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Constraints on polyploid evolution: a test of the minority cytotype exclusion principle

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Polyploid evolution is often considered a mechanism of instant speciation; yet the establishment of rare tetraploids within diploid populations may be constrained by a frequency-dependent mating disadvantage (minority cytotype exclusion principle). I tested this hypothesis using experimental populations of *Chamerion angustifolium* (Onagraceae) that contained different proportions of tetraploids and diploids. Fitness, measured as total seed production over the entire flowering season, was calculated from a census of flower number and estimates of ovule number per flower and proportion of seed set per fruit. The fitness of tetraploids relative to diploids was frequency dependent, increasing from 0.4, when tetraploids were rare, to 0.7 when at 50% and 1.15 when they were in the majority (67%). This pattern exists because of a negative relationship between tetraploid frequency and seed set per fruit in diploids. Seed set in tetraploids was independent of cytotype frequency. The frequency-independent effect in tetraploids reflects higher assortative mating, partly because of non-random patterns of bee visitation. Bees visited a disproportionately high number of diploid inflorescences; however, the proportion of successive flights between tetraploids increased above random expectations as the frequency of tetraploids decreased. These results provide the first experimental test of frequency-dependent fitness in diploid–polyploid mixtures and suggest an important role for more gradual, population processes governing the evolution of polyploidy in natural populations.

Keywords: fireweed; frequency dependence; phenology; pollinator visitation; reproduction; polyploid

1. INTRODUCTION

A central condition in most models of speciation is that genetic divergence can only occur between populations in geographical isolation. Speciation in sympatry is considered less likely, primarily because either recurrent gene flow will prevent genetic divergence or, when hybrids are partially inviable or sterile, a selective disadvantage will be experienced by the rare genotype (Barton 1989). The minority genotype will be less fit not because it is less well adapted, but because it is less common, has fewer potential mates, and in a randomly mating population, will be involved in more ineffectual matings. Under such frequency-dependent selection, both genotypes cannot coexist in a stable equilibrium; rather, the population will become fixed for the majority genotype.

Polyploidy, chromosome multiplication above the diploid complement, is believed to be one of the most significant mechanisms of evolution and speciation in plants (Levin 1983; Thompson & Lumaret 1992). Estimates vary, but between 35 and 70% of all flowering plants are associated with a chromosome multiplication event (Grant 1981; Masterson 1994). Furthermore, because polyploids arise instantaneously and cause wholesale changes throughout the genome that result in partial or complete post-zygotic reproductive isolation, they are generally offered as a classical example of rapid, sympatric speciation; an exception to the general rule (Futuyma 1998). However, the success of polyploids in populations of their diploid progenitors is not necessarily automatic. This is because, unless polyploids exhibit complete assortative mating upon their origin, rare polyploids will be subject to the same frequency-dependent forces as any other genotype, a process referred to by Levin (1975) as minority cytotype disadvantage. It is for this reason that most evolutionary models of polyploidy find the conditions for polyploid evolution to be relatively restrictive (Fowler & Levin 1984; Felber 1991; Rodrîguez 1996). Unfortunately, the population mechanisms of species diversification through polyploidy are not sufficiently understood to explain how such forces are mitigated.

In *Chamerion angustifolium* (fireweed), diploid ($2n = 2x = 36$) and tetraploid ($2n = 4x = 72$) individuals are usually geographically separated in North America. Throughout most of their range, diploids occur at higher latitudes than tetraploids (Mosquin 1966, 1967). However, their ranges overlap in a narrow zone along the southern border of the boreal forest and in the northern Rocky Mountains. Even within this zone of overlap, diploids tend to occur at higher altitudes. While most populations are uniform for one cytotype, nearly 40% of all sites contain diploids and tetraploids in intermediate frequencies (Husband & Schemske 1998; B. C. Husband, unpublished data). In mixed populations, diploids and tetraploids are heterogeneously distributed but frequently occur in close proximity (Husband & Schemske 1998). The identification of mixed populations raises questions about the processes that regulate coexistence of diploids and tetraploids and whether coexistence is constrained by frequency-dependent selection.

Here, I test the minority cytotype exclusion hypothesis using experimental populations of *C. angustifolium* (Onagraceae) that differ in cytotype frequency. Using this approach, I addressed the following three questions. First, does cytotype fitness increase with its frequency in a population? If minority cytotype exclusion operates in mixed cytotype populations of *C. angustifolium*, I would expect seed production to increase in tetraploids and decrease in diploids as the proportion of tetraploids rises. Second, what is the threshold frequency at which

Received 14 September 1999 *Accepted* 20 October 1999

tetraploid fitness equals that of diploids? Assuming there are no inherent differences in viability and fertility between cytotypes, the threshold should be near 50%. Third, to what extent do flower asynchrony and pollinator preferences cause assortative mating and thus weaken frequency-dependent selection acting on either cytotype?

