

Population differentiation and hybrid success in *Campanula americana*: geography and genome size

L. F. GALLOWAY & J. R. ETTERSON

Department of Biology, University of Virginia, Charlottesville, VA, USA

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Abstract

Populations within a species may diverge through genetic drift and natural selection. Few studies report on population differentiation in autopolyploids where multiple gene copies and the ratio of cytoplasmic to nuclear genes differ from diploids and may influence divergence. In autotetraploid *Campanula americana* we created hybrids between populations that differed in geographic proximity and genome size. Differences in genome size (up to 6.5%) did not influence hybrid performance. In contrast, hybrid performance was strongly influenced by population proximity. F1 hybrids between distant populations performed poorly relative to their parents while hybrids between proximate populations outperformed their parents. Outbreeding depression was strongest for juvenile traits. The expression of outbreeding depression often differed between reciprocal hybrids indicating interactions between nuclear and cytoplasmic genes contribute to population differentiation. Because plants were grown under greenhouse conditions, the outbreeding depression was likely due to genetic (underdominance or loss of additive-by-additive epistasis) rather than ecological factors.

Gene flow in plants occurs largely via the movement of pollen and seeds. These mechanisms result in gene movement that is substantially narrower than the geographical range of most species. As a consequence of limited gene exchange, populations become differentiated due to a combination of natural selection and genetic drift (Wright, 1943). Genetic divergence between physically separated populations may lead to allopatric speciation. However, in most cases population differentiation is not sufficient to result in reproductive isolation. Studies of partial isolation are of interest because they provide insight into the genetic architecture that underlies local scale differentiation (e.g. Armbruster *et al.*, 1997; Edmands, 1999; Fenster & Galloway, 2000a). Recent studies suggest that this mirrors the genetic architecture of species differences (e.g. Coyne & Orr, 1998; Fishman & Willis, 2001). An understanding of population differentiation is also of interest to conserva-

tion and restoration biologists. The preservation and restoration of biodiversity requires knowledge of patterns of genetic differentiation within species (reviewed in Hufford & Mazer, 2003).

Experimental hybridization is a powerful tool for exploring the magnitude and genetic architecture of population differentiation. For example, random genetic drift can lead to the fixation of alternate alleles among populations. Hybridization of these populations will result in hybrid vigor (heterosis) if loci express overdominance or if alleles fixed in either population are mildly deleterious and recessive. This increase in performance is typically greatest in first generation (F1) hybrids (Lynch & Walsh, 1998). Alternately, inter-population hybrids may express outbreeding depression, underperforming relative to their parents. Under natural conditions, outbreeding depression may be due to a loss of local adaptation to either parental environment ('ecological' mechanism, cf. Montalvo & Ellstrand, 2001). Outbreeding depression may also be due to the genetic mechanisms of epistasis and underdominance.

Outbreeding depression caused by epistasis results from the loss of gene interactions that enhance fitness in each population and is a consequence of combining

Correspondence: L. F. Galloway, Department of Biology, University of Virginia, Charlottesville, VA 22904-4328, USA.

Tel.: 434 982 5010; fax: 434 982 5626;

e-mail: lgalloway@virginia.edu

Present address: J. R. Etterson, Department of Biology, University of Minnesota, Duluth, MN 55812, USA

genomes. Recent work suggests that epistatic gene complexes vary not only among species but also among populations of the same species (e.g. Armbruster *et al.*, 1997; Edmands, 1999; Fenster & Galloway, 2000a; Keller *et al.*, 2000; Galloway & Fenster, 2001). Fitness loss in hybrids because of epistatic incompatibilities underlies the Dobzhansky–Muller theory of speciation (reviewed in Turelli & Orr, 2000). While this form of outbreeding depression is expected to be most pronounced in recombinant hybrid generations, favourable additive-by-additive epistasis may be lost in F1 hybrids (Lynch, 1991). Genetic interactions may also occur between the nuclear and cytoplasmic genomes. Functional interactions between nuclear and cytoplasmic genes may lead to coadaptation, e.g. cytochrome *c* (nuclear) and cytochrome *c* oxidase (mitochondrial, Edmands & Burton, 1999). If there is variation of these coadapted genes among populations, intraspecific hybrids will have reduced fitness (Rawson & Burton, 2002). Such interactions between nuclear and cytoplasmic genes have also been implicated in interspecific incompatibilities in a growing number of plant species (Levin, 2000; Tiffin *et al.*, 2001).

