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INTRASPECIFIC CYTOTYPIC VARIATION AND COMPLICATED GENETIC STRUCTURE IN THE *PHLOX AMABILIS–P. WOODHOUSEI* (POLEMONIACEAE) COMPLEX[1]

SHANNON D. FEHLBERG[2,4] AND CAROLYN J. FERGUSON[3]

[2]Desert Botanical Garden, 1201 North Galvin Parkway, Phoenix, Arizona 85008 USA; and
[3]Herbarium and Division of Biology, Kansas State University, Manhattan, Kansas 66506 USA

- *Premise of the study:* Polyploidy is widely recognized as an important process in the evolution of plants, but less attention has been paid to the study of intraspecific polyploidy, including its prevalence, formation, taxonomic implications, and effect on genetic diversity, structure, and gene flow within and among individuals and populations. Here we studied intraspecific ploidy level variation in the *Phlox amabilis–P. woodhousei* complex to determine the amount and distribution of cytotypic and genetic variation present and measure the extent of gene flow among species, cytotypes, and populations.
- *Methods:* Flow cytometry and microsatellite analyses were used to ascertain cytotypic variation, genetic diversity, and population structure within and among eight populations of *P. amabilis* and 10 populations of *P. woodhousei* from Arizona and New Mexico.
- *Key results:* Our analyses support the recognition of *P. amabilis* and *P. woodhousei* as two distinct species. Both species exhibit cytotypic variation with geographically structured diploid, tetraploid, and hexaploid populations, and genetic analyses suggest a combination of auto- and allopolyploidy in their formation. Diploid, tetraploid, and most hexaploid populations within species share much of their genetic variation, while some hexaploid populations are genetically distinct. All populations maintain moderately high genetic diversity and connectivity, and genetic structure is strongly influenced by geography.
- *Conclusions:* This study highlights the potential for complicated patterns of genetic variation relative to cytotypic variation and provides evidence for the role of cytotypic variation and geographic isolation in shaping diversity, differentiation, and potentially speciation in the *P. amabilis–P. woodhousei* complex.

Key words: allopolyploidy; autopolyploidy; cytotype; flow cytometry; gene flow; microsatellites; *Phlox*; Polemoniaceae; polyploidy; population genetics.

Polyploidy plays an important role in the diversification of many angiosperm groups and has been common throughout angiosperm evolutionary history (reviewed by Otto and Whitton, 2000; Wendel and Doyle, 2005; Soltis et al., 2007). As ploidy level patterns within plant groups are increasingly assessed, particularly through the recent, wide use of flow cytometry (Suda et al., 2007), it has become clear that cytotypic variation within recognized plant species is more widespread than previously thought. In some cases, different cytotypes may be reproductively isolated and may actually represent cryptic species (Soltis et al., 2007), while in other cases there may be substantial gene flow or shared ancestral variation among cytotypes (Ramsey et al., 2008). Focused studies of population genetic structure tied to patterns of ploidy level variation are central to our understanding of the effects of polyploidy on plant diversification and speciation.

Recent estimates of the frequency of intraspecific ploidy level variation indicate that 12–13% of angiosperm species harbor multiple cytotypes (Soltis et al., 2007; Wood et al., 2009). Different cytotypes may occur allopatrically, parapatrically, or in mixed populations. For example, diploid and tetraploid *Ranunculus adoneus* have generally nonoverlapping regional distributions, and when both cytotypes occur on the same site, they are spatially segregated (Baack, 2004). However, other groups show complex patterns of distribution, where cytotypes have different habitat preferences and are generally allopatric, but populations of mixed cytotypes are found in contact zones (Husband and Schemske, 1998; Suda et al., 2004; Halverson et al., 2008; Šingliarová et al., 2011). Morphological studies of species harboring cytotypic variation have led to the discovery of subtle differences among cytotypes, usually in floral and fruit characters (Perny et al., 2005; Španiel et al., 2008; Cires et al., 2009). In some cases, these morphological differences lead to shifts in pollinators, potentially limiting gene flow between cytotypes. Diploid and tetraploid *Heuchera grossulariifolia* attract different suites of floral visitors (Thompson and Merg, 2008), and pollinator fidelity plays an important role in the reproductive isolation of diploid and tetraploid *Chamerion angustifolium* (Husband and Sabara, 2004; Kennedy et al., 2006). Morphological or physiological differences among cytotypes may also lead to the occupation of different ecological niches (Johnson et al., 2003; Suda et al., 2004). Such ecological differentiation among cytotypes may result in endemism or rarity of one or more cytotypes (e.g., Garcia et al., 2008; Cires et al., 2009; Balao et al., 2010). Furthermore, cytotypic variation and

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[4] Author for correspondence (e-mail: sfehlberg@dbg.org)

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ecological differentiation in rare species is of particular importance when planning recovery actions that include reintroduction or population augmentation (Severns and Liston, 2008).

Studies of the genetic characteristics of species with cytotypic variation have revealed complicated evolutionary histories and varying genetic outcomes. While many studies report that higher ploidy levels were likely formed through autopolyploidy (i.e., genome doubling within a species; reviewed in Soltis et al., 2007; Jiang et al., 2009), other studies found evidence that populations of higher ploidy levels were most likely formed through allopolyploidization events with other taxa (Ricca et al., 2008; Balao et al., 2010). However, classification into strict taxonomic autopolyploidy and allopolyploidy may be complicated by processes such as hybridization and lineage sorting in the evolutionary history of the complex being studied (Wendel and Doyle, 2005; Garcia-Jacas et al., 2009). At the population genetic level, different cytotypes may be poorly differentiated, indicating an ongoing exchange of genes among ploidy levels, recent reproductive isolation accompanied by lack of lineage sorting, and/or support for autopolyploid formation (Halverson et al., 2008; Ramsey et al., 2008). Alternatively, different cytotypes may exhibit clear genetic differentiation, indicating a reduction of gene flow and possible reproductive isolation among ploidy levels (see examples in Soltis et al., 2007; Ricca et al., 2008; Balao et al., 2010). Determining the extent of gene flow among ploidy levels can have important implications for species delimitation as a reduction in gene flow may lead to significant differentiation (Petit et al., 1999; Soltis et al., 2007).

