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Research

Postzygotic isolation varies by ploidy level within a polyploid complex

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Summary

- Whole genome duplication is considered to be a significant contributor to angiosperm speciation due to accumulation of rapid, strong interploid reproductive isolation. However, recent work suggests that interploid reproductive isolation may not be complete, especially among higher order cytotypes. This study evaluates postzygotic reproductive isolation among three cytotypes within a polyploid complex.
- We conducted reciprocal crosses using two diploid and two hexaploid populations each crossed to tetraploid populations spanning the geographic and phylogenetic range of the *Campanula rotundifolia* polyploid complex. Interploid and intrapopulation crosses were scored for fruit set, seed number, germination proportion and pollen viability. Postzygotic isolation was calculated for each cross as the product of these fitness components. A subset of offspring was cytotyped via flow cytometry.
- Postzygotic isolation was significantly lower in tetraploid–hexaploid crosses than diploid–tetraploid crosses, mostly due to substantially higher germination among tetraploid–hexaploid crosses. Tetraploid–hexaploid crosses produced pentaploids exclusively, whereas diploid–tetraploid crosses produced both triploids and tetraploids in high frequencies.
- Postzygotic isolation was weaker among higher order polyploids than between diploids and tetraploids, and unreduced gametes may facilitate diploid–tetraploid reproduction. This incomplete postzygotic isolation could allow ongoing interploid gene flow, especially among higher order polyploids, which may slow divergence and speciation in polyploid complexes.

Introduction

Polyploidization is considered one of the primary mechanisms of sympatric plant speciation (Bolnick & Fitzpatrick, 2007; Wood *et al.*, 2009) due to its induction of rapid, strong reproductive isolation between diploids and related polyploids (Husband & Sabara, 2003; Rieseberg & Willis, 2007; Ramsey, 2011). However, diploids and polyploids frequently exist in complexes in which interploid gene flow has been observed (Stebbins, 1942; Petit *et al.*, 1999), suggesting that the magnitude of interploid reproductive isolation may be overestimated.

In particular, recent work suggests that reproductive barriers between higher order polyploids may be weaker than those between diploids and polyploids (Hersch-Green, 2012; Sonnleitner *et al.*, 2013; Hülber *et al.*, 2015). If interploid reproductive isolation is incomplete, gene flow between cytotypes could slow diversification and speciation among polyploid lineages (Costa *et al.*, 2014). Polyploids are an important component of angiosperm diversity, with *c.* 35% of genera containing polyploids and polyploid complexes (Otto & Whitton, 2000; Wood *et al.*, 2009). Yet despite this prevalence, broad patterns of polyploid evolution are not well-understood and relative diversification rates of polyploid lineages are still subject to debate (Mayrose *et al.*, 2011; Soltis *et al.*, 2014; Kellogg, 2016). Quantification of postzygotic interploid isolation and comparison of diploid–tetraploid reproductive barriers to those between higher order cytotypes will improve our understanding of interploid gene flow and its influence on polyploid diversification.

The mechanisms by which interploid mating can occur may differ depending on the cytotype of each parent. In diploid–tetraploid matings, fusion of a $1n$ haploid gamete from the diploid parent with a $2n$ diploid gamete from the tetraploid parent results in a triploid embryo. These embryos face triploid block, substantial developmental defects caused by parental genomic imbalance and meiotic irregularities (Marks, 1966; Köhler *et al.*, 2010). However, unreduced gametes offer an alternative mechanism of diploid–tetraploid reproduction (Bretagnolle & Thompson, 1995). If an unreduced $2n$ gamete from the diploid parent fuses with a reduced $2n$ gamete from the tetraploid parent, the resultant tetraploid embryo would not be subject to the decreased fitness inherent to triploid block. This mechanism of interploid compatibility has been studied as a route to neopolyploid formation (Ramsey & Schemske, 1998; Schatlowski & Köhler, 2012) but rarely as a source of ongoing interploid gene flow.

Postzygotic reproductive barriers between higher order polyploids may not be as strong as in diploid–tetraploid systems.

When diploid angiosperms reproduce, both a diploid embryo and a triploid endosperm are formed. The endosperm contains a 2 : 1 ratio of maternal to paternal genomes (Birchler, 2014), and this ratio is the same in most homoploid systems regardless of ploidy. A 2 : 1 genomic ratio is critical for normal endosperm development; substantial deviation leads to aberrant endosperm development and little to no germination (Haig & Westoby, 1991; Scott *et al.*, 1998; von Wangenheim & Peterson, 2004). The magnitude of deviation from a 2 : 1 parental genomic ratio in the endosperm, known as parental genomic imbalance, is associated with the severity of aberrant development. Because the magnitude of genomic imbalance in tetraploid–hexaploid hybrids is approximately one third less than in diploid–tetraploid hybrids (Sonnleitner *et al.*, 2013) these higher-ploidy hybrids may experience fewer endosperm defects and thus may have higher fitness than diploid–tetraploid hybrids. In addition, the magnitude of imbalance is always less when maternal ploidy is greater, so we expect crosses with maternal ploidy excess to germinate more frequently. Finally, high rates of inviable or aneuploid pollen in odd-ploidy hybrids appear to be less frequent in pentaploids than triploids (Costa *et al.*, 2014), again supporting greater success of interploid reproduction in higher order polyploids.

