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GENETIC STRUCTURE AND OUTCROSSING RATES IN *VIOLA PEDUNCULATA* (VIOLACEAE), A CALIFORNIA ENDEMIC VIOLET LACKING CLEISTOGAMOUS FLOWERS

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ABSTRACT

Although most North American violet species (*Viola*, Violaceae) produce both showy chasmogamous (CH) flowers and inconspicuous cleistogamous (CL) flowers, some species lack the ability to manufacture the automatically self-pollinated CL flowers. Given that such flowers are considered beneficial as a back-up method of seed production when pollinators are scarce, the ecological and genetic implications of this absence remain unknown. In the current study, we focused on the California endemic violet, *Viola pedunculata* Torr. & A. Gray, which produces only CH flowers within its habitat in prairies and oak savannahs. Using microsatellites, we quantified the population genetic structure of three mainland populations and one island population in Southern California. We then used allozymes to estimate the outcrossing rate within a single population. Consistent with its production of CH flowers, levels of genetic variation were moderate to substantial within the species ($A_p = 6.66$, $H_o = 0.39$), with low but significant structure detected ($\Theta = 0.11$). Furthermore, the high outcrossing rate (0.86) suggests that insect pollinators are frequent enough to ensure adequate seed set. These values were similar to those of a morphologically similar stemmed violet, *Viola pubescens* Aiton, which produces both CH and CL flowers. Overall, these results are consistent with substantial outcrossing occurring in *V. pedunculata* through CH flowers, leading to gene flow among populations and potentially counteracting effects of genetic drift.

Key Words: Allozymes, chasmogamy, cleistogamy, microsatellites, *Viola pedunculata*.

Breeding systems are critical components of a population's ability to reproduce, and in plants, these systems often involve the evolution of floral traits to maximize mating opportunities and fertilization success. In angiosperms, the chasmogamous/cleistogamous (CH/CL) breeding system has evolved multiple times (Culley and Klooster 2007), in part because it increases fitness by facilitating continued reproduction in the face of fluctuating resources, such as light levels and pollinator availability (Lloyd 1984; Schoen and Lloyd 1984). In this system, two different types of flowers are often produced on the same individual, often at different times or locations. These consist of open, showy CH flowers that typically appear under high light conditions when insect pollinators are frequent, and closed, self-pollinated CL flowers which are produced when light levels are low and pollinators are rare. CH flowers are advantageous in that they promote outcrossing and thus gene flow within and among populations, while CL flowers are considered a back-up mechanism of reproduction in the event that CH flowers fail to set seed, thereby extending the reproductive window of CH/CL species during the growing season (Culley and Klooster 2007).

Given the direct fitness advantages of producing both floral types, some plant species have paradoxically lost the ability to produce CL flowers. Although this loss may ultimately increase the resources allocated to CH reproduction, such a reproductive system could be risky if pollination of CH flowers is not assured. As one example, the majority of violets produce both floral types (Culley and Klooster 2007), but several violets lack CL flower production and are completely chasmogamous. The loss of CL flowers is considered a derived condition given that several related lineages that are successive sisters to North American violets, such as *Hybanthus* (Brizicky 1961) and the South American sections *Leptidium* (Becker 1907) and *Chilenium* (Reiche 1896), do produce both floral types. Interestingly, CH-only violet species all tend to occur in relatively constant environments that do not vary substantially in light levels and/or pollinator availability. For example, *V. pedata* L. and *V. pedunculata* Torr. & A. Gray occur in open prairies (in which season cleistogamy may also conflict with summer dormancy) while several Hawaiian taxa (e.g., *V. chamissoniana* Gingins) occupy mesic forests that retain an intact forest canopy or open bogs exposed to consistent light levels (*V. maviensis*; H. Mann; Wagner et al. 1990; Havran et al. 2009). In addition, CL flowers are also absent in the pansies (e.g., *V. tricolor* L.), *Viola* section *Melanium* in Europe, and the rostrate violets of Section *Andinium* in South America which are

confined to treeless, mostly Alpine habitats. In contrast, CH/CL species such as *V. pubescens* Aiton (Culley 2002) and *V. canadensis* L. (Culley 2000) as well as most other temperate species inhabit temperate deciduous forests in which light levels and pollinator abundance dramatically fluctuate throughout the year as the forest overstory leafs out in early spring. Similarly, *Viola grahamii* Benth. is a subtropical species that is CH/CL and lives in forest environments with a strong dry/wet seasonality (Cortés-Palomec 2005).