2. MATERIAL AND METHODS

(a) *Study organism*

C. angustifolium L. Holub, formerly *Epilobium angustifolium*, is an insect-pollinated, herbaceous perennial distributed widely in the Northern Hemisphere and found primarily in open or disturbed habitats. It is self-compatible, although selfed offspring rarely survive to maturity due to high inbreeding depression (Husband & Schemske 1997). Plants produce tall, narrow racemes consisting of between ten and 20 open flowers. In North America, *C. angustifolium* consists of diploid, triploid and tetraploid individuals (Mosquin 1966; Husband & Schemske 1998). In general, diploids are found at high latitudes and altitudes (Mosquin 1966; Flint 1980). While diploids and tetraploids are mostly allopatric, mixed populations do occur in the zone of contact, along the Rocky Mountains. Experimental crosses between diploids and tetraploids yield significantly less seed than within-cytotype pollinations, indicating that, regardless of maternal parent, hybrid triploids are largely, though not completely, inviable (T. L. Burton and B. C. Husband, unpublished data).

Diploid and tetraploid plants used in this experiment were derived from seed collected from a uniform diploid and uniform tetraploid population on the Beartooth Highway, Wyoming (identified as D2 and T26 in Husband & Schemske 1998). Open-pollinated seed families were collected from 30–50 plants in each population and five to ten progeny per family were germinated and grown in 1 gal (US) pots in the Department of Botany greenhouse. Based on previous studies of the mating system in this plant, the maternal plants sampled in the field are highly outbred (Husband & Schemske 1995, 1998).

(b) *Experimental design*

To examine the influence of cytotype frequency on cytotype fitness, I created ten experimental populations of *C. angustifolium*, differing in the proportion of tetraploids. All populations consisted of 30 plants, each from a different maternal parent. Two populations were randomly assigned to each of five different experimental treatments: 0, 10, 15, 20 or 30 tetraploids, with the remainder being diploid. Seven of the populations were planted in isolated regions of the University of Guelph Arboretum. Three others were established in local gardens nearby. Plants were arranged in a 5×6 grid, with 30 cm between each plant and their pots sunk into the soil approximately 15 cm. All populations were separated by a minimum of 150 m to minimize pollen contamination. Plants were placed at the array locations on 23 June 1996 and removed on 13 August 1996 after flowering was completed. All plants were watered every second day and fertilized every two weeks during that period of time.

(c) *Fitness measures*

Mean fitness of diploids and tetraploids was estimated for each replicate population from three fertility components: total flower number per plant, ovule number per flower and seed number per ovule. The number of flowers produced was censused completely in all plants of all experimental populations. Every second day, a tape marker was placed at the bottom of the lowest open flower on the main reproductive stem and on each axillary branch. The number of flowers between the new marker and the previous marker was counted, as was the number of flowers that were currently open. This allowed me to estimate the total number of flowers produced over the growing season, as well as to determine the two-day period in which any flower was fertilized.

To estimate ovule number and seed production per ovule, all fruit were harvested as they matured. Four fruit were then randomly drawn from each plant, one from each quartile of its flowering period. The seeds and unfertilized ovules were cleaned of their comose and counted. Seed set was estimated as the proportion of ovules that had developed into mature seeds for each fruit.

In addition, I estimated the number of pollen grains per anther. Ten plants from each cytotype present in each population were sampled. One anther per plant was collected just prior to dehiscence and placed in 1 ml of 70% ethanol. The number of pollen grains from two 0.1 ml aliquots per anther was counted using a haemocytometer.

Flower number per plant and ovule number per flower were not expected to differ among treatments and therefore a *t*-test comparison between diploids and tetraploids was conducted on data pooled from all experimental populations. Mean seed set in diploids and tetraploids was calculated separately for each population. The relationship between mean seed set per cytotype and cytotype frequency was then tested using a model I linear regression. Total seed production for each individual was calculated as the product of its flower number, ovule number per fruit and proportion seed set per fruit. Relative fitness of tetraploids was then calculated as the mean total seed production of tetraploids expressed as a proportion of mean total seed production for diploids in each treatment that contained both cytotypes (33, 50 and 67% tetraploid).

