

# Genome duplication and the evolution of physiological responses to water stress

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## Summary

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**Key words:** adaptation, *Chamerion angustifolium*, gas exchange, hydraulic conductance, polyploidy, soil moisture, xylem cavitation.

- Whole-genome multiplication, or polyploidy, is common in angiosperms and many species consist of multiple cytotypes that have different physiological tolerances. However, the relative importance of genome duplication vs post-duplication evolutionary change in causing differentiation between cytotypes is not known.
- We examined the water relations of *Chamerion angustifolium*, a herbaceous perennial in which diploid and tetraploid cytotypes occupy different niches. To differentiate between the effects of genome duplication and evolutionary changes that followed polyploidization, we compared extant diploids and tetraploids with experimentally synthesized neotetraploids.
- Tetraploids had 32% higher xylem hydraulic conductivity ( $K_H$ ) than neotetraploids and 87% higher  $K_H$  than diploids, but vulnerability to water stress induced cavitation and gas exchange sensitivity to water potential did not differ among cytotypes. Nevertheless, tetraploids took 22% and 30% longer to wilt than neotetraploids and diploids. A simple hydraulic model suggested that tetraploids deplete soil moisture to a greater degree than neotetraploids and diploids before reaching leaf water potentials that cause stomatal closure.
- We conclude that the different physiological tolerances and distribution of diploid and tetraploid *C. angustifolium* are unlikely to be caused solely by genome duplication. The enhanced ability of tetraploids to survive water stress likely evolved after polyploidization.

## Introduction

Whole-genome multiplication, or polyploidy, has occurred throughout the evolutionary history of angiosperms, with elevated chromosome numbers in at least 70% of all species (Grant, 1981; Masterson, 1994; Otto & Whitton, 2000; Cui *et al.*, 2006). Polyploidy can arise from the duplication of genomes of a single species (autopolyploidy) or of hybrids between two different species (allopolyploidy) (Levin, 2002). Because polyploids are reproductively isolated from their progenitors by postzygotic barriers to gene flow, polyploidy is also an engine of sympatric speciation (Ramsey & Schemske, 2002; Husband & Sabara, 2004; Baack & Stanton, 2005; Soltis *et al.*, 2007). However, in order for polyploids to form stable populations, they must overcome the negative effects of inter-cytotype mating following chromosome doubling (Levin, 1975; Husband, 2000). Newly formed polyploids can persist in nature if they can either outcompete their diploid progenitors, ensure

assortative mating or have distinct physiological tolerances that allow them to exploit new niches (Levin, 1983; Fowler & Levin, 1984).

The most immediate effect of chromosome doubling is an increase in cell size (Stebbins, 1971; Masterson, 1994), which has consequences for physiological function and the tolerance of abiotic stress. For example, increased stomatal guard cell size with increased ploidy can increase leaf gas exchange (Li *et al.*, 1996). However, differences in physiological tolerance are not consistently predictable from genome copy number. Polyploids can be less (Baldwin, 1941) or more (Garbutt & Bazzaz, 1983; Watanabe, 1986; Li *et al.*, 1996) tolerant of low soil moisture, less (Mosquin, 1967; Schaefer & Miksche, 1977; Hardy *et al.*, 2000) or more (Lewis & Terrell, 1962; Sharma & Dey, 1967) cold tolerant and less (Rothera & Davy, 1986; Brammel & Semple, 1990) or more (Fukuda, 1967) shade tolerant than diploids. These conflicting observations suggest that in addition to the direct effects of chromosome doubling,

physiological differences between polyploids and their diploid progenitors could be caused by evolutionary changes following polyploidization (Ramsey & Schemske, 2002). Nevertheless, little is known about the relative importance of genome duplication vs post duplication evolutionary change in causing physiological differentiation among cytotypes.

In addition to guard cell size, polyploidy can increase xylem conduit size (Pockman & Sperry, 1997), which could influence the regulation of water transport (Zimmermann, 1983). Physiological theory indicates that increased xylem cell size could make polyploids either more sensitive or less sensitive to water stress than diploids (Maseda & Fernandez, 2006; McDowell *et al.*, 2008). Larger xylem conduits increase whole-plant hydraulic conductivity, or the physical capacity of the roots and stems to supply leaves with water (Zimmermann, 1983; Tyree & Ewers, 1991), which increases stomatal opening (Mencuccini, 2003). If increased xylem conduit size is accompanied by increased pore size in the pit membrane that separates adjacent conduits (Sperry & Hacke, 2004), then polyploids may be more sensitive to water stress than diploids. This is because the water transport pathway is vulnerable to cavitation through air-seeding, or the aspiration of air bubbles into water-filled conduits (Zimmermann, 1983; Tyree & Sperry, 1989). Cavitation results in a vapor- or air-filled conduit that no longer carries water, which decreases leaf water supply, and ultimately causes death if transpiration is not restricted by stomatal closure (Tyree & Ewers, 1991). Because air seeding depends on the surface tension of the meniscus in each pore, a less negative water potential is required to cause cavitation with increasing pore size (Jarbeau *et al.*, 1995). By contrast, if increased xylem conduit size has no effect on xylem vulnerability to cavitation, increased whole-plant hydraulic conductivity could allow polyploids to transpire at the same rate as diploids but at a less negative leaf water potential (Whitehead *et al.*, 1984; Tyree & Ewers, 1991; Mencuccini & Grace, 1995; Maherali & DeLucia, 2001). As a result, polyploids could take longer than diploids to reach leaf water potentials that cause xylem cavitation during drought. Despite the potential for genome duplication to affect xylem conduit size, xylem structure and function have not been studied as mechanisms of differential drought adaptation between diploids and polyploids.

*Chamerion angustifolium* (Onagraceae) is a herbaceous perennial with a circumpolar distribution, consisting of both diploid ( $2n = 2x = 36$ ) and tetraploid ( $2n = 4x = 72$ ) populations (Mosquin, 1967). Tetraploids are considered to be autopolyploids based on morphological, cytological and genetic evidence (Mosquin, 1967; Husband & Schemske, 2000; Y. Roy *et al.*, unpublished). *Chamerion angustifolium* is widely distributed in North America, most often occurring in open and disturbed habitats. Diploids and tetraploids are primarily allopatric, with most diploids

occurring at higher latitudes and elevations than tetraploids (Soltis *et al.*, 2007). Because of the wide and distinct distributions of the cytotypes, *C. angustifolium* has been highlighted as an important model system in the study of polyploid speciation (Soltis *et al.*, 2007). Although the microhabitats of each cytotype are not well-characterized, the specialization of each cytotype on different elevations suggests that their microclimate tolerances differ. For example, lower elevations typically experience increased atmospheric and soil water deficits because of higher temperature and a longer growing season (Maherali & DeLucia, 2000; McKay *et al.*, 2001). These microclimate differences, and the observation that tetraploids flower later than diploids (Husband, 2000) suggests that tetraploids are more tolerant of water deficits (McKay *et al.*, 2003) than diploids.

