EVIDENCE FOR HYBRIDIZATION BETWEEN TWO SYMPATRIC VIOLET SPECIES, *VIOLA GRAHAMII* AND *V. HOOKERIANA* (VIOLACEAE), IN CENTRAL MEXICO

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ABSTRACT

Viola hookeriana and *V. grahamii* (Violaceae) are closely related species in section *Viola*, subsection *Mexicanae* occurring in mountainous habitats across much of central Mexico. In various locations, these species grow sympatrically, and the occurrence of many individuals with intermediate morphological characters has suggested hybridization between these species. Using a combination of morphological, ecological, and molecular evidence we evaluated two mixed populations of *V. grahamii* and *V. hookeriana* in mountains surrounding Lake Pátzcuaro in the state of Michoacán to determine the potential of distinct hybrid morphologies, the strength of phenological differences and ecological preferences among the species and putative hybrid, and to evaluate the level of gene flow between the two species. Our results indicate that hybridization is occurring and that hybrids are morphologically distinct from the parental taxa. Pre-mating isolation is weak and has favored extensive hybridization due to largely overlapping blooming times, weak ecological isolation and absence of spatial isolation. Hybrids are morphologically and genetically closer to *Viola grahamii*, but do show unique alleles as well.

RESUMEN

Viola hookeriana y *V. grahamii* (Violaceae) son dos especies pertenecientes a la Subsección *Mexicanae* dentro de la Sección *Viola* del género *Viola*. Estas especies crecen simpátricamente en varias regiones en el centro del México y la presencia de individuos con características intermedias ha sugerido la existencia de hibridización entre las especies. Usando una combinación de datos morfológicos, ecológicos y moleculares evaluamos dos poblaciones mixtas de *V. grahamii* y *V. hookeriana* en las montañas cercanas al lago de Pátzcuaro en el estado de Michoacán en México para determinar la presencia de morfologías características a los híbridos, el grado de diferencias fenológicas y ecológicas entre las especies y el nivel de flujo génico entre ellas. Nuestros resultados indican la presencia de híbridos y que estos son morfológicamente distintos a los padres. Los mecanismos de aislamiento entre las especies parentales no son muy fuertes y esto ha favorecido la hibridización extensa entre ellas debido a tiempos de floración similares, muy poco aislamiento ecológico y la ausencia de separación espacial entre las especies. Los híbridos son morfológicamente y genéticamente mas similares a *Viola grahamii*, pero muestran aleleos que son únicos a ellos.

Hybridization is a common phenomenon in nature, as evidenced by an estimated 70,000 natural interspecific plant hybrids worldwide (Rieseberg & Ellstrand 1993; Judd et al. 1999), and the fact that between 16 and 37% of the plant families reported in different floras contain at least one hybrid taxon (Ellstrand et al. 1996). In some cases, hybridization can lead to the creation of hybrid species and/or introgressants between hybrid derivatives and the parental taxa (Arnold 1992; Rieseberg 1995). The genus *Viola* L. (Violaceae), which comprises about 525–600 species (Clausen 1964; Ballard et al. 1999), is well known for its taxonomic problems due to hybridization and introgression, as well as complex patterns of variation in individual traits (Brainerd 1924; Anderson 1954; Rus-

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sell 1955; Ballard 1994; Krahulcová et al. 1996; Gil-ad 1998; Neuffer et al. 1999; Marcussen & Borgen 2000; Calderón de Rzedowski 2001; Harmaja 2003).

Analysis of herbarium specimens from central Mexico has indicated that hybridization is likely occurring among species belonging to *Viola* subsection *Mexicanae* (Ballard 1994). *Viola* subsection *Mexicanae* W. Becker (sensu Ballard et al. 1999) is a monophyletic group consisting of eight stoloniferous or rosette-forming species: *Viola hemsleyana* G. Calderón, *V. grahamii* Benth., *V. oxyodontis* H.E. Ballard, *V. humilis* H.B. & K., *V. hookeriana* H.B. & K., *V. beamanii* G. Calderón, *V. guatemalensis* W. Becker, *V. nannei* Polak. (Ballard & Sytsma 2000; Ballard et al. 1999), and an additional undescribed species (Ballard, per. obs.). Instances of hybridization have been identified from several localities in central Mexico occurring between *Viola grahamii* and four other members of the group, *V. guatemalensis*, *V. hemsleyana*, *V. hookeriana* and *V. humilis*. Occurrences of these putative hybrids appear to be restricted however to the immediate areas where both parents occur sympatrically.

Viola grahamii (known in Mexico as "hoja de pasmo" or "pensamiento del cerro") is a frequent to locally abundant perennial distributed across much of Mexico. Specifically, it occurs in mountainous regions from northern Mexico south to northern Guatemala, and is one of the most widely distributed members of the subsection *Mexicanae* (Fig. 1). It thrives in dry to mesic sandy loam under varied forest canopy and along stream banks at elevations of 1950–3600 meters (Ballard 1994; Calderón de Rzedowski 2001), and often forms large mats connected via aboveground stolons. It produces chasmogamous flowers at the beginning of the rainy season (June) for approximately four weeks and cleistogamous flowers for several months afterward (June–December) until the onset of the dry season (Cortés-Palomec 2005).

As previously mentioned, one of the four species with which *Viola grahamii* appears to hybridize is *V. hookeriana* (known in Mexico as "violeta"). *Viola hookeriana* is an infrequent to locally common perennial generally growing in small isolated populations across northern and central Mexico (Fig. 1). It grows in mesic loam under mixed deciduous and coniferous forest canopy at elevations between 1700–2500 meters (Ballard 1994; Calderón de Rzedowski 2001). Reproduction occurs via both chasmogamous and cleistogamous flowers and follows a similar pattern to that seen in *V. grahamii* (Cortés-Palomec, pers. obs.).

Viola hookeriana and *V. grahamii* are divergent in several characters: growth habit, flower structure, and foliage, and can be separated without difficulty in both herbarium specimens and living populations (Table 1) (Ballard 1994). The putative hybrids have been identified in areas of overlapping distribution of the two species (Fig. 1), and are recognizable as exhibiting intermediate morphologies (Ballard 1994).

The goal of our study was to assess the presence and extent of hybridization between *V. grahamii* and *V. hookeriana* in natural populations, and identify intrinsic pre-mating isolation mechanisms that may be functioning between the two species. We examined morphological and phenological differences between the two species, characterized the ecological factors governing where individual species and putative hybrids occur, and surveyed genetic patterns to identify gene flow between species in two study sites in central Mexico where *V. grahamii* and *V. hookeriana* intermingle extensively.

