GENETIC RELATIONSHIPS, MORPHOLOGICAL DIVERGENCE AND ECOLOGICAL CORRELATES IN THREE SPECIES OF THE *VIOLA CANADENSIS* COMPLEX IN WESTERN NORTH AMERICA

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Genetic Relationships, Morphological Divergence and Ecological Correlates in Three Species of the *Viola canadensis* Complex in Western North America (209 pp.)

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Viola flettii, *Viola cuneata* and *Viola ocellata* are sister species within the *Viola canadensis* complex (Violaceae). All are endemics of western North America, growing in widely divergent ecological environments. During the summers of 1998, 1999, 2001 and 2002, leaf material for DNA extraction was collected from 26 populations of the three species, including much of their range. Analysis of *V. flettii* DNA using intersimple sequence repeat (ISSR) markers showed a great deal of diversity with percent polymorphic loci (P) of 65% and a disjunction between northern and southern populations. Statistical analysis of collected ecological data from *V. flettii* indicated a microhabitat effect of greater elevation and more southernly aspect leading to lowered genetic diversity and population size, respectively. Preserving the genetic diversity in *V. flettii* by protecting populations in both regions with emphasis on those at more optimal microhabitats will aid in maintaining the current fitness ability of this endemic species. Nested Clade Analysis (NCA) of Polymerase chain reaction - restriction fragment length polymorphisms (PCR-RFLP) data from chloroplast regions of all three species showed no distinct groupings based on taxon assignment, potentially indicating past hybridization and chloroplast capture during the early stages of speciation or subsequently and repeatedly within Pleistocene refugia harboring all three species. Contrary to the

evidence suggesting hybridization and interspecific gene flow, ecological, environmental, leaf morphology and leaf angle data all show the three *Viola* species to be strongly distinct, supporting the idea that the three species are morphologically and ecologically well differentiated.

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Chapter 1: Overview and Introduction

Viola fletti, *Viola cuneata* and *Viola ocellata* are endemic species to different areas of western North America. They are morphologically differentiated and adapted to very different environmental conditions. They also represent three versions of rarity as classified by Rabinowitz (1981). All three species occur in geographic areas where endemism is common due to past historic events.

All three species are members of the Canadensis complex which along with the widespread *V. canadensis* has a number of endemic species. Based on ITS phylogeny *V. canadensis* was found to be of recent origin while and the endemic species were palo-endemic (Ballard *et al* 1997). Basal among these palo-endemics is the clustering the three Pacific Northwest endemics, *Viola fletti*, *Viola cuneata* and *Viola ocellata* studied here. Thus the group was thought to have a presumably ancient origin speciating perhaps a few million years ago, and with the known presence of glacial refugia in area as late as the Pleictocene ossibly a unique genetic structuring among and between the three species.

Viola flettii occurs in rock crevices and talus slopes in alpine and subalpine environments of the Olympic Mountains in Washington State. Its growing season is short, lasting only from June to August, but temperatures can remain extremely low. Populations are found in the rain shadow in the eastern half of the Olympic Mountains, from elevations of 1,340 to 1,980 meters, and may be more common on the south, drier sides of mountains. The Olympic Mountains possess steep moisture gradients and sharp elevational gradients that produce a variety of microhabitats (Peterson *et al* 1997). *Viola flettii* has both a very small range (only the eastern portions of the Olympic Mountains)

and a limited distribution within that range, being present in small, isolated populations limited to specific microhabitats.

Viola cuneata occurs in serpentine areas of the mountains in Northern California and Southwestern Oregon. Serpentine substrates are known for high Magnesium and heavy metal (Iron, Nickel, Chromium and Cobalt) concentrations, low Calcium concentrations, and low nutrient levels. The landscape is often barren, with rocky soil and only a thin forest overstory of serpentine-restricted conifers. *Viola cuneata* has an elevational range of 365 to 1,525 meters, occurring on higher elevations in the southern half of its range. There appears to be a trend towards ecological differentiation between southern and northern populations, and both locations were studied. Large populations (greater than 1,000 individuals) of the species exist and the species is common in specific habitats of Josephine County Oregon. *Viola cuneata* has small range, however, can be common in particular locations within that range.