We report how xylem structure and function and physiological performance under water stress are affected by polyploidy in *C. angustifolium*. To differentiate between the direct effects of genome duplication *sensu stricto* and subsequent evolutionary changes (Ramsey & Schemske, 2002), we compared extant diploids and tetraploids with neotetraploids synthesized by treating extant diploids with colchicine (Blakeslee, 1941; Chen & Tang, 1945; Husband *et al.*, 2008). We addressed the following four questions. Do tetraploids and neotetraploids have larger stomata and xylem conduits than diploids? Do differences in xylem anatomy influence xylem hydraulic conductivity and the vulnerability of cytotypes to water stress induced cavitation? Does the regulation of gas exchange in response to water stress differ between cytotypes? Do tetraploids and neotetraploids differ in their ability to tolerate water stress in comparison with diploids? If genome duplication was primarily responsible for altering physiological responses to water stress, then we would expect tetraploids and neotetraploids to have similar physiological phenotypes and responses to water stress. If evolutionary change following genome duplication was an important factor in determining the phenotypes of extant tetraploids, then we would expect tetraploids and neotetraploids to have distinct physiological phenotypes and responses to water stress.

## Materials and Methods

### Source populations, experimental design and growth conditions

To minimize the effects of habitat and historical differences on phenotypes, we used diploid and tetraploid *C. angustifolium* L. Holub from three mixed ploidy populations in the Canadian Rocky Mountains: Rampart Creek (52°02.498' N, 116°51.835' W), Coleman (50°18.540' N, 114°36.748' W) and Continental Divide (51°13.673' N, 116°02.910' W). Seeds were collected from open-pollinated maternal plants of each cytotype at each site.

Neotetraploid plants were synthesized by treating diploid seedlings from > 250 diploid families with colchicine (Blakeslee, 1941; Chen & Tang, 1945; Martin, 2008). About 30 seeds from each maternal family were germinated in a Petri dish containing moist filter paper and then treated with a 0.2% colchicine solution for 12 h. Seedlings were rinsed with distilled water and transplanted into trays with 1-inch (*c.* 2.5 cm) square plugs filled with Promix (Premier Tech, Rivière du-Loup, QC, Canada). At the rosette stage leaf tissue was sampled from each plant and screened using flow cytometry (Bennett *et al.*, 2000; Dart *et al.*, 2004) to determine if plants were tetraploid. The neotetraploids were transplanted into plastic cylindrical 15-cm pots filled with Promix. After flowering, plants were screened again with flow cytometry to exclude any that had reverted to diploidy. Fertile plants with tetraploid inflorescences were crossed to generate first-generation neotetraploid seed.

To establish the experiment, we selected a subset of 10 diploid and nine tetraploid (three or four from each population) families. To ensure that differences between neotetraploids and diploids were not caused by different genetic backgrounds, we selected 13 neotetraploid families that had at least one parent from the selected diploid families. Thirty seeds from each family were sown in 1-inch square plug trays filled with Pro-Mix BX (Premier Tech), covered with a plastic lid to maintain high humidity, and placed in a climate controlled growth room (16 h light cycle at 24°C). After 10 d, six to eight germinated seedlings per maternal family were planted in 15-cm pots filled with Pro-Mix BX, and fertilized with 5 g of Nutricote Total NPK 13 : 13 : 13 slow-release fertilizer (Plant Products, Brampton, ON, Canada). Pots were randomly positioned on a single glasshouse bench, watered every other day, and exposed to supplemental light to maintain 16 h days. Mortality following transplantation reduced sample sizes in some families to two or three individuals. After 10 wk growth, when plants had begun flowering, we began physiological measurements.

### Stomatal anatomy

To determine stomatal length and density, we made a mould of the abaxial surface of a single randomly selected mature leaf on 32–46 individuals of each cytotype using polyvinylsiloxane dental impression material ('Extrude' Medium; Kerr Manufacturing Co., Orange, CA, USA) and used the hardened mould as a cast for clear nail polish. The surface area (cm<sup>2</sup>) of each sampled ovate leaf was calculated as the product of its maximum length and width. We measured stomatal length on the nail polish impression as the average distance in µm between the junctions of the guard cells (Malone *et al.*, 1993; Maherali *et al.*, 2002) for a total of 10 stomata per leaf. We measured stomatal density on the nail polish impression as the average number of stomata

in two randomly selected, 0.8-mm<sup>2</sup> viewing areas per leaf. The measurements and counts were made using a light microscope interfaced with a Nikon Coolpix4500 digital camera, and ImageJ (US National Institute of Health; <http://rsb.info.nih.gov/ij/>).

### Hydraulic conductivity and vulnerability to xylem cavitation

Vulnerability to water stress induced xylem cavitation was measured as the reduction in hydraulic conductivity of a stem as a function of xylem pressure (a 'vulnerability curve'; Sperry *et al.*, 1988) created by air-injection (Sperry & Saliendra, 1994). Xylem cavitation was induced by successively increasing positive air pressure on a stem segment inside a double-ended pressure chamber (PMS Instruments, Corvallis, OR, USA). The positive air pressure necessary to cause a decrease in hydraulic conductivity is an estimate of the magnitude of xylem tension that causes cavitation in dehydrated stems (Sperry & Saliendra, 1994).