The loss of CL flowers in *Viola* and other taxa is expected to directly impact the mating system as it will affect rates of outcrossing within and between populations, and thus has the potential to alter the population genetics. Furthermore, long term persistence of populations may be endangered if CL flowers are not present to ensure seed production in case of pollinator failure. Overall, CH-only species are expected to exhibit greater outcrossing with higher levels of genetic variation than in other species in which a portion of the mating system consists of self-pollination through CL flower production. Despite these expectations, genetic studies of CH-only species remain severely limited to nonexistent (Batista and Sosa 2002), relative to the growing number of investigations of species that produce both floral types (e.g., Knight and Waller 1987; Lesica et al. 1988; Cole and Biesboer 1992; Sun 1999; Culley and Wolfe 2001; Culley and Grubb 2003; Cortés-Palomec et al. 2006; Culley et al. 2007).

We address this gap in our knowledge by focusing on the population genetics and outcrossing rate of the yellow violet, *Viola pedunculata*, which belongs to section *Chamaemelanium* Ging. of the Violaceae. Also known as California golden violet or johnny jump-up, *V. pedunculata* is a herbaceous perennial commonly found in the relatively undisturbed prairies and oak savannahs of the Californian mainland, the Channel Islands, and Baja California (Baldwin et al. 2012). The species grows from a rhizome located at least 15 cm deep and produces orange-yellow CH flowers from March until May (lacking the CL flowers often found in *Viola*) before becoming dormant in late summer (Baldwin et al. 2012). Pollination is likely through insect visitation, primarily Hymenoptera, as has been seen in other stemmed violets in North America (e.g., Beattie 1972, 1974; Culley 2002). The species is diploid with $n = 6$ (Baker 1949). We used recently developed microsatellite markers (Culley 2005) to examine patterns of genetic diversity and structure among populations, and also used traditional allozyme markers to quantify outcrossing rates. We then compared this information to populations of another perennial, stemmed yellow violet, *V. pubescens* Aiton var. *scabriuscula* Schwein. ex Torr. & A. Gray (data obtained from Culley and Yadav, in prep.), which has similar vegetative morphology but produces both CH and CL flowers and inhabits the temperate deciduous forest of eastern North America (Culley 2002).

Materials and Methods

Study Sites

Four populations of *V. pedunculata* were selected in Southern California, USA for analysis of population genetic structure (Fig. 1). One population was located on Santa Catalina Island, which is situated 32 km from mainland California and is a member of the Southern Channel Islands. The other three populations were situated on the mainland. Two of these populations were located at Santa Rosa Plateau Ecological Reserve (Santa Rosa A and B), owned by Riverside County Parks. The last population inhabits Starr Ranch Sanctuary, owned and managed by the Audubon Society. All sites consist of oak savannah or Mediterranean scrubland with populations typically located in open, high-light areas.

Population Genetics

During February 2005, leaf tissue was collected from 33 to 42 individuals from each of the four populations. Samples were temporarily stored on ice, transported to the laboratory at the University of Cincinnati, and stored at −80°C until DNA was extracted as described in Culley and Wolfe (2001).