Viola ocellata occurs throughout the coastal mountain from central California to central Oregon in a variety of forested areas including redwoods. These forests have well-developed organic soils and higher moisture and shade compared to *V. flettii* and *V. cuneata*. *Viola ocellata* is found from elevations of 0 to 1,067 meters (Munz 1959). It appears to be the most physiologically generalist of the species, and is found on limestone outcrops as well as serpentine areas. A form of rarity called sparsity is represented by a species with a large range that still occurs infrequently throughout it. While the range of *V. ocellata* covers much of the costal ranges, it occurs only

sporadically throughout it in small, isolated populations, and is exemplified by the "sparsity" form of rarity.

Objectives. Smaller, isolated and peripheral *V. flettii* populations were expected to show less genetic diversity and more unique alleles with the Intersimple Sequence-Repeat (ISSR) marker system. The populations at summits and in more extreme environments were predicted to have reduced vigor and fitness, reduced genetic diversity and increased genetic differentiation. Evidence of gradients in vigor and genetic diversity correlating with population and community ecological characters was sought.

Chloroplast differentiation, which is maternally inherited, was compared between the three closely related endemics *V. flettii*, *V. cuneata* and *V. ocellata* and its implication in the evolution and speciation of these species was attempted. A high level of between population genetic differentiation and low level of within population genetic differentiation was predicted overall, with populations at the geographic center of species range showing the opposite in relation to the rest of the surveyed populations. Populations in closer proximity were expected to be more genetically similar.

By characterizing morphology, habitat and evolutionary ecology environmental factors that may have spurred diversification and evolution of these three species was sought. Soil material from *V. cuneata* sites should differ from that of *V. fletti* and *V. ocellata* in having lower Calcium and higher Magnesium and heavy metals (Iron, Nickel and Chromium). Leaf morphology between species was predicted to differ as well as leaf base morphology, leaf angle, elevational ranges and percent overstory coverage. The manipulation of leaf angle, such that it was held opposite from naturally found, was

predicted to cause a raise in leaf temperature for *V. cuneata* and lower leaf temperatures for *V. flettii* and *V. ocellata*. Associations between phylogenetic data and ecological and climate data was expected to display associations.

In synthesis, these studies should provide a clearer picture of the evolution and past history of the three *Viola* species, ranging from the genetic diversity and ecology of populations of *V. flettii*, to characterization of morphological and ecological differentiation in all three species and their context as key components of speciation.

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Chapter 2: The Conservation Biology of the Olympic Mountain Endemic *Viola flettii*

Abstract

Viola flettii (Violaceae) is endemic to the Olympic Mountains in Washington State where it is limited to isolated populations in potentially rare and specific microsites. The population diversity and genetic structure of *V. flettii* were examined using Intersimple sequence repeat (ISSR) markers; three ISSR primers yielded 60 loci, all of which were polymorphic. Population vigor was estimated from leaf numbers and population size. Environmental variables of elevation and aspect were documented for each population. Genetic diversity estimates and population vigor were analyzed with respect to ecological site variables to infer relative fitness and microhabitat influences on the maintenance of genetic diversity. Most populations were genetically distinct, and all populations fell into two genetically divergent clusters roughly representing two geographic areas of the Olympic Peninsula. Populations expressed a great deal of unexpected genetic diversity. Substantial differentiation of populations despite sometimes short distances between them, relatively small size in most populations, and long-term maintenance of genetic differences between two sets of populations perhaps dating back to Pleistocene separation, suggest relatively low gene flow among populations. Populations at the upper elevational limit of the species tend to have lower genetic diversity, indicating possible genetic erosion due to reduced fitness. Substantial population vigor and reproductive success of most populations permit this narrow endemic to persist successfully despite limited geographic distribution, patchy

occurrence, narrow preference for rock substrates and predominately small population size.

Introduction

Viola flettii Piper (Violaceae) is an endemic plant of the Olympic Mountains in Washington State. It is a small, stemmed perennial herb that grows in rock crevices and talus slopes in subalpine and alpine areas. Potentially high microhabitat fidelity, complete restriction to certain well developed rock exposures, harsh climatic conditions, small size of most colonies and substantial geographic isolation among them, make this endemic plant potentially prone to extreme inbreeding, strong population subdivision with low inter-population gene flow, and local extinctions.

The goal of this study was to examine potential correlations among population genetic diversity, reproductive output, morphological differences, population size, and ecological site characteristics. Several other endemic species, most of them listed as imperiled at the state or federal level, grow near or with *Viola flettii*. Therefore, studies of this violet may provide insights into the population ecology and status of other Olympic Peninsula endemics of similar subalpine and alpine habitats.