Interploid reproduction is likely to be subject to the same genetic incompatibilities that affect homoploid reproduction. As populations become geographically isolated and accrue genetic divergence, Dobzhansky–Muller incompatibilities can arise and prevent hybridization following secondary contact. This effect has been well-studied in homoploid systems (Moyle *et al.*, 2004; Nosrati *et al.*, 2011). Although empirical data are sparse, genetic incompatibilities may arise more quickly in polyploids, either through reciprocal resolution of redundant gene copies (Lynch & Force, 2000; though see Muir & Hahn, 2015) or faster fixation of adaptive mutations in diverging polyploid lineages (Otto & Whitton, 2000). If polyploids accumulate genetic incompatibilities more rapidly than diploids, Dobzhansky–Muller incompatibilities may occur faster in polyploid lineages. Therefore, we may expect higher order polyploids to demonstrate more postzygotic reproductive isolation as genetic divergence increases than diploids and tetraploids. However, this effect may be difficult to detect given the strong postzygotic isolation expected due to genomic imbalance in interploid mating.

In order to elucidate patterns of interploid postzygotic isolation, we quantify postzygotic barriers among three cytotypes – diploid, tetraploid and hexaploid – of an outcrossing autopolyploid complex. Using an autopolyploid system allows us to reduce the conflating effects of parental genomic incompatibilities or heterosis that can occur in allopolyploids. We use a series of controlled crosses to investigate the following questions: in a complex comprising diploids, tetraploids and hexaploids, do tetraploids exhibit different levels of postzygotic isolation when crossed with diploids than with hexaploids? Do interploid crosses show a difference in postzygotic isolation depending on parental cross-direction? Does increasing genetic divergence between populations increase interploid postzygotic isolation and are any patterns similar across cytotypes? Is there evidence of interploid reproduction via unreduced gametes?

Materials and Methods

Campanula rotundifolia exists as an autopolyploid complex comprising three dominant cytotypes: diploid ($2n = 34$ chromosomes), tetraploid ($2n = 68$ chromosomes) and hexaploid ($2n = 102$ chromosomes) (Kovanda, 1966; Stevens *et al.*, 2012). Interploid reproduction between these three cytotypes, particularly diploid–tetraploid and tetraploid–hexaploid reproduction, could experience different postzygotic isolation with respect to both parental ploidy and cross-direction. Endosperm genomic imbalance is expected to be greater for diploid–tetraploid crosses than tetraploid–hexaploid crosses. When a tetraploid dam is crossed to a diploid sire, the genomic ratio is 4 : 1 due to fertilization of a $2n = 4$x maternal polar nucleus by a $1n = 1$x pollen grain. The reciprocal cross ratio is 1 : 1 ($2n = 2$x polar nucleus and $1n = 2$x pollen grain). These cross ratios, 4 : 1 and 1 : 1, represent 19% and 25% deviation, respectively, from expected parental genomic dosages of 2 : 1. By contrast, the endosperm genomic ratios in 6x–4x and 4x–6x crosses are 3 : 1 and 4 : 3, representing 12% and 15% deviation, respectively, from the homoploid parental genomic dosages. Parental genomic imbalance and the severity of developmental defects are therefore expected to be reduced in tetraploid–hexaploid crosses relative to diploid–tetraploid crosses, and when the maternal ploidy is greater.

Campanula rotundifolia is a short-lived, self-incompatible, perennial wildflower that is thought to have originated in Central or Eastern Europe. It has a circumboreal distribution, and is common throughout much of Europe as far north as Svalbard and as far south as northern Spain, westward to Ireland and eastward to western Russia (Stevens *et al.*, 2012). In North America, it is common in calcareous rocky outcrops and sandy lakeshores across the northern latitudes, and can extend in isolated alpine populations along the Appalachian and Rocky Mountains as far south as North Carolina and Mexico, respectively (Giblin, 2005). Due to a hypothesized recent range expansion as well as cytotypic and morphological complexity, *C. rotundifolia* is taxonomically complex, consisting of numerous named species, subspecies and varieties. For the purposes of this study, a broad definition including *C. rotundifolia* and its allies is used, roughly corresponding to the *C. rotundifolia* polytomy present in Mansion *et al.* (2012).