MATERIALS AND METHODS

Site Establishment and Sampling

Two study sites were established in the mountains to the north of Lake Pátzcuaro in the

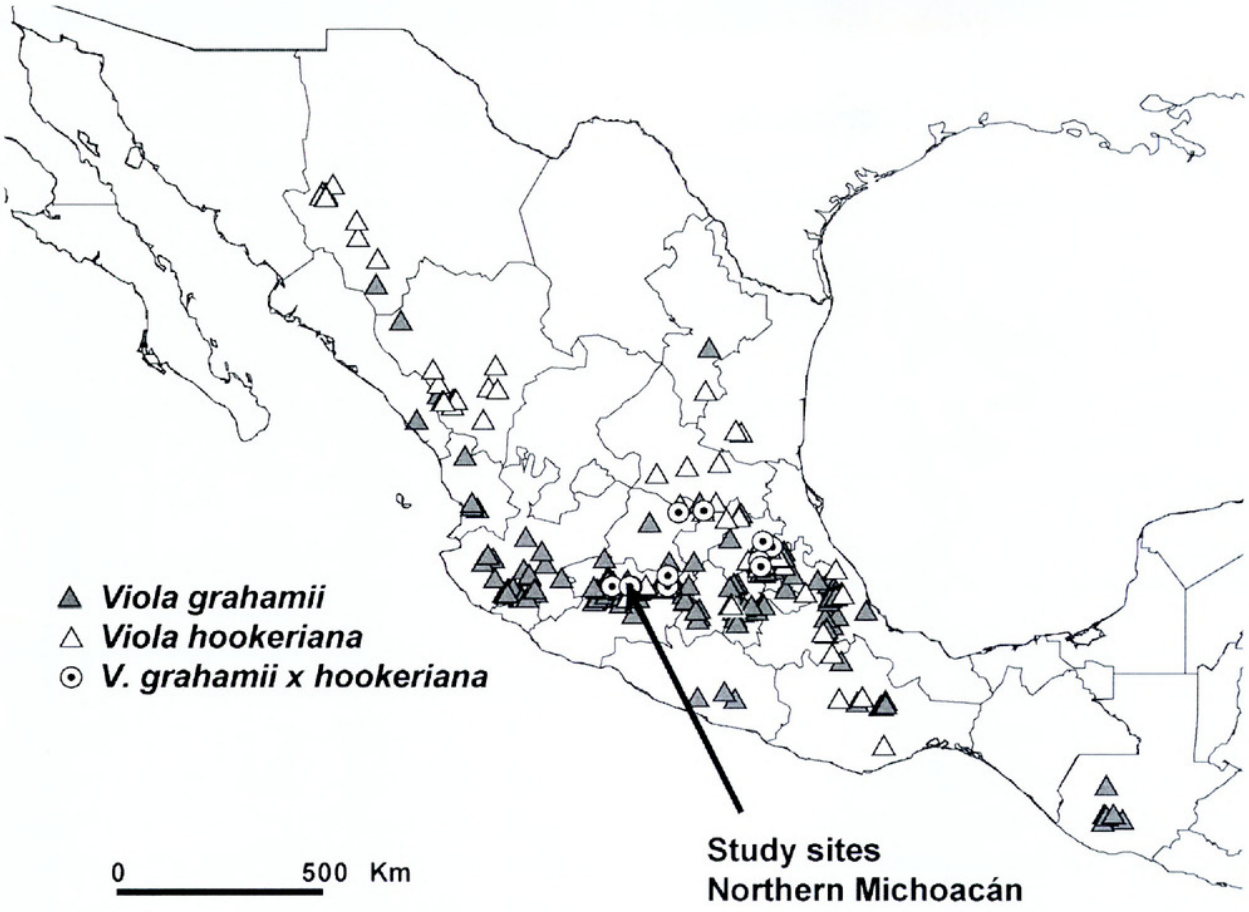

FIG. 1. Distribution of *Viola grahamii*, *V. hookeriana*, and *V. grahamii* × *hookeriana* across Mexico and northern Guatemala. Distribution from Ballard (unpublished data).

municipality of Quiroga, Michoacán, Mexico (Fig. 1). The sites were established in early June 2000 and observations were made weekly over a period of five weeks during the summer rainy season when chasmogamous flower production was most extensive. The two specific sites were chosen due to the presence of abundant populations of the two species and their putative hybrids as identified through a survey of the area conducted earlier in the spring. The first site (Site A) [19°41'57" N; 101°32'27" W] was situated on the lower southern slopes of Mt. Zirate, above the town of Santa Fe de la Laguna. The second site (Site B) [19°42' N; 101°35' W] was located 8 km to the west of Santa Fe de la Laguna (Km 48 on the road from Quiroga to Zacapu (MX-15)). While both sites were located in superficially similar environments, they differed in the level of disturbance and abundance of violet species. *Viola grahamii* was abundant in both sites, but areas of visually pure *V. hookeriana* separated from *V. grahamii* were found only in Site A.

In Site A, four 20 m long transects were established. Two transects were arbitrarily placed through the middle of a visually "pure" population of the parental species (A-T1VG in a *V. grahamii* population and A-T4VH in a *V. hookeriana* population). Two additional transects (A-T2HY and A-T3HY) were placed through areas where putative hybrid plants intermingled with the parental taxa. Ten quadrats (0.5 m^2) were randomly established along each transect with position determined using a random number table. In Site B the same procedure was followed except that only three transects were positioned resulting in a total of 30 quadrats (B-T1VG for *V. grahamii*, B-T3HY of mostly *V. hookeriana* and a mixture of *V. grahamii* and hybrids, and B-T2HY consisting mostly of putative hybrids).

TABLE 1. Comparison of morphological characters used to distinguish between *Viola grahamii* and *V. hookeriana* (on the basis of Ballard 1994). The characters of hybrids are not included since they are not consistent (i.e., hybrids show varying combinations of traits, making them difficult to characterize).