Between-population crosses may also perform poorly because of underdominance (Macnair & Christie, 1983; Schierup & Christiansen, 1996). Although underdominance is typically assumed to result from allele interactions at individual loci (e.g. Schierup & Christiansen, 1996), chromosomal rearrangement is an example of a larger scale mechanism that can produce underdominance. In this case, individuals heterozygous for their chromosome arrangement may be partially sterile because of recombination within the altered regions (King, 1993). The magnitude of underdominance attributable to either mechanism is likely to increase with population differentiation.

Polyploidy is a genetic change resulting in chromosome doubling within species (autopolyploidy) or of interspecific hybrids (allopolyploidy). Polyploidy is common; genomic studies have revealed widespread evidence of ancient genome doubling in addition to more recent ‘neopolyploids’ (Soltis & Soltis, 2003). However, there are few explicit studies of population differentiation in polyploids. This is surprising since Werth & Windham (1991) proposed that silencing of the same gene in different populations in polyploids composed of differentiated chromosome sets (i.e. allopolyploid), will result in 25% of F1 hybrids in which neither copy of the gene is functioning (see also Lynch & Force, 2000). In this manner post-zygotic isolation may accrue rapidly between allopolyploid populations. Are expectations the same for autopolyploids where chromosome copies are more similar? If chromosome copies segregate independently resulting in tetrasomic inheritance, gene silencing may not be possible, as it would regularly produce gametes without gene function. However, if the genome has become ‘diploidized’ such that chromosomes pair at

meiosis and inheritance is disomic, differential gene silencing is likely to contribute to population differentiation and post-zygotic isolation. The multiple genomes of polyploids also allow for functional divergence of the gene copies (reviewed in Soltis & Soltis, 2003). Although this may enhance population differentiation, the potential for retention of the original function while acquiring new function may maintain compatibility of differentiated populations. Studies of population differentiation in polyploids, in particular autopolyploids, are needed for comparison with diploids to reveal evolutionary patterns associated with polyploidy.

In this paper we report on the performance of interpopulation hybrids in the herbaceous autotetraploid *Campanula americana*. Flow cytometry confirmed ploidy level and indicated variation in genome size among the focal populations. Therefore we explored both the contribution of geographical location and genome size to hybrid performance. Specifically, we compare offspring of within- and between-population crosses to address the following questions: (1) Does hybrid performance vary with the geographical distance between populations? (2) Do differences in genome size between populations affect hybrid performance? (3) Are there asymmetries in the performance of reciprocal hybrids? (4) Finally, does the expression of hybrid vigor or outbreeding depression vary over the life cycle?

Materials and methods

Study species

Campanula americana L. (= *Campanulastrum americana* Small) Campanulaceae, is an herbaceous plant with a broad distribution in eastern North America ranging from Florida to Ontario, and from the east coast to Kansas. Previous common-environment studies have found variation for morphological and phenological characters among populations that span a north–south transect (Kalisz & Wardle, 1994) and among populations from southwest Virginia (Galloway, 2001; Galloway *et al.*, 2003). *Campanula americana* is a predominately outcrossing, insect-pollinated annual or biennial that typically grows on steep slopes or disturbed areas (Galloway *et al.*, 2003).

According to published chromosome counts and anecdotal information, the ploidy level of *C. americana* is uncertain. Studies report that *C. americana* is either diploid (haploid chromosome number $n = 17$; Rogers, 1965), tetraploid ($n = 29$; Gadella, 1964), or hexaploid ($n = 51$; Sugiura, 1942). A base chromosome number of 17 is common in the genus *Campanula* (Gadella, 1964). A haploid chromosome count of 29 suggests a tetraploid in which five chromosomes have been either lost or fused. While ploidy levels may vary among populations, it is also possible that one or more of these chromosome counts were in error (T. Lammers, personal communi-

cation). Indirect evidence of ploidy level derived from protein electrophoresis indicates populations in south-west Virginia are autotetraploid (Galloway *et al.*, 2003).

Measurement of nuclear DNA content

Genome size was measured for ten populations of *C. americana* including five populations from Virginia, three from North Carolina, and one from Indiana and Missouri (see Table 1 for locations). One of these populations was from the county that yielded the original chromosome count of 17 (Rogers, 1965, Alleghany Co.), and another population was from adjacent Grayson Co. in Virginia.