Molecular analysis was performed in 2008 using nine of the 12 microsatellites (*Vpub-1,4,7,9,11,16,21,57,69*) previously developed for *Viola pubescens* (Culley 2005) and which amplified well in this species. PCRs were conducted using the QIAGEN Multiplex Kit (Qiagen, Inc., Valencia, CA) using two primer groups, as described in Culley (2005). PCR was performed in 10 μL reactions, using the following components: 5 μL Multiplex PCR Master Mix, 1 μL primer mix (0.2 μM of each primer in TE), dH_2O and 12–16 ng DNA. Forward primers were tagged using either 6-FAM, NED, VIC or PET. PCR was carried out using the following thermocycler settings: 95°C for 15 min, 27 cycles consisting of 94°C for 30 s, 57°C for 90 s and 72°C for 60 s, with a final extension of 60°C for 30 minutes. Amplified fragments were then analyzed using the LIZ 500 internal size standard on a 3730 xl sequencer (Applied Biosystems, Fortune City, CA) at the Cornell University Bio-Resource Center. The resulting fragment data were then analyzed using GeneMarker

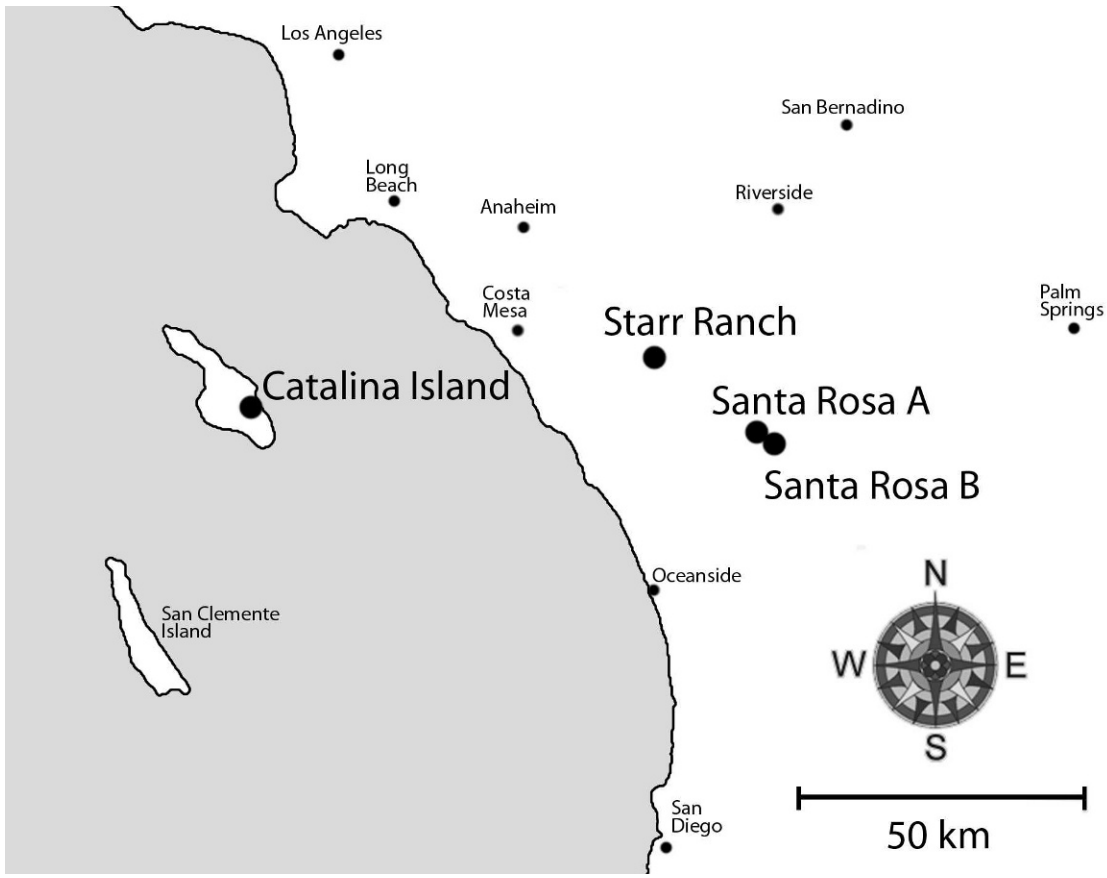

FIG. 1. Populations of *Viola pedunculata* in Southern California: Catalina Island, Santa Rosa A, Santa Rosa B and Starr Ranch. Nearby cities are shown in smaller font.

vers. 1.75 (SoftGenetics, State College, PA) to identify alleles for each locus.