Character	*Viola grahamii*	*Viola hookeriana*
Stolon position	above ground	underground
Stolon nodes	present	absent
Stipule adnation	free or adnate	free
Pubescence on petioles and abaxial surface of leaves	present [abundant]	absent
Leaf shape	ovate-oblong to elliptic	broadly ovate to reniform
Length of pedicels (cm)	3–12	5–21
Calyx pubesence	ciliate	glabrous
Corolla color	white	white, sometimes purplish
Elevation	1950–3600 m	1700–2500 m

For transects representing "pure" parental taxa at each study site, three plants that were not connected via stolons were randomly selected in each of the 10 quadrats. One leaf was removed from each of the three plants and dried in silica gel for later DNA extraction. A second leaf, the largest of each individual, was collected and labeled to correspond to the DNA sample. This leaf was pressed and used for subsequent morphological analysis. The same methodology was used for plants in the putative hybrid/mixed-taxon transects, except that 5 individuals were sampled per quadrat.

Phenology and Pollinator Visitation

In each of the 70 quadrats (40 from Site A, 30 from Site B), the number of individuals present per quadrat and the number of individuals with open chasmogamous flowers were recorded weekly during the five weeks of field observations to interpret phenology. The percentage of flowering individuals, those bearing chasmogamous flowers at some point during the study, was calculated for each visit for the two species and for the putative hybrids to allow for a comparison of phenology among the parental taxa and putative hybrids at both sites. Quadrats with more than one taxon were recorded separately for each taxon and analyzed as separate observations. A Kolmogorov-Smirnov two-sample test was performed in NCSS (Hintze 1999) to compare phenological patterns among taxa at each of the two sites.

Pollinator visitation was assessed during weekly visits to Site A, which was the only site to have clearly differentiated populations of *V. grahamii* and *V. hookeriana*. A total of 40 hours of direct observations of the flowering individuals were recorded with special emphasis made to observe instances of pollinator movement between or among differing taxa. The hours of pollinator observation were equally divided between two daily periods relating to potential insect activity based on the times when previous observations in the area suggested the potential pollinators would be more active, between 8:00 and 11:00 am, and between 12:00 and 3:00 pm.

Ecological Characterization

To determine if the taxa grow in modally different microhabitats, infer whether ecological isolation might reduce gene flow between the species and the putative hybrids, and understand the local distribution of putative hybrids relative to the parents, an ecological characterization of microhabitat in the 70 quadrats was conducted. Soil N, P, K con-

centrations and pH were evaluated using a LaMotte™ Combination Soil Test Kit (LaMotte, Chestertown, MD). Values were given in exact units for pH while values for N, P, and K were determined via a colorimetric test procedure from which concentration could be estimated within a set range, 5–75 ppm for N, 5–100 ppm for P, and 50–200 ppm for K. Due to the inability of obtaining exact concentration values for N, P, and K, these values were range standardized and analyzed in rank form.

Percent soil moisture was recorded using the gravimetric method described by Hadley and Levin (1967), with the modification that soil mass was determined after one week of air drying. Light availability as a direct means of inferring canopy closure was measured using a Model-C spherical densiometer (Vora 1988). Quadrats with more than one taxon were recorded separately for each taxon and analyzed as separate observations. Data were standardized and an analysis of variance was performed in NCSS to test for differences among *V. hookeriana*, *V. grahamii* and putative hybrids.

Morphological Analysis

From the pressed leaves, measurements were taken of the 0°, 30°, 60°, 90°, 120°, 150° and 180° radii, following the procedure outlined by Ballard and Wujek (1994). The center of the leaf was considered to be the point along the midrib opposite the widest part of the leaf blade. Presence or absence of leaf pubescence on the abaxial surface of the leaf, a characteristic considered diagnostic for separating *V. hookeriana* (glabrous) and *V. grahamii* (pubescent), was also recorded. Measurements were standardized and a discriminant analysis (Manly 1994) was performed, both including and excluding visually identified putative hybrids to determine overlap in leaf characters among the two taxa and putative hybrids. The analysis was performed in NCSS using an automatic variable selection procedure. Measurements at 30° and 120° were excluded from the analysis due to colinearity of the data. The canonical scores generated from these analyses were used in an analysis of variance (ANOVA) with a Fisher's LSD pairwise comparison analysis (Zar 1996).

Genetic Analysis

To test the presence of genetic differences among the parental taxa and identify potential gene flow within the putative hybrids, biparentally inherited nuclear markers, inter-simple sequence repeats (ISSRs) were used. ISSR data have proven to be highly effective in detecting hybridization and/or gene flow among closely related species, and to test the hybrid speciation hypothesis (Wolfe et al. 1998a, b; Wolfe & Randle 2001; Archibald et al. 2004; James & Abbott 2005). Additionally these markers are useful in studies of differentiation among closely related species for they are able to utilize some of the variability present at microsatellite loci without the need to develop species-specific microsatellite primers, an advantage when working with species which have not been well-studied genetically.

Genomic DNA was extracted from the silica gel-dried leaves using a Wizard Genomic DNA purification kit (Promega, Madison, WI). Five different ISSR primers (McCauley & Ballard 2002) were initially screened to identify primers which would produce consistent and scorable polymorphic bands among the taxa. Two primers were selected: Wolfe #99B [$(CA)_6GG$] and HB#15 [$(GTG)_3GC$]. These were used for amplification of ISSR products in replicated 25 µl reactions consisting of 1 µg µl^{-1} diluted genomic DNA, 19 µl autoclaved distilled water, 2.4 µl 10× PCR Buffer (Gibco BRL), 2 µl $MgCl_2$ (50 mM, Gibco BRL), 2 µl of dNTP mix (10 mM, Fisher), 0.5 µl of BSA (Bovine Serum Albumin, 4 µg µl^{-1}, Fisher),

0.25 µl primer and 0.25 µl of Taq polymerase (5 U μl^{-1}, Gibco BRL). The polymerase chain reaction was performed in a Stratagene RoboCycler 96 with hot-top (Stratagene Inc, La Jolla, CA) and programmed for 2 min. at 94°C; 40 (primer Wolfe #99b) 44 (primer HB #15) × 30 sec. at 94°C, 45 sec. at 44°C, 1 min. 30 sec. at 72°C; 20 min at 72°C.