The genus *Phlox* L. (Polemoniaceae) presents excellent opportunities to explore polyploidy and its evolutionary implications. Polyploidy has long been noted in *Phlox* ($x = 7$; Flory, 1934; Meyer, 1944; Levin, 1966, 1968; Smith and Levin, 1967; Levy and Levin, 1974), and the genus has received much attention from evolutionary biologists generally (e.g., Wherry, 1955; Grant, 1959; Levin and Smith, 1966; Levin, 1967, 1975, 1978; Levin and Schaal, 1970; Ferguson et al., 1999; Ferguson and Jansen, 2002). *Phlox* comprises ca. 60 species of annual and perennial herbs distributed predominantly in North America with a center of diversity in the western United States. Some taxa are thought to be entirely tetraploid (examples in Smith and Levin, 1967; Levy and Levin, 1975), and other recognized species harbor cytotypic variation (Smith and Levin, 1967; Fehlberg and Ferguson, in press; C. J. Ferguson et al., unpublished data).

The present study focuses on *P. amabilis* Brand and *P. woodhousei* (A. Gray) E. E. Nelson, two narrowly ranging, parapatric species endemic to coniferous forests and shrublands in Arizona (and in the latter case, adjacent New Mexico). These two species are closely related (based on chloroplast and nuclear sequence data; C. J. Ferguson et al., unpublished data) and similar in gross morphology, sharing an upright perennial growth form, thick linear-elliptic leaves, and notched petals. They differ notably in position of reproductive parts: the style of *P. amabilis* is 7–15 mm long, with the stigma placed among the anthers near the opening of the corolla tube; while the style of *P. woodhousei* is 2–5 mm long, with the stigma placed well below all of the anthers, which in turn are all included within the corolla tube (Wilken and Porter, 2005). Though reproductive biology of these species in particular has not been studied, *P. amabilis* and *P. woodhousei* are likely self-incompatible like most *Phlox* species (the annual *P. cuspidata* is one exception; see Levin, 1978, 1993). The most recent monographer of *Phlox*, E. T. Wherry (1955), emphasized style length in classification to the extent that *P. amabilis* and *P. woodhousei* were placed in different sections of the genus with *P. woodhousei* considered a geographically isolated subspecies of another short-styled taxon, *P. speciosa* Pursh, which ranges in the Pacific Northwest from northern California to southern British Columbia, and east to western Montana. However, short styles and associated characters have evolved multiple times in the genus (Ferguson et al., 1999; Ferguson and Jansen, 2002), and workers since Wherry have not suggested a taxonomic affinity between *P. woodhousei* and any other short-styled *Phlox* taxa (e.g., Cronquist, 1984; Wilken and Porter, 2005; Locklear, 2011). Cronquist (1984), emphasizing the overall similarity of *P. amabilis* and *P. woodhousei*, grouped both entities into a broad *P. amabilis* that included variation in the reproductive characters described above, yet later workers have maintained the two taxa as distinct (e.g., Wilken and Porter, 2005; Locklear, 2011). Chromosome numbers of these taxa have only recently been examined, and it was found that both *P. amabilis* and *P. woodhousei* occur in diploid, tetraploid and hexaploid populations (Fehlberg and Ferguson, in press). The *P. amabilis–P. woodhousei* complex is therefore a focused system to study population genetic structure in light of cytotypic variation. Using flow cytometry and analyses of microsatellite markers, we specifically addressed the following questions: (1) What is the pattern of cytotype distribution for each species at local and regional spatial scales? (2) How much genetic diversity is present and how is it distributed? (3) What is the extent of gene flow among species, cytotypes, and populations? (4) Are *P. amabilis* and *P. woodhousei* genetically distinct? (5) What is the most likely mode of polyploid formation (auto- or allopolyploidy)? These findings will provide insight on the relationship between intraspecific cytotypic variation and genetic structure, allow evaluation of species boundaries, and set the stage for detailed ecological and broader genetic investigations.

MATERIALS AND METHODS

Sampling—A total of 172 samples of *P. amabilis* from eight populations in Arizona, and 239 samples of *P. woodhousei* from 10 populations in Arizona and New Mexico were collected for microsatellite analysis (Fig. 1; Table 1). *Phlox amabilis* is a species of conservation concern with S2 and G2 conservation rankings (Arizona Game and Fish Department, 2005; NatureServe, 2011), and populations sampled represent its entire range and ca. 75% of known populations. *Phlox woodhousei*, while narrowly distributed, is more common, and sampled populations were selected to cover its general geographic range. At each sampling location (referred to as populations), several leaves from 23 or 24 spatially separated individuals were collected (except for two populations of *P. amabilis*, which only consisted of 13 and 15 clearly distinct, large individuals) and stored separately in silica gel for DNA extraction. In addition, several leaves from one to nine individuals were collected and stored at 4°C for nuclear extraction for flow cytometry. Voucher specimens for each population were deposited at the Kansas State University Herbarium (KSC; Appendix 1).

Determination of nuclear DNA content and cytotype—DNA content was measured by flow cytometry in one to nine individuals from each population. For each individual sample, ca. 100–300 mg of leaf tissue was placed into a petri dish with 1.5 mL of chilled chopping buffer, modified from Bino et al. (1993) as described by Davison et al. (2007). Leaf tissue was finely chopped with a new razor blade, and the resulting suspension was filtered through 30 μm nylon mesh into a 1.5 mL microcentrifuge tube. Following centrifugation at 500 × *g* for 7 min, the supernatant was discarded, the pellet was resuspended in 700 μL propidium iodide staining solution (50 mg/mL; BioSure, Grass Valley, California, USA), and 2 μL of chicken erythrocyte nuclei singlets were added (CEN internal standard; BioSure). Samples were protected from light and stored on ice for at least 30 min before analysis on a Becton Dickinson (Franklin Lakes, New Jersey, USA) FACS Calibur flow cytometer at the Kansas State University Flow Cytometry Facility. The amount of fluorescence was measured for ca. 10000 nuclei per sample. Resulting histograms were visually inspected for the presence of clear nuclear populations from the sample and CEN internal standard, and mean peak values were calculated using the program Cell Quest (Becton Dickinson). Results for mean peak values were only used when the coefficient of variation was less than 5%. Nuclear DNA content was calculated as the sample mean peak value divided by the CEN internal standard mean peak value multiplied by the 2C-value of the CEN internal standard (2.5 pg; following Dolezel and Bartos, 2005). DNA ploidy level was inferred for each sample based on the calculated DNA content.

Chromosome count data were also obtained from several samples to provide a reference for flow cytometry data (see Suda et al., 2007; Fehlberg and Ferguson, in press). A modified version of B. L. Turner's pollen mother cell squash technique (Jones and Luchsinger, 1986) was used. Developing floral buds were collected at the same time as sampling for population genetic and flow cytometry studies or at the same localities the following year (Table 1; voucher specimens are likewise deposited at KSC; Appendix 1).