Measures of genetic variation within and among populations were calculated using the Genetic Data Analysis (GDA) software package (Lewis and Zaykin 1999). The following statistics were computed for each population: number of alleles per locus (A), number of alleles per polymorphic locus (A_p), percentage of polymorphic loci (P_p; 95% criteria), expected heterozygosity (H_e), observed heterozygosity (H_o) and the fixation index (F). Significance from zero was tested for each statistic with a Paired Students t-test (each value paired with zero). All populations were tested for Hardy-Weinberg equilibrium and linkage disequilibrium between loci, using the sequential Bonferroni correction (Rice 1989).

Genetic differentiation among populations was calculated in GDA using θ (Weir and Cockerham 1984), an analog of F_{st} which takes into account small and unequal population sizes. The inbreeding coefficient (f), an analog of F_{is}, was calculated as well. Confidence intervals for θ and f used to determine significance from zero were generated by bootstrapping across loci using 1000 replicates (i.e., a value is significant if the upper and lower confidence intervals do not include zero). A Mantel test was then used to examine potential correlations between pair-wise genetic distances and geographic distance between populations, using GenAlEx vers. 6.41 (Peakall and Smouse 2006). To visualize genetic relationships among populations and individuals, an Unweighted Pair Group Method with Arithmetic Mean (UPGMA) phenogram of populations was first constructed using pair-wise calculations of θ generated from GDA. To visualize relationships among individuals within populations, a Principal Coordinates Analysis (PCA) was conducted in GenAlEx, based on pair-wise genetic distances using an algorithm adapted from Orloci (1978). Population differentiation was also analyzed separately in GenAlEx with a hierarchical analysis of variance (AMOVA) using θ values calculated for all population pairs.

Outcrossing Rate

The rate of outcrossing in *V. pedunculata* was determined via allozyme analysis of offspring

TABLE 1. GENETIC VARIABILITY IN POPULATIONS OF *VIOLA PEDUNCULATA* IN SOUTHERN CALIFORNIA. Mean sample size per locus (N), mean number of alleles per locus (A), mean number of alleles per polymorphic locus (A_p), percentage of polymorphic loci (P_p, 95% criteria), expected heterozygosity (H_e), observed heterozygosity (H_o), and the fixation index (F). Significance from zero was tested for each mean statistic with a Paired Students t-test (each value paired with zero); *P ≤ 0.001, **P ≤ 0.0001.

Population	N	A	Ap	P_p	H_e	H_o	F
Catalina	35.5	5.67	7.83	66.67	0.460	0.435	0.055
Santa Rosa A	40.9	3.67	5.60	55.56	0.391	0.396	−0.014
Santa Rosa B	34.1	4.56	7.00	55.56	0.398	0.365	0.085
Starr Ranch	39.4	4.00	6.20	55.56	0.408	0.385	0.056
Mean	**38.2**	**4.47***	**6.66***	**58.33****	**0.414****	**0.395****	**0.046**

germinated from naturally produced seeds. Fruits were collected from eight plants located in the Santa Rosa A location during February 2005 (mature fruits were not yet available at the other populations) and immediately transported to the University of Cincinnati greenhouse facility where 45 seeds were extracted and planted at two different time periods – two weeks and then one month following collection. After first removing each elaisome to minimize fungal growth during the germination process, seeds were treated in the following way, modified from Blaxland (1996). For each fruit, all seeds were spread onto damp 10 cm filter paper and sprinkled lightly with a small amount of gibberellic acid powder (Sigma Chemical Co., St. Louis, MO). The paper was folded and then placed in a sealed container along with a small amount of water to maintain humidity, and stored at 4°C. Germination started about two weeks later and continued for about one month. Seeds were checked every few days and those with a protruding radicle were removed and transplanted into Pro-Mix® BX planting media (Premeir Tech Horticulture, Rivière-du-Loup, Québec, Canada).