(d) *Factors affecting premating isolation*

Bee foraging patterns were recorded for 0.5 h every second day in each population, for as long as at least 25 out of the 30 plants in the population were flowering. Observations were concentrated on three bees (leaf-cutter bee, bumble-bee, honeybee) as they were the most frequent visitors. For each foraging bout into the population, I recorded the general category of bee, the sequence of visits to plants in the population and the number of flowers visited per inflorescence. Pollinator visitation was described in three ways: pollinator preference, pollinator flight sequence and flowers per inflorescence. Pollinator preference is defined as a disproportionate number of visits to one cytotype, and was calculated as the log of the proportion of visits to tetraploids. A preference value of zero indicates no preference; a negative value indicates a preference for diploids, while a positive value reflects a disproportionately high number of visits to tetraploids. Pollinator flight sequences were broken down into four categories: between diploids ($2x$–$2x$), diploid to tetraploid ($2x$–$4x$), tetraploid to diploid ($4x$–$2x$) and between tetraploids ($4x$–$4x$). I calculated the frequency of these sequences for each treatment, and compared them statistically with a random expectation. Finally, I compared the number of flowers visited per inflorescence. These values were compared among cytotypes and treatments using a 2×3 factorial ANOVA.

Overlap in flowering between diploid and tetraploids was estimated for the pooled sample of 300 plants by calculating the

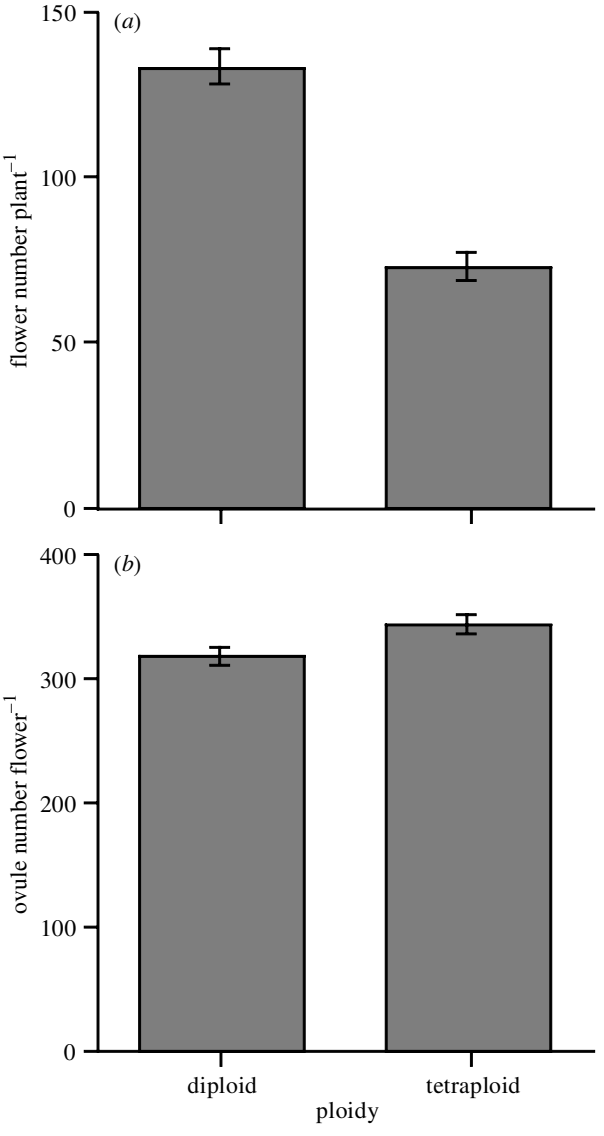

Figure 1. Relationship between ploidy (diploid versus tetraploid) and (*a*) flower number per plant and (*b*) ovule number per flower in *C. angustifolium*. Differences between ploidies were significant for both variables.

number of diploid and tetraploid plants flowering at each census period (every second day), expressed as a percentage of the total flower days across all census periods. This index of overlap was calculated by (i) determining the sum of all open flowers for a given cytotype across the entire growing season, (ii) expressing the number open on any given census day as a proportion of this total, and (iii) superimposing the values for both cytotypes and determining which cytotype occurs in the lowest proportion for each census period. These minimum values were summed to give the percentage overlap between diploids and tetraploids.