Microsatellite analysis—DNA was isolated from dried leaf samples using a small-scale CTAB extraction method modified from Doyle and Doyle (1987) and Loockerman and Jansen (1996). Genetic variation was assessed using five microsatellite loci developed in our laboratory: PHL28, PHL33, PHL68, PHL98, and PHL113 (Fehlberg et al., 2008). Primer sequences for PHL28 are as follows: forward (5′-GTTGCCACCTCACAGATTCC-3′) and reverse (5′-AATTGGGCGGTAAAAATGAA-3′). Primer sequences for PHL33, PHL68, PHL98, and PHL113 are described by Fehlberg et al. (2008). Amplification products from each locus for several individuals were cloned and sequenced to confirm that the intended microsatellite locus was being amplified. General amplification and genotyping procedures followed that described by Fehlberg et al. (2008).

When microsatellite loci are genotyped in polyploid individuals that are not homozygous or fully heterozygous, the alleles are not completely codominant, and it is not possible to know the copy number of each observed allele. For example, a tetraploid individual with an observed phenotype of alleles AB (peaks at A and B) could have a genotype of AABB, AAAB, or ABBB. Although electropherogram peak height can sometimes be used to estimate allele copy number (e.g., Esselink et al., 2004), this is often too difficult in higher level polyploids (Obbard et al., 2006a; Jorgensen et al., 2008; Helsen et al., 2009) and was not feasible for our study. In addition, it is not known where these polyploid populations lie along the spectrum of auto- and allopolyploidy (i.e., disomic and polysomic inheritance; Obbard et al., 2006a). Therefore, the best option for our study was to score microsatellites as presence–absence data, and there is substantial precedent for using this approach (e.g., Jorgensen et al., 2008; Andreakis et al., 2009; Helsen et al., 2009; DeWalt et al., 2011; Kirk et al., 2011; Sampson and Byrne, 2012). For presence–absence scoring, the alleles at each microsatellite locus were treated as multiple independent dominant loci, and each allele (or locus) was scored as present or absent (Rodzen et al., 2004). To supplement and corroborate results obtained from the analysis of the presence-absence data set, we also scored and analyzed all six diploid populations (three for each species) as codominant allele data. Both the presence–absence and codominant allele microsatellite data matrices are available in the Dryad data repository (http://dx.doi.org/10.5061/dryad.3rn6323d).

Data analysis—Genetic diversity statistics were calculated for all populations (presence–absence data set) and diploid populations (codominant data set) using the program GENALEX version 6.2 (Peakall and Smouse, 2006). For the presence–absence data set, statistics included total number of alleles (N_a), mean N_a per locus, total number of private alleles (P_a), mean P_a per locus, unbiased expected heterozygosity (H_e, excluding monomorphic loci), genetic differentiation (Φ_{PT}, Peakall and Smouse, 2006; significance based on 1000 permutations), and genetic distance (D_s, Nei, 1972; and binary genetic distance, Huff et al., 1993). Calculations of genetic differentiation based on genetic distances derived from presence–absence data sets have been shown to be little affected by ploidy level, and therefore are likely to be informative (Obbard et al., 2006b). For the codominant data set, statistics included unbiased expected heterozygosity (H_e), fixation index (F), genetic differentiation (F_{ST}, Weir and Cockerham, 1984; and Φ_{PT}, Peakall and Smouse, 2006; significance based on 1000 permutations), number of migrants (N_m), and deviation from Hardy–Weinberg equilibrium.

Genetic diversity statistics for the presence–absence data set were also calculated using a Bayesian method implemented in the program HICKORY version 1.1 (Holsinger et al., 2002), which avoids many of the assumptions used in the previous analyses. HICKORY was designed specifically for the analysis of dominant data sets, and it does not require prior knowledge of the magnitude of inbreeding, assume Hardy–Weinberg equilibrium, or treat presence–absence data as haplotypes (Holsinger et al., 2002). Genetic diversity (H_s) and genetic differentiation (Θ_I, comparable to F_{ST} of Wright (1951); and Θ_{II}, comparable to F_{ST} of Weir and Cockerham (1984)) were calculated using default settings under four different models: (1) full model, which includes priors for inbreeding (f), differentiation (Θ), and the mean of the allele frequency distribution across populations (Π_i); (2) $f = 0$ model, which assumes no inbreeding; (3) $\Theta = 0$ model, which assumes no population differentiation; and (4) f free model, which chooses values of f at random from its prior distribution to incorporate uncertainty in the magnitude of inbreeding. Following the application of each model to the data, the deviance information criterion (DIC) was used to evaluate the fit between the data and a particular model and to choose among models (Holsinger et al., 2002; Spiegelhalter et al., 2002).

In addition to diversity statistics, genetic structure was examined in the presence–absence data set in four ways. First, the apportionment of genetic variation within and among populations and species was calculated using an analysis of molecular variance (AMOVA) based on binary genetic distances. Second, the relationship between binary genetic distances and the natural log of geographic distances between populations was evaluated using a Mantel test. Third, major patterns in the genetic data were detected and visualized using principle coordinate analysis (PCoA) of pairwise genetic distances between individuals and populations. This approach has been suggested as an appropriate way to compare genetic diversity across populations of different ploidy levels (Kloda et al., 2008). All of these analyses were performed with GENALEX. Finally, a Bayesian clustering analysis was performed in the program STRUCTURE version 2.3 (Pritchard et al., 2000). The likelihood of K, where K is the number of distinct genetic clusters, was calculated for $K = 2$ to 18 using a no admixture model, correlated allele frequencies, and no prior population information. Each value of K was evaluated with six independent runs of 500000 iterations preceded by a burn-in of 50000 iterations. To determine the most likely value of K, we examined log probabilities [$L(K)$; Pritchard et al., 2000] and the change in log probabilities [$\Delta L(K)$; Evanno et al., 2005]. Clusters were aligned and averaged using the program CLUMPP version 1.1 with the Greedy algorithm and 1000 permutations of randomized input order (Jakobsson and Rosenberg, 2007). Resulting assignments were visualized using the program DISTRUCT version 1.1 (Rosenberg, 2004).