It was hoped that correlations between genetic diversity and site ecology would reveal critical new information about the population biology conditions that promote high genetic diversity in the area. Genetic diversity is important for population and species survival, especially in relation to changing weather conditions. The discovery of site characteristics that promote greater genetic diversity and fitness will aid in protection and conservation by identifying factors to note and monitor in the field. The study had the potential to increase our knowledge concerning environmental influences and genetic constraints or consequences on endemism in alpine plants generally. Clarification of

genetic variation patterns and population biology of *V. flettii* would also provide insights into evolution of species in the *Viola canadensis* complex in North America.

Species Studied. *Viola flettii* is a member of the *Viola canadensis* complex (Violaceae). It is a small robust, perennial herb that consists of an underground rootstock and a rosette of leaves that produces a stem with cauline leaves and flowers. It is found in rock crevices and talus slopes in subalpine and alpine areas of the Olympic Mountains. The species blooms from June to August, is presumably insect pollinated (probably by bees), and has a reddish-violet corolla with a yellow throat. Other *Viola* species have limited seed dispersal by ants and/or explosive seed capsules (Beattie & Lyons 1975), and the same may be true of *V. flettii*.

Viola flettii is endemic to the Olympic Peninsula and is legally protected in the Olympic National Park and Olympic National Forest. Populations are located in the drier eastern portions of the mountains from elevations of 1340m to above 1980m. The species has not previously been studied and little is known about its population biology or genetic diversity.

Endemic Species and Genetic Diversity. Narrow niche, low genetic diversity and limited dispersal can lead to endemism in plants (Kruckeberg & Rabinowitz 1985). *Viola flettii* possesses all of these characteristics. Studies have shown that endemic species commonly have lower genetic diversity than widespread ones (Karron 1981, Karron 1987, Soltis & Soltis 1991). This is thought to be due to small population sizes, low gene

flow, inbreeding depression, founder effects and historical bottlenecks (Ellstrand & Elam 1993, Westerbergh & Saura 1994, Godt *et al.* 1996, Gerard *et al.* 1998). Low genetic diversity is thought to lower fitness and thus affect the survival of individuals, populations and possibly even species. Small population size and low genetic variation have been correlated with offspring fitness (Oostermeijer *et al.* 1994).

The fixation of deleterious alleles can cause a threat of extinction in small populations, especially where a once-larger population becomes highly fragmented (Lande 1994). Outcrossing, to a limited extent, is still present even in primarily selfing plants, where it may provide the genetic variation needed for changing environments (Schemske & Lande 1985). Inbreeding depression has been shown to be significantly correlated with primary selfing rate, and self-fertile plants have reduced negative impacts from inbreeding depression on fitness in terms of seed production, germination and survival (Husband & Schemske 1996).

However, it is possible that species occurring naturally in small, sparsely distributed populations have adapted genetic systems to deal with the problems of inbreeding (Barrett & Kohn 1991). Inbreeding can lead to purging of the genetic load, causing a rebound in fitness (Crnokrak & Barrett 2002). Selfing as a form of inbreeding can eliminate lethal and semi-lethal mutations, but recurrent population bottlenecks and pollinator failure could lead to similar results (Schemske & Lande 1985). Short-term genetic purging may not eliminate weakly deleterious alleles (Willis 1999) and may be inconsistent (Byers & Waller 1999). Additive genetic variance contributes to the evolutionary load in continuously reproducing populations (Lande & Shannon 1996).

Viola flettii is expected to have low genetic diversity within populations and substantial differentiation among them, due to the small size and often relatively great geographic distance between the populations as well as the low likelihood of extensive gene flow by pollinator movement or seed dispersal. However, this is a hypothesis which requires testing. Even if the species does manifest low genetic diversity and high inter-population differentiation, it is still possible that it has adapted ways to counteract any potentially disadvantageous consequences. Comparisons of island populations with mainland populations of *Aquilegia canadensis* showed no difference in the association between population size and reproductive output, even though island populations have a greater level of isolation (Mavraganis & Eckert 2001). No difference in fitness due to inbreeding depression was found between central and isolated peripheral populations in *Clarkia concinna* (Groom & Preuninger 2000). Small populations may, in fact, be important for conservation as ecological stresses, even at low levels, induce the loss of heterozygosity more slowly than populations in more benign environments, i.e., those in the primary range of the species (Lesica & Allendorf 1992).