3. RESULTS

(a) *Fitness*

Over the 53-day flowering period, tetraploids produced nearly half the number of flowers produced by diploids (diploid: mean = 134.1, s.e. = 5.2; tetraploid: mean = 68.2, s.e = 4.2; $F_{1,298} = 100.7$, $p < 0.0001$; figure 1). This difference was related to the fact that diploid inflorescences were more often branched (96% of diploid and 63% of tetraploid inflorescences had branches), although flower number on the primary stem also differed among cytotypes (diploid: mean = 55.1, s.e. = 1.6; tetraploid: mean = 41.5, s.e = 1.8; $F_{1,298} = 31.1$, $p < 0.0001$). With respect to gamete production, tetraploids produced more ovules per flower (diploid: mean = 319.3, s.e. = 7.1; tetraploid: mean = 343.51, s.e. = 7.7; $F_{1,503} = 5.35$, $p < 0.05$; figure 1) but produced the same number of pollen grains per anther (diploid: mean = 1526, s.e. = 118.9; tetraploid: mean = 1409, s.e. = 100.5; $t = 0.76$, $n_{2x} = 20$, $n_{4x} = 28$, $p > 0.25$).

Seed set per fruit averaged 0.221 (s.e. = 0.009, $n = 505$ fruits, 158 plants). Mean seed set in diploids did not differ from that in tetraploids (diploid: mean = 0.228, s.e. = 0.01; tetraploid: mean = 0.213, s.e. = 0.01; $F_{1,503} = 0.70$, $p > 0.25$). However, seed set in diploids and tetraploids was affected differently by cytotype frequency (figure 2). Diploid seed set ranged from 0.089, when diploids were in the minority (67% tetraploids), to 0.291 in populations that were completely diploid (figure 2). There was a significant linear relationship between seed set in diploids and the frequency of tetraploids in the population (linear regression: $F_{1,6} = 8.4$, $r^2 = 0.59$, $p < 0.03$; deviation from linear regression: $F_{2,4} = 0.28$, $p > 0.75$). In contrast, seed set in tetraploids showed no relationship with cytotype frequency (linear regression: $F_{1,6} = 0.17$, $r^2 = 0.03$, $p > 0.50$; deviation from linear regression: $F_{2,4} = 1.2$, $p > 0.25$). The patterns observed for diploids and tetraploids were even stronger when data only from the time-period in which 80% of plants were flowering (12–27 July) were used (linear regression: diploids, $F_{1,6} = 14.4$, $r^2 = 0.71$, $p < 0.01$; tetraploids, $F_{1,6} = 0.15$, $r^2 = 0.024$, $p > 0.70$). Based on seed set alone, the mean fitness of tetraploids relative to that of diploids for the 33, 50 and 67% tetraploid treatments was 0.73, 1.28 and 1.94, respectively (figure 3).

Average total seed production, calculated as the product of flower number, ovule number per flower and seed set per fruit was 9762 for diploids and 4990 for tetraploids. Expressed relative to total seed production in diploids, the fitness of tetraploids increased from 0.40, when tetraploids were rare (i.e. $4x = 33\%$), to 0.69, when tetraploid and diploids were at equal frequencies ($4x = 2x = 50\%$), and 1.15 when tetraploids were in the majority ($4x = 67\%$; figure 3).

(b) *Pollinator preference*

The primary insect visitors to *C. angustifolium* were bumble-bees, honeybees and leaf-cutter bees. For all insect observations combined, an average of 6.0 foraging bouts were observed per 30 min observation period, while the number of inflorescences visited was 61.6, or 10.2 inflorescences per foraging bout. No differences in either variable were found among the population treatments (number of foraging bouts: $F_{4,5} = 2.9$, $p > 0.10$; number of inflorescences per foraging bout: $F_{4,5} = 0.38$, $p > 0.75$). Out of the inflorescences visited by bees in each array, a disproportionately high number were diploid, as indicated by the mean pollinator preference index of -0.125. The tendency to visit more diploids than expected did not differ among population treatments ($F_{2,3} = 0.032$, $p > 0.90$).

Bees visited an average of 3.6 (s.e. = 0.23) flowers on diploid inflorescences, statistically similar to that on tetraploids (mean = 3.3, s.e. = 0.16; $F_{1,6} = 1.1$, $p > 0.25$),