Leaf tissue from these seedlings was ground and analyzed via starch-gel electrophoresis, as described in Culley and Wolfe 2001 (see also Culley and Grubb 2003) for the following four allozyme systems: aminopeptidase (AMP; EC 3.4.11.1), glucose-6-phosphate isomerase (GPI; EC 5.3.1.9), malate dehydrogenase (MDH; EC 1.1.1.37), and phophoglucomutase (PGM; EC 5.4.2.2). A total of six loci were resolved (AMP-1, AMP-2, GPI, MDH, PGM-1, and PGM-2). The resulting data were analyzed with the MLTR software package (Ritland 1990) to determine the outcrossing rate in the population. Maternal genotypes were estimated using the most likely maternal genotype (Ritland 1990) to generate multilocus (t_m) and single-locus (t_s) outcrossing rates; a positive difference between t_m and t_s indicates biparental inbreeding. Allozyme markers were used for this analysis, not microsatellite markers (developed later and described above), because allozymes were readily available at this time and had been used in other violets (e.g., Culley and Wolfe 2001).

RESULTS

Population Genetics

Using the nine primer sets that successfully amplified in *V. pedunculata*, six loci (*Vpub-1, 7, 11, 16, 21, 57*) were polymorphic, while *Vpub-4* and *Vpub-69* were monomorphic for a single allele and *Vpub-9* exhibited fixed heterozygosity across all populations. Of all loci, only three from Catalina Island showed significant deviations from Hardy-Weinberg equilibrium after a Bonferroni correction. Linkage disequilibrium was also minimal, only being detected for two pair of loci within Santa Rosa A (*Vpub-7*/*Vpub-21* and *Vpub-16*/*Vpub-21*) and a single pair of loci at Starr Ranch (*Vpub-7*/*Vpub-57*).

Overall, the sampled populations of *V. pedunculata* showed significant and moderate to substantial levels of genetic variation (Table 1), with a mean number of alleles for all loci of 4.47 and for polymorphic loci of 6.66. An average 58.3% of loci were polymorphic and the observed heterozygosity was 0.39. With the exception of Santa Rosa A, all of the populations had lower levels of observed heterozygosity than expected, averaging 0.41 (Table 1). The Catalina Island population showed the greatest number of private alleles (22), compared to populations at Santa Rosa A (4), and Santa Rosa B and Starr Ranch (5 each). Both the mean fixation index (F = 0.046; Table 1) and the inbreeding coefficient within populations were minimal (f = 0.045; Table 2) and were not significant from zero.

Significant genetic structure was detected overall among the sampled populations (θ = 0.1067; CI = 0.068–0.150; Table 2) and also for each pairwise comparison of populations (Table 3). Populations at Catalina Island and Santa Rosa B showed the greatest difference (θ = 0.142). The Catalina Island population also showed high level differentiation between all other populations, while Santa Rosa A and Santa Rosa B showed the least difference. The AMOVA also indicated substantial but lower levels of genetic structuring among populations (Table 4), with the majority of variation (75%) found within individuals and only 10% and 14%

TABLE 2. ESTIMATES OF F-STATISTICS IN FOUR POPULATIONS OF *VIOLA PEDUNCULATA* IN SOUTHERN CALIFORNIA. Weir and Cockerham's f and θ (analogs of Wright's F_{IS} and F_{ST}), and the upper and lower 95% confidence intervals (CI) obtained by bootstrapping over loci (exclusion of zero within the range of CIs indicates significant difference from zero). *** indicates a monomorphic locus.

Locus	f	θ
Vpub-1	0.030	0.182
Vpub-4	***	***
Vpub-7	0.085	0.109
Vpub-9	0.748	−0.012
Vpub-11	0.060	0.007
Vpub-16	0.084	0.124
Vpub-21	−0.055	0.112
Vpub-57	0.005	0.177
Vpub-69	***	***
Mean	**0.045**	**0.114**
Upper CI	0.106	0.150
Lower CI	−0.009	0.068

found among populations and among individuals, respectively. There was a strong but nonsignificant correlation of geographic and genetic distances, as determined by the Mantel test (r = 0.880, P = 0.090) in GenAlEx.

As expected, the two Santa Rosa populations grouped together in the UPGMA phenogram (Fig. 2), followed by Starr Ranch, and then the Catalina Island population. The PCA indicated substantial genetic overlap among individuals from the mainland populations, and Catalina Island individuals were most dissimilar to the other populations (Fig. 3). In this analysis, the first and second principal coordinate axes explained 27.9% and 20.3% of the total variation, respectively.