RESULTS

Cytotypic variation and distribution—Measurements of nuclear DNA content by flow cytometry revealed that *P. amabilis* and *P. woodhousei* were primarily made up of diploid, tetraploid, and hexaploid populations (Table 1). Average DNA content was calculated when multiple individuals from a single location were measured. This DNA content ranged from 8.36–9.01 pg for diploids, 16.62–17.63 pg for tetraploids, and 24.08–27.03 pg for hexaploids. The coefficients of variation observed for each sample peak ranged from 1.50 to 4.66%. Variation of DNA content within populations was not detected with the exception of the *P. woodhousei* Sharp Creek population where both tetraploid and putative pentaploid (21.31 pg) individuals were found; however, cytotypic variation within populations may have been underestimated due to limited sampling in most populations. Results from chromosome counts confirmed ploidy levels of several samples (see Table 1 and footnotes).

The distribution of cytotypes is shown in Fig. 1. Diploid populations of *Phlox amabilis* occurred toward the western portion of the range, both in the Santa Maria Mountains northwest of Prescott and the Grand Canyon-Parashant plateau north of the Grand Canyon. Tetraploid populations occurred somewhat farther east near the city of Prescott and in the Mt. Trumbull area directly north of the Grand Canyon. Hexaploid populations occurred at the eastern edge of the range near the city of Williams and west of the San Francisco Peaks and are possibly associated with magnesium rich igneous rock formations (S. D. Fehlberg,

TABLE 1. *Phlox amabilis* and *P. woodhousei* sampling localities, number of individuals sampled per site, mean nuclear DNA content as measured by flow cytometry, and inferred DNA ploidy level. Sites where the inferred DNA ploidy level is supported by meitoic chromosome counts are indicated with superscripts.

Species	Pop ID		Population name	County	State	West	North	N_{micro}	N_{fc}	DNA (pg)	SD	Ploidy
Phlox amabilis	1	BR	Black Rock	Mojave Co.	AZ	−113.75	36.80	24	1	8.70	—	2*x*
	2	CW	Camp Wood	Yavapai Co.	AZ	−112.96	34.79	24	2	8.36	0.23	2*x*
	3	DV	Death Valley Springs	Mojave Co.	AZ	−113.25	36.36	24	5	16.98	1.86	4*x*
	4	HM	Hobble Mountain	Coconino Co.	AZ	−112.02	35.50	24	1	24.61	—	6*x*
	5	KL	Kaibab Lake	Coconino Co.	AZ	−112.16	35.27	15	3	26.38	0.27	6*x*
	6	MM	Mingus Mountain	Yavapai Co.	AZ	−112.15	34.67	24	1	24.08	—	6*x*
	7	TB	Thumb Butte	Yavapai Co.	AZ	−112.55	34.54	24	4	8.48	0.20	2*x* [a]
	8	WL	Watson Lake	Yavapai Co.	AZ	−112.42	34.58	13	3	17.62	0.27	4*x*
P. woodhousei	9	BW	Bill Williams	Coconino Co.	AZ	−112.17	35.19	24	1	9.01	—	2*x*
	10	MC	McFadden Peak	Gila Co.	AZ	−110.95	33.91	24	5	17.12	0.8	4*x*
	11	OC	Oak Creek	Coconino Co.	AZ	−111.74	35.05	24	1	8.88	—	2*x*
	12	R1	Reserve 1	Catron Co.	NM	−108.79	33.62	24	1	16.62	—	4*x*
	13	R2	Reserve 2	Catron Co.	NM	−108.72	33.56	24	—	—	—	—
	14	SA	Sierra Ancha	Gila Co.	AZ	−110.80	34.18	23	1	27.03	—	6*x*
	15	SC	Sharp Creek	Gila Co.	AZ	−111.00	34.31	24	4	17.03	0.05	4*x*
									5	21.31	0.44	5*x*
	16	SH	Show Low	Navajo Co.	AZ	−110.08	34.20	24	4	17.62	0.68	4*x*
	17	STO	Stoneman Lake	Coconino Co.	AZ	−111.52	34.78	24	3	8.90	0.44	2*x* [a]
	18	STR	Strawberry	Coconino Co.	AZ	−111.50	34.44	24	4	17.63	0.55	4*x* [b]

Notes: N_{micro} = number of individuals sampled for microsatellite analysis; N_{fc} = number of individuals sampled for flow cytometry

[a] $N = 7$; specimen vouchers, Kansas State University Herbarium (KSC): TB, CF 775; STO, SDF 51307-1.

[b] $N = 14$; specimen voucher (KSC): STR, SDF 51207-3.

personal observations). Most sampled populations of *P. woodhousei* were tetraploid and occurred along the Mogollon Rim to the easternmost portion of the range in New Mexico. Populations of *P. woodhousei* in closest geographical proximity to *P. amabilis* (i.e., in the northwest portion of the range near Williams and Oak Creek) were diploid. A single detected *P. woodhousei* hexaploid population occurred in the Sierra Ancha Wilderness area north of Globe.