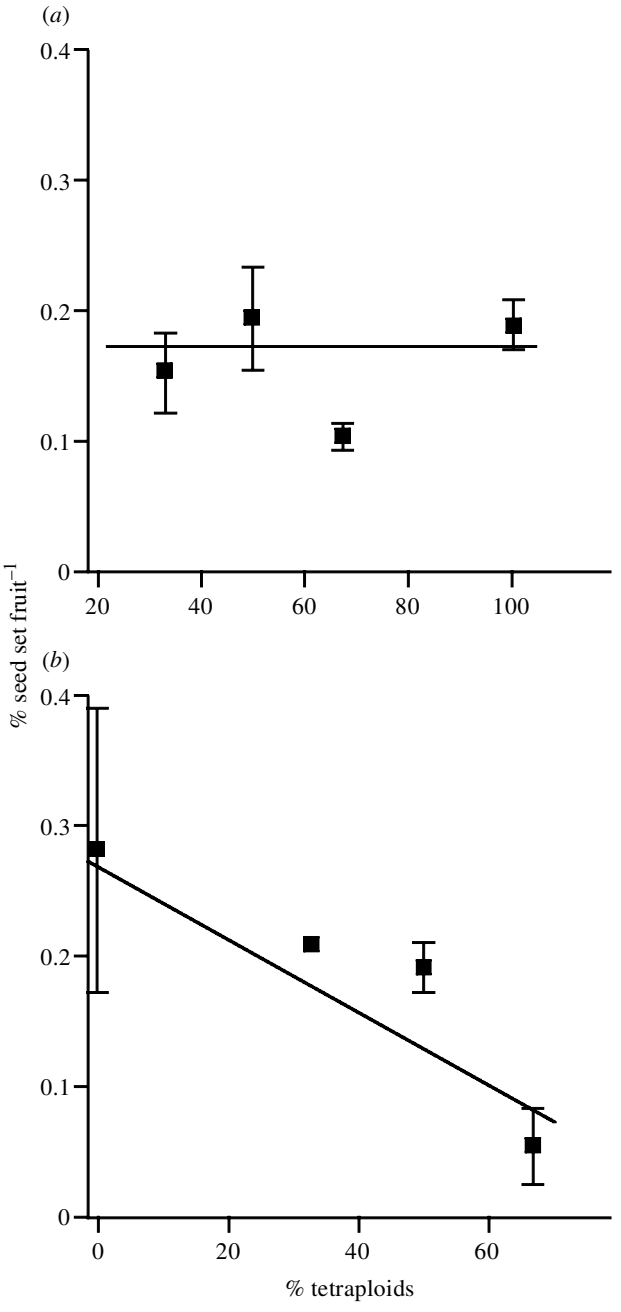

Figure 2. Relationship between proportion of seed set per fruit and percentage of tetraploids in the population for diploid and tetraploid *C. angustifolium*. (*a*) 4*x*, (*b*) 2*x*. Means (s.e.) of all replicate populations are reported, along with the least-squares regression line.

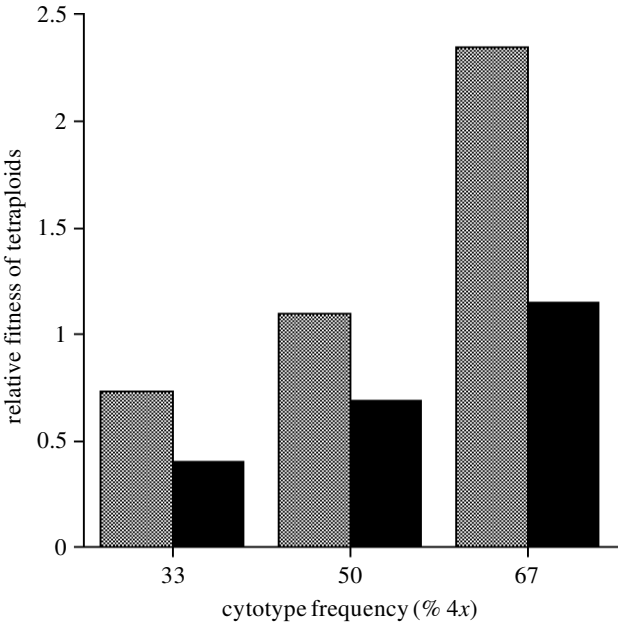

Figure 3. Relative fitness of tetraploids (mean fitness of 4*x*/mean fitness of 2*x*) as a function of the percentage of tetraploids in the population. Shaded bars depict values based on seed set alone. Black bars represent values based on total seed production per plant (i.e. seed set per fruit × number of ovules per flower × number of flowers per plant).

despite there being differences in the number of open flowers between cytotypes (diploid: mean = 16.1; tetraploid: mean = 9.7; $F_{1,6} = 19.9$, $p < 0.01$). The proportion of flowers on an inflorescence that were visited was different among ploidy levels (diploid: mean = 0.23, tetraploid: mean = 0.35, $F_{1,6} = 11.2$, $p < 0.02$); however, variation among cytotype frequency treatments ($F_{2,6} = 0.46$, $p > 0.50$) and the ploidy × treatment interaction ($F_{2,6} = 0.86$, $p > 0.47$) were not significant.