Elevational Effects. Vegetative reproduction and self-pollination are predicted to be more common in stressful environments such as high elevation sites. Leaves and stems are commonly smaller in higher elevation plants (Emery *et al.* 1994, Cordell *et al.* 1998). Reproductive responses of plants to elevational gradients have been linked to environmental influences (Bauert 1993). Self-pollination may be more common at higher elevations due to limited pollinator activity, as has been shown in *Glycine clandestina*

(Schoen & Brown 1990). However, this is not always the case, as a study of *Saxifraga oppositifolia* at different elevational levels has shown (Gugerli 1998). In investigations on *Stellaria longipes*, gradients in morphology related to genetic similarity more than elevational gradients, while harsher environmental conditions at higher elevations caused less phenotypic plasticity in morphology (Emery *et al.* 1994). Leaf morphology of *Metrosideros polymorpha* was found to be genetically constrained, whereas physiological differences were plastic at different elevations (Cordell *et al.* 1998).

The Olympic Mountains. The Olympic Mountains are located in the northwest corner of Washington State on the Olympic Peninsula. They comprise a coastal range that has been separated from the rest of the continent by glaciers during the Pleistocene glacial advances, leading to the stranding of populations and the evolution of several highly restricted species in the region. Eight plant species are endemic to the Olympic Peninsula and six additional plant species are endemic to the Olympic Peninsula plus nearby Victoria Island. *Viola flettii* is likely a relic species with a formerly broader range, which survived past glaciations by adapting to the harsh alpine conditions present at high elevations in the Olympic Mountains (Peterson *et al.* 1997). A strong southwestern to northeastern rainfall gradient throughout the Olympic Peninsula, together with heterogeneous bedrock substrates and diverse topography, provides an extensive patchwork of microclimates throughout the region to support the growth and diversification of various angiosperm groups (Peterson *et al.* 1997).

The populations of *V. flettii* present in the Olympic Peninsula are found primarily in the eastern and central parts of the mountains in the rainshadow of the mountain range (Figure 2.1). Populations are located in both the Olympic National Park and the Olympic National Forest. There are four main mountainous areas where the species grows: Skokomish-Duckabush and Wynochee areas in the southeast, Constance-Buckhorn areas in the east, Gray Wolf-Dosenwallips and Hurricane Ridge areas in the northeast, and Olympus-Bailey and Quinault areas in the center. Populations are found at elevations of 1340m to above 1980m.

Intersimple Sequence Repeat (ISSR) Markers. The molecular method chosen for this study is Intersimple Sequence Repeat (ISSR) markers, which are RAPDs-like in accessing anonymous variation throughout the nuclear genome and thus circumventing the challenge of characterizing individual loci that other individual microsatellite loci and certain other approaches require. Unlike Random Amplified Polymorphic DNA (RAPD) markers, however, ISSR primers amplify the intervening stretch of DNA lying between a pair of proximal tandem repeat areas (microsatellites) on complementary strands, usually 750-3500 bp distance from each other. Microsatellites are very short (usually 10-20 basepair) stretches of DNA that are "hypervariable" and characterized by mono-, di- or trinucleotide repeats. ISSR primers specifically have di- or trinucleotide tandem repeats embedded within them, and thus only anneal to nuclear regions.

ISSR markers are dominant and are scored as present or absent, yielding data which are amenable to phenetic (distance) methods of analysis. Only small amounts of

tissue are needed for this technique, and dried material can be used. For species, such as *V. flettii*, that are difficult to access and grow in remote locations, obtaining fresh leaf material for isozymes or other equivalent methods is impractical or impossible. The ease of silica gel-preservation makes the ISSR approach particularly desirable.

Most ISSR studies have revealed sufficient to abundant loci for genetic "fingerprinting" and estimation of genetic diversity using only two to four primers (Hodkinson *et al* 2002, McCauley & Ballard 2002, Crawford 1997, Marsh & Ayers 2002, Ge *et al* 2003, Smith & Bateman 2002, Esselman *et al* 1999). ISSR markers have proven to reveal greater levels of diversity than allozymes (Essellman *et al* 1999), are usually more sensitive in accessing higher levels of variation, but are more repeatable than RAPD markers (Marsh & Ayers 2002). They are not as good as AFLP markers (Hodkinson *et al* 2002) for accessing genetic diversity in species with potentially low variation, but they are substantially cheaper and far less complex to apply to a given study species. Given various constraints, the ISSR approach was selected as the genetic method of choice for this study.

Objectives. The following objectives were pursued as testable hypotheses in this study.