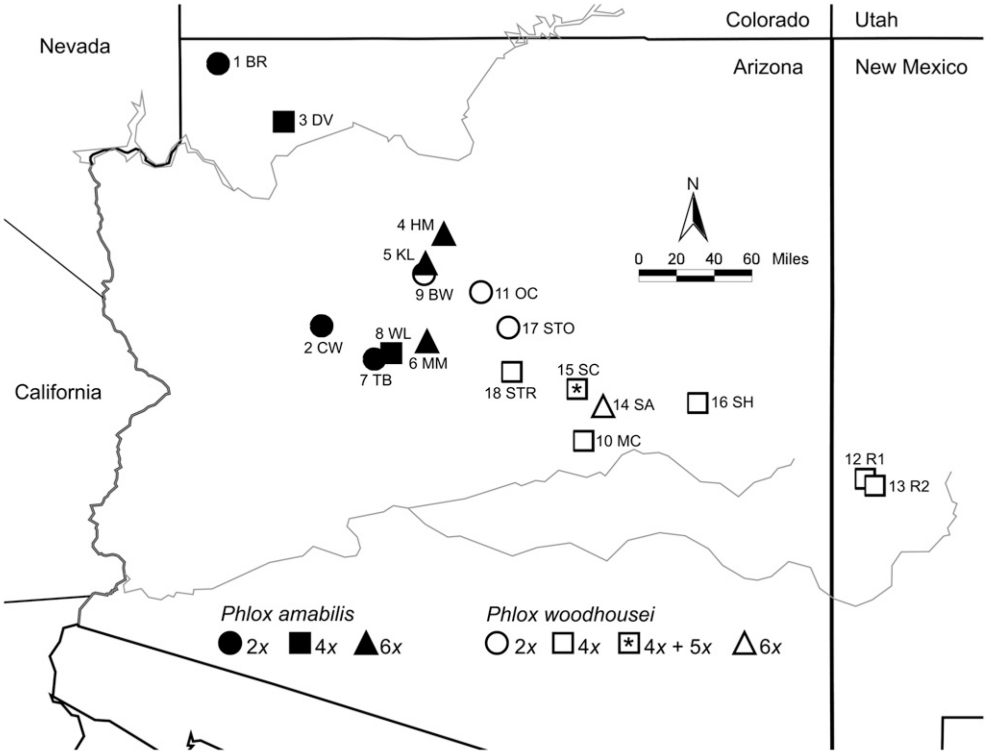

Fig. 1. Sampling localities for *Phlox amabilis* and *P. woodhousei*. *Phlox amabilis* sites are shown in solid shapes, and *P. woodhousei* sites are shown in open shapes. Sites with diploids are indicated by circles, tetraploids by squares, mixed tetraploids and pentaploids by a starred square, and hexaploids by triangles. Numbers and abbreviations for localities are defined in Table 1.

Genetic diversity— A total of 212 alleles was found across all microsatellite loci and populations, with 170 found in *P. amabilis* and 141 in *P. woodhousei*. The mean number of alleles per locus across all loci and populations was 10.1 in *P. amabilis* with a range of 5.4–15.0, and 10.2 in *P. woodhousei* with a range of 7.0–13.2 (Table 2). For *P. amabilis*, the greatest numbers of alleles were found in three hexaploid populations, Mingus Mountain, Kaibab Lake, and Hobble Mountain (Table 2). For *P. woodhousei*, there was no clear pattern for the greatest numbers of alleles. A total of 66 private alleles were found across all loci and populations, 44 in *P. amabilis* and 22 in *P. woodhousei*. The mean number of private alleles per locus was 1.1 in *P. amabilis* with a range of 0.0–2.2, and 0.44 in *P. woodhousei* with a range of 0.0–0.8 (Table 2). The greatest numbers of private alleles across all samples were found in two geographically separated *P. amabilis* populations, Black Rock and Death Valley Spring, and in one hexaploid *P. amabilis* population, Hobble Mountain (Fig. 1; Table 2). Most *P. woodhousei* populations did not have more than two private alleles (Table 2).

For the presence–absence data set, mean expected heterozygosity calculated across all polymorphic loci and populations was $H_e = 0.19$ in *P. amabilis* with a range of 0.15–0.30, and 0.20 in *P. woodhousei* with a range of 0.14–0.26 (Table 2). The Bayesian estimates of genetic diversity calculated without assumptions for inbreeding and Hardy–Weinberg equilibrium were similar for all populations ($H_s = 0.24$) and for each species (*P. amabilis* $H_s = 0.24$ with a range of 0.19–0.33; and *P. woodhousei* $H_s = 0.24$ with a range of 0.19–0.31; Table 2). Populations were moderately differentiated with $\Phi_{PT} = 0.305$ among all populations combined, 0.266 among *P. amabilis* populations, and 0.248 among *P. woodhousei* populations (Table 3). Bayesian estimates of genetic differentiation (under the full model; discussed below) were similar with $\Theta_{II} = 0.233$ among all populations combined, 0.257 among *P. amabilis* populations, and 0.234 among *P. woodhousei* populations (Table 3). Pairwise population comparisons for Nei's genetic distance and Φ_{PT} indicated that populations of different species were not necessarily more genetically distinct or differentiated than populations of the same species (Appendix S1, see Supplemental Data with the online version of this article).

A comparison of the four models used to calculate genetic diversity and differentiation in HICKORY based on the deviance information criterion (DIC) indicated that the full model, which incorporates inbreeding and population differentiation, was the best fit for the data (DIC = 5231.91; Appendix S2, see online Supplemental Data). The slightly lower value for the $f = 0$ model (DIC = 5246.72) indicates that inbreeding has little effect on population structure, but the much lower value for the $\Theta = 0$ model (DIC = 14683.60) indicates that there is evidence for significant differentiation among populations.

For the codominant data set, mean expected heterozygosity calculated across all loci and diploid populations was $H_e = 0.744$, and calculated across diploid populations of each species separately was 0.682 for *P. amabilis* and 0.805 for *P. woodhousei*. Diploid populations of *P. amabilis* were moderately differentiated with $F_{ST} = 0.224$ and $N_m = 0.866$, and $\Phi_{PT} = 0.238$ and $N_m = 0.764$ (Table 3). Diploid populations of *P. woodhousei* were poorly differentiated with $F_{ST} = 0.076$ and $N_m = 3.051$, and $\Phi_{PT} = 0.086$ and $N_m = 2.644$, probably due to their close geographic proximity (Fig. 1; Table 3). Mean fixation indices were high across both *P. amabilis* populations ($F = 0.682$) and *P. woodhousei* populations ($F = 0.805$), and most loci in diploid populations deviated significantly from Hardy–Weinberg equilibrium (60% of all loci in some populations after Bonferroni correction, data not shown), possibly indicating inbreeding or undetected null alleles. Inbreeding is a more likely explanation given life history characteristics and the lack of a consistent signal of null alleles at specific loci across all populations. In general, patterns of diversity and differentiation were similar between presence–absence and codominant data sets.

TABLE 2. Descriptive statistics for each population and across all populations of *Phlox amabilis* and *P. woodhousei.* Genetic diversity measures were calculated from a binary data set where microsatellite alleles were coded as present or absent.