Pollinator flights between inflorescences were classified as 4*x*–4*x*, 4*x*–2*x*, 2*x*–4*x* or 2*x*–2*x* for all populations containing both cytotypes. The distribution of flights among the four categories deviated from the random expectation (based on the frequencies of cytotype in the population) for all treatments (goodness-of-fit test: 33% 4*x*, $G = 23.2$, d.f. = 3, $p < 0.01$; 50% 4*x*, $G = 77.9$, d.f. = 3, $p < 0.01$; 67% 4*x*, $G = 48.7$, d.f. = 3, $p < 0.01$; figure 4). Regardless of the frequency of cytotypes in the population, there was an excess of flights between diploid plants and a deficiency of flights between tetraploid plants. Flights from diploid to tetraploid, or tetraploid to diploid were similar to random expectations. When the number of flights between flowers on the same inflorescence was included in the analysis of flight sequences, the distribution of flights deviated strongly from random expectations (goodness-of-fit test: 33% 4*x*, $G = 1385$, d.f. = 3, $p < 0.001$; 50% 4*x*, $G = 1785.6$, d.f. = 3, $p < 0.001$; 67% 4*x*, $G = 1478.0$, d.f. = 3, $p < 0.001$) as a result of an excess of flights between tetraploids and between diploids but a deficiency of flights between flowers of different cytotypes (figure 4). The excess of 2*x*–2*x* flights far exceeds random expectations regardless of the frequency of 4*x* in the population. The excess of 4*x*–4*x* flights is greatest when 4*x* are rare and smallest when they are common (figure 4).

(c) *Phenology*

Out of the 300 plants used in the experimental arrays, ten did not flower during the experiment; eight of these plants were tetraploid. On average, diploids started flowering eight days earlier than tetraploids; however, both cytotypes completed flowering at the same time (figure 5). The flowering curves for diploids and tetraploids overlapped by 80%. Overlap in individual experimental populations ranged from 76 to 90% and did not differ among treatments (one-way ANOVA; $F_{2,3} = 4.2$, $p > 0.10$).

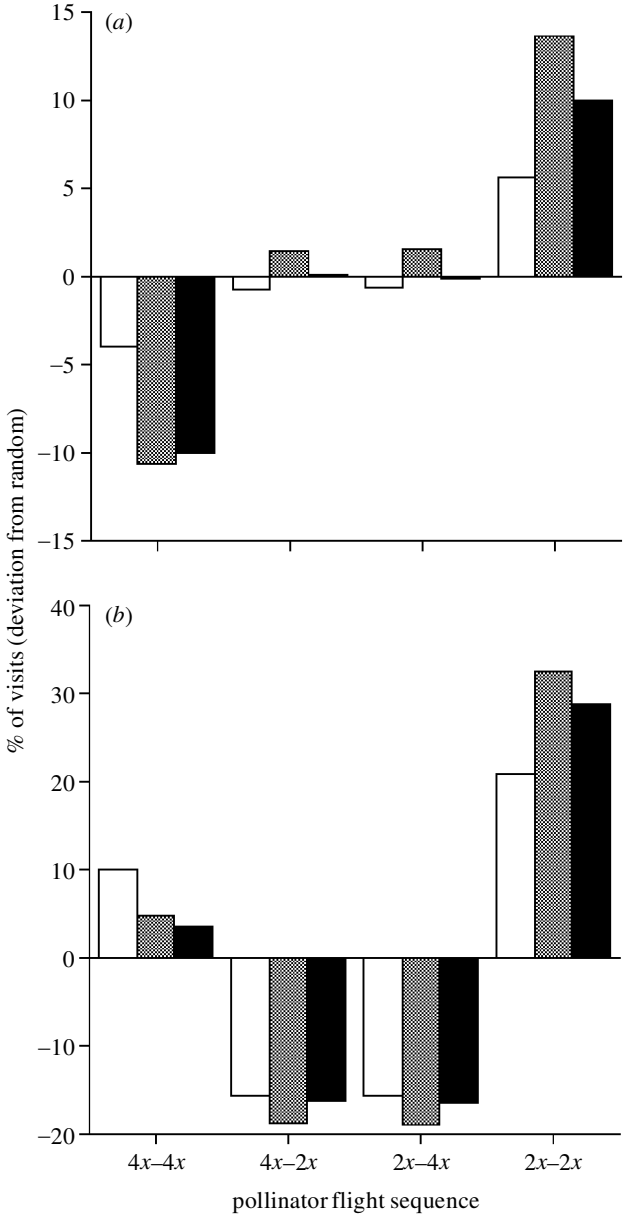

Figure 4. Percentage of pollinator flights, expressed as the deviation from random expectations, between all possible pairs of cytotypes ($2x$–$2x$, $2x$–$4x$, $4x$–$2x$ and $4x$–$4x$). (*a*) Results are based on pollinator flights between inflorescences only. (*b*) Results include pollinator flights between flowers on the same inflorescence as well as between inflorescences.