PCR products were electrophoresed in a 1.3% agarose gel in 0.5 × TBE buffer with flanking 250 bp ladders (Gibco BRL). Gels were stained with a solution of ethidium bromide in 0.5 × TBE buffer for 20 minutes and imaged under UV light. Gel images were analyzed with BioMax ID image analysis software (Version 2.0.3, Eastman Kodak Company, Rochester, NY) to identify and size fragments. Fragments were scored as present (1) or absent (0), with fragments comigrating at identical rates (± 10 base pairs, the general limit of resolution for agarose gels) considered equivalent. Statistics concerning fragment occurrence, including total fragment number, distribution across taxa, polymorphic and fixed fragments, and number of fragments shared among taxa per site were calculated using TFPGA 1.3 (Miller 1997).

Analysis used three discrete methods to evaluate relationships and similarity among the taxa and putative hybrids. UPGMA cluster analysis and Principal Coordinates Analyses (PCoA) were performed both within and between the two sites using NTSYS ver. 2.02j (Applied Biostatistics Inc.). An Analysis of Molecular Variance (AMOVA) was additionally performed in a hierarchical fashion among species and sites using WINAMOVA 1.55 (Excoffier 1993). All analyses were performed according to the methods described in McCauley and Ballard (2002).

RESULTS

Phenology and Pollinator Visitation

The parental species expressed divergent but non-significant tendencies in blooming time; in fact, in Site A *V. hookeriana* bloomed first, whereas in Site B it bloomed later (Fig. 2). The Kolmogorov-Smirnov two-sample test showed no significant differences among the blooming times of the hybrids and the parental taxa in either site (p values = 1 in some pairwise comparisons) (Fig. 2). No pollinators were observed visiting violets at any time during this study.

Ecology

All three taxa inhabited modally different microhabitat conditions influenced by light and certain nutrients, in all cases where there were significant differences between the taxa, the hybrids grew in intermediate conditions to the parental species. The ANOVA of the ecological factors revealed significant differences in light ($p = 0.02$), P ($p = 0.04$) and K ($p = 0.03$) among *V. hookeriana*, *V. grahamii* and the putative hybrids; moisture ($p = 0.32$), pH ($p = 0.86$) and N ($p = 0.62$) did not differ significantly. *Viola hookeriana* grew in higher concentrations of P, lower concentrations of K and more shaded environments than *V. grahamii* (Table 2).

Leaf Morphology

Two hundred and eighty eight individuals were classified into three groups corresponding to the two parental taxa and the putative hybrid individuals (Fig. 3). The discriminant analysis indicated three marginally distinct groups (Fig. 4), with the GLM ANOVA and the Fisher's LSD Multiple Comparison analysis indicating that they were well-supported and significant ($p < 0.05$) groups.

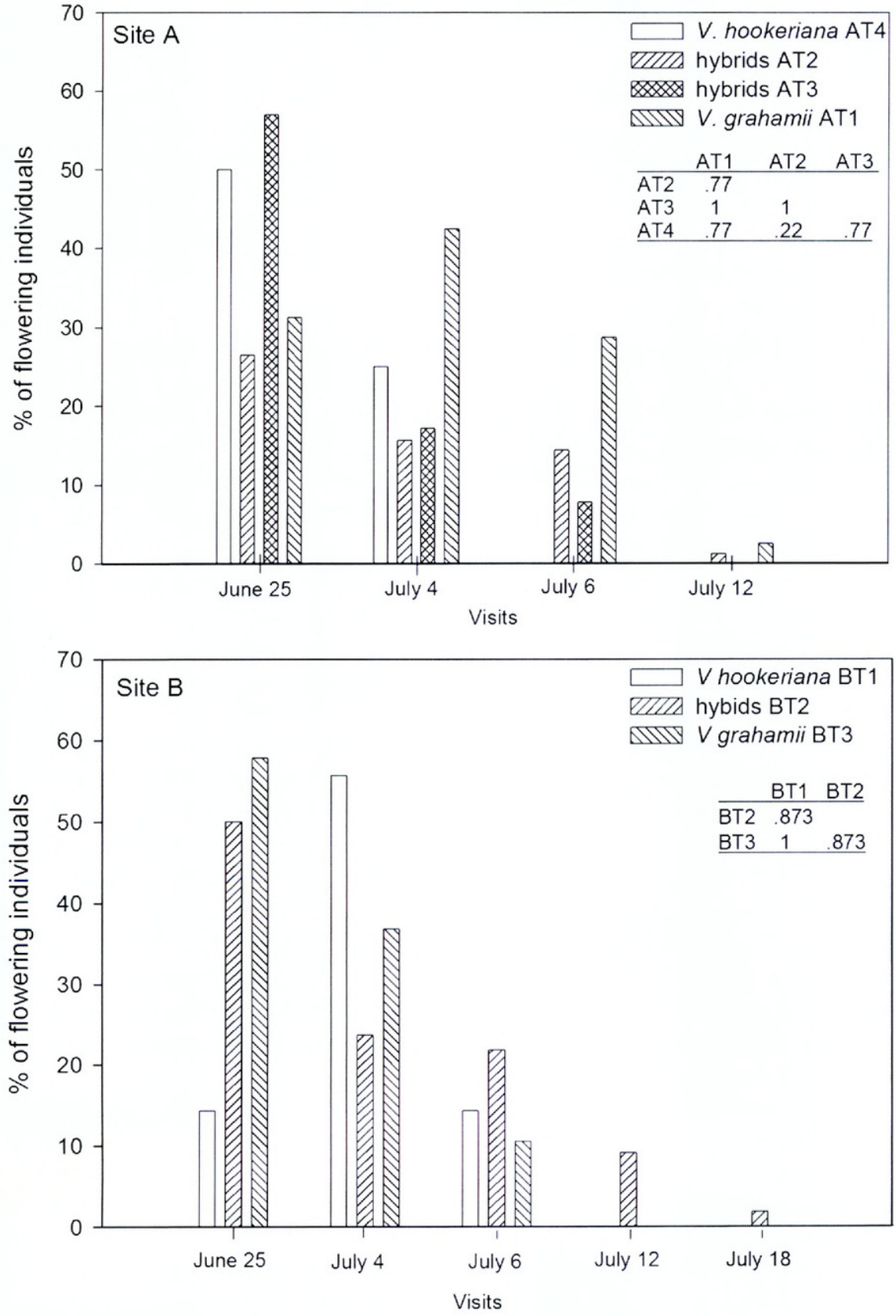

FIG. 2. Histogram of phenology for flowering individuals in two sites of *Viola grahamii*, *V. hookeriana* and *V. grahamii* × *hookeriana*. Abundance of flowering individuals is summarized by transects. *P*-values depicting strength of similarities or differences among taxa using a Kolmogorov-Smirnov two-sample test are shown in the box. VH = *V. hookeriana*, VG = *V. grahamii*, HY = hybrids. Total individuals sampled are as follows (number of reproductive individuals): Site A: VH(AT1)=24(12); HY(AT2)=151(76); HY(AT3)=232(134); VG(AT4)=146(77). Site B VG=35(19); HY=95(55); VH =142 (33).