Population	Ploidy level	Total N_a	Range N_a/locus	Mean N_a/locus	Total P_a	Range P_a/locus	Mean P_a/locus	H_e	H_s
Phlox amabilis									
1BR	2*x*	47	4–14	9.4	11	0–7	2.2	0.15	0.19
2CW	2*x*	30	3–9	6.0	2	0–1	0.4	0.18	0.22
3DV	4*x*	58	6–24	12.0	9	0–5	1.8	0.17	0.22
4HM	6*x*	62	8–22	12.4	10	0–4	2	0.21	0.25
5KL	6*x*	68	7–20	13.6	6	0–3	1.2	0.18	0.23
6MM	6*x*	75	8–33	15.0	4	0–4	0.8	0.17	0.22
7TB	2*x*	36	3–12	7.2	2	0–1	0.4	0.18	0.23
8WL	4*x*	27	3–9	5.4	—	—	—	0.30	0.33
All *P. amabilis*		170	3–33	10.1	44	0–7	1.10	0.19	0.24
P. woodhousei									
9BW	2*x*	44	7–13	8.8	2	0–1	0.4	0.17	0.21
10MC	4*x*	65	8–20	13.0	4	0–2	0.8	0.18	0.23
11OC	2*x*	50	7–13	10.0	2	0–1	0.4	0.14	0.19
12R1	4*x*	57	8–15	11.4	2	0–1	0.4	0.19	0.24
13R2	—	35	5–9	7.0	1	0–1	0.2	0.25	0.29
14SA	6*x*	43	7–14	8.6	—	—	—	0.26	0.29
15SC	4*x*	39	5–10	7.8	—	—	—	0.26	0.31
16SH	4*x*	66	11–17	13.2	5	0–2	1	0.18	0.23
17STO	2*x*	50	7–14	10.0	2	0–1	0.4	0.16	0.20
18STR	4*x*	60	9–18	12.0	4	0–2	0.8	0.18	0.23
All *P. woodhousei*		141	5–20	10.2	22	0–2	0.55	0.20	0.24

Notes: N_a = number of alleles; P_a = number of private alleles; H_e = unbiased expected heterozygosity as calculated in GENALEX; H_s = genetic diversity as calculated in HICKORY.

Table 3. Genetic differentiation among populations of *Phlox amabilis* and *P. woodhousei* calculated for all populations using the presence–absence data set and for diploid populations using the codominant data set. Calculations for Φ_{PT}, F_{ST}, and N_m were performed with GENALEX, and calculations for Θ_I and Θ_{II} were performed with HICKORY using the full model.

Genetic differentiation among:	Φ_{PT}	N_m	Θ_I	Θ_{II}	F_{ST}	N_m
All populations combined	0.305*	—	0.203	0.193	—	—
All *Phlox amabilis* populations	0.266*	—	0.233	0.229	—	—
All *P. woodhousei* populations	0.248*	—	0.227	0.205	—	—
All diploid populations combined	0.238*	0.798	—	—	0.219*	0.890
All diploid *P. amabilis* populations	0.247*	0.764	—	—	0.224*	0.866
All diploid *P. woodhousei* populations	0.086*	2.644	—	—	0.076*	3.051

Note: N_m = number of migrants. *Values significant at $P = 0.001$.

Genetic structure—Results from the AMOVA indicated that much of the observed variation was due to differences within populations (69%) rather than difference among populations (24%), and 7% of the observed variation was due to differences between species (Table 4). There was a significant correlation between genetic and geographic distances when all populations were tested ($r = 0.283$, $P = 0.001$), and when populations of each species were tested separately ($r = 0.267$, $P = 0.001$ for *P. amabilis* and $r = 0.332$, $P = 0.001$ for *P. woodhousei*).

Several overall patterns were apparent from PCoA analysis of individuals and populations (Fig. 2). First, *P. amabilis* and *P. woodhousei* were genetically distinct with low levels of shared variation detected among certain populations (Fig. 2A). In addition, diploid, tetraploid, and hexaploid populations did not appear to be genetically distinct from one another, with the exception of one hexaploid *P. amabilis* population, Hobble Mountain, and one hexaploid *P. woodhousei* population, Sierra Ancha (Fig. 2). Results from individual and population-level analyses were similar.

Results from Bayesian clustering analysis (Fig. 3) were similar to that of PCoA. Examination of the log probabilities $L(K)$ and the change in log probabilities $\Delta L(K)$ revealed $K = 2$ as the uppermost division of structure. In all $K = 2$ models from six independent runs, the first cluster was comprised of *P. amabilis* individuals, and the second cluster of *P. woodhousei* individuals (results not shown). The next division of structure indicated by $L(K)$ and $\Delta L(K)$ was $K = 6$. Individual assignments into the six clusters from two of the most likely models from six independent runs of $K = 6$, and individual assignments into the six clusters averaged across all independent runs of $K = 6$ are shown in Fig. 3. In all $K = 6$ models, *P. amabilis* and *P. woodhousei* were genetically distinct with low levels of shared variation (with one exception discussed below). Within *P. amabilis*, one hexaploid population, Hobble Mountain, was genetically distinct and comprised a separate cluster, while all other populations were placed into two clusters primarily correlated with geography. One cluster was comprised of hexaploid and tetraploid populations located in the southeastern portion of the range (Kaibab Lake, Mingus Mountain, and Watson Lake), while the other cluster was comprised of diploid and tetraploid populations located in the northwestern portion of the range (Campwoods, Black Rock, and Death Valley Spring). Diploid population Thumb Butte is more centrally located and was variously assigned to each of the two clusters described above or to its own cluster in independent runs of $K = 6$. Within *P. woodhousei*, tetraploid populations from New Mexico (Reserve 1 and 2) clustered with the hexaploid population, Sierra Ancha, although population Reserve 2 was variously assigned to its own cluster in some independent runs of $K = 6$. The clustering of these populations together likely results from their shared dissimilarity with the remaining populations rather than their similarity to one another. The remaining tetraploid populations and all diploid populations were variously assigned to a single cluster or to separate clusters correlating with ploidy level and geography. The first of the separate clusters was comprised of tetraploid populations located in the central portion of the range (McFadden Peak, Sharp Creek, and Strawberry). The second of the separate clusters was comprised of diploid populations located at the western edge of the range (Stoneman, Oak Creek, and Bill Williams). High levels of shared genetic variation were indicated between one *P. woodhousei* population (Show Low) and populations of *P. amabilis*.

Table 4. Hierarchical analysis of molecular variation (AMOVA) in *Phlox amabilis* and *P. woodhousei* illustrating the proportion of variation attributable to differences between species, among populations within species, and within populations.

Source	df	Sum of squares	Variance component	Percentage of variation
Between species	1	197.90	0.68	7
Among populations within species	16	990.60	2.41	24
Within populations	393	2758.83	7.02	69

DISCUSSION

Cytotypic variation and distribution—*Phlox amabilis* and *P. woodhousei* each include diploid, tetraploid, and hexaploid populations within their narrow geographic ranges (see also Fehlberg and Ferguson, in press). While cytotypic variation has previously been documented in some widespread *Phlox* taxa (e.g., *P. pilosa*; Smith and Levin, 1967), it is particularly intriguing to find it in narrowly distributed species. Based on our sampling, different cytotypes occur both allopatrically and parapatrically, with the exception of one mixed *P. woodhousei* population composed of tetraploids and putative pentaploids (discussed below). Overall, cytotypic variation in *P. amabilis* and *P. woodhousei* exhibits broad geographic patterns (Fig. 1), suggesting that cytotypes are differentiated ecologically to some extent and that ecological factors could play a role in reproductive isolation of cytotypes within species.