Nuclear DNA content was assayed using flow cytometry following the protocol of Costich *et al.* (1991) on an average of 10 greenhouse-grown plants per population (range 7–14). Two 50 mg leaf tissue samples were collected from newly emerged leaves on the morning of the assay and kept on ice with 1 mL of extraction buffer. The extracts were filtered and spun in a high-speed centrifuge for 30 s. The supernatant was discarded and the pellet resuspended in 150 μ L of the extraction buffer with added dyes and chicken red blood cells (CRBC, see Costich *et al.*, 1991). This 150 μ L suspension consisted of approximately 1000 whole plant nuclei with 1000 CRBC nuclei serving as an internal DNA standard. The samples were incubated for 15 min at 37 °C and stored on ice until assayed. Plant nuclei suspensions were analysed using a flow cytometer (514 nm). An estimate of the nuclear DNA content for each sample was determined by dividing the mean fluorescence of the *C. americana* nuclei by the mean for the CRBC in the same sample and then multiplying by 2.33, the nuclear DNA content of CRBC (in pg). Samples were run in random order over 4 days. DNA content was analysed using analysis of variance with population as a fixed effect and day included as a blocking factor.

Between-population hybrids

Two sets of three populations each were sampled to create inter-population hybrids (Table 1). All pair-wise crosses were conducted among IN, VC, and NC (trio 1) and among IN, VSW1, and VSW2 (trio 2). Five genotypes

from each population were used in between-population crosses. Reciprocal F1 hybrids were created using the same individuals as pollen donors in one crossing direction and pollen recipients in the other. Ten genotypes were crossed pair-wise within populations to produce parental genotypes in the same environment as the hybrids. This resulted in 18 cross-types, nine for each trio of populations (3 within-population + 3 between-population \times 2 crossing directions).

Performance of the cross-types was evaluated under controlled conditions. Between 50–60 seeds were selected across genotypes from each cross type. The seeds were individually surface-sown onto potting medium (Metro-mix 200; Scotts-Sierra Horticultural Products, Marysville, OH, USA) in 30 mm plug tray cells. Seed location was fully randomized and trays were placed in a 21 °C day/14 °C night growth chamber (12 h days). Seeds were kept moist and germination success and timing were scored for 32 days. The number of rosette leaves and the length of the longest leaf was then measured (measurements were inadvertently delayed for a week in the trio 1). An index of juvenile size was calculated as the product of these characters (leaf number \times leaf size). Plants were then moved into a 5 °C cold room (12 h days) for 6 weeks to stimulate flowering. Following cold stratification, plants were transplanted into pots and moved to a greenhouse where supplemental lights increased day length to 16 h. The number of days to flowering and survivorship from germination to flowering was recorded. At flowering, adult size was estimated as the sum of plant height and total branch length.

Reproductive traits were scored on flowering individuals. Anthers and ovaries were harvested from two early flowers. For plants in trio 1, the number of pollen grains per flower was determined using a particle counter (cf. Etterson & Galloway, 2002). Pollen viability was scored for both trios as the fraction of pollen grains stained out of 300 using lactophenol-aniline blue (Kearns & Inouye, 1993). The number of ovules per ovary was counted following storage in the freezer. Finally, a single pollination was conducted on each plant using pollen from another member of the same cross-type. The resulting seeds were counted and reflect a combination of maternal and paternal reproductive success.

Table 1 Least-square means of nuclear DNA content (SE) for 10 populations of *Campanula americana*. All pair-wise crosses were conducted for two trios of populations: VC, NC, IN (underlined) and VSW1, VSW2, IN (bold).

Population	Location	pg DNA
<u>VC</u>	Wintergreen, Augusta Co, Virginia	4.43 (0.05)
VSW1	Salt Pond Mountain (Rt 700), Giles Co, Virginia	4.29 (0.05)
VSW2	Salt Pond Mountain (Rt 613), Giles Co, Virginia	4.18 (0.05)
VSW3	Eggleston, Giles Co, Virginia	4.21 (0.05)
VS	Grayson Highland State Park, Grayson Co, Virginia	4.35 (0.05)
<u>NC</u>	Blue Ridge Parkway, Alleghany Co, North Carolina	4.16 (0.05)
NCC1	Balsam Mt. Rd., Swain Co, North Carolina	4.12 (0.05)
NCC2	Balsam Mt. Rd., Swain Co, North Carolina	4.19 (0.05)
<u>IN</u>	Bloomington, Monroe Co, Indiana	4.29 (0.06)
MO	Columbia, Boone Co, Missouri	4.36 (0.05)