TABLE 2. Means and SE for selected ecological parameters among *V. hookeriana* (VH), *V. grahamii* (VG) and hybrids (HY). Superscript letters indicate a significant difference for a particular parameter among the taxa. Moisture and light are expressed as percents. P, N and K values are along a scale of 1 to 7 (1=very low, 7= high).

Taxon	Moisture	Light	pH	P	N	K
VH	26(±0.1)	16(±0.4)[a]	6.40(±0.14)	5.32(±0.49)[a]	1.28(±0.10)	6.44(±0.16)[a]
VG	24(±1.1)	29(±0.3)[b]	6.51(±0.12)	3.82(±0.37)[b]	1.14(±0.09)	7.0(±0.13)[b]
HY	25(±0.1)	29(±0.3)	6.43(±0.10)	4.40(±0.33)	1.18(±0.08)	6.81(±0.12)

Genetic Data

A total of 242 *Viola* individuals were examined genetically with ISSRs: 33 *Viola hookeriana* in Site A and 19 in Site B, 65 *V. grahamii* individuals in Site A and 35 in Site B, and 48 putative hybrid individuals in Site A and 42 in Site B. In Site A, 70 ISSR fragments were resolved vs. 53 in Site B. The majority of these were identical between the two sites, resulting in a total of 75 unique and scorable fragments for the study sites together. The relative frequency of individual fragments ranged from less than 10% of individuals (rare to infrequent) to 100% (ubiquitous). Distribution of fragments across taxa (Table 3) showed that some were specific to one of the parental taxa (e.g., five fragments were species-specific for *V. grahamii*, and three were specific for *V. hookeriana*) whereas others were shared among taxa. Most of the shared fragments occurred between *V. grahamii* and the putative hybrids (e.g., 17 fragments in Site B). Nine fragments were specific to the putative hybrids.

UPGMA cluster analysis (Fig. 5) showed strong separation of the two study sites. All individuals diverged substantially between Site A and Site B, regardless of taxon. The PCoA confirmed these discrete site differences (Fig. 6A). Additional PCoA analyses were carried out separately on the two sites [Site A (Fig. 6B) and Site B (Fig. 6C)]. In both sites, *V. grahamii* and the putative hybrids showed the most extensive genetic overlap. In Site A, *V. hookeriana* mostly segregated from the other taxa, although some individuals clustered with putative hybrid derivatives, and others with *V. grahamii*. In Site B (Fig. 6C) two distinct groups of *V. hookeriana* were evident, with some putative hybrids occurring within each of them. In all cases, putative hybrid individuals were widely placed and substantially overlapping in distribution with the parental taxa, together expressing a greater level of genetic diversity.

The AMOVA (Table 4) showed that most of the variation was within morphologically defined taxa (69%), although significant differences were detected between sites and among taxa. Variation between sites accounted for 27% of the total variance. Differences among parental taxa and hybrids accounted for only 3.72% of the variance. The same pattern was observed when the two sites were analyzed separately. In Site A, 94.5% of the variance was due to variation within the taxa; 95.3% in Site B. If the hybrids were eliminated, variation was slightly reduced within taxa (90.32% and 89.17%); however, levels of variation within the species themselves remained high.

DISCUSSION

Our results show conclusively that hybridization is occurring between *V. grahamii* and *V. hookeriana*. Phenetic analysis of leaf shape characters in the species, more exploratory than exhaustive, showed separation among *V. hookeriana*, *V. grahamii* and the hybrids. Hybrid individuals formed a distinct group from the parental taxa suggesting that leaf

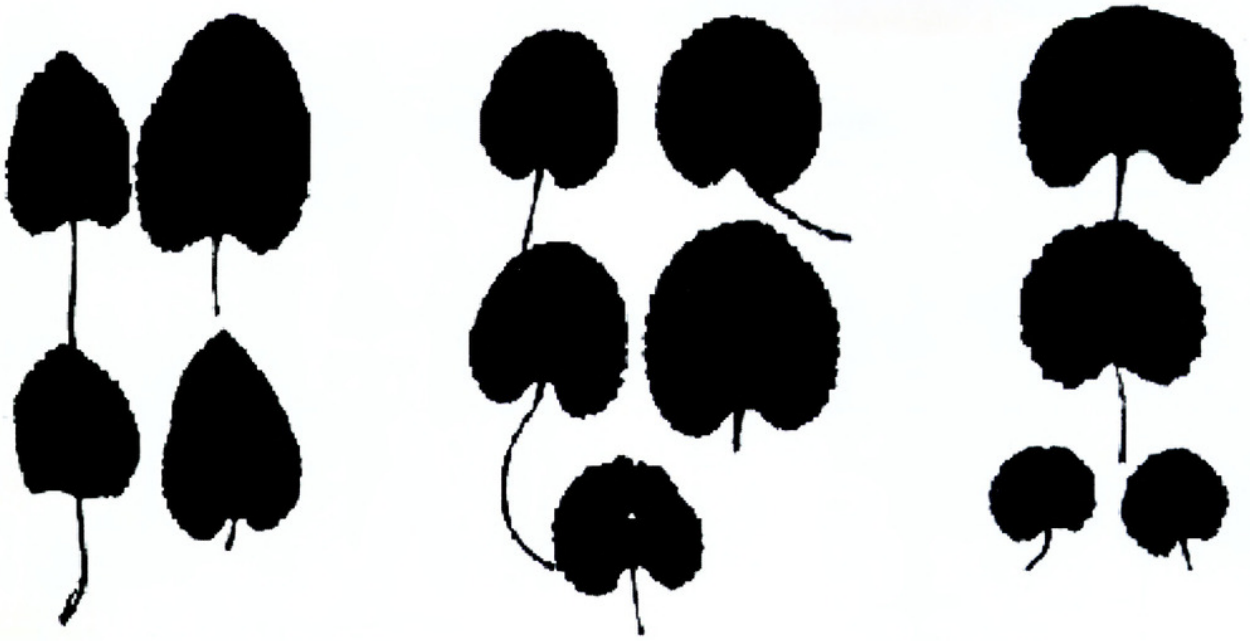

FIG. 3. Representative leaves of *Viola grahamii V. grahamii* μ *hookeriana*, and *V. hookeriana*.