1. Smaller isolated (peripheral) populations of *V. flettii* were predicted to possess less genetic diversity than larger populations.
2. Peripheral populations of *V. flettii* were predicted to have a significantly greater proportion of unique alleles.

3. Populations of *V. flettii* on the summits (i.e., at the highest elevations) were predicted to be phenotypically and genetically different from populations in the mountain passes (at lower elevations), expressing reduced plant vigor and size, reduced fitness as expressed in reproductive output, reduced genetic diversity within populations, and greater differentiation among populations.

4. Vigor, reproductive success and genetic diversity were predicted to correlate with population and community ecological characters along an elevational gradient.

Methods

Collection Sites and Times. Field data were collected from the Olympic Mountains during June and July of 1999 and July of 2001. Study sites included six populations, three from the Olympic National Park (1999) and three from the Olympic National Forest (2001) (Figure 2.1). Most populations were separated by sufficient geographic distance (mean of 23.3 Km and median of 14.9 Km) to render negligible any considerations of active gene flow. (This may not be entirely true in the case of the Blue Mountain study sites because of their comparatively close proximity--1.12 Km apart).

Genetic Diversity. One small leaf was collected from each of 30 plants per population (Table 2.1), where possible, and stored in microcentrifuge tubes with silica gel. Since this species has never been observed to reproduce asexually, each individual was considered a genetic individual for the purpose of this study. DNA was extracted from the leaves in the lab using a Wizard® Genomic DNA Purification System (Promega), which helped eliminate the problem of polysaccharides in the final extraction, making the final DNA solution less viscous and easier to accurately pipette.

After screening a series of primers that have be found to work well with other *Viola* species, three primers, 844A [$(CT)_8AC$], 17899B [$(CA)_8GG$] and HB10 [$(GA)_6CC$], were selected to generate genetic diversity estimates. The DNA was amplified using an adaptation of Wolfe's Master Mix (Wolfe *et al.* 1998). The amplification program used with a RoboCycler (Stratagene RoboCycler Gradient 96 Hot Top Combo) was: 94°C for 2 minutes; 40 cycles of: 94°C for 30 seconds, 44°C for 45

seconds, 72°C for 1 min 30 seconds; 72°C for 20 minutes; and 4°C soak forever. Ten ml of the PCR products were electrophoresed with a 250 basepair ladder on a 1.5% agarose "maxi-gel" rig with ethidium bromide stain to separate fragments (Figure 2). Kodak Biomax 1D Image Analysis software was used to identify and obtain molecular weight (converted to basepair length) of bands. Data were scored in a present/absence matrix for analysis.

A series of programs (Apostol *et al.* 1996: RAPDPLD, RAPDFST and RAPDPLOT) created by Bill Black for analyzing RAPD data, an analogous dominant marker system, were used to calculate linkage disequilibrium and F_{ST} values for the entire species and each population, to generate Nei and Li's similarity values, and matching values for cluster analyses. Phylip's Neighbor program (Felsenstein 1995) was used for a bootstrapped Unweighted Paired Group Method with Arithmetic mean (UPGMA) cluster analysis, as well as a neighbor-joining tree of individuals. Percent polymorphic loci (P), ϕ_{st} and genetic distance values were calculated. A mantel test of genetic distance versus geographic distance in kilometers was preformed with TFPGA (Miller 2002). GenAlEx (Peakall & Smouse 2002) was used to perform an Analysis of Molecular Variance (AMOVA) with permutation analysis using populations as well as populations and regions in two separate analyses, since other analyses suggested the existence of southern and northern genetic groupings. A Principal Coordinates Analysis (PCoA) of the data was performed with NTSYS (Rohlf 2002) using the Dice coefficient, in order to examine broader genetic relationships among populations that could not be portrayed adequately from cluster analysis or neighbor-joining dendrograms.

Ecological and Elevation Data. In the summers of 1999 and 2001, 30 plants were sampled in each population where possible (Table 2.1). On each plant, leaf number, flower number and fruit number were recorded as measures of vigor and fecundity. Estimated number of plants per population, elevation, location, aspect and date were recorded as environmental variables at each of the six study sites.

The following exceptions were made in data collection, mostly due to small population sizes. In 1999, the population at Eagle Point (VF3) contained only 13 individuals, 6 adults and 7 juveniles (2 juveniles were too small to collect any material from). The populations at Mount Townsend (VF4) and Mount Ellinor (VF5) both collected in 2001 also had fewer than 30 individuals. Mount Townsend (VF4) had 20 plants, and the visitation date was too early in the season to record peak flowering, so the distinction between adults and juveniles was difficult to evaluate. The population at Mount Ellinor (VF5) was rather small, with only 8 juveniles, all with only a few leaves.