Life history and phenological data were analysed using analysis of variance. Differentiation among parental populations was determined by comparing the offspring of the within-population crosses in each trio using one-way ANOVA. Analyses of the inter-population hybrids tested cross-type with nine levels as the independent factor. If performance varied among cross-types, linear contrasts were conducted to evaluate (1) if the mean performance of the F1 hybrids was equal to the average of the parental populations (expected if only genes of additive effect differ between the populations, Lynch & Walsh, 1998) and (2) whether performance differed between the reciprocal hybrids. As seeds were produced in a common greenhouse environment, differences between reciprocal hybrids are likely due to cytoplasmic genetic effects rather than maternal environmental effects. Separate analyses were conducted for each trio because the plants were grown at different times. A sequential Bonferroni adjustment was applied to the contrasts conducted on the same trait. To meet the assumptions of ANOVA, rosette size, days to flower and seed number were square-root transformed, branch length natural-log transformed, and pollen viability logit-transformed. The probability of germination and of survivorship was analysed using a log-linear analysis assuming a binomial distribution and a logit link (PROC GENMOD, SAS Institute, 2000) and linear contrasts were conducted analogous to those in the ANOVA.

Results

Nuclear DNA content

Although no differences in ploidy level were detected, nuclear DNA content varied among populations. Nuclear DNA content ranged from 4.12 pg DNA in central North Carolina to 4.43 in central Virginia, a 7.5% difference

(Table 1). Using the tetraploid population (VSW1) as a reference (Galloway *et al.*, 2003), any diploid populations would be expected to have a DNA content of one-half that of VSW1 and any hexaploid populations a 1.5-fold increase. Lack of these large-scale differences among populations indicates that all populations are tetraploid. Nevertheless, there was significant variation in DNA content among populations ($F_{9,102} = 4.41$, $P < 0.001$), as well as across days of analysis ($F_{3,102} = 249.58$, $P < 0.001$).

Between-population hybrids

The expression of juvenile traits and survivorship differed little between parental IN and eastern populations (Table 2). However, outbreeding depression was expressed in juvenile traits in F1 hybrids between most populations (Table 3). Crosses between IN and all eastern populations produced seeds that germinated more slowly, less frequently, and became smaller rosettes than expected based on the average of their parents (Table 3, Figs 1 and 2). Similarly, survivorship from germination to flowering was on average less in hybrid individuals than the parental populations for most crosses between IN and the eastern populations (Table 3, Fig. 3).

Although parental populations differed for many adult and reproductive traits (Table 2), these traits expressed less outbreeding depression than juvenile traits in hybrids between IN and eastern populations (Table 3). For example, the only later-life trait to express outbreeding depression in crosses between IN and VC was pollen viability (mean viability: F1 85%, mid-P 92%; Table 3) and in crosses between IN and VSW2, seed production (Fig. 4). The cross between IN and NC resulted in modest outbreeding depression: hybrids flowered 10% later, produced 11% less pollen that had a 6.5% reduction in viability, and pollinations resulted in fewer seeds

	F/χ^2	IN	VC	NC	F/χ^2	IN	VSW1	VSW2
Juvenile traits								
Prob. of germination	1.83	–	–	–	14.17***	a	a	b
Days to germination	1.34	–	–	–	11.42***	b	a	a
Rosette size	1.23	–	–	–	1.42	–	–	–
Adult traits								
Survivorship	0.69	–	–	–	0.53	–	–	–
Days to flower	16.02***	b	b	a	3.17*	ab	b	a
Branch length	19.49***	a	b	a	4.95**	b	b	a
Reproductive traits								
Ovule number	7.62***	a	b	ab	4.68*	b	ab	a
Pollen number	34.27***	a	b	c	N.A.			
Pollen viability	0.50	–	–	–	4.58*	b	a	ab
Seeds per fruit†	3.72*	–	–	–	11.06***	b	a	a

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

†d.f. 2,69 (IN,VC,NC); d.f. 2,83 (IN,VSW1,VSW2).

Table 2 Comparison of juvenile, adult and reproductive traits between populations of *Campanula americana* grown under greenhouse conditions. F -values (ANOVA) or χ^2 -values (log-linear analysis) are given for trios of three populations used in crosses (d.f. 2,106–151); means different at $\alpha = 0.05$ in Tukey multiple comparison tests are indicated by different letters.

Table 3 Comparison of intraspecific F1 hybrids to midparent values for juvenile, adult and reproductive traits in *Campanula americana*. *F*-values (ANOVA) or χ^2 -values (log-linear analysis) are given for linear contrasts comparing the average of the reciprocal hybrids to the average of the parental populations; values in bold are significant at $P < 0.05$ following a sequential Bonferroni adjustment.