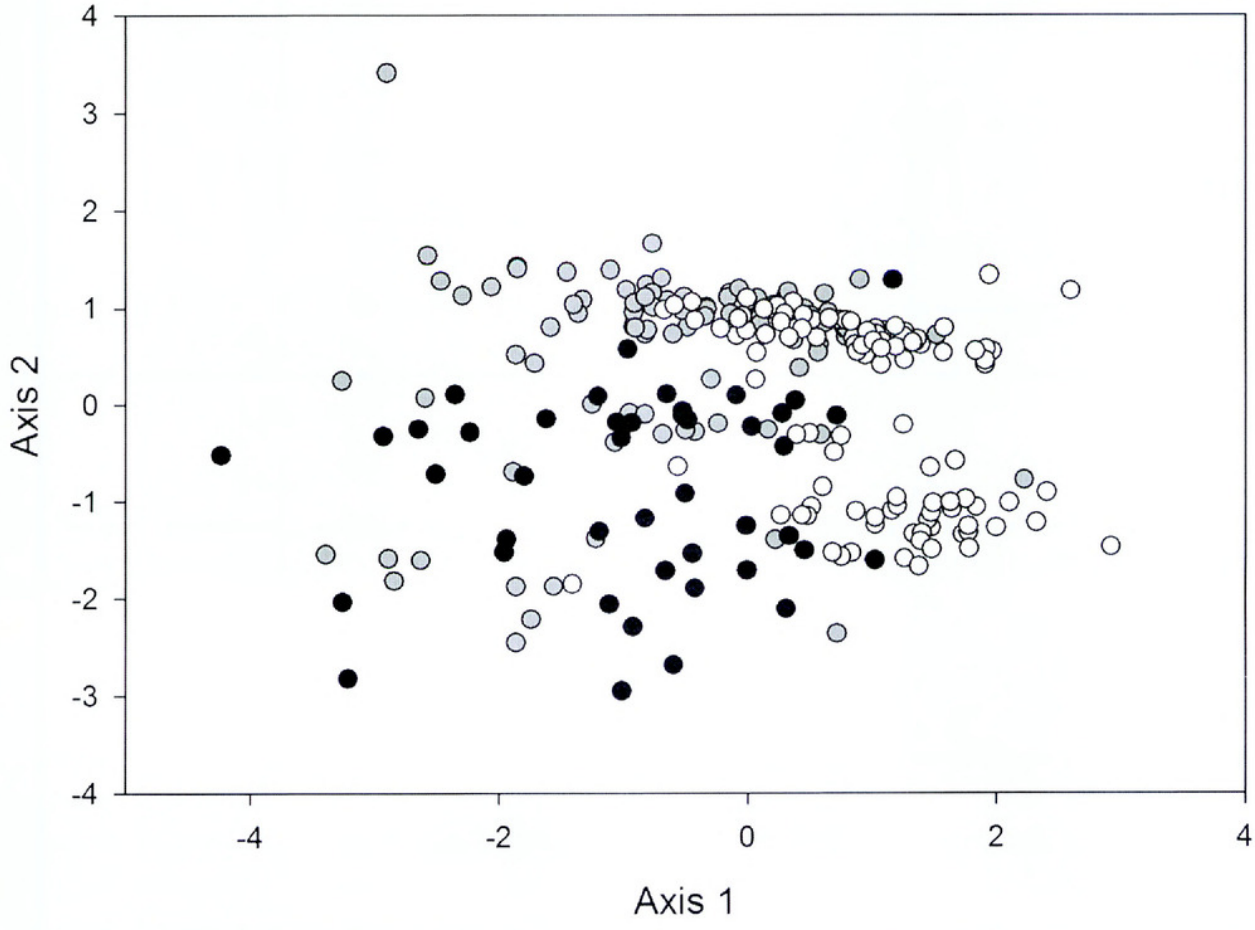

FIG. 4. Results of discriminant analysis of leaf shape from individuals of *Viola grahamii* (white dots), *V. hookeriana* (black dots) and *V. grahamii* × *hookeriana* (gray dots).

TABLE 3. Number of ISSR bands found per site per taxon (*V. hookeriana*, *V. grahamii* and hybrids), as well as number of bands shared among the taxa.

Number of bands	SITE A	SITE B
Viola hookeriana	3	1
Viola grahamii	5	5
hybrids	3	9
V. hookeriana and hybrids	1	2
V. grahamii and hybrids	11	17
All individuals	46	20