Cytotypes are nonuniformly distributed throughout the range. Diploids occur in Central and Eastern Europe as well as extreme Northern Europe. Limited diploid populations have been reported in North America (Löve & Löve, 1966), but repeated efforts to resample these populations have proven to be unsuccessful (B. Sutherland, pers. obs.). Hexaploids are limited to the British Isles in Europe (Stevens *et al.*, 2012) and in central and western North America (B. L. Sutherland, unpublished). Tetraploids are the dominant cytotype and are common throughout the distribution, with multiple known contact zones with both diploids and hexaploids. Given the skewed geographic and phylogenetic distribution of cytotypes, it is not possible to compare all three cytotypes within the same geographic region. Therefore, to assess the effects of both ploidy and genetic divergence on postzygotic isolation, we chose 11 tetraploid

populations spanning the geographic and genetic range to cross against two diploid and two hexaploid 'test' populations (Supporting Information Table S1). Therefore, crosses should be viewed as demonstrating potential for reproduction, bearing in mind that prezygotic barriers untested in this study would exist in natural populations. Fruits were solicited from local collectors familiar with *C. rotundifolia* and were harvested in 2006 and 2012 (Table S1). Seeds from 30 source populations were obtained from throughout the distribution – 16 from Europe and 14 from North America. Crossing populations chosen from these samples included two North American hexaploids, two Central European diploids, seven European tetraploids and four North American tetraploids.

Before performing controlled crosses, we established the parental generation for all 15 study populations. Two hundred seeds were planted from each population in 40 cells of five seeds. Each cell contained seeds from the same family, and families were sampled evenly across all available for a given population, typically 10–15 families, but as high as 23. Seeds were surface sown on a moist 3 : 1 mix of Sunshine growth medium and Turface soil conditioner, then covered with a very thin layer of dry soil. Seeds were cold-stratified at 4°C for 14 d to improve germination (Drake & Ewing, 1997), then moved to a growth chamber with a 12-h light : dark cycle at 24°C : 15°C. Germination, defined here as full emergence of both cotyledons and shedding of the seed coat, was scored every 2 d for 6 wk. Once all five potential germinants had emerged in a cell, or after 6 wk had elapsed, germinants were thinned randomly to one per cell. After 6 wk of germination, plants were returned to 4°C for 6 wk for vernalization. Following vernalization, 20 germinants of each tetraploid population and 40 from each test population were transplanted into conetainers and grown in the glasshouse where additional light was used to extend day length to 16 h.

In order to investigate interploid postzygotic isolation, the 11 tetraploid populations were each reciprocally crossed to two diploid (23 and 25) and two hexaploid (10 and 13, Table S1) 'test' populations. A total of 88 cross-types were created (11 4x populations × 2 test ploidies × 2 test populations/ploidy × 2 cross-directions). Within each tetraploid population and test diploid or hexaploid population, maternal and paternal plants were chosen randomly. Six to eight pollinations were performed per cross-type for a total of 560 experimental pollinations. In addition, 150 intrapopulation pollinations were performed, 10 per population. For each pollination, a bud was chosen before anthesis and emasculated by physically removing young anthers. Buds were then monitored daily for opening of the stigmatic lobes. Once stigmatic surfaces were exposed, a surplus of donor pollen was brushed onto the maternal flower.

Four fitness traits were measured to assess postzygotic isolation: fruit set, seed number, germination proportion and pollen viability. Fruit set was defined as the presence of a visibly inflated fruit at maturation, *c.* 3 wk after pollination, and was scored as a binary character. Seed number per fruit was scored by opening ripe fruits before dehiscence and counting all mature seeds, defined here as medium to dark brown and commensurate in size with those of intraploid crosses. Once counted, 25 replicates of five seeds each were sown for all cross-types, evenly distributed across all families. For cross-types with fewer seeds, all available seeds were sown. In many cases, fruits yielded only a couple of seeds, too few for planting. A total of 1374 replicates were planted across all cross-types, averaging 16 replicates per cross-type with a range of 3–25. As with parental populations, F_1 germination was scored every 2 d for 6 wk. Up to 20 seedlings were selected from all germinants within a given cross-type for growth to maturity. Due to poor germination of some cross-types, only 896 seedlings were transplanted, averaging 10 F_1s per cross-type. When sufficient germinants were available, one seedling was selected randomly for transplant per replicate. Survival rates were high throughout the experiment; all cross-types experienced at least 95% survival, and no discernible patterns were found, so survivorship was not included as a fitness trait.

In order to get a measure of F_1 fertility, we quantified the number of viable pollen grains on a subset of F_1 plants. Pollen was sampled more thoroughly in diploid–tetraploid F_1s because they showed considerable variation in cytotype (see the Results section), and we wanted to collect robust samples of each cytotype. Pollen was scored in *c.* 60% of diploid–tetraploid F_1s, but only 30% of tetraploid–hexaploid F_1s. Just before anthesis, anthers were removed from one flower per chosen plant. Anthers were dried for 72 h at room temperature, then stained with 60 µl 1% lactophenol blue solution. Stained samples were stored at least 1 wk before counting, but could be stored in the staining solution indefinitely. To count pollen grains, samples were vortexed thoroughly, then 10 µl of pollen suspension was pipetted onto a microscope slide and covered. Four randomly chosen fields of view were selected from the slide, and all stained and unstained pollen grains were counted from each slide. To calculate the number of viable pollen produced by each individual, the average proportion of stained (viable) pollen was calculated and multiplied by the total number of pollen counted from the four views.