4. DISCUSSION

This study showed that, in experimental populations, relative seed production of tetraploid *C. angustifolium* varies with cytotype frequency. The strength of this relationship is enhanced for the period of greatest flowering overlap. These results clearly indicate that minority cytotype exclusion can operate in natural populations as a result of infertility of triploid progeny in $2x \times 4x$ matings. In the absence of additional counteracting factors, such selective forces will act to constrain the establishment of tetraploids (the rare cytotype) and inhibit coexistence of tetraploids and diploids in sympatry.

This study represents the first experimental test of frequency-dependent fitness in naturally occurring diploid and tetraploid plants. However, studies of competition between diploid and tetraploid populations have provided evidence that bears indirectly on minority cytotype processes. For example, Maceira *et al.* (1993) found that diploid and tetraploid *Dactylis* grown in association had reduced seed production compared with pure stands and that both cytotypes were affected equally. In addition, studies of phenotypic selection on flowering time in sympatric populations of diploid and tetraploid *Anthoxanthum alpinum* indicated that fitness varies as a function of the degree of flowering overlap (Bretagnolle 1999). Results from both studies probably reflect the effects of increased between-cytotype pollinations and low viability of offspring from $2x \times 4x$ fertilizations. Research on domesticated species has also provided support for frequency-dependent fitness. Hagberg & Ellerstrom (1959) monitored changes in cytotype frequencies over three years in diploid–tetraploid mixtures of rye and found that the minority cytotype declined. However, a similar study in corn (Cavanah & Alexander 1963) showed a systematic decline in tetraploids, regardless of its initial frequency.

According to theoretical models (Levin 1975; Felber 1991), in the absence of environmentally based viability and fertility differences between diploids and tetraploids, the cytotypes should have equal fitnesses when they occur in a 1:1 ratio. In contrast, tetraploid *C. angustifolium* had a lower fitness (relative fitness of 0.7) than diploids when at equal frequencies; moreover, the frequency-dependent effects were not symmetrical as cytotype frequencies deviated from 1:1. Fitness of tetraploids exceeded that of diploids by 15% when populations comprised 67% tetraploids, but was 60% less than diploids when tetraploids occurred at a frequency of 33%. Assuming a linear relationship between seed set and cytotype frequency, the estimated threshold frequency of tetraploids at which both cytotypes have similar fitnesses would be 61%. This pattern would be expected if diploids had an inherent fitness advantage over tetraploids, beyond the effect of cytotype frequency. In my study, diploids had higher ovule production per plant as a result of nearly twice the flower production of tetraploids.

Frequency-dependent effects on relative fitness in *C. angustifolium* are the result of seed set variation in diploids, but not tetraploids. Results for diploids suggest that the three conditions of the minority cytotype exclusion model have been met. First, tetraploid pollen is deposited on diploid stigmas in proportion to the frequency of tetraploids. Second, tetraploid pollen must be as competitive as diploid pollen on diploid stigmas, and third, triploid progeny from $2x \times 4x$ matings are less viable than within-cytotype matings. But why do tetraploids show no sign of frequency-dependent seed set? Several factors may be at play. First, a relationship may actually exist but I cannot detect it due to a lack of power. This explanation is probably unlikely since I was able to detect a significant relationship for diploids under the same sampling regime. Second, tetraploids may be less affected by cytotype frequency because diploid pollen transfer to or pollen competition on tetraploid stigmas is less effective than the reciprocal intercytotype pollination. Regardless of the mechanisms, the end result suggests that tetraploids experience a higher rate of assortative