	IN-VC	IN-NC	VC-NC	IN-VSW2	IN-VSW1	VSW1-VSW2
Juvenile traits d.f. 1,331–372						
Prob. of germination	24.84***	15.20***	0.18	20.01***	48.48***	3.49†
Days to germination	70.57***	8.02**	4.13*	37.70***	94.22***	4.20*
Rosette size	66.12***	31.07***	6.13*	7.95**	12.47***	5.69*
Adult traits d.f. 1,324–329						
Survivorship	6.78**	0	0.85	30.69***	26.45***	2.19
Days to flower	3.56†	5.96*	0.13	0.17	2.50	0.36
Branch length	0.66	1.11	0	1.30	1.78	5.72*
Reproductive traits d.f. 1,194–323						
Ovule number	2.75†	0.48	0.02	0.04	0.11	1.34
Pollen number	0.30	4.36*	2.23	–	–	–
Pollen viability	7.07**	4.67*	0.06	1.29	2.00	5.52*
Seeds per fruit	2.01	10.48**	0.88	24.68***	0.73	5.89*

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; † $0.05 < P < 0.1$.

(Table 3, Fig. 4). A small percentage of individuals sired by VSW1 on IN plants survived to flowering (Fig. 3). As a consequence, statistical tests for adult and reproductive traits for this cross are based largely on one crossing direction, and therefore the results in Table 3, indicating that hybrids do not differ from the parents, are not informative about overall hybrid performance.

Less outbreeding depression was expressed in the intermediate distance cross between VC and NC despite differences between the parental populations for adult and reproductive traits (Table 2). The probability of germination and survivorship in the hybrids did not differ from the expected mid-parent average (Table 3,

Figs 1 and 3). In addition, the hybrids had similar timing of flowering, branch length, pollen viability, and pollen, ovule, and seed numbers as the average of their parental populations (Table 3, also Fig. 4). However, hybrids between these intermediate-distance populations germinated 9% more slowly and formed on average smaller rosettes than their parental populations (Table 3, Fig. 2).

Hybrids between the nearby south-west Virginia populations (VSW1 and VSW2) expressed hybrid vigor for many traits. They germinated more rapidly and became larger rosettes than their parents (Table 3, Fig. 2). This hybrid vigor was also found for adult size (15% increase in branch length) and pollen viability (5% greater). However, the seed production of F1 individuals was slightly less than the average of their parental populations (Table 3, Fig. 4). For the remaining traits

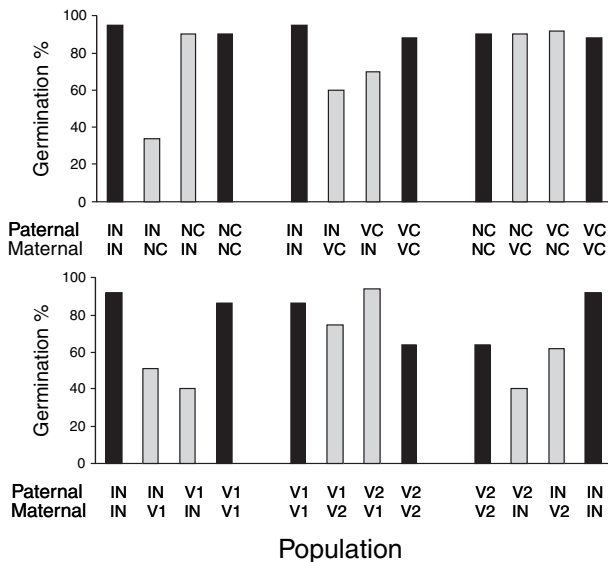


Fig. 1 Percentage germination for hybrids between populations of *Campanula americana*. Reciprocal F1 hybrids (grey bars) are shown between their parental populations (black bars). See Table 1 for population locations (V1 = VSW1, V2 = VSW2).

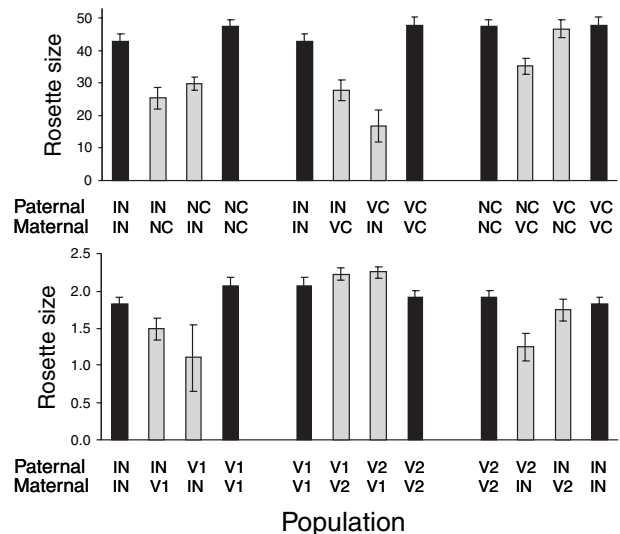


Fig. 2 Size of juvenile rosettes (SE) for hybrids between populations of *Campanula americana*. See Fig. 1 for details.