Stem segments (15 cm long), located within 20 cm of the root collar, were isolated by first removing soil from roots on seven randomly selected plants per cytotype (two or three per population), and placing plants in a water-filled sink. Segments were cut under water and the axillary branches removed. To prevent water from leaking out of axillary branch openings, the entire segment was wrapped tightly in Parafilm (e.g. Sperry *et al.*, 2005). Stem segments were then immersed in a 4°C water bath for 2–3 h to reduce latex emissions. Each segment was cut again at each end and was notched (0.5–1 mm deep) at the midpoint of the segment with a razor blade to provide an entry point for air and then placed into the pressure chamber (Sperry & Saliendra, 1994). Segments were inserted into vinyl tubing that was attached to a Xylem meter (Xylem Embolism Meter; Bronkhorst, Montigny les Cormeilles, France; Cochard, 2002) which utilizes a high-resolution liquid mass-flowmeter to measure the volume of water flow. Axial hydraulic conductivity of stem segments was measured under laboratory irradiance conditions (< 15 µmol m<sup>-2</sup> s<sup>-1</sup>) by perfusing them at a hydrostatic pressure of 10 kPa with filtered (0.2 µm) distilled water. We noted that the use of pure water, rather than a KCl solution, can underestimate  $K_H$  because of hydrogels in the pit membrane (Zwieniecki *et al.*, 2001). Volume flow rates ( $Q$ , kg s<sup>-1</sup>) typically reached steady state within 5 min, after which data were recorded. Hydraulic conductivity ( $K_H$ , kg m MPa<sup>-1</sup> s<sup>-1</sup>) was expressed as the volume flow rate divided by the pressure gradient (MPa m<sup>-1</sup>) and corrected to water viscosity at 20°C. Following hydraulic measurements, we calculated stem area ( $A_S$ ) of each sample by subtracting the cross-sectional area of pith from the cross-sectional area of the entire segment. We calculated specific hydraulic conductivity ( $K_S$ ) as  $K_H/A_S$ .

In preliminary experiments, perfusing a stem with high pressure to dissolve existing emboli had no effect on hydraulic conductivity. Therefore, we assumed that xylem was saturated and the initial measurement of hydraulic conductivity before the induction of cavitation was recorded as the maximum hydraulic conductivity ( $K_{\max}$ ). Hydraulic conductivity was measured again at a hydrostatic pressure of 10 kPa after each pressurization. During measurement of hydraulic conductivity, air pressure in the chamber was maintained at 30 kPa to prevent the refilling of embolized conduits by the perfusing solution. Per cent loss in conductivity (PLC) following each pressurization of the chamber was calculated as  $PLC = 100 \times ((K_{\max} - K_h)/K_{\max})$ , where  $K_h$  is the hydraulic conductivity of the segment measured after each chamber pressurization. Vulnerability curves were fit with an exponential sigmoid equation (Pammenter & Vander Willigen, 1998):

$$PLC = \frac{100}{[1 + \exp(a(\Psi - b))]} \quad \text{Eqn 1}$$

where  $\Psi$  is the negative of the injection pressure,  $a$  is a measure of the degree to which conductivity responds to injection pressure or tension (curve slope) and  $b$  represents the  $\Psi$  at which a 50% loss in conductivity occurs ( $\Psi_{50}$  or curve displacement along the  $x$ -axis). Coefficients  $a$  and  $b$  were estimated using the nonlinear regression procedure in SYSTAT 8.0 (Systat Software, Chicago, IL, USA).

### Xylem anatomy

Xylem conduit diameter was measured on stem segments used for hydraulic measurements. Stem cross-sections were made with a safety razor blade (Electron Microscopy Sciences, Hatfield, PA, USA) and stained with 0.1% toluidine blue. The cross-sections were viewed with a light microscope (Leica DM2500, Wetzlar, Germany) interfaced with digital camera and desktop computer. Using OPENLAB 4.0.3 software (Improvisation Inc., Waltham, MA, USA) we measured two randomly selected vascular bundles on each cross-section. We measured the diameter of the shortest and longest side of each conduit and recorded the total number of vessels in each vascular bundle. Hydraulic diameter ( $D_h$ ) of lumens was calculated as  $D_h = (2x^2y^2/(x + y))^{1/2}$  for vessels, where  $x$  and  $y$  are the short and long sides of the conduit, respectively (Lewis & Boose, 1995).

### Effect of stem length on hydraulic conductivity

A stem shortening experiment (Chiu & Ewers, 1993; Sperry *et al.*, 2005) was used to determine if cytotype hydraulic conductivity was affected by differences in vessel length. If genome duplication increases cell size, vessel

length as well as vessel diameter could increase with ploidy. Shorter vessels could reduce stem hydraulic conductivity of a given stem length because water would have to pass through more end walls between axially connected conduits. By contrast, longer vessels would increase hydraulic conductivity because of reduced contributions of end wall resistance to water flow. Six randomly selected individuals per cytotype were harvested and prepared for hydraulic conductivity measurements as described earlier, except that 16-cm long stem segments were used, and attached to the Xylem meter. After stable volume flow rates were established, we calculated  $K_H$ , and then cut stems with a razor blade in 2 cm increments. After each cut, we waited for volume flow rates to stabilize and then recalculated  $K_H$ . Measurements were ended when stems were 2 cm long. The response of hydraulic conductivity to stem length was described using a power function using SIGMAPLOT 8.0 (Systat Software, San Jose, CA, USA).

### Gas exchange and response to water stress

To determine how cytotypes responded to a reduction in soil moisture, we randomly selected 10–13 individuals of each cytotype and measured steady-state leaf gas exchange before the cessation of watering and then remeasured gas exchange on the same individuals three and 5 d after watering had ceased. Gas exchange was measured on the youngest fully expanded leaf of each individual at saturating irradiance ( $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) with an open gas-exchange system (LI-6400; Li-Cor Inc., Lincoln, NE, USA) between 09 : 00 and 12 : 00 h EST. During measurements, incident irradiance was provided by red–blue light-emitting diodes and cuvette  $\text{CO}_2$  concentration was maintained at  $400 \mu\text{mol mol}^{-1}$  to reflect prevailing ambient conditions. A Peltier cooling module maintained leaf temperature at approximately ambient conditions (25–30°C) during each measurement period. The leaf-to-air vapor pressure deficit ( $D$ ) increased from 1 kPa to 3 kPa during the course of measurements, as stomata closed with increasing water stress. To calculate  $g_s$  we used a boundary layer conductance of  $1.42 \text{ mol m}^{-2} \text{ s}^{-1}$ , which was calculated on the basis of leaf area and fan speed using the energy balance algorithms of the LI-6400. Following enclosure in the leaf cuvette, leaves reached steady-state values (e.g. when the coefficients of variation of  $\text{CO}_2$  and  $\text{H}_2\text{O}$  within the chamber were < 0.25%) within 5 min. After each gas exchange measurement, leaf water potential ( $\Psi$ ) was measured with a pressure chamber (Plant Moisture Status Instrument Company, Corvallis, OR, USA). Gas exchange responses to leaf water potential were described using an exponential decay function using SIGMAPLOT 8.0 (Systat Software).