Cytotypes of a subset of F_1 plants were estimated via flow cytometry to confirm expected ploidy (Otto 2-step protocol; Otto, 1990). Because F_1s from diploid–tetraploid crosses showed considerable variation in cytotype, sampling was skewed toward diploid–tetraploid F_1s in order to capture accurate estimates of cytotypic diversity. A total of 432 plants were cytotyped, 36% of the diploid–tetraploid F_1s and 12% of the tetraploid–hexaploid F_1s. Approximately 20 mg each of radish (*Raphanus sativus* 'Saxa': DNA content 1.11 pg/2C) and soybean (*Glycine max* 'Polanka': DNA content 2.50 pg/2C) were used as either internal standards (co-chopped with sample tissue) or external standards (prepared separately and analyzed to calibrate machine parameters for use throughout a single session). Approximately 30 mg of fresh *C. rotundifolia* leaf tissue was collected from basal rosettes and chopped finely into 1 ml of Otto I buffer. The sample then passed through a 30-µm filter and was incubated at room temperature for *c.* 1 h. Otto II buffer, containing 50 µg ml^{-1} of RNAse A and 50 µg ml^{-1} of propidium iodide, was then added and incubated for 10–15 min before visualization. Samples were analyzed using a FACSCalibur flow cytometer. We compared the relative fluorescence of unknown samples to our internal and

external standards to estimate relative DNA quantity. Ploidy levels were assigned to be the nearest whole-number multiple of the ratio of fluorescence between a diploid *C. rotundifolia* and each standard. Because the haploid genome size for *C. rotundifolia* is *c.* 1.1 Gb, there was no overlap in fluorescence peaks between each cytotype (2x–6x).

Because initial cytotype results for diploid–tetraploid F_1s did not match our predictions of uniformly 3x offspring, a second round of pollinations was performed for approximately one-third of populations (underlined taxa in Table S1) to demonstrate repeatability of our results. Eight pollinations were performed for each cross-type. Up to 50 seeds per cross-type were planted in replicates of five seeds each and germinated as above. Once sufficient leaf tissue was available, plants were cytotyped as above for a total of 240 additional individuals.

In order to calculate genetic distances, a maximum-likelihood phylogeny was generated using RADseq data for 28 populations of *C. rotundifolia* that included the 15 populations used in this study. A presence threshold of 12 populations out of 28 was set, resulting in a DNA dataset consisting of 25 762 SNPs and surrounding invariant positions which were concatenated into one continuous sequence per population (B. L. Sutherland, unpublished). Using a GTR+gamma model of nucleotide substitution, a maximum-likelihood tree was generated using RAxML (Stamatakis, 2014). Pairwise differences per nucleotide (π) were then calculated using MEGA v.5.2.2 (Tamura *et al.*, 2011).

Statistical analysis

Postzygotic isolation was defined as the difference in performance between within-population intraploid crosses and interploid crosses for four fitness components: fruit set (scored as a binary trait), seed number, germination proportion and viable pollen number. To standardize measures, mean values for each of the four components were calculated for all intraploid crosses, then interploid values for individual offspring were divided by intraploid means. Seed number was assumed to be influenced mainly by ovule production in the maternal plant, so seed number from each maternal population was used as the intraploid value. For fruit set, germination and pollen viability, an average of both parental populations was calculated and used as the intraploid value. The product of all four components was calculated to produce a relative cumulative fitness for each cross. Postzygotic isolation was calculated as one minus cumulative fitness – lower fitness values therefore lead to higher isolation rates, and vice versa.

We used a generalized mixed model (PROC GLIMMIX, SAS 9.3; SAS Institute Inc., Cary, NC, USA) to evaluate postzygotic isolation in interploid crosses. Analyses were performed on each fitness component as well as on cumulative fitness. Cross-direction (whether the maternal plant was high or low ploidy) was included as a main effect. We used test population as a fixed effect with four levels, for example, two hexaploid and two tetraploid populations, and tested the *a priori* hypothesis of ploidy with an independent contrast that compared the diploid–tetraploid results to the tetraploid–hexaploid results. Because our goal was to sample the variation present in tetraploids, tetraploid population was included as a random effect. For pollen viability, an additional analysis compared F_1s by cytotype combining all cross-types; that is, pollen viability of triploid vs tetraploid offspring for all diploid–tetraploid crosses. Finally, we used ANCOVA to assess whether the genetic distance between crossed populations influenced F_1 performance for each trait. We first calculated an average performance of each cross-type for each trait and regressed it on genetic distance (π) including the fixed effects of test population, cross-direction and their interaction. We also included the interactions between the fixed effects and genetic distance. These interactions indicate whether any effects of genetic distance on fitness depend on test population or cross-direction.

