishment. *Heredity* 94: 538–546.

Barker, M. S., N. Arrigo, A. E. Baniaga, Z. Li, and D. A. Levin. 2016. On the relative abundance of autopolyploids and allopolyploids. *New Phytologist* 210: 391–398.

Barringer, B. C. 2007. Polyploidy and self-fertilization in flowering plants. *American Journal of Botany* 94: 1527–1533.

Barringer, B. C., and L. F. Galloway. 2017. The reproductive ecology of diploid and tetraploid *Galax urceolata*. *American Midland Naturalist* 177: 299–308.

Bender, A. 1999a. Polüploidiseerimise mõju punase ristiku õite mõõtmetele, tolmeldava entomofauna liigilisele koosseisule ja arvukusele. *Agraarteadus* 4: 9–23.

Bender, A. 1999b. Risttolmlemise Tagajana. *Agraarteadus* 4: 24–37.

Butterfass, T. 1987. Cell-volume ratios of natural and of induced tetraploid and diploid flowering plants. *Cytologia* 52: 309–316.

Buyukkartal, H. N. 2008. Causes of low seed set in the natural tetraploid *Trifolium pratense* L. (Fabaceae). *African Journal of Biotechnology* 7: 1240–1249.

Buyukkartal, H. N. 2013. Polar nuclei behaviour in the mature megagametophyte of the natural tetraploid *Trifolium pratense* (Fabaceae). *Turkish Journal of Botany* 37: 537–541.

Cavalier-Smith, T. 1978. Nuclear volume control by nucleoskeletal DNA, selection for cell volume and cell growth rate, and the solution of the DNA C-value paradox. *Journal of Cell Science* 34: 247–278.

Chae, W. B., S. J. Hong, J. M. Gifford, A. L. Rayburn, J. M. Widholm, and J. A. Juvik. 2013. Synthetic polyploid production of *Miscanthus sacchariflorus*, *Miscanthus sinensis*, and *Miscanthus* x *giganteus*. *Global Change Biology Bioenergy* 5: 338–350.

Chen, J. F., J. Staub, J. Adelberg, S. Lewis, and B. Kunkle. 2002. Synthesis and preliminary characterization of a new species (amphidiploid) in *Cucumis*. *Euphytica* 123: 315–322.

Elçi, S. 1982. The utilization of genetic resource in fodder crop breeding. Eucarpia, Fodder crop section 13-16 September, Aberystwyth. U.K.

Felber, F. 1991. Establishment of a tetraploid cytotype in a diploid population - effect of relative fitness of the cytotypes. *Journal of Evolutionary Biology* 4: 195–207.

Fowler, N. L., and D. A. Levin. 1984. Ecological constraints on the establishment of a novel polyploid in competition with its diploid progenitor. *American Naturalist* 124: 703–711.

Fowler, N. L., and D. A. Levin. 2016. Critical factors in the establishment of allopolyploids. *American Journal of Botany* 103: 1236–1251.

GBIF Secretariat. 2017. GBIF Backbone Taxonomy. Checklist Dataset. Website: https://doi.org/10.15468/39omei. Accessed (via GBIF.org) 11 April 2018.

Glick, L., N. Sabath, T. L. Ashman, E. Goldberg, and I. Mayrose. 2016. Polyploidy and sexual system in angiosperms: is there an association? *American Journal of Botany* 103: 1223–1235.

Godsoe, W., M. A. Larson, K. L. Glennon, and K. A. Segraves. 2013. Polyploidization in *Heuchera cylindrica* (Saxifragaceae) did not result in a shift in climatic requirements. *American Journal of Botany* 100: 496–508.

Hansen, L. N., and E. D. Earle. 1994. Novel flowering and fatty acid characters in rapid cycling *Brassica napus* L. resynthesized by protoplast fusion. *Plant Cell Reports* 14: 151–156.

Husband, B. C., and H. A. Sabara. 2003. Reproductive isolation between autotetraploids and their diploid progenitors in fireweed, *Chamerion angustifolium* (Onagraceae). *New Phytologist* 161: 703–713.

Husband, B. C., and D. W. Schemske. 2000. Ecological mechanisms of reproductive isolation between diploid and tetraploid *Chamerion angustifolium*. *Journal of Ecology* 88: 689–701.

Husband, B. C., S. J. Baldwin, and H. A. Sabara. 2016. Direct vs. indirect effects of whole-genome duplication on prezygotic isolation in *Chamerion angustifolium*: implications for rapid speciation. *American Journal of Botany* 103: 1259–1271.

Jersáková, J., S. Castro, N. Sonk, K. Milchreit, I. Schödelbauerová, T. Tolasch, and S. Dötterl. 2010. Absence of pollinator-mediated premating barriers in mixed-ploidy populations of *Gymnadenia conopsea* s.l. (Orchidaceae). *Evolutionary Ecology* 24: 1199–1218.

Kennedy, B. F., H. A. Sabara, D. Haydon, and B. C. Husband. 2006. Pollinator-mediated assortative mating in mixed ploidy populations of *Chamerion angustifolium* (Onagraceae). *Oecologia* 150: 398–408.

Kron, P., J. Suda, and B. C. Husband. 2007. Applications of flow cytometry to evolutionary and population biology. *Annual Review of Ecology Evolution and Systematics* 38: 847–876.

Levin, D. A. 1975. Minority cytotype exclusion in local plant populations. *Taxon* 24: 35–43.

Levin, D. A. 2019. Why polyploid exceptionalism is not accompanied by reduced extinction rates. *Plant Systematics and Evolution* 305: 1–11.