Outcrossing Rate

Nearly all seeds from the Santa Rosa Plateau population germinated successfully (95%). The multilocus outcrossing rate obtained from eight maternal field plants was 0.861 (i.e., 86% of the progeny resulted from outcross-pollination). This was similar to the single-locus estimate of outcrossing (0.862), indicating lack of biparental inbreeding in the population.

DISCUSSION

The substantial levels of genetic variation along with the low but significant structure detected in *Viola pedunculata* are consistent with its method of reproduction through only showy CH flowers. Furthermore, the high outcrossing rate estimated in at least the one population measured of the species suggests that insect pollinators are frequent enough to ensure adequate seed set. Despite lacking CL flowers and thus a back-up mechanism of seed production if pollinators become scarce (Culley and Klooster 2007), *V. pedunculata* appears to be suffering no detrimental effects to its genetic pool at the current time, at least in the populations sampled here. This is especially important given that the species inhabits a landscape in Southern California that is becoming increasingly fragmented by human population growth and urbanization. However, given that pollinators can also be impacted by habitat fragmentation over time (e.g., Buchmann and Nabhan 1997; Cranmer et al. 2011), any subsequent alteration of pollinator services to *V. pedunculata* has the future potential to reduce outcrossing rates and decrease levels of genetic variation over time. Consequently, we advocate continued conservation of the species and protection of its habitat in Southern California.

Given that *V. pedunculata* does not produce CL flowers, we expected to find higher levels of genetic variation and less genetic structuring in the species (due to increased gene flow following outcrossing) compared to a similar violet with both floral types, *V. pubescens* (Culley and Yadav, unpublished data) located within a similar-sized geographic area in southwestern Ohio. This was indeed the case based on similar microsatellite loci (Table 5), with *V. pedunculata* consistently having higher values than *V. pubescens* for A (4.47 vs. 3.08) and A_p (6.66 vs. 3.23), but with similar values for H_o (0.385 vs. 0.392) and H_e (0.414 vs. 0.405); in contrast, *V. pedunculata* exhibited a substantial lower value of P_p than *V. pubescens* (58.33% vs. 84.72%).

TABLE 3. PAIRWISE COMPARISONS BETWEEN POPULATIONS OF GEOGRAPHIC DISTANCES (KM; UPPER RIGHT MATRIX) AND GENETIC DISTANCES (LOWER LEFT MATRIX), CALCULATED AS θ. *P < 0.005, **P < 0.001 as generated from 9999 permutations of the data.

	Catalina Island	Starr Ranch	Santa Rosa A	Santa Rosa B
Catalina Island	–	84.66	107.03	108.52
Starr Ranch	0.128**	–	28.48	28.80
Santa Rosa A	0.127**	0.083**	–	2.37
Santa Rosa B	0.142**	0.104**	0.017*	–

TABLE 4. AMOVA RESULTS INDICATING THE SOURCES OF VARIATION FOR THE FOUR POPULATIONS OF *VIOLA PEDUNCULATA* BASED ON Θ VALUES.

Source	df	SS	MS	Est. Var.	%
Among pops.	3	63.92	21.31	0.239	10
Among indiv.	155	365.86	2.36	0.325	14
Within indiv.	159	272.00	1.71	1.711	75
Total	**317**	**701.78**	–	**2.274**	**100**

Although both species exhibited significant population differentiation, structuring was less pronounced in *V. pedunculata* compared to *V. pubescens*, as determined by θ (0.107 vs. 0.224). In addition, the inbreeding coefficient was statistically equivalent to zero, indicating a lack of inbreeding overall in these populations. Taken together, these results are consistent with substantial outcrossing in the California violet through the CH flowers, leading to gene flow among populations and counteracting effects of drift. Although selfing may occur through geitonogamy (see below), it does not happen to the extent found in CH/CL species (e.g., Culley 2002). In particular, θ in *V. pedunculata* (0.11) was less than the mean values for other species (Table 5) with mixed breeding systems (0.26) and even outcrossing species (0.22; Table 5; Nybom 2004) but similar to a CH-only violet from the Canary Islands (0.10; Batista and Sosa 2002), suggesting that the California violet is capable of gene flow.