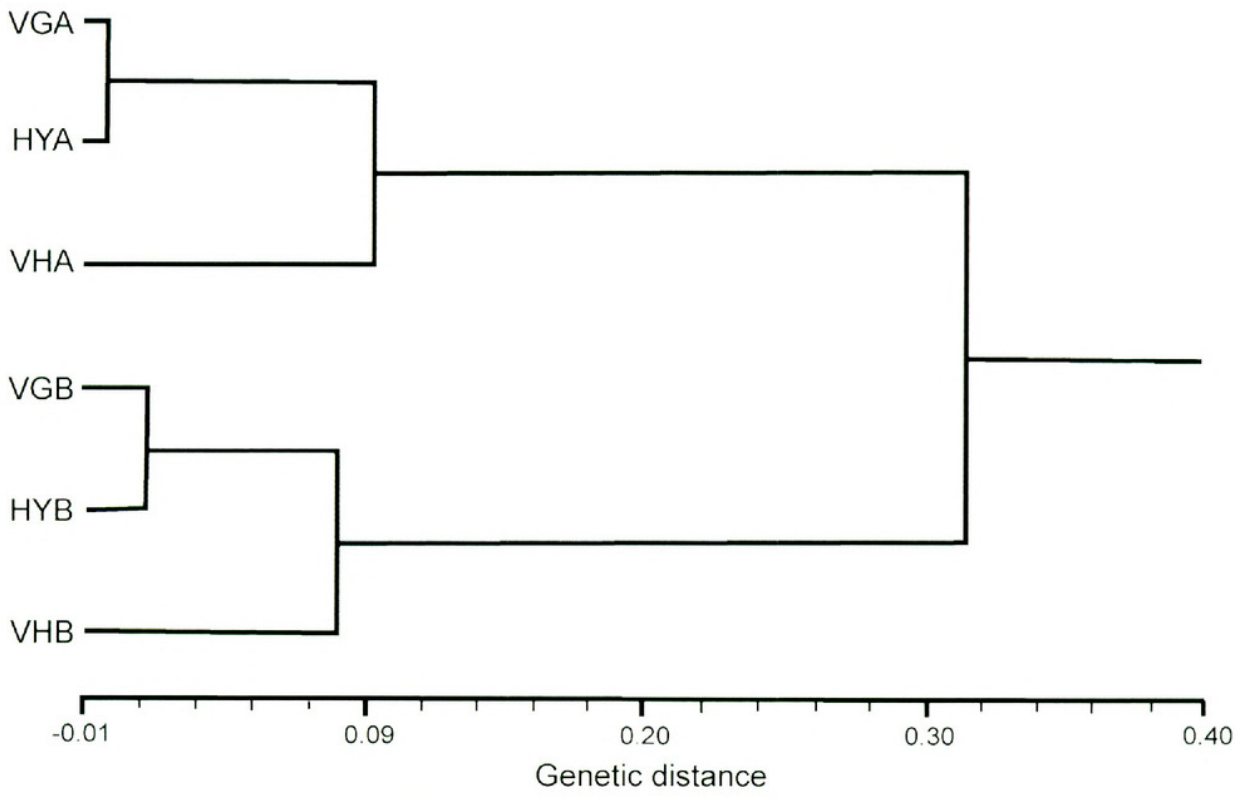

FIG. 5. UPGMA dendrogram depicting relationships among taxa and transects. Similarity compared using the AMOVA-derived PhiST distance matrix. (VHA = *Viola hookeriana* in Site A, HYA = hybrids in Site A, VGA = *V. grahamii* in Site A; the same format for Site B).

traits are useful in distinguishing the hybrids from the pure species, similar to the findings seen in other *Viola* (Russell 1954; Jonsell et al. 2000). While these differences appear to follow a clear pattern, our potential discovery of introgressant individuals may actually blur the distinction to some degree. Extensive gene flow was confirmed, and has proceeded to the extent that the two sites harbored substantially different genotypic combinations. At both sites, numerous morphologically "pure" parental individuals of the two species were demonstrated to be cryptic hybrid derivatives, with a preponderance of these resembling *V. grahamii*. High levels of genetic variability within taxa may be the product of long-continued gene flow. The results from AMOVA suggest that around 95% of the genetic variation is due to variability within each taxon. While this high level of variation could be partly artifactual, owing to rapid evolution of ISSR primer sites, it is

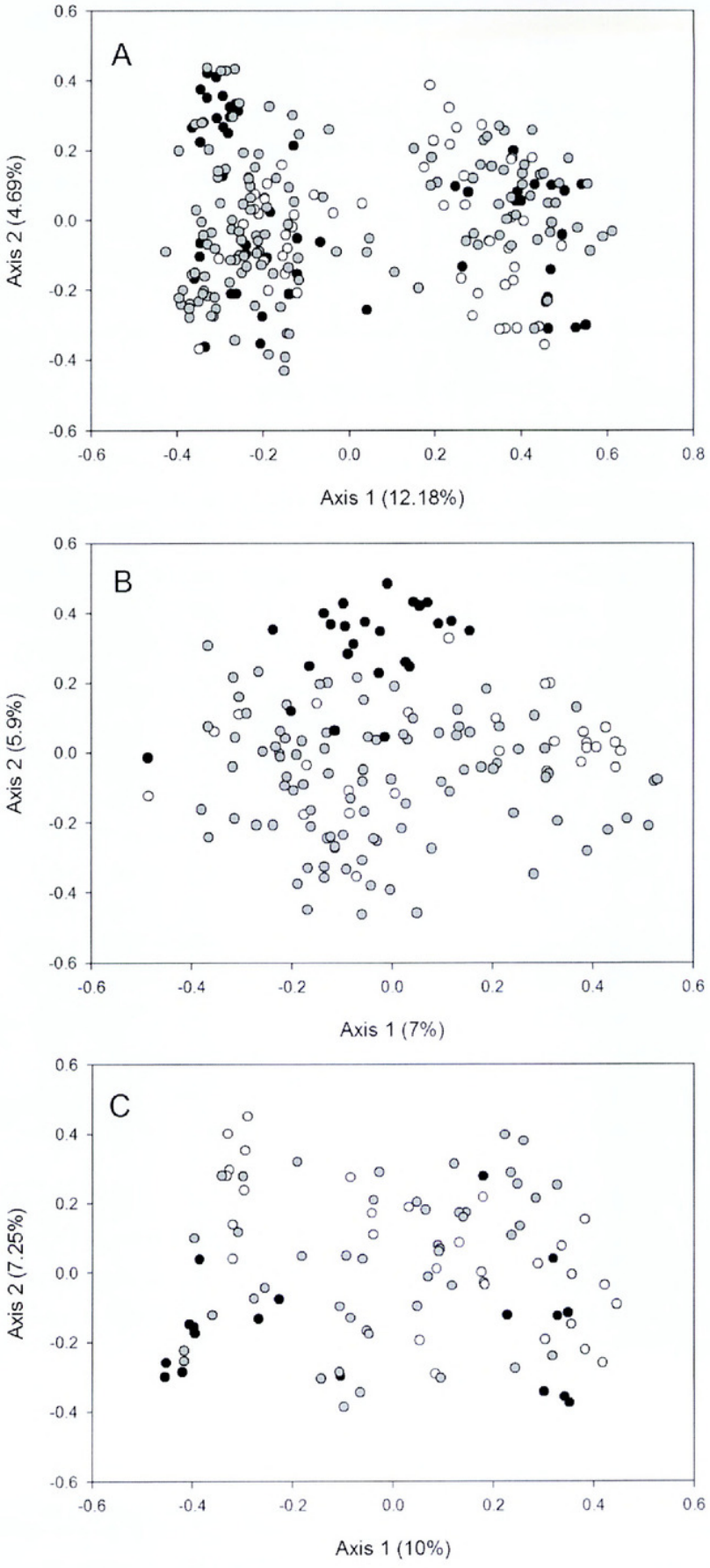

FIG. 6. **A)** Principal Coordinate Analysis (PCoA) of genetic data using the Jaccard similarity coefficient for individuals collected in both sites (A and B). **B)** PCoA of individuals collected in Site A. **C)** PCoA of individuals collected in Site B. Percentages on each axis indicate percentage of total variation explained by that axis. *Viola grahamii* (white dots), *V. hookeriana* (black dots) and *V. grahamii* × *hookeriana* (gray dots).