Results

F_1 performance varied among fitness components (Table 1; Figs 1, S1). Fruit set and seed number were both greater when the paternal parent had the higher ploidy (Fig. 1a,b), whereas germination and pollen viability did not depend on the direction of the cross. There was a striking difference in germination between offspring of diploid–tetraploid crosses and tetraploid–hexaploid crosses (Fig. 1c). On average, seeds from crosses with hexaploids had over six times greater germination than those from crosses with diploids. Fruit set and pollen viability was slightly higher among F_1s from tetraploid–hexaploid crosses than diploid–tetraploid crosses (Fig. 1a,d).

Postzygotic isolation was lower when tetraploid plants were crossed with hexaploids than with diploids, regardless of cross-direction (Table 1; Fig. 2). Diploid–tetraploid crosses experienced well over 90% reduction in fertile F_1s relative to intraploid crosses and over 95% reduction in three of four diploid cross-types. By contrast, although tetraploid–hexaploid crosses experienced substantial postzygotic isolation, they were capable of producing more fertile F_1s than diploid crosses, with reductions ranging from only 71.6% to 84.3%. Cross-direction did not affect RI.

F_1s from tetraploid–hexaploid crosses were almost uniformly pentaploid (Fig. 3). However, cytotype frequencies for diploid–tetraploid crosses differed strongly from the expectation of uniform triploids. Only *c.* 47% of all diploid–tetraploid F_1s were triploid; another 43% of these offspring were tetraploid, 8% were diploid and 1.5% pentaploid. Follow-up diploid–tetraploid crosses confirmed these results. Among follow-ups, 52% were triploid, 45% were tetraploid and 2.3% were diploid. No directional effect was observed in tetraploid crosses to diploids or hexaploids. Pollen fertility was assessed for triploid and tetraploid F_1s from diploid–tetraploid crosses, and pentaploid F_1s from tetraploid–hexaploid crosses. Tukey post-hoc comparisons found that pollen fertility differed with respect to cytotype, with tetraploid F_1s significantly more pollen fertile than either triploids or pentaploids, and with pentaploids significantly more fertile than triploids (Fig. 4; $F_{2,231} = 5.03$, $P = 0.0073$).

Genetic distance between populations only influenced fruit set of interploid crosses (Table S2). Greater genetic distance in diploid–tetraploid crosses increased fruit set, explaining 19.2% of

Table 1 Analysis of variance for fitness components and cumulative fitness of *Campanula rotundifolia* interploid crosses

Source	df	Fruit set	Seed set	Germination	Pollen viability	Cumulative fitness
Test population	3	2.71*	2.09	56.87***	1.34	8.05***
Cross-direction	1	26.12***	30.85***	2.79^{+}	0.82	0.32
Test pop′ direction	3	1.2	0.48	0.83	1.37	0.94
Ploidy contrast	1	6.6*	0.02	162.29***	4.00*	21.10***
Error		549	549	473	183	401

Test population refers to the diploid and hexaploid populations crossed to each tetraploid population, and cross-direction refers to the relative ploidy of each parent in a cross, that is, if the maternal plant was a greater ploidy (4x-2x and 6x-4x crosses) or a lesser ploidy (2x-4x and 4x-6x crosses). Ploidy contrast compares the two diploid and two hexaploid populations using an *a priori* test. Tetraploid population was included in the model as a random effect. Table lists *F*-values, *P*-values: +, < 0.1; *, < 0.05; **, < 0.01; ***, < 0.001.