Mable, B. K. 2004. Polyploidy and self-compatibility: is there an association? *New Phytologist* 162: 803–811.

Marks, G. E. 1966. The origin and signficance of intraspecific polyploidy: experimental evidence from *Solanum chacoense*. *Evolution* 20: 552–557.

Martin, S. L., and B. C. Husband. 2012. Whole genome duplication affects evolvability of flowering time in an autotetraploid plant. *PLoS One* 7(9): e44784.

Muntzing, A. 1936. The evolutionary significance of autopolyploidy. *Hereditas* 21: 263–378.

Nghiem, C. Q., C. E. Harwood, J. L. Harbard, A. R. Griffin, T. H. Ha, and A. Koutoulis. 2011. Floral phenology and morphology of colchicine-induced tetraploid *Acacia mangium* compared with diploid *A. mangium* and *A. auriculiformis*: implications for interploidy pollination. *Australian Journal of Botany* 59: 582–592.

Oswald, B. P., and S. L. Nuismer. 2011. Neopolyploidy and diversification in *Heuchera grossulariifolia*. *Evolution* 65: 1667–1679.

Parrott, W. A., and R. R. Smith. 1986. Recurrent selection for 2n pollen formation in red-clover. *Crop Science* 26: 1132–1135.

Pegoraro, L., D. Cafasso, R. Rinaldi, S. Cozzolino, and G. Scopece. 2016. Habitat preference and flowering-time variation contribute to reproductive isolation between diploid and autotetraploid *Anacamptis pyramidalis*. *Journal of Evolutionary Biology* 29: 2070–2082.

Pinar, N. M., H. N. Buyukkartal, and H. Colgecen. 2001. Pollen and seed morphology of diploids and natural tetraploids of *Trifolium pratense* L. (Leguminosae). *Acta Biologica Cracoviensia Series Botanica* 43: 27–32.

Porturas, L. D., and K. A. Segraves. 2020. Data from: Premating isolation in neopolyploid red clover *(Trifolium pratense)*. *Dryad Digital Repository*. https://doi.org/10.5061/dryad.98sf7m0fc.

Porturas, L. D., T. J. Anneberg, A. E. Curé, S. Wang, D. M. Althoff, and K. A. Segraves. 2019. A meta-analysis of whole genome duplication and the effects on flowering traits in plants. *American Journal of Botany* 106: 469–476.

R Development Core Team. 2016. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing.

Ramsey, J. 2011. Polyploidy and ecological adaptation in wild yarrow. *Proceedings of the National Academy of Sciences USA* 108: 7096–7101.

Ramsey, J., and T. S. Ramsey. 2014. Ecological studies of polyploidy in the 100 years following its discovery. *Philosophical Transactions of the Royal Society of London B* 369: 15–19.

Ramsey, J., and D. W. Schemske. 1998. Pathways, mechanisms, and rates of polyploid formation in flowering plants. *Annual Review of Ecology and Systematics* 29: 467–501.

Rausch, J. H., and M. T. Morgan. 2005. The effect of self-fertilization, inbreeding depression, and population size on autopolyploid establishment. *Evolution* 59: 1867–1875.

Reisberg, L. H., and J. H. Willis. 2007. Plant speciation. *Science* 317: 910–914.

Robertson, K., E. E. Goldberg, and B. Igic. 2011. Comparative evidence for the correlated evolution of polyploidy and self-compatibility in Solanaceae. *Evolution* 65: 139–155.

Roccaforte, K., S. E. Russo, and D. Pilson. 2015. Hybridization and reproductive isolation between diploid *Erythronium mesochoreum* and its tetraploid congener *E. albidum* (Liliaceae). *Evolution* 69: 1375–1389.

Rodriguez, D. J. 1996. A model for the establishment of polyploidy in plants. *American Naturalist* 147: 33–46.

Schneider, C. A., W. S. Rasband, and K. W. Eliceiri. 2012. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods* 9: 671–675.

Soltis, D. E., R. J. A. Buggs, J. J. Doyle, and P. S. Soltis. 2010. What we still don't know about polyploidy. *Taxon* 59: 1387–1403.

Stebbins, G. L. 1971. Chromosomal evolution in higher plants. London, UK: Addison-Wesley.

Szadkowski, E., F. Eber, V. Huteau, M. Lodé, O. Coriton, E. Jenczewski, and A. M. Chèvre. 2011. Polyploid formation pathways have an impact on genetic rearrangements in resynthesized *Brassica napus*. *New Phytologist* 191: 884–894.

Taylor, N. L., and R. R. Smith. 1979. Red clover breeding and genetics. *Advances in Agronomy* 31: 125–154.

Taylor, N. L., M. K. Anderson, K. H. Quesenberry, and L. Watson. 1976. Doubling the chromosome number of *Trifolium* species using nitrous oxide. *Crop Science* 16: 516–518.

Tulay, E., and M. Unal. 2010. Production of colchicine induced tetraploids in *Vicia villosaroth*. *Caryologia* 63: 292–303.

Van Drunen, W. E., and B. C. Husband. 2018. Immediate vs. evolutionary consequences of polyploidy on clonal reproduction in an autopolyploid plant. *Annals of Botany* 122: 195–205.

Vleugels, T., I. Roldan-Ruiz, and G. Cnops. 2015. Influence of flower and flowering characteristics on seed yield in diploid and tetraploid red clover. *Plant Breeding* 134: 56–61.

Wood, T. E., N. Takebayashi, M. S. Barker, I. Mayrose, P. B. Greenspoon, and L. H. Rieseberg. 2009. The frequency of polyploid speciation in vascular plants. *Proceedings of the National Academy of Sciences USA* 106: 13875–13879.