TABLE 4. Analysis of Molecular Variance (AMOVA) of 242 individuals collected from both sites (A and B), based on 2000 permutations. Mean squares (MDS), variance component and percentage of variance as well as P values are presented. A nested design was performed.

Source of variation	df	MDS	Variance component	Percentage	*p*
Between sites	1	286.41	2.30	26.79	< 0.0005
Among taxa	4	18.38	0.32	3.72	< 0.0005
Within taxa	236	5.98	5.98	69.49	< 0.0005
	241				

more likely that hybridization and subsequent gene flow have proceeded to the point that many morphologically "pure" parental individuals are actually products of extensive gene flow, bridging the gap between parents and hybrids and obscuring taxon boundaries.

Additional genetic evidence suggesting that the hybridization between *V. grahamii* and *V. hookeriana* has likely been occurring over a long period of time is the presence of ISSR fragments unique to the hybrids. It has been suggested by various authors that later-generation backcrosses might contain only a small number of the fragments from the parental species (O'Hanlon et al. 1999). If this is the case, the development of fragments that are "specific" to the hybrids are best interpreted as the result of later generation recombination and long-term post-hybridization genetic reorganization, as suggested by O'Hanlon et al. (1999). Studies of introgression in German violets conducted by Neuffer et al. (1999) using RAPDs describe a similar phenomenon in the *V. riviniana* Rchb.—*V. reichenbachiana* Jord. ex Bor. complex, but the fact that no unique hybrid bands were found led them to conclude that the hybrid complex had a recent origin. Directional backcrossing has also been suggested towards *V. lutea* subsp. *sudetica* (Willd.) Nyman in hybrids of this species and *V. tricolor* L. (Krahulcová et al. 1996). In this study, the presence of many "hybrid-specific" bands suggested a significant time component in the formation of hybrid derivatives as well as differentiation in some. Our results suggest that introgression is occurring between *V. grahamii* and *V. hookeriana*, and that hybridization might have taken place thousands of years in the past or may occur only rarely, but high levels of interfertility and appropriate site conditions have allowed the hybrids sufficient time to backcross with the parental taxa.

Hybridization has likely been favored by the absence of a temporal reproductive isolation between the two species. Despite a minor, non significant, shift in blooming times of the two species (between populations) they have greatly overlapping blooming times, which provide extended opportunities for inter-taxon crossing. This small shift in blooming may be related to differences in microsite characteristics and levels of human perturbation such as wood cutting for local uses, which were more pronounced in Site B than in Site A. The differences in canopy openness between the two sites due to human disturbance can also explain why *V. hookeriana* was not common in Site B, while *V. grahamii* was very abundant. Open environments with more light favor *V. grahamii*, which grows best in open-canopy forest or open sites while *V. hookeriana* is most abundant in more shaded environments, mainly under the forest canopy. Other differences were found in terms of soil requirements; *V. hookeriana* grows in higher concentrations of P and lower concentrations of K than does *V. grahamii*. Soil nutrients in the area however are highly heterogenous (Cortés-Palomec 2005), and while they may be affecting the distribution

of *V. hookeriana* and *V. grahamii*, the hybrids appear to survive in various soil conditions, accounting for their wider local distribution in each site, especially in areas with moderate to heavy levels of disturbance. It is commonly suggested that since hybrid individuals have partial genotypes from both parental species they can potentially grow in both parental habitats as well as in their own "intermediate" niche and even lead to the extinction of the parental taxa if one of them is rare (O'Hanlon et al. 1999; Levin et al. 1996). In fact, hybrids between *V. tricolor* and *V. lutea* subsp. *sudetica* have been suggested to have a wider range of distribution than both parental species (Krahaulcová et al. 1996). In addition, disturbance can lead to "hybridization of the habitat" (Anderson 1954; Rieseberg & Ellstrand 1993), resulting in a mosaic of microhabitat conditions that favor hybrids and in extreme cases may cause the local extinction of one or both of the parental taxa (Rieseberg & Ellstrand 1993; Rhyme & Simberloff 1996). Hybrids in this case can be compared to alien plants in the sense that they are new elements in the flora that can threaten the original flora of the region (Neufer et al. 1999). Perturbation was high in both study sites, mainly due to human activity (i.e., livestock grazing and timber harvest). If we consider our sampling of the study sites as representative for the region, then the hybrids may be said to be as abundant or even more abundant locally in the zone of sympatry than the parental taxa. The heavy genetic overlap of hybrids with *V. grahamii* may threaten that species with local genetic swamping, but since it is widely distributed and occurs as pure populations across large regions of Mexico and northern Guatemala that is probably not a problem. Given the large number of hybrid individuals between the two species at the sites, and the comparatively small number of *V. hookeriana* plants at any given site, however, the latter species may indeed become imperiled (Levin et al. 1996).

Sharing of similar flowering times has likely favored pollen movement between the two species, however pollinator activity would play a key role in successfully moving the pollen. During the year this study took place, several potential pollinators were seen in the area of study, however none of them were observed visiting *Viola*, a finding which seems to be abnormal. Similar observations on blooming individuals of one of the species (*V. grahamii*) over the next two subsequent summers did however reveal the presence of the golden banded skipper (*Autochton cellus* Boisduval and Le Conte, Hesperiidae) and an undescribed species of bee of the genus *Dianthidium* (*Adanthidium*) in the Megachilidae (Griswold, T. pers. com.). The skipper does not seem to be very specific and visits several species showing white flowers (Cortés-Palomec 2005), but the bee does appear to only frequent *Viola* and it could be favoring gene flow between these two species. More specific studies of the pollinators would be needed to better understand their behavior.

In conclusion, we have shown that hybridization occurs between *Viola grahamii* and *V. hookeriana*, and that the hybrids are morphologically distinct and intergrading from the parental taxa. Genetically, despite a larger similarity to *V. grahamii*, the hybrids have unique alleles. Ecologically, hybrids have a wider distribution and environmental tolerance than any of the parental taxa, and at least in the area of study they are much more abundant than *V. hookeriana*. Similar flowering phenologies and effective pollen movement between the species has likely favored hybridization over a long period of time between the two species.

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