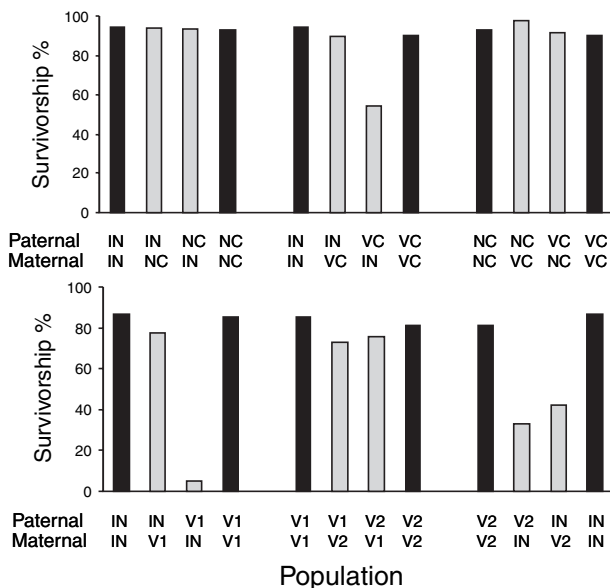


Fig. 3 Survivorship from germination to flowering of hybrids between populations of *Campanula americana*. See Fig. 1 for details.

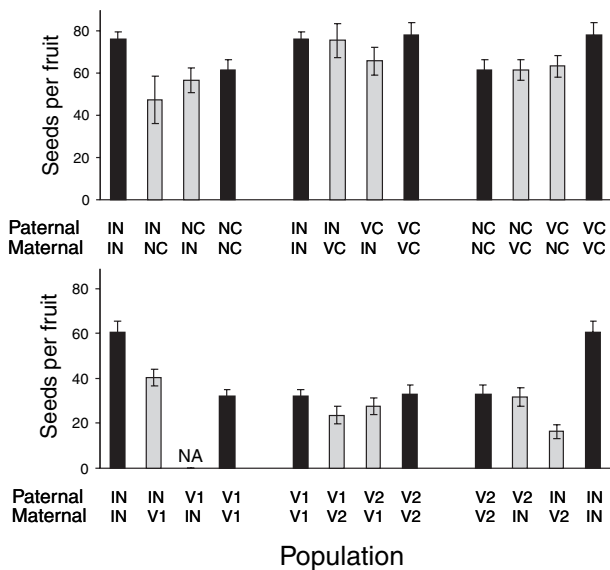


Fig. 4 Number of seeds (SE) produced following within-cross pollinations for hybrids between populations of *Campanula americana*. See Fig. 1 for details.

(probability of germination, survivorship, timing of flowering, and ovule number) the F1 hybrids between VSW1 and VSW2 did not differ from the expected mid-parent value (Table 3, Figs 1 and 3).

Performance of the reciprocal F1 hybrids for juvenile, adult and reproductive traits differed for one third of the crosses (Table 4). For many crosses the F1 hybrids

performed more poorly when IN was the maternal plant than when an eastern population served as the maternal plant. This pattern held for all traits in which the reciprocal hybrids differed in crosses between IN and both VC and VSW1 (Table 4, Figs 2 and 3). It was also found for all juvenile traits and flowering date for hybrids between IN and VSW2 (Table 4, Figs 1 and 2). The reciprocal hybrids had similar performance more often in crosses between populations separated by shorter distances (VC-NC, VSW1-VSW2; Table 4).