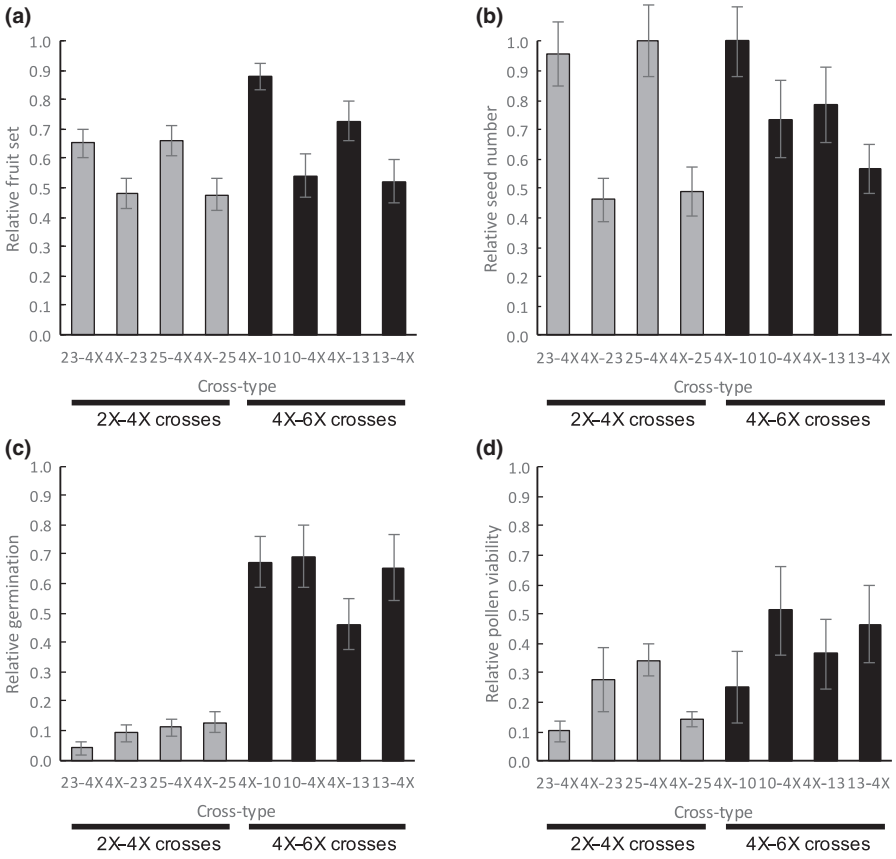

Fig. 1 Relative fitness of interploid crosses in *Campanula rotundifolia*. Two diploid (23, 25) and two hexaploid (10, 13) test populations were each crossed to 11 tetraploid (4x) populations; crosses list maternal plant first. (a) Fruit set, (b) seed number per fruit (crosses that did not set fruit excluded), (c) proportion germination, and (d) viable pollen all reported relative to mean parental values. Bars denote ± SE.

the variance, but had little effect on tetraploid–hexaploid fruit set (Table S2; Fig. S2).

Discussion

We hypothesized that within a polyploid complex, higher order cytotypes would experience weaker interploid postzygotic isolation than diploids and tetraploids. We found that tetraploids experienced 22% weaker postzygotic isolation when crossed with hexaploids than when crossed with diploids, and that this difference was due primarily to large differences in germination. A surprisingly high percentage of offspring from diploid–tetraploid crosses were fertile tetraploids; however, low germination of diploid–tetraploid F_1s limited the effect of these tetraploids on postzygotic isolation. The weaker postzygotic isolation between higher order cytotypes is consistent with ongoing field data (B. L. Sutherland, unpublished) which show that interploid offspring are relatively common between tetraploids and hexaploids, but virtually absent between diploids and tetraploids in both experimental and natural mixed-ploidy populations. The finding that interploid postzygotic isolation is weaker among higher order cytotypes supports the possibility of greater gene flow and reduced divergence within polyploid complexes.

Diploid–tetraploid crosses experienced over 96% postzygotic isolation in both cross-directions, whereas postzygotic isolation between tetraploids and hexaploids ranged from 76% to 83%. Germination had the strongest effect of all fitness components on postzygotic isolation; significantly more seeds germinated from

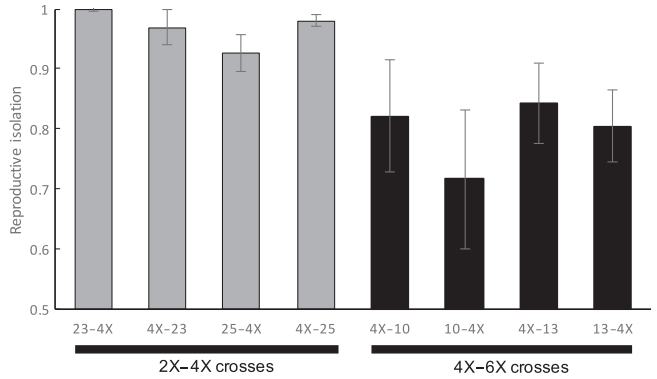

Fig. 2 Index of postzygotic reproductive isolation for interploid crosses of *Campanula rotundifolia*. Two diploid (23, 25) and two hexaploid (10, 13) test populations were each crossed to 11 tetraploid (4x) populations. Reproductive isolation was calculated as the product of relative fruit set, relative seed number, relative germination proportion, and relative pollen fertility. Bars denote ± SE.

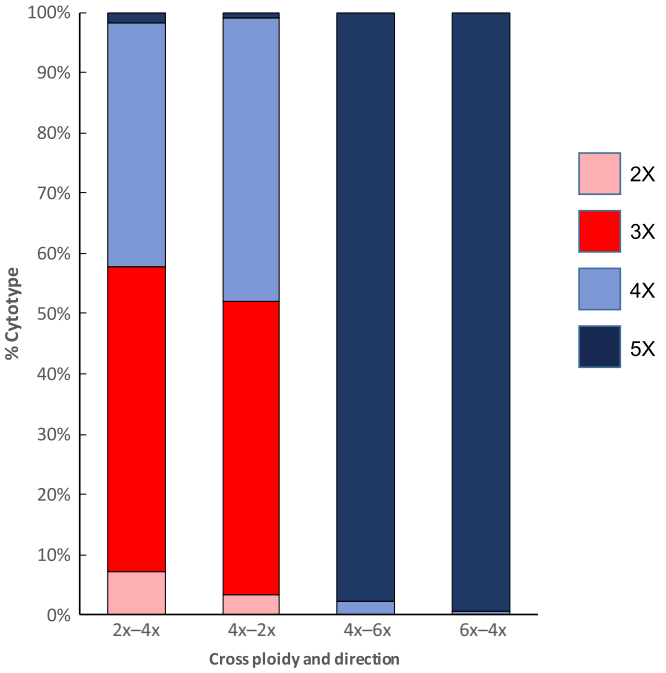

Fig. 3 Combined cytotype composition of F_1 offspring from two rounds of interploid crosses. Diploid and hexaploid *Campanula rotundifolia* test populations were reciprocally crossed to 11 tetraploid populations. Maternal ploidy is listed first.