Normality was tested for leaf number, flower number and fruit number data. Due to the differing dates of collection for flower number and fruit number, they were combined into reproductive structure number. The small number of sampled populations, as well as lack of normality or successful correction of normality with transformations, limited the statistical testing available for the correlation of genetic and ecological data. The difference in data type and limited number of studied populations made non-parametric tests the only statistical option. Pearson and Spearman correlations with pair-wise deletions were performed for elevation, Beers-transformed aspect (Beers *et al*

1966), ϕ_{st}, percent polymorphic loci (P), mean leaf number and mean reproductive structure number using NCSS.

Results

Genetic Diversity. Sixty loci were found with three ISSR primers (21 loci for primer 844A, 17 loci for primer 17899B, and 21 loci for primer HB10), all of which were polymorphic (Table 2.2). Overall species percent polymorphic loci (P), with a 95% criterion, was 65% P for individual populations ranged from 56.67% for the Mount Ellinor population to 30% for the Eagle Point and Mount Townsend populations. Overall theta st (ϕ_{ST}) for *V. flettii* as a species was 0.445 (+/- 0.038), and the population ϕ_{IS} values ranged from 0.759 for the Blue Mountain and Eagle Point populations to 0.367 for Marmot Pass population (Table 2.2). The Analysis of Molecular Variance (AMOVA) for populations showed significant differentiation, with 51% attributed to among populations, while the AMOVA for populations and regions showed significant differentiation, with 25% attributed to among regions, 29% among populations in regions, and 49% within populations (Table 2.3). In the Linkage Disequilibrium analysis, which is an extension of Fisher's exact probability test on contingency tables, the average disequilibrium caused by random genetic drift [D(IS)] was much greater (95%) than the average disequilibrium caused by epistasis (4.4%). The mantel test results had a p-value of 0.301, rejecting a relationship between genetic and geographic distances. The northern region contained 10 unique alleles while the southern only possessed 9.

Structuring of Genetic Diversity. UPGMA clustering from both the matching index and similarity index revealed distinct groups of northern populations (Near Blue Mountain, Blue Mountain and Eagle Point) and southern ones (Mount Townsend, Mount Ellinor,

and Marmot Pass). These analyses further showed the Near Blue Mountain, Blue Mountain and Eagle Point populations to be genetically distinct from the other three pouplations, and the Mount Townsend population to be largely distinct from Marmot Pass and Mount Ellinor (Figure 2.3). Neighbor-joining trees showed similar results (not shown), although the trends were not as clear. The PCoA portrayed the separation between northern and southern populations well, with the Mount Townsend population separating from the Mount Ellinor and Marmot Pass populations along the second and third axes (Figure 2.4).

Both the cluster analysis and PCoA indicated that the two populations at the Blue Mountain area, which are the closest geographic populations (1.12 Km distance between), are the most genetically distinct among the six investigated. A few individuals in the Eagle Point and Blue Mountain populations shared some alleles and consequently intermingled (in the cluster dendrogram) or abutted (in the PCoA ordination). Conversely, the geographically remote Mount Ellinor and Marmot Pass populations included individuals with substantial numbers of shared alleles and intermingled to a substantial extent in both analyses; Mount Townsend individuals appeared to share fewer alleles and to overlap to a lesser extent with the first two populations. The Marmot Pass population was the largest sampled and the Mount Ellinor population, the smallest (and likely the newest based on only juveniles present at the site), and the two are geographically further apart than any other pair of proximal populations. Intermingling of individuals in both would suggest that Mount Ellinor represents a fragmentation derivative from Marmot Pass.

Ecological and Elevational Trends. Elevation was negatively correlated with percent polymorphic loci (P) (Table 2.4). Aspect was positively correlated with $theta_{st}$ (ϕ_{st}). Mean leaf number was positively correlated with population size, $theta_{st}$ and mean reproductive structure number. Mean reproductive structure number was also correlated with $theta_{st}$. $Theta_{st}$ was negatively correlated with P. Simple scatter plots of elevation versus mean leaf number and population size show slightly bell shaped curves (Figure 2.5), reflecting that vigor and population size express their maximal responses at middle elevations.