Discussion

Performance of intraspecific hybrids in *C. americana* depended on the proximity of populations. First generation hybrids between Indiana and eastern populations (>550 km apart) performed more poorly than their parents for probability of germination, timing of germination, juvenile size and survivorship. In contrast, hybrids between nearby populations in south-western Virginia (1.5 km apart) performed equivalent to or better than their parents for the same traits. Hybrids between intermediate-distance populations expressed outbreeding depression that was more modest in magnitude and only for a subset of traits. Many studies have found that intraspecific F1 hybrids display heterosis and outperform parental populations, e.g. probability of flowering (Luijten *et al.*, 2002), fitness (Fenster & Galloway, 2000a, b), and biomass (Keller *et al.*, 2000). When reduced performance of intraspecific F1 hybrids is found, it is often associated with either loss of adaptation to local environmental conditions (e.g. Schmitt & Gamble, 1990; Waser *et al.*, 2000; Montalvo & Ellstrand, 2001), more highly differentiated inter-varietal crosses (e.g. Levy, 1991; Montalvo & Ellstrand, 2001), or outcrossing highly inbreeding species (e.g. Parker, 1992; Fischer & Matthies, 1997; Affre & Thompson, 1999; Quilichini *et al.*, 2001). There is limited evidence from prior studies for genetically based outbreeding depression in intraspecific F1 hybrids of predominately outcrossing species, as was found in this study (e.g. Montalvo & Ellstrand, 2001; Stacey, 2001).

In contrast to what we observed for geography, there was no relationship between hybrid performance and population differentiation of DNA content. For example, the DNA content of VSW1 and IN were the same and yet there was substantial outbreeding depression in population hybrids. In contrast, VC had 6.5% more DNA than NC and yet for most traits hybrids performed on average as well as their parents. Therefore, although DNA content varied among populations, differences were not associated with the loss or gain of genes that influence cross-compatibility. This result is consistent with other studies that have demonstrated that changes in DNA content between populations and species are typically because of differences in the copy number of mobile genetic elements (e.g. Kalendar *et al.*, 2000; reviewed in

Table 4. Comparison of the performance of reciprocal F1 hybrids for juvenile, adult and reproductive traits in *Campanula americana*. *F*-values (ANOVA) or χ^2 -values (log-linear analysis) are given; values in bold are significant at $P < 0.05$ following a sequential Bonferroni adjustment.

	IN-VC	IN-NC	VC-NC	IN-VSW2	IN-VSW1	VSW1-VSW2
Juvenile traits	d.f. 1,331–372			d.f. 1,283–324		
Prob. of germination	1.10	38.06***	0.15	4.88*	1.17	8.53**
Days to germination	11.69***	1.17	0.09	32.83***	14.98***	0.34
Rosette size	8.03**	1.23	7.39**	4.02*	1.51	0.02
Adult traits	d.f. 1,324–329			d.f. 1,227–232		
Survivorship	10.78***	0.01	1.89	0.39	29.56***	0.09
Days to flower	5.00*	12.06***	0.01	6.94**	–‡	0.02
Branch length	6.84**	0.09	0.02	4.11*	–	0.37
Reproductive traits	d.f. 1,194–323			d.f. 1,217–222		
Ovule number	2.43	0.39	0.72	7.60**	–	0.09
Pollen number	0.08	0.36	2.35	–	–	–
Pollen viability (%)	0.01	3.03†	0.27	1.56	–	0.93
Seed per fruit	0.34	2.12	0.01	9.82**	–	0.69

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; † $0.05 < P < 0.1$.

‡Adult traits for IN-VSW1 not tested due to small sample size of one crossing direction.

Bennetzen, 2002), and therefore not expected to contribute to reproductive isolation.

Expression of outbreeding depression changed over the life cycle. Fourteen of the 15 comparisons of juvenile traits revealed outbreeding depression (excluding the southwest Virginia populations that expressed heterosis). In contrast, outbreeding depression was found in only four of 15 adult-trait comparisons and five of 13 reproductive-trait comparisons. One possible explanation for decreased outbreeding depression over the life cycle is that the initial hybrid seed pool was heterogeneous, containing poorly performing individuals and individuals with average performance. If the poorly performing individuals did not survive to flower, the remaining plants would perform comparably to the parental populations for adult traits. We evaluated this hypothesis by analysing the juvenile performance of individuals that survived to adulthood. The surviving individuals, however, did not differ in juvenile trait expression relative to the average of all plants (results not shown). Therefore, changes in outbreeding depression over the life cycle are not likely to be due to selection operating within a generation. Instead the accumulation of incompatibilities between populations appears to change over ontogeny (see also Stacey, 2001).