Gene flow between species and taxonomic implications—*Phlox amabilis* and *P. woodhousei* are readily distinguished by all analyses of genetic variation. Each species is separated in PCoA analysis, clustering analysis clearly assigns them to two different groups, and 7% of genetic variation is attributable to differences

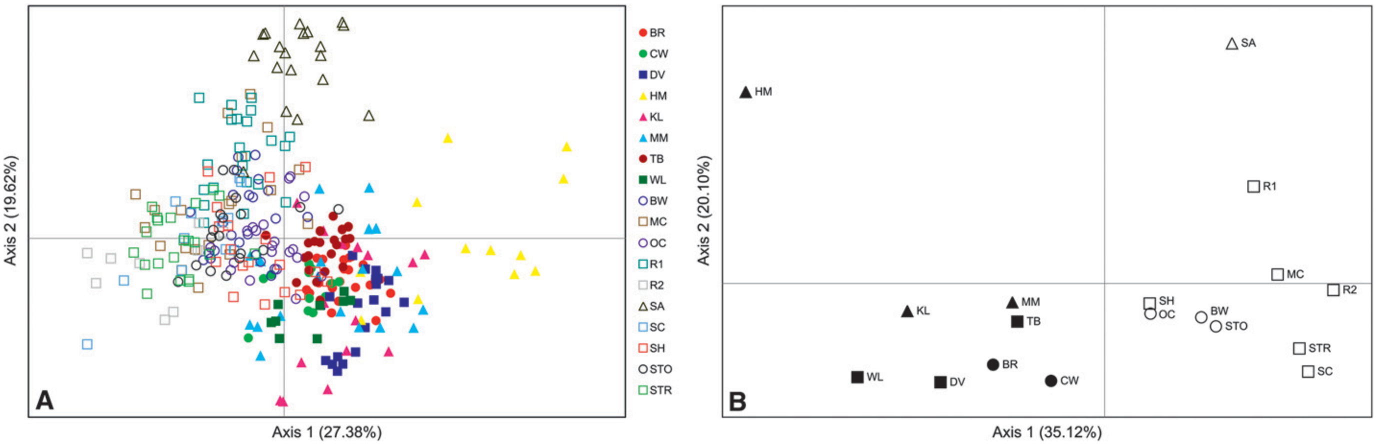

Fig. 2. Principal coordinates analysis of microsatellite variation among (A) individuals and (B) populations of *Phlox amabilis* (solid shapes) and *P. woodhousei* (open shapes). (A) Principle coordinate axis 1 explains 27.38% of the variation, and axis 2 explains 19.62%. (B) Axis 1 explains 35.12% of the variation, and axis 2 explains 20.10%. Diploids are indicated by circles, tetraploids by squares, and hexaploids by triangles. Abbreviations for localities are defined in Table 1.

between species (Figs. 2, 3; Table 4). Genetic findings thus support the recognition of these distinct species (see Wherry, 1955; Wilken and Porter, 2005; Locklear, 2011), rather than Cronquist's (1984) broad view of a single species with size variation in reproductive characters. Although these species can be distinguished genetically, they share a number of alleles and pairwise population values between species for genetic distance and differentiation are generally low. In addition, the genetic variation observed in one of the *P. woodhousei* populations (Show Low) is shared across both species (Figs. 2, 3). These observed genetic similarities between species are most likely due to their recent divergence and shared ancestral variation (see Helsen et al., 2009).

Gene flow among cytotypes and polyploid formation—The presence of multiple cytotypes within these two species poses questions of the origins of the polyploid populations as well as patterns of genetic structure. Genetic clustering and ordination analyses provide support for a combination of auto- and allopolyploidy in the *P. amabilis–P. woodhousei* complex. Tetraploid and some hexaploid populations likely formed through autopolyploidy and experience ongoing gene exchange with diploid populations as evidenced by their genetic similarity (see Halverson et al., 2008; Ramsey et al., 2008). For example, one *P. amabilis* tetraploid population (Death Valley Spring) and two diploid populations (Black Rock and Camp Wood) overlap in PCoA analysis, are clearly assigned to a single genetic group in clustering analysis, and have low pairwise genetic distances and differentiation (Figs. 2, 3; online Appendix S1). Likewise, in *P. woodhousei*, the three diploid populations and nearby, predominantly tetraploid populations (McFadden, Sharp Creek,

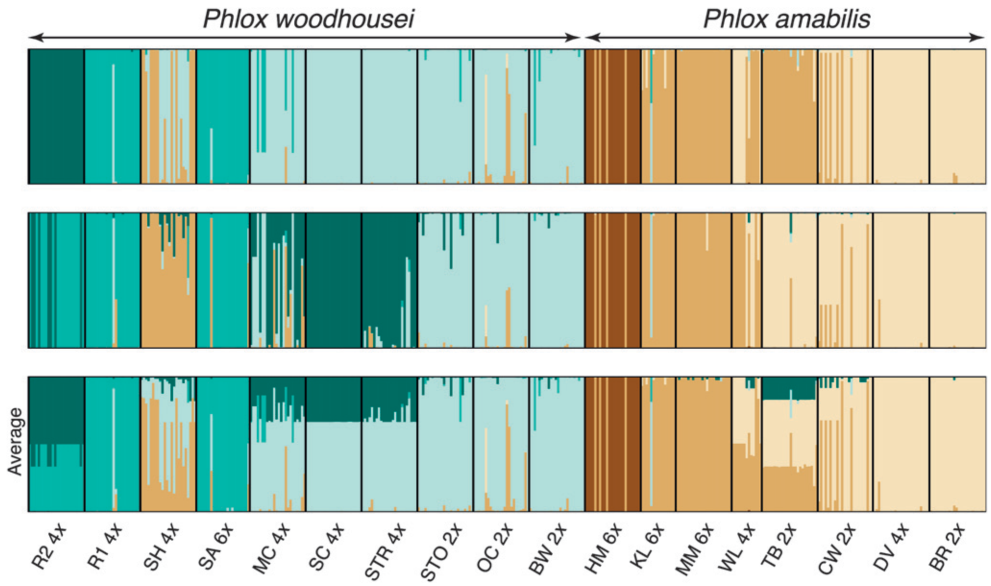

Fig. 3. Bayesian clustering analysis of population structure in *Phlox amabilis* and *P. woodhousei*. Individuals are grouped by population. Individual assignments into $K = 6$ clusters from two of the most likely models, and individual assignments into $K = 6$ clusters averaged across all independent runs are shown. Abbreviations for localities are defined in Table 1 and are presented in order of their geographic locality from east to west.