As expected, the Catalina Island population was the most genetically distinct from the mainland populations although there was no significant relationship between genetic and geographic distances. Not only was there a much higher number of unique alleles present in the Catalina Island population, but its individuals tended to cluster for the most part, away from other mainland individuals in the PCA. This was further reinforced by the UPGMA phenogram in which the Catalina population was the most dissimilar. In contrast, the two populations of *V. pedunculata* at the Santa Rosa Plateau consistently clustered together, both in the PCA and the UPGMA. In addition, they exhibited the lowest pair-wise θ value. Sampling additional populations, including on more islands, would contribute to a better understanding of forces affecting the genetic structure of *V. pedunculata*.

Although substantial, the outcrossing rate detected in *V. pedunculata* indicates that some selfing does occur within the sampled populations. Given that there was little, if any, evidence of biparental inbreeding, it is instead likely that individual flowers are capable of geitonogamy or delayed self-pollination. The latter has been detected in the morphologically similar *V. pubescens* in which the stigma moves to effect self-fertilization in flowers that have not been visited by insect pollinators after several days (Culley 2002). It would be helpful if the flowering phenology of *V. pedunculata* could be examined more carefully in future studies to determine if this also occurs in this species. The outcrossing rate in *V. pedunculata* is also consistent with other violets in which both CH and CL flowers are produced, including *V. pubescens* (0.40 for 1996, 0.93 for 1997; Culley 2002). Consequently, outcrossing in this California violet remains similar to other species that produce self-pollinated, CL flowers. However, the selfing rate reported here is only for a single season, and rates may differ annually depending upon environmental conditions such as pollinator availability (Culley 2002). It would be informative to determine if outcrossing rates in *V. pedunculata* remain relatively consistent over time or vary over multiple growing seasons.

To our knowledge, this is one of the first genetic studies of a CH-only violet (but see Batista and Sosa 2002) and there is an urgent need for additional studies of other similar species, which happen to be especially common in the California flora. For example, Little (1993) lists seven of 33 *Viola* species that reportedly lack CL flowers,

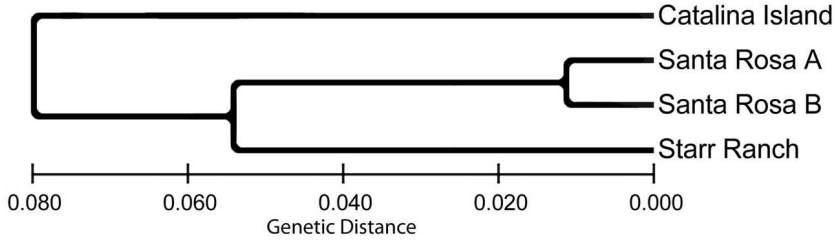

FIG. 2. Phenogram based on Unweighted Pair Group Method with Arithmetic Mean (UPGMA) for four populations of the California violet, *Viola pedunculata*, using pairwise θ values.