Similarly, fewer differences in performance of reciprocal hybrids were evident for reproductive traits than juvenile characters. Reciprocal hybrids differed for nine of 18 juvenile-trait comparisons, seven of 16 adult-trait comparisons, but only two of 15 reproductive-trait comparisons. Reciprocal hybrids have equal nuclear genetic contributions from both parental populations but differ in the origin of their cytoplasmic genomes. Consistent trait expression across maternal populations, regardless of cross-type, would indicate strictly cytoplasmic differentiation. Alternatively, differences between reciprocal hybrids and between hybrids and maternal populations, as found in this study, indicate that cyto-

plasmic genes influence trait expression in combination with nuclear genes and that both genomes contribute to population differentiation. Reduced performance of specific cyto-nuclear combinations suggests coadaptation of the genomes. Cyto-nuclear interactions have also been found to influence performance in intraspecific and interspecific hybrids in a number of other species (Burke *et al.*, 1998; Galloway & Fenster, 1999; Campbell & Waser, 2001; Willett & Burton, 2001).

The presence of variegated and albino seedlings in crosses that demonstrated outbreeding depression further suggests population differentiation of cyto-nuclear interactions. For example, 25.5% of seedlings from the cross between Indiana and VSW1 had chlorophyll deficiencies. In some cases these led to reduced growth but in many individuals expression of other traits was not apparently affected. Similar patterns were seen in seedlings from crosses between Indiana and VSW2 as well as Indiana and central Virginia (13.7 and 16.9% chlorophyll mutants respectively). In contrast, no reduced chlorophyll seedlings were found in within-population crosses. Chlorophyll variegation is commonly found in interspecific hybrids and is due to incompatibilities between cytoplasmic and nuclear genomes (Herrmann *et al.*, 2003).

The expression of outbreeding depression in inter-population hybrids is not associated with population differentiation of phenotypic traits in this study. Although there was genetic variation for almost all adult and reproductive traits among populations, these characters expressed little outbreeding depression. Hybrid trait expression near the mid-parent value is typically thought to indicate population differentiation for genes with additive effects. However, it is also possible that trait expression of hybrids similar to the parental average is because of the combined effects of dominance (enhancing hybrid performance) and loss of positive epistasis (reducing hybrid performance; Lynch, 1991). In contrast,

juvenile traits showed little differentiation among populations yet hybrids expressed outbreeding depression or heterosis. This indicates that populations differ in the genetic or allelic combinations that yield the same phenotypes. Genetic differences between populations for juvenile traits may include dominance, underdominance, and additive-by-additive epistasis (Lynch, 1991). It is likely that dominance played the major role in short-distance hybrids where heterosis was detected. In other hybrids, outbreeding depression was expressed as underperformance relative to either or both parental populations. Both underdominance and additive-by-additive epistasis may contribute to outbreeding depression, but recombinant hybrid generations are required to distinguish the effects of these intra- and inter-locus interactions (see Lynch & Walsh, 1998).

Greater outbreeding depression for juvenile traits than later life characters allows further insight into the genetic architecture of population differentiation. For example, underdominance in F1 hybrids because of differences in chromosomal arrangement between populations is not expected until the onset of reproduction, therefore it is unlikely to underlie the outbreeding depression found here. Alternately, studies of inbreeding depression may illuminate changes in the expression of outbreeding depression throughout the life cycle. Inbreeding depression is strong early in the life cycle of predominantly outcrossing taxa but not selfing taxa, suggesting it is due to the expression of early acting lethal mutations (Husband & Schemske, 1996). In contrast, inbreeding depression for later life traits is thought to be due to the accumulation of weakly deleterious mutations (Husband & Schemske, 1996). If population differentiation for juvenile traits similarly involves genes of large effect (i.e. mismatched developmental genes) whereas differentiation of adult and reproductive traits involves genes with relatively minor effects, then the expected pattern of outbreeding depression would match that found in the current study.

In summary, only crosses between populations of *C. americana* separated by a very short distance produced the hybrid vigor found so often in between-population crosses in plants. Outbreeding depression was found in crosses between populations that span only one-quarter of the range of the species. Because plants were raised in the greenhouse, poor performance was not due to a lack of adaptation by hybrids to either parental environment (i.e. ecological effects). Rather poor hybrid performance appears to be due genetic interactions. A comparison of reciprocal hybrids reveals that interactions between cytoplasmic and nuclear genes influence trait expression. In addition, poor hybrid performance relative to the mid-parent value indicates the either underdominance or additive-by-additive epistasis contribute to genetic differences between populations. While the spatial scale of cross-compatibility is not known for very many plant taxa

(Fenster & Galloway, 2000a; Levin, 2000; Montalvo & Ellstrand, 2001; Edmands, 2002), a short-lived species that occupies disturbed areas such as *C. americana* might be expected to demonstrate relatively broad-scale genetic differentiation. The extent to which genome duplication influences the magnitude of outbreeding depression in *C. americana* is not known and additional studies of population differentiation in polyploids are needed.

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