Discussion

Genetic Diversity. ISSR markers are a relatively new technique for genetic diversity estimation, so comparisons of results with other studies on endemic plant species is necessarily limited. The technique also increases the degree of variation revealed by other approaches such as protein electrophoretic variation (Essellman *et al* 1999). Our results, compared with other ISSR studies on endemic species, suggest that *V. flettii* may possess slightly more genetic diversity than is common or expected (although only a few such studies are available). The pattern of population differentiation is also somewhat more extensive than that found in other endemics (Crawford *et al* 2001, Marsh & Ayers 2002, Ge *et al* 2003).

Smaller isolated (peripheral) populations of *V. flettii* were predicted to possess less genetic diversity than larger populations. The three largest populations showed greater amounts of genetic diversity and percent polymorphic loci than smaller populations, as expected. Smaller populations in *V. flettii* were shown to be more highly differentiated than larger ones, with low gene flow, substantial levels of selfing (probably through cleistogamy), increased opportunities for genetic drift, and increased potential effects of environmental stresses, likely fostering divergence of small populations.

Peripheral populations of *V. flettii* were predicted to have a significantly greater proportion of unique alleles. The peripheral population at Mount Ellinor (population 5) in the extreme south of the species range did possess greater unique alleles. The next

most peripheral population at Eagle Point (population 3) in the far northwest of the range, however, shared much of its alleles with the other two northern populations at and near Blue Mountain. Northern populations, which are closer geographically, showed barely more unique alleles than southern populations. Thus, overall the presence of greater unique alleles in perpherial populations seems to not always be the case in *V. flettii*.

Populations of *V. flettii* on the higher elevations were predicted to be phenotypically and genetically different from populations at lower elevations. Both elevation, and not as strongly, aspect were correlated with genetic diversity, lending support to the idea that site characteristics may exert substantial influence on population size, maintenance and genetic differentiation. Populations from higher elevations had smaller population sizes and lower genetic variability.

Ecological parameters can affect plants differently at different life history stages. The harsh conditions of high elevations may particularly limit seedling establishment due to the lack of suitable microsites for early plant growth. The short alpine growing season and the slow growth rate of *V. flettii* suggest that several seasons may be required following successful seedling establishment in order for individual plants to reach reproductive age. How a plant's immediate growing conditions affect subsequent mature plant survival and fecundity is likely an important feature of population success. Despite the operation of genetic drift, the inverse correlation of elevation with genetic polymorphism, P, argues for erosion of genetic diversity at higher elevations by environmental selection pressures.

Vigor, reproductive success and genetic diversity were predicted to correlate with population and community ecological characters along an elevational gradient. Microsite conditions such as elevation and aspect have substantial influences on the maintenance of populations and their genetic diversity. As aspect increased from north to south, population size decreased, indicating that south-slope populations experience additional stresses that reduce survivorship, population vigor, reproductive success or a combination of these. Leaf number increased with population size, indicating that individual vigor is related to population vigor, perhaps by increased biomass that ultimately translates into increased fecundity. As leaf number increased so did reproductive structures, meaning larger plants were more reproductively capable.

As expected, P is inversely correlated with ϕ_{st}, representing the relationship between overall species genetic diversity in contrast to among-population genetic differentiation. The strong inverse correlation between population genetic differentiation and population genetic diversity has been postulated and demonstrated in other studies (e.g., Ellstrand & Elam 1993). The positive correlation of leaf number and reproductive structure with ϕ_{st} may be a consequence of past events, or of increasing current isolation and/or genetic drift. Mean leaf number increased as genetic variability increased, and as leaf number is an estimator of plant vigor, this displays a possible fitness response due to greater genetic diversity (and, indirectly, greater population heterozygosity). Population size was correlated positively with mean leaf number, suggesting that population size, population genetic diversity and collective individual fitness have a cumulative synergistic influence on each other.

These results suggest that environmental influences of elevation and aspect, and perhaps many secondary ones such as substrate temperature or local precipitation pattern that were not directly measured, play a key role in determining where the species may grow, how well it can establish populations through time, how fit those populations will be, and also how genetically diverse they will eventually become. Thus, local microsite or microhabitat conditions serve as a selective filter to limit population size, fitness and genetic variation, over a relatively small elevational range of ca. 520 meters.