and Strawberry) overlap in PCoA analysis, are assigned to a single genetic cluster, and are very similar genetically (Figs. 2, 3; online Appendix S1). Their occasional grouping into separate genetic clusters correlating with cytotype is more likely due to geographic proximity than patterns of ploidy level variation. The occurrence of a *P. woodhousei* population with mixed cytotypes (tetraploid and pentaploid, Sharp Creek) suggests that there may be an unsampled hexaploid *P. woodhousei* population in the region that is genetically similar.

In contrast, genetically unique hexaploid populations may represent entities formed through allopolyploidy. One hexaploid population of each species (*P. amabilis* population Hobble Mountain and *P. woodhousei* population Sierra Ancha) is genetically distinct from other populations of the same species in PCoA analysis and genetic clustering (Figs. 2, 3). This genetic distinctiveness primarily reflects differences in allele frequencies, but is also due to the presence of unique alleles in the Hobble Mountain population. This population harbors the greatest number of private alleles per locus as compared to other populations, possibly due to the introduction of alleles from outside the *P. amabilis*–*P. woodhousei* complex. Furthermore, the genetic distinctiveness observed in these hexaploid populations could also reflect genetic isolation from populations of other cytotypes (Ricca et al., 2008; Balao et al., 2010). Further study including other upright *Phlox* species in the region (particularly members of *P. longifolia* Nutt. s.l.; Wilken and Porter, 2005) will improve our understanding of overall population genetic and phylogeographic relationships in this group. When this information is combined with further morphological study, the hypothesis of cryptic species of allopolyploid origin within *P. amabilis* and *P. woodhousei*, as currently circumscribed, can be evaluated.

Genetic diversity, structure, and gene flow within and among populations—The overall pattern of genetic variation in *P. amabilis* and *P. woodhousei* is one of diverse and interconnected populations with a strong influence of geography on genetic structure. Populations of both species are characterized by moderately high genetic diversity as indicated by the total number of alleles and private alleles, number of alleles per locus, and average gene diversity, which is similar to values found in short-lived perennials, endemics and outcrossing species ($H_s = 0.20$, 0.20 and 0.27, respectively; Nybom, 2004; Table 2). Populations of each species also appear to be connected by moderate levels of localized gene flow or dispersal. Genetic differentiation is similar to that typically found in endemics and outcrossing species ($\Phi_{PT} = 0.26$ and 0.27; and $\Theta_{II} = 0.18$ and 0.22, respectively), and lower than that found in short-lived perennials ($\Phi_{PT} = 0.41$ and $\Theta_{II} = 0.32$; Nybom, 2004; Table 3). AMOVA analysis indicates that the majority of observed differences are due to differences within populations rather than differences among populations, and genetic distance and differentiation values are low in most pairwise population comparisons. Taken together, these values are generally consistent with life history characteristics of *P. amabilis* and *P. woodhousei* and a lack of barriers to gene flow or dispersal across the ranges of both species (historic and/or contemporary). Furthermore, observed high values for both genetic diversity and population connectivity in these endemic species (including numerically small *P. amabilis* populations) are encouraging from a conservation standpoint (Booy et al., 2000; Amos and Balmford, 2001).

It appears that much of the genetic differentiation that is present can be explained by the current geographic distribution of populations. Strong geographic structuring is evidenced by positive correlations between genetic and geographic distances, genetic clustering of populations in close geographic proximity to one another, greater genetic differentiation of geographically isolated populations (such as the *P. amabilis* populations north of the Grand Canyon, BR and DV, and the New Mexico populations of *P. woodhousei*, R1 and R2; online Appendix S1), and more private alleles in some of the isolated populations (*P. amabilis* populations north of the Grand Canyon; Table 2). The generally high levels of shared variation among populations within species coupled with little shared variation among populations between species support recognition of *P. amabilis* and *P. woodhousei* as species harboring cytotypic variation—we did not detect evidence for reproductively isolated autopolyploid entities (see Soltis et al., 2007). Detailed morphological and ecological study of populations in the future (with increased sampling) will yield further insights into the processes of diversification within these species.

Conclusions—This work represents a focused case study of genetic variation relative to ploidy level patterns in two closely related species of the southwestern United States; *Phlox amabilis* and *P. woodhousei* are genetically distinct species with moderately high levels of genetic diversity, and each include diploid, tetraploid, and hexaploid populations. These populations have possibly been formed through a combination of auto- and allopolyploidy, and some are genetically distinct (and may represent cryptic taxa). In addition, some populations are experiencing the effects of genetic isolation as a result of geographic separation, and/or a lack of gene flow among cytotypes. This focused study contributes to our understanding of the roles of polyploidy and gene flow in diversification of the genus *Phlox* and provides the basis for future work with respect to phylogeny, morphology, ecology, and taxonomy of *Phlox*. Furthermore, it demonstrates that intraspecific polyploids can be cryptic, contribute to complex patterns of evolution, and potentially provide the basis for diversification and speciation.

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APPENDIX 1. Voucher information for *Phlox* samples included in this study. Population abbreviations are defined in Table 1. Voucher specimens are deposited at the Kansas State University Herbarium (KSC).

Taxon: Population, collector voucher number.

Phlox amabilis: **BR**, SDF 51707-1; **CW**, SDF 51607-1, CF 780; **DV**, SDF 51807-2, 50708-4; **HM**, SDF 51407-2; **KL**, SDF 50508-1; **MM**, SDF 51507-1, 50308-1; **TB**, SDF 51507-2, CF 775; **WL**, SDF 50208-4.

P. woodhousei: **BW**, SDF 51407-1; **MC**, SDF 51207-1, 50108-1; **OC** SDF 51307-2, CF 770; **R1**, SDF 50807-1; **R2**, SDF 50907-1; **SA**, SDF 51107-2; **SC**, SDF 51207-2, 43008-2; **SH**, SDF 51107-1, 42908-2; **STO**, SDF 51307-1, 50108-3; **STR**, SDF 51207-3, 50108-2.