After the last day of gas exchange measurements, we continued to measure water potential on each plant each day

until leaves began to wilt. After gas exchange measurements ended, water potential was measured on small axillary shoots to minimize negative effects on plant evaporative surface area. We recorded the day that leaves on each plant wilted and the day that plants died. Plants were judged to have died when all leaves were wilted and dry to the touch.

### Statistical analyses

Because many response variables such as hydraulic conductivity and vessel diameter can covary with plant size (Maherali *et al.*, 1997), statistical comparisons between cytotypes were made by analysis of covariance (ANCOVA, Sokal & Rohlf, 1995) with stem diameter as the covariate. When size did not have a significant effect, we removed it from the model and analysed data with a one-way ANOVA, followed by a Fisher's LSD *post hoc* test.

Because stomatal density and size are negatively correlated and because stomatal density and size scale with leaf size (Tichá, 1982), we used linear regression to examine the strength of these pairwise relationships. We used ANCOVA to examine whether slopes of these pairwise relationships differed among cytotypes. In the ANCOVA, we tested differences among slope coefficients by including the cytotype  $\times$  (stomatal or leaf) size interaction term in each model. If this term was significant, then we rejected the null hypothesis that slope coefficients between cytotypes were homogeneous (Sokal & Rohlf, 1995). We also tested for differences in stomatal density, stomatal size and leaf size between cytotypes with ANOVA and Fisher's LSD *post hoc* tests. Statistical differences among cytotypes for relationships that were fitted with nonlinear regression (i.e. the response of  $K_H$  to stem length, vulnerability curves, and gas exchange responses to water potential) were determined by comparing whether 95% confidence limits around regression coefficients overlapped among cytotypes. All data met assumptions for regression, ANCOVA or ANOVA. Statistical analyses were done with SYSTAT 8.0 or SIGMAPLOT 8.0.

Experimental populations of each cytotype consisted of specific maternal families. However, many of the physiological measurements were time consuming and we could not sample with sufficient replication at the family level to allow this term to be included in the ANOVA model. In order to determine cytotype effects, we randomly selected

individuals within each cytotype without regard to family affiliation. We also note that because of the small sample size, the statistical power to detect physiological differences among cytotypes was low. In addition, our use of sympatric populations provides a relatively conservative estimate of phenotypic differences among cytotypes. Physiological differences among cytotypes could be more apparent in a comparison of allopatric populations.

### Results

Stomatal characters differed among diploid, neotetraploid and tetraploid cytotypes. Neotetraploids had 34% larger stomata than diploids and 16% larger stomata than tetraploids ( $MS = 20.79$ ,  $F_{2,110} = 5.21$ ,  $P = 0.007$ ; Table 1). Neotetraploids had 33% lower stomatal density than diploids and 12% lower stomatal density than tetraploids ( $MS = 851.5$ ,  $F_{2,110} = 8.72$ ,  $P = 0.0003$ ), suggesting that there was a trade-off between stomatal length and stomatal density across cytotypes. Stomatal size and stomatal density were also negatively correlated in neotetraploids and tetraploids (Fig. 1a, Table 2).

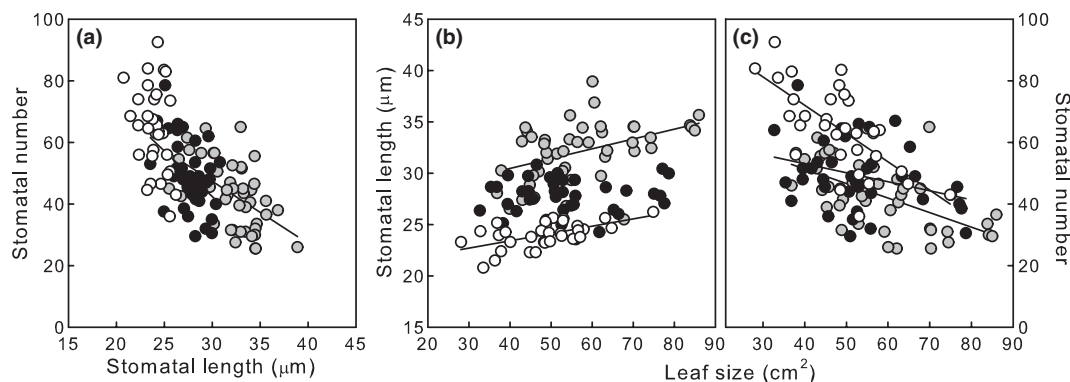
Leaf size varied among cytotypes ( $MS = 772.5$ ,  $F_{2,113} = 5.29$ ,  $P = 0.006$ ). Neotetraploids had larger leaves than diploids, with tetraploids having intermediate leaf size (Table 1). Variation in leaf size was correlated with stomatal characters within cytotypes in some cases. Stomatal length increased with leaf size in diploids and neotetraploids, but these two traits were unrelated in tetraploids (Fig. 1b, Table 2). Stomatal density was negatively correlated with leaf size in all three cytotypes, but the relationship was strongest in diploids and neotetraploids, in comparison with tetraploids (Fig. 1c, Table 2).

Xylem structure and function differed among cytotypes (Table 3). Tetraploids had 87% higher hydraulic conductivity ( $K_H$ ) than diploids and 32% higher  $K_H$  than neotetraploids ( $MS = 2.31 \times 10^{-11}$ ,  $F_{2,18} = 4.46$ ,  $P = 0.027$ ). Similarly, tetraploids had 81% higher specific hydraulic conductivity ( $K_S$ ) than diploids and 12% higher  $K_S$  than neotetraploids ( $MS = 0.67$ ,  $F_{2,17} = 4.55$ ,  $P = 0.026$ ). The response of hydraulic conductivity to stem shortening did not differ among cytotypes, suggesting that vessel length distributions also did not differ among cytotypes (Fig. 2a). Hydraulic vessel diameter distributions largely overlapped

**Table 1** Mean ( $\pm 1$  SE) stomatal characters and leaf size of diploid, neotetraploid and tetraploid *Chamerion angustifolium*

Trait	Diploids	Neotetraploids	Tetraploids
<i>n</i>	32	46	39
Stomatal length ( $\mu\text{m}$ )	24.0 $\pm$ 0.22 a	32.11 $\pm$ 0.42 b	27.77 $\pm$ 0.28 c
Stomatal number	64.28 $\pm$ 2.42 a	43.14 $\pm$ 1.58 b	49.05 $\pm$ 1.77 b
Leaf size ( $\text{cm}^2$ )	48.34 $\pm$ 1.80 a	57.43 $\pm$ 1.94 b	53.54 $\pm$ 1.98 ab

Significant differences between cytotypes ( $P \leq 0.05$ ) for each trait are indicated by different letters within rows. Statistical differences were assessed with ANOVA and Fisher's LSD *post hoc* tests. Stomatal number was determined on a viewing area of 0.8 mm<sup>2</sup>.