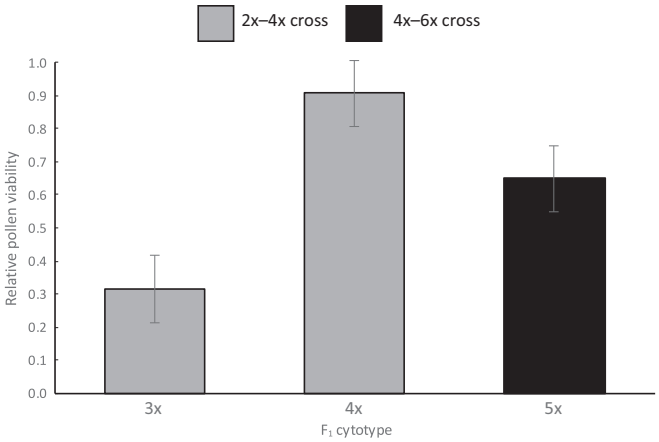

Fig. 4 Pollen viability of offspring of diploid–tetraploid and tetraploid–hexaploid crosses in *Campanula rotundifolia*. Offspring of interploid crosses was separated by cytotype and pollen counts were pooled across crosses. Bars ± SE.

tetraploid–hexaploid crosses than from diploid–tetraploid crosses (61.9% and 9.2%, respectively). Differences in interploid germination were likely caused by parental genomic imbalances in developing seeds and embryos. In interploid *Arabidopsis* crosses, reduced germination was found in diploid–tetraploid F_1s, and attributed to aberrant development of endosperm caused by overexpression of genes from the parent with genomic excess (Slotte *et al.*, 2008; Stoute *et al.*, 2012). Crosses between tetraploids and hexaploids are expected to have reduced parental genomic imbalance (Sonnleitner *et al.*, 2013). This reduced imbalance between higher ploidy levels suggests that endosperm development in F_1s derived from tetraploid–hexaploid crosses have fewer developmental defects than those from diploid–tetraploid crosses, allowing for greater seed viability, which is congruent with our markedly higher germination proportions among tetraploid–hexaploid F_1s.

Although no parental cross-direction effect on cumulative fitness was observed, three fitness components differed with cross-direction. Germination was marginally higher in interploid crosses in which the maternal ploidy was greater. By contrast, fruit set and seed number were greater in crosses when the higher-ploidy parent was paternal. These opposing effects of parental direction among traits likely explain why we observed no directional effect on postzygotic isolation. Parental genomic imbalance may explain the observed directional effects of individual traits. In Brassicaceae, interploid crosses with maternal genome excess often create more viable seed than crosses with paternal genome excess (Dilkes & Comai, 2004; Stoute *et al.*, 2012), which is consistent with our germination results. However, the higher fruit set and seed number observed in this study for crosses with a paternal genomic excess is congruent with observations from other systems. Paternal genomic excess has been linked with higher fruit set and seed number in other systems (Haig & Westoby, 1991; but see Greiner & Oberprieler, 2012). This is thought to result from later seed abortion when the paternal genome is in excess, leading to phenotypically normal but inviable seeds, whereas maternal excess aborts seed development early enough that phenotypically normal seeds are not formed.

Genetic distance played a limited role in postzygotic isolation within the *C. rotundifolia* complex. Although tetraploid populations chosen for this study sampled broadly from the phylogeny and spanned the geographic range of the species, there was a limited effect of genetic distance between populations on fitness of crosses. We expected that postzygotic isolation would increase with greater genetic distance, as has been observed in homoploid systems (Moyle *et al.*, 2004; Nosrati *et al.*, 2011). We also expected that tetraploid–hexaploid crosses would accumulate

genetic incompatibility more quickly with increasing genetic distance than diploid–tetraploid crosses due to resolution of redundant gene copies and faster mutation rates among higher cytotypes (Lynch & Force, 2000; Otto & Whitton, 2000). However, we found no support for these expectations. There are several possible explanations for the lack of any genetic distance effect on postzygotic isolation. First, it is possible that the magnitude of postzygotic isolation imparted by ploidy change masks any underlying effect of genetic distance. Alternatively, decreases in fitness may only become apparent in F_2 recombinant hybrid individuals (Ramsey *et al.*, 2003). Lastly, populations chosen for this study, particularly those from North America, have relatively few fixed nucleotide differences in chloroplast DNA, suggesting that populations have not diverged sufficiently to accumulate Dobzhansky–Muller incompatibilities.