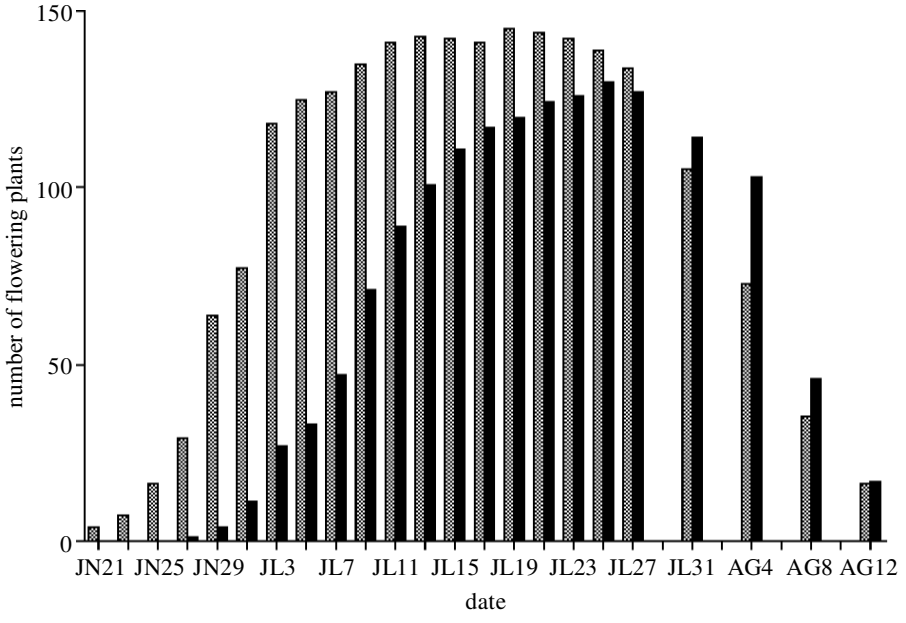

Figure 5. Number of diploid (shaded bars) and tetraploid (black bars) plants flowering over an eight-week period. Data from all experimental populations ($n = 150$ diploids, $n = 150$ tetraploids) are included.

mating and hence lose fewer ovules to between-cytotype pollinations.

Two factors leading to higher assortative mating in tetraploids were examined in this study. Higher assortative mating would be expected in tetraploids if their flowering period is longer than diploids but completely encompasses the entire diploid flowering period. In fact, the opposite is observed. The tetraploid flowering period was encompassed within that of the diploids, and, in addition, the diploids flowered for approximately ten days prior to any tetraploids. Therefore, flowering asynchrony is insufficient to cause higher assortative mating in tetraploids.

The second factor affecting assortative mating is pollen transfer, as reflected in pollinator visitation and flight sequences. Assortative mating in tetraploids could be caused by an excess of visits between tetraploid flowers or by a greater number of $4x$–$2x$ flights compared with $2x$–$4x$ flights. My results indicate no difference in the number of reciprocal flights between diploids and tetraploids. Moreover, there was an excess of visits to diploid but not tetraploid inflorescences. However, when one considers the flights between flowers on each inflorescence as well as the flights between inflorescences (figure 4*b*), we find there to be an excess number of flights between $4x$ flowers, which would promote assortative mating. Furthermore, the excess is greatest when tetraploids are rare and smallest when tetraploids are common. This shift in pollinator behaviour is small but may help to counteract the effects of the diploid cytotype when it is common. Clearly, other factors must be operating to neutralize the minority cytotype effect in tetraploids. Additional research on relative siring abilities and pollen transfer will hopefully clarify the difference in frequency dependence between diploid and tetraploid seed set.

The data from this study highlight the importance of population processes governing the establishment of tetraploids or the coexistence of diploids and tetraploids in natural populations. But if minority cytotype exclusion can operate in populations, as indicated here, what then favours the coexistence of diploids and tetraploids in so many natural populations? The answer to this question is beyond the scope of this study, although the strength of minority disadvantage suggests that attributes unique to natural populations must promote greater assortative mating and hence reduce pollinations between cytotypes. Admittedly, the experimental populations here were simple in design, with regular spacing between plants and no microhabitat differences associated with the two cytotypes. Both of these factors may lead to greater spatial patchiness and, combined with short pollinator flight distances, could enhance assortative mating. Regardless of the specific mechanisms, it is clear that polyploid evolution may depend on ecological factors that can overcome frequency-dependent selection. Without these forces operating polyploid evolution may be considerably slower than has often been portrayed.

I thank Tracy Burton, Helen Kubiw and Alison Sutton for field assistance, Ms Francis Schwenger, Ric Jordan and Arboretum staff for the space to set up the populations, Chris Hussel and Mary Henry for countless hours of seed cleaning and counting, and the Natural Sciences and Engineering Research Council of Canada for financial support.

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As this paper exceeds the maximum length normally permitted, the author has agreed to contribute to production costs.