**Fig. 1** The relationship between (a) stomatal number per unit area ( $0.8 \text{ mm}^2$ ) and stomatal length, (b) stomatal length and leaf size, and (c) stomatal number and leaf size for diploid (open circles), neotetraploid (tinted circles) and tetraploid (closed circles) cytotypes of *Chamerion angustifolium*. Mean differences between each cytotype are shown in Table 1 and regression equations and their statistical significance are shown in Table 2. Regression lines were drawn for statistically significant relationships.

**Table 2** Regression equations describing the bivariate relationships between stomatal size, stomatal number and leaf size in diploid (2x), neotetraploid (Neo) and tetraploid (4x) cytotypes of *Chamerion angustifolium*

y	x	Cytotype	Regression parameters				ANCOVA	
			b (SE)	a	$r^2$	P	F	P
Stomatal number	Stomatal length	2x	-3.37 (1.89)	145	0.10	0.085	0.58	0.56
		Neo	-2.03 (0.48)	108	0.29	<b>0.0001</b>		
		4x	-2.96 (0.93)	131	0.22	<b>0.003</b>		
Stomatal length	Leaf size	2x	0.07 (0.02)	20.6	0.33	<b>0.0006</b>	3.38	<b>0.04</b>
		Neo	0.10 (0.03)	26.6	0.19	<b>0.003</b>		
		4x	0.006 (0.02)	27.4	0	0.79		
Stomatal number	Leaf size	2x	-0.91 (0.18)	108	0.47	<b>0.00002</b>	4.16	<b>0.02</b>
		Neo	-0.47 (0.10)	70.2	0.32	<b>0.00005</b>		
		4x	-0.29 (0.13)	64.7	0.11	<b>0.04</b>		

Equations follow the form:  $y = bx + a$ . Analysis of covariance (ANCOVA) was used to determine if slopes differed statistically between cytotypes. Statistically significant regression slopes and slope differences between treatments (at  $P < 0.05$ ) are in bold type.

(Fig. 2b) and the effect cytotype on mean hydraulic vessel diameter was marginally significant ( $MS = 20.68$ ,  $F_{2,16} = 3.12$ ,  $P = 0.07$ ). However, neotetraploids and tetraploids each had *c.* 20% larger mean hydraulic vessel diameters than diploids (Table 3). The overall effect of cytotype on vessel number per vascular bundle was marginally significant ( $MS = 3042$ ,  $F_{2,16} = 2.79$ ,  $P = 0.09$ ). Neotetraploids and tetraploids had similar vessel number per vascular bundle but both neotetraploids and tetraploids had *c.* 26% fewer vessels than diploids. Neotetraploids and tetraploids had larger stem diameter than diploids at the time of hydraulic measurements ( $MS = 0.48$ ,  $F_{2,18} = 7.57$ ,  $P = 0.004$ ), but stem diameter did not explain any significant variation in any xylem structure and function across cytotypes ( $P = 0.17$ – $0.74$ ).

Vulnerability to water stress-induced cavitation did not differ across cytotypes (Fig. 3a). The mean ( $\pm 1$  SE) water potential (MPa) at which a 50% loss in conductivity occurred ( $\Psi_{50}$ ) was  $-1.59 \pm 0.084$ ,  $-1.63 \pm 0.064$  and  $-1.66 \pm 0.055$  for diploids, neotetraploids and tetraploids,

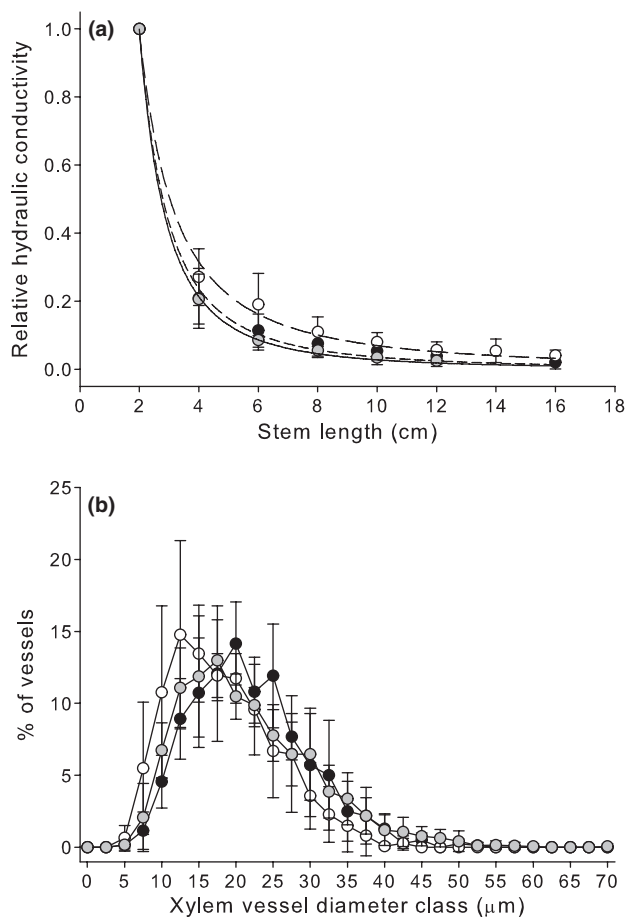
respectively. There were also no differences among cytotypes in the response of gas exchange to decreasing leaf water potential. Stomata were very sensitive to water stress, which resulted in complete stomatal closure and the cessation of photosynthesis at a leaf water potential of  $-1.5$  MPa (Fig. 3b,c).