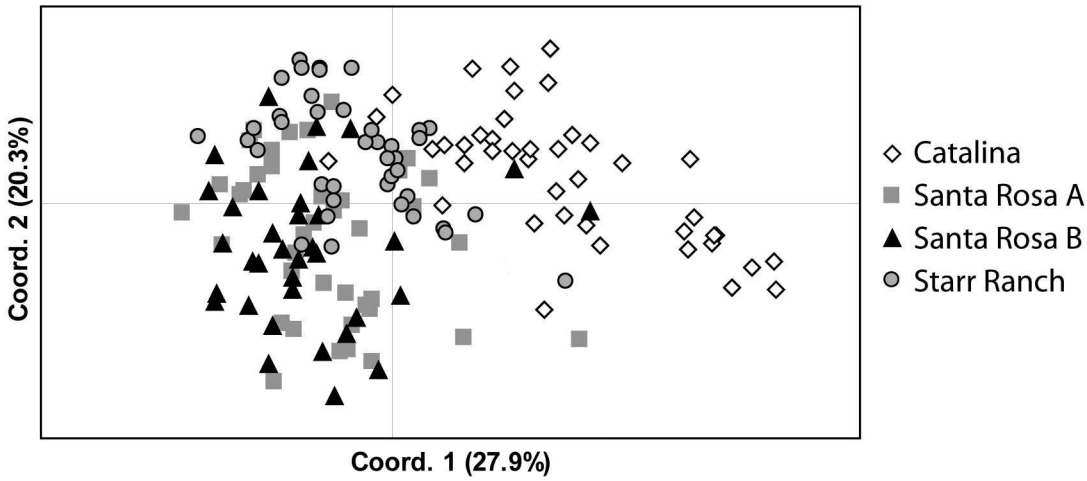

FIG. 3. Distribution of individuals from the four populations in ordination space according to the Principal Coordinates Analysis (PCA), based on the pair-wise genetic distances using an algorithm adapted from Orloci (1978) in GenAlEx vers. 6.41. The first two axes explain a combined 48.2% of the variation in the data.

TABLE 5. COMPARISON OF GENETIC VARIATION PARAMETERS GENERATED FROM CODOMINANT GENETIC MARKERS (MICROSATELLITE OR ALLOZYME) FOR *VIOLA* AND OTHER PLANT SPECIES WITH SIMILAR LIFE HISTORY TRAITS.

Species	Breeding system	Marker	*A*	*Ap*	*P*	H_e	H_o	F_{st} or θ	Reference
V. palmensis	CH	allozyme	1.53	–	43.2	0.16	0.10	0.10	Batista and Sosa 2002
V. pubescens	CH/CL	allozyme	2.19	2.86	50.0	0.23	0.25	0.34	Culley and Grubb 2003
V. pubescens	CH/CL	microsat	3.08	3.23	84.72	0.40	0.39	0.22	Culley and Yadav, in prep.
V. pedunculata	CH	microsat	4.47	6.66	58.33	0.41	0.39	0.11	current study
Various	selfing	microsat	–	–	–	0.41	0.05	0.42	Nybom 2004
Various	mixed	microsat				0.60	0.51	0.26	Nybom 2004
Various	outcrossing	microsat	–	–	–	0.65	0.63	0.22	Nybom 2004

although further studies under field conditions are warranted to confirm this. All species are found in consistent light environments, either in high light (grassy slopes, marshes) or low light environments (pine forests). These species consist of the following (habitat descriptions taken from Little (1993): *V. arvensis* Murray (abandoned fields), *V. beckwithii* Torr. & A. Gray (vernally moist places among shrubs or beneath pines), *V. douglasii* Steud. (found on vernally moist flats, grassy slopes, often on serpentine soils), *V. hallii* A. Gray (vernally moist areas, open forest, grassy hills, flats, chaparral, often serpentine or gravelly soil), *V. nephrophylla* Greene (shady areas in moist or swampy ground, lake margins, yellow-pine forest), *V. primulifolia* L. (marshes, bogs), and *V. tomentosa* M.S. Baker & J.C. Clausen (dry, gravelly places in open pine forest). However further investigation is warranted as CL flowers have been reportedly produced in other parts of the North American range for both *Viola primulifolia* (Ballard 2007) and *V. nephrophylla* (Voss 1985); that later species was also reported by Munz (1974) in California to produce CL flowers but on shorter peduncules than CH flowers.

Violet species in other areas that lack CL flowers include the Hawaiian violets (e.g., *V. chamissoniana, V. helenae* Forbes & Lydgate, *V. maviensis*; Wagner et al. 1990; Ballard and Systma 2000), as well as *V. pedata* (Culley and Klooster 2007). Studies of these species, as well as continued genetic investigations of CH/CL violets, will help inform our understanding of the genetic implications of the evolutionary loss of cleistogamy within the genus. In California, such studies also have the added benefit of enhancing the conservation of the endemic species, several of which are rare and in danger of encroachment from human activities.

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