Cytotypic frequencies for interploid hybrids differed substantially from expected values. Interploid crosses are expected to yield intermediate cytotypes: triploids in diploid–tetraploid crosses, and pentaploids in tetraploid–hexaploid crosses. Although tetraploid–hexaploid crosses consistently had pentaploid offspring, only 50% of diploid–tetraploid offspring were triploid and over 44% were tetraploid. Unreduced gametes have been associated with neopolyploid formation in other systems (Ramsey, 2007) and are likely to have mediated neotetraploid formation in this study. Previous work suggests that unreduced ovules are more likely than unreduced pollen to generate neopolyploids (Ramsey, 2007). However, the high frequencies of tetraploids observed in both diploid–tetraploid crossing directions suggest that both types of unreduced gametes can facilitate neotetraploid formation in *C. rotundifolia*. Although diploid–tetraploid crosses yielded high relative rates of tetraploid germinants, the absolute number of germinants for diploid–tetraploid crosses was much lower than for tetraploid–hexaploid crosses. Poor viability of triploid offspring is likely to explain both low germination of diploid–tetraploid crosses as well as the high relative rate of tetraploid formation; because tetraploid seeds are not subject to parental genomic imbalance, a greater proportion of those seeds germinate relative to their triploid siblings. Therefore, although unreduced gamete formation occurs at a low rate, the diploid–tetraploid germinants in this study were effectively enriched for neotetraploids.

Although unreduced gametes can occur in any ploidy level, fusion of an unreduced (2x) gamete from a diploid with a reduced gamete from a tetraploid is the most likely to result in viable offspring. Not only does this combination yield even-ploidy offspring, duplication of the diploid genome effectively ameliorates parental genomic imbalance. Fusion of an unreduced (4x) gamete from a tetraploid parent with a reduced gamete from a diploid parent would result in considerable parental genomic imbalance likely to cause reduced germination and survival. Likewise, for tetraploid–hexaploid crosses, unreduced hexaploid gametes (6x) fused with reduced tetraploid gametes (2x), would likely be inviable due to highly unbalanced parental genomes. However, unreduced tetraploid gametes (4x) with reduced hexaploid gametes (3x) would yield less parental genomic imbalance. Although these embryos are predicted to survive, none were found in this study, perhaps due to the high success of the more frequent 5x offspring.

The differential patterns of interploid postzygotic isolation found in the present study, as well as preliminary data from natural populations, suggest that rates of gene flow vary among cytotypes. If these patterns are widespread, interploid gene flow could constrain divergence within polyploid complexes, particularly among higher order cytotypes. Increased interploid gene flow among higher cytotypes may also help explain why pentaploids are found more commonly in mixed tetraploid–hexaploid populations than triploids are found in mixed diploid–tetraploid populations (e.g. Stevens *et al.*, 2012; Hülber *et al.*, 2015; B. L. Sutherland, unpublished). Our results also caution against assumptions of little to no gene flow in diploid–tetraploid populations based on absence of triploids. Triploid block is a powerful barrier to interploid gene flow via triploid intermediates. The diploid–tetraploid crosses in our study would, in almost all cases, experience essentially complete postzygotic isolation without formation of fertile tetraploids. Although tetraploid offspring were few in absolute terms, the fact that they comprised a substantial proportion of diploid–tetraploid offspring suggests that they may represent an alternative path to unidirectional gene flow from diploids to tetraploids. Focusing solely on the presence of triploids as a marker of interploid reproduction overlooks the potential effect of neotetraploid formation.

Interploid reproductive isolation is often considered to be strong enough to act as a sympatric speciation mechanism between related cytotypes, to the point of being dubbed 'instant speciation' (Coyne & Orr, 2004). Although this may be true in some taxa, our findings indicate that postzygotic barriers between higher order cytotypes may be lower than previously assumed, and suggest that unreduced gametes may mediate reproduction between diploids and tetraploids. By focusing on individual postzygotic barriers we establish that germination greatly affects the role that ploidy level plays on interploid postzygotic isolation. If naturally occurring mixed-ploidy populations show similar differences in postzygotic isolation, the potential for differential rates of gene flow is substantial. So far, our work in natural populations aligns with the results presented herein. Greater gene flow among higher cytotypes, if found, could help explain the persistence of polyploid complexes and provide an alternative hypothesis for the finding of lower than expected diversification in some polyploid lineages.

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Author Contributions

B.L.S. designed and performed the research, collected and analyzed the data, and drafted the manuscript; L.F.G assisted with experimental design, data analysis and interpretation, and drafting the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Postzygotic fitness components of interploid crosses in *Campanula rotundifolia* compared to intrapopulation crosses.

Fig. S2 ANCOVA regressing fruit set against genetic distance with respect to test-population.

Table S1 Accessions of *Campanula rotundifolia* and allied species used for this study

Table S2 ANCOVA on cross-type means for fitness components vs genetic distance

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