Cytotypes differed in the amount of time it took leaves to wilt after watering was suspended ( $MS = 7.65$ ,  $F_{2,28} = 4.87$ ,  $P = 0.015$ ; Fig. 4a,b). On average, tetraploids took 22% and 30% longer to reach water potentials that caused wilting than neotetraploids and diploids. Similarly, tetraploids took 18% and 28% longer to die than neotetraploids and diploids, though the overall effect of cytotype on time to death was marginally significant ( $MS = 15.3$ ,  $F_{2,29} = 2.97$ ,  $P = 0.07$ ; Fig. 4c). Despite differences in time to wilting, all cytotypes wilted at approximately the same leaf water potential ( $MS = 0.010$ ,  $F_{2,21} = 0.273$ ,  $P = 0.76$ ; Fig. 4d). Differences in time to wilting and death were not associated with any intrinsic differences in plant size among cytotypes ( $MS = 0.224$ ,  $F_{2,32} = 0.243$ ,  $P = 0.79$ ; Fig. 4e).

**Table 3** Mean ( $\pm 1$  SE) stem size, hydraulic and anatomical characters of diploid, neo-tetraploid and tetraploid *Chamerion angustifolium*

Trait	Diploids	Neotetraploids	Tetraploids
<i>n</i>	5–7	7	7
Stem diameter (mm)	3.37 $\pm$ 0.09 a	3.8 $\pm$ 0.11 b	3.84 $\pm$ 0.08 b
Hydraulic conductivity, $\times 10^{-6}$ ( $K_H$ ; kg m MPa $^{-1}$ s $^{-1}$ )	4.17 $\pm$ 0.86 a	5.90 $\pm$ 0.95 ab	7.80 $\pm$ 0.76 b
Specific hydraulic conductivity ( $K_S$ ; kg m $^{-1}$ MPa $^{-1}$ s $^{-1}$ )	0.77 $\pm$ 0.09 a	1.24 $\pm$ 0.17 b	1.39 $\pm$ 0.15 b
Mean hydraulic vessel diameter ( $\mu$ m)	16.95 $\pm$ 1.31 a	20.32 $\pm$ 0.98 b	20.58 $\pm$ 0.94 b
Vessel number	183 $\pm$ 21 a	134 $\pm$ 9.8 b	135 $\pm$ 13.9 b

Significant differences ( $P \leq 0.05$ ) between cytotypes for each trait are indicated by different letters within rows. Statistical differences were assessed with ANOVA and Fisher's LSD post hoc tests. Vessel diameter and number were measured on two randomly selected vascular bundles per stem.



**Fig. 2** The relationship between relative stem hydraulic conductivity ( $K_H$ ) and stem length for diploid (open circles), neotetraploid (tinted circles) and tetraploid (closed circles) cytotypes of *Chamerion angustifolium*. Data were fit with a power function (a). Hydraulic vessel diameter distributions (in 2.5- $\mu$ m classes) for stems of diploid, neotetraploid and tetraploid cytotypes (b).

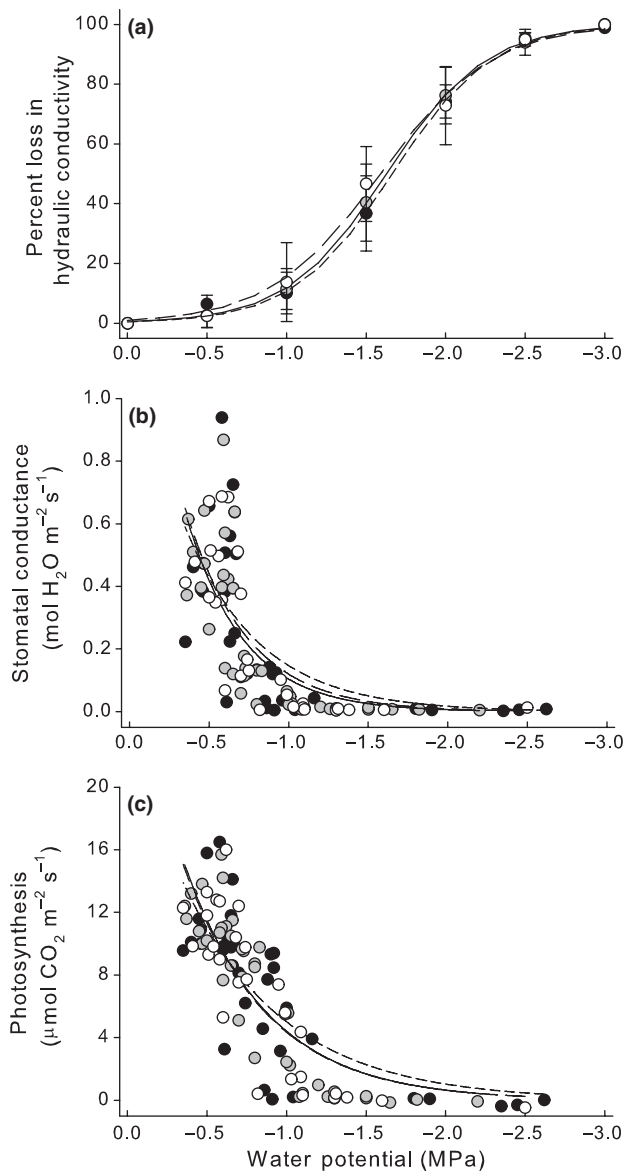
## Discussion

We found partial support for the hypothesis that genome duplication would cause neotetraploids to resemble tetraploids. For example, neotetraploids had stomatal phenotypes similar to tetraploids, and both polyploids had larger

and fewer stomata than diploids (Table 1), a result that was consistent with previous observations (Masterson, 1994; Levin, 2002). Nevertheless, the stomatal characters of neotetraploids and tetraploids differed when comparing allometric relationships among stomatal size, stomatal number and leaf size (Tichá, 1982). We found that the slope of the relationship between stomatal length and leaf size was similar between neotetraploids and their diploid progenitors, but there was no relationship between stomatal length and leaf size in tetraploids (Fig. 1, Table 2). Similarly, the relationship between stomatal number and leaf size was much stronger for diploids and neotetraploids than for tetraploids.

Comparisons of xylem structure and function among cytotypes also provided partial support for the hypothesis that genome duplication would cause neotetraploids to resemble tetraploids. Xylem anatomy was similar between neotetraploids and tetraploids, and polyploids generally had wider hydraulic vessel diameters than diploids (Fig. 2b, Table 3). The increase in hydraulic vessel diameter appeared to be responsible for the increase in hydraulic conductivity ( $K_H$ ) of polyploids relative to diploids. Based on the Hagen–Poiseuille law for liquid flow through circular tubes, in which hydraulic conductivity is proportional to the 4th power of the mean vessel lumen radius (Zimmermann, 1983), tetraploids should have 117% higher  $K_H$  than diploids. This value was similar to the actual difference between diploids and tetraploids (*c.* 87%; Table 3). The lower than predicted difference is expected because xylem vessels are not circular, and possess extra sources of hydraulic resistance such as pits in the axial and end walls when compared with ideal capillaries (Ewers, 1985; Lewis & Boose, 1995).

Despite similarities in hydraulic vessel diameter among polyploids, tetraploids had higher  $K_H$  than neotetraploids. The slightly larger lumens of tetraploids relative to neotetraploids should have resulted in *c.* 5% greater water transport capacity, but tetraploids had 32% higher  $K_H$  than neotetraploids. The difference was not associated with vessel length or vessel number per vascular bundle, which were similar across polyploids (Fig. 2a, Table 3). It is possible



**Fig. 3** Vulnerability to water stress-induced xylem cavitation in diploid (open circles), neotetraploid (tinted circles) and tetraploid (closed circles) cytotypes of *Chamerion angustifolium* shown as the response of per cent loss in hydraulic conductivity to decreasing xylem water potential (a). The response of stomatal conductance to H<sub>2</sub>O vapor (b) and net CO<sub>2</sub> fixation by photosynthesis (c) to decreasing xylem water potential in the three cytotypes.

that tetraploid vessels had higher pit conductivity than neotetraploids (Sperry & Hacke, 2004), but the similarity in air seeding pressures that caused cavitation among cytotypes makes this explanation unlikely. Higher  $K_H$  in tetraploids relative to neotetraploids may be associated with lower end wall resistivity (Sperry *et al.*, 2005) or possibly higher density of vascular bundles in the stem.

Differences in vessel size and  $K_H$  among cytotypes did not influence vulnerability to water stress-induced xylem cavitation (Fig. 3a, Table 2). Comparative studies suggest that

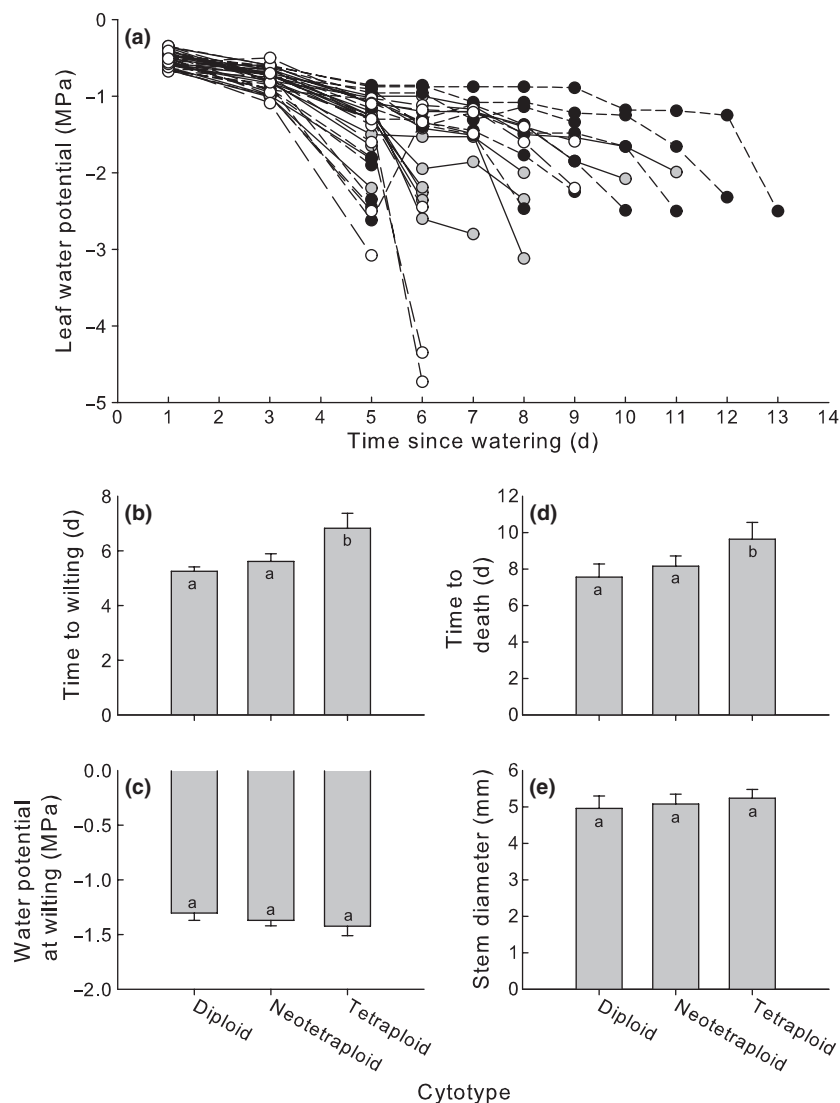
there is a trade-off between maximizing hydraulic efficiency of xylem vessels and minimizing vulnerability to water stress-induced cavitation (Tyree *et al.*, 1994), although the relationship may be driven by the pattern of phylogenetic relatedness among species in the sample (Maherali *et al.*, 2004). Because we experimentally increased conduit size through genome duplication in *C. angustifolium*, we were able to compare the effects of increased conduit size on vulnerability to cavitation among populations with the same genetic background. The lack of an effect of increased conduit size on air-seeding pressures in a comparison of neotetraploids and their diploid progenitors suggests that increased vessel conductivity may not necessarily trade off with vulnerability to water stress-induced xylem cavitation.

Differences in stomatal size, stomatal number, and leaf size among cytotypes had no apparent effect on gas exchange because maximum stomatal conductance and photosynthesis did not differ among cytotypes (Fig. 3b,c). However, the similarity of vulnerability curves among cytotypes was consistent with how gas exchange responded to declining water potential after watering was stopped. Stomata for all three cytotypes were equally sensitive to water stress, with complete stomatal closure and wilting occurring at water potentials approaching -1.5 MPa. These minimum water potentials were similar to other reports for *C. angustifolium* (Carroll *et al.*, 2001). Stomatal closure was also complete at c. 20% cavitation (Fig. 3b), indicating that stomata close well in advance of hydraulic failure in stems. As a result, stomatal behavior in *C. angustifolium* is strongly isohydric compared with other angiosperms (McDowell *et al.*, 2008).

Despite the apparent similarity of xylem vulnerability to cavitation and gas exchange responses to water stress, tetraploids were able to maintain gas exchange and survive for longer periods than both neotetraploids and diploids after watering ceased (Fig. 4a–d). The longer survival in response to water stress by tetraploids may be explained by higher hydraulic conductivity, a hypothesis that can be evaluated through a simple hydraulic model:

$$E = (K_H/A_L) \times (\Psi_{\text{soil}} - \Psi_{\text{leaf}}) \quad \text{Eqn 2}$$

in which transpiration rate ( $E$ ) is proportional to the hydraulic conductivity per unit leaf area ( $K_H/A_L$ ) and the water potential gradient from the soil to leaf ( $\Psi_{\text{soil}} - \Psi_{\text{leaf}}$ ) (Whitehead *et al.*, 1984; Tyree & Ewers, 1991; McDowell *et al.*, 2008). Because all cytotypes had the same maximum  $g_s$  and plants were grown in the same glasshouse environment,  $E$  should not differ among cytotypes. In addition, the allometric relationship between leaf area and stem cross-sectional area does not differ between tetraploids and diploids (A.E. Walden & H. Maherali, unpublished). Assuming that  $E$  and  $A_L$  are constant, 87% higher  $K_H$  for tetraploids than diploids indicates that tetraploids could support the same  $E$  as diploids at a 47% smaller water



**Fig. 4** Changes in leaf water potential during the course of a 13-d dry-down experiment in diploid, neotetraploid and tetraploid cytotypes of *Chamerion angustifolium*. Water potential was measured on each plant until leaves wilted (a; diploid, open circles; neotetraploid, tinted circles; tetraploid, closed circles). Days to wilting (b), days to death (c), water potential at wilting (d) and stem diameter (e) of plants used in the dry-down experiment. Significant differences between cytotypes are indicated by different letters in (b–e).

potential gradient. As a result, tetraploids could deplete soil moisture to a greater degree before reaching leaf water potentials that cause stomatal closure and wilting, which would delay wilting relative to diploids. Such a mechanism of enhanced water uptake and delayed wilting could facilitate the longer times required for flowering in tetraploids relative to diploids (Husband, 2000). The combination of later flowering and delayed response to water stress might explain why tetraploids are able to exploit niches with warmer temperatures, increased water limitation and longer growing seasons at lower altitudes.

Nevertheless, increased  $K_H$  may not be solely responsible for the ability to withstand water stress. For example, neotetraploids, which had 42% higher  $K_H$  than diploids, were only able to survive 8% longer than diploids after watering was stopped. The limited effect of  $K_H$  on time to wilting and death in neotetraploids may be because this trait was measured on stems, which represent only one portion of the

water transport pathway. Other organs, such as petioles and leaves, can contribute far greater resistance to water flux (Tyree & Ewers, 1991; Sack & Holbrook, 2006). If the hydraulics of petioles and leaves were less strongly affected by genome duplication than stems, whole-plant hydraulic conductivity may have been quite similar for diploids and neotetraploids. Limits on the effect of stem hydraulic traits on survival were also observed in the comparison between tetraploids and diploids. For example, the predicted 47% lower water potential gradient in tetraploids suggests that this cytotype should have survived water stress for nearly twice as long as diploids. However, tetraploids only survived 30% longer than diploids.

In addition to the effects of increased xylem conduit size on vulnerability to water stress, cytotypes with higher ploidy and cell size may also be more vulnerable to freezing-induced cavitation (Pockman & Sperry, 1997; Martinez-Vilalta & Pockman, 2002). Upon freezing, dissolved gases in xylem

sap are forced out of solution and form air bubbles which can block water transport after thawing (Zimmermann, 1983; Sperry *et al.*, 1994; Davis *et al.*, 1999). Larger xylem conduits tend to be more vulnerable to freezing-induced cavitation because larger bubbles form in these conduits (Ewers, 1985). The vulnerability of a xylem conduit to freezing increases dramatically when conduit diameter is  $> 30 \mu\text{m}$  (Davis *et al.*, 1999), which suggests that *C. angustifolium* cytotypes should be relatively invulnerable to freezing-induced cavitation because mean conduit diameters were  $\leq 20 \mu\text{m}$  (Table 3). However, maximum conduit diameters were above the  $30 \mu\text{m}$  threshold for all three cytotypes, and 8%, 18% and 21% of vessels in diploids, tetraploids and neotetraploids, respectively, were  $> 30 \mu\text{m}$  (Fig. 2b). Because tetraploids and neotetraploids had more than twice the number of conduits than diploids in the larger size classes, and because large vessels disproportionately contribute more water transport capacity, they may be especially vulnerable to freezing induced cavitation. A greater susceptibility to freezing-induced cavitation could exclude *C. angustifolium* tetraploids and neotetraploids from high elevation sites.

Genome duplication caused stomatal and xylem cell size in neotetraploids to resemble that of tetraploids, but genome duplication itself was not sufficient to allow neotetraploids to survive water stress to the same degree as tetraploids. Therefore, the different physiological tolerances and distribution of tetraploid *C. angustifolium* are unlikely to be caused solely by genome duplication. We suggest that genome duplication, by increasing xylem vessel size and vulnerability to freezing, would have made neotetraploids less competitive at high elevation sites occupied by diploids. However, the ability to survive water stress under warmer temperature and longer growing seasons at lower elevations found in extant tetraploids likely evolved through natural selection on plant water relations after polyploidization. The greater frequency of recombination, genomic redundancy and weaker pleiotropy that is associated with genome duplication (Otto & Whitton, 2000; Sémon & Wolfe, 2007; Martin, 2008) could have also allowed new tetraploid populations to respond more quickly to natural selection than their diploid progenitors. In conclusion, our results support the hypothesis that neopolyploidy can cause plants to differ phenotypically from diploid progenitors, but might not be the primary cause of ecotypic differentiation between extant diploids and tetraploids (Ramsey & Schemske, 2002).

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