Structuring of Genetic Diversity. The high genetic diversity seen in *V. flettii* might be the result of fragmentation and range diminution from a formerly much broader ancestral range; the results are inconsistent with population establishment from a small number of initial immigrants from the *V. canadensis* complex and the well known consequences of founder effect. Past regional separation of populations, likely the result of Pleistocene alpine glacial advances over the central mountain ranges, has led to differentiation into two genetic groups of *V. flettii* populations, northern and southern. During the last glacial period, the Olympic Mountains were only partly covered by glaciers, and are thought to have served as a refugium for various species (Peterson *et al* 1997). From the clear separation of populations into two geographic regions, it would appear that *V. flettii*'s range was indeed bisected by central summit glaciers, and that the species' low gene flow and dispersal since then have not been sufficient to counteract the lingering effects of previous isolation and population fragmentation. The Gray Wolf Ridge and the valleys of Gray Wolf River and Dungeness River separate the northern populations from the

Mount Townsend and Marmot Pass populations. This high elevation ridge and its associated valley systems could have contained one or more glaciers separating the northern and southern sets of populations. The fact that *V. flettii* presently contains a great deal of genetic diversity in both northern and southern regions would presumably reflect the substantial variation with the populations must have initially contained.

The lack of a strong association between genetic diversity and geographic distance, the great genetic difference between the two geographically nearest populations, the substantial levels of among-population differentiation, and the high value assigned to genetic drift, all point to low levels of active gene flow. Gene flow restrictions caused by limited pollen and seed dispersal, and the likelihood of significant to high levels of selfing, have probably maintained or even increased the differentiation of populations. Other studies have indeed found limited pollination of species in the area, likely caused by limited pollinator activity overall (Campbell 1987).

Conservation of *Viola flettii*. Certainly, because the regions show a great deal of differentiation between them, protecting populations in both southern and northern regions is important. Protection of individual populations should receive higher priority in that region. In the south, the population from Marmot Pass appears to contain much of the unique diversity of the southern region, thus protecting this population should be a priority. From the ecological data it appears that elevations at the middle of the species range and populations with more southernly aspects have greater genetic diversity and genetic differentiation. These characteristics should also be considered when protecting

populations, in the thought that promoting greater genetic variability promotes greater species fitness and allows the species more genetic resources in dealing with climate change.

Conclusions. A great deal more remains to be studied about this alpine *Viola* species. It is a restricted endemic, with isolated populations, that contain a large amount of genetic diversity. Its more ancient past appears to be well preserved in the pattern of genetic diversity and differentiation, revealing a possible history of isolation and fragmentation under the spread of Pleistocene alpine glaciers, variable effects of drift and environmental selective pressures, probable significant selfing rates, and typically very small population sizes which encourage further among-population differentiation and genetic isolation. This complex organismal scenario merits future research. It also shows that within a narrow elevational range, microsite and microhabitat selection can apparently occur to reduce population size, fecundity and genetic diversity. What the exact components of selection are, and how these impact the morphology, physiology, germination, seedling survivorship and reproduction of *V. flettii*, deserve further investigation.

Chapter 2 Tables and Figures

Table 2.1. Locational and ecological data collected for *V. flettii* populations. Elevation based on map data, aspect taken with a compass, population size count values except for Marmot Pass which was estimated, and average leaf number calculated from 30 or as many plants as available.

Population	Location	Population Size	Elevation (m)	Aspect (degrees)	Mean Leaf Number	SE
1	Near Blue Mountain	62	1760.8	215	16.4	1.707
2	Blue Mountain	87	1612	110	17.3	2.210
3	Eagle Point	14	1891	70	4.5	0.671
4	Mount Townsend	25	1705	250	6.5	0.497
5	Mount Ellinor	9	1364	70	3.4	0.242
6	Marmot Pass	100	1782.5	180	6.6	0.408

Table 2.2. Polymorphic loci (P) and Φst for *V. flettii* populations. Calculated from ISSR present/absence data.

Population	P	Φ_{IS} (jackknifed)	SE Φ_{IS}
1	40%	0.747	0.010070
2	48.3%	0.759	0.010199
3	30%	0.759	0.010199
4	30%	0.630	0.012652
5	56.7%	0.408	0.016267
6	41.7%	0.367	0.011748
Overall	65%	Φ_{ST} = 0.445	SE of Φ_{ST} =0.004906

Table 2.3. AMOVA results for *V. flettii* ISSR data with and without regions included. Southern region included populations 4 (Mount Townsend), 5 (Mount Ellinor) and 6 (Marmot Pass). Northern region included populations 1 (Near Blue Mountain), 2 (Blue Mountain) and 3 (Eagle Point).

Source	Df	SS	MS	Est. Var.	Statistic	Value	Prob.
Among Populations	5	454.349	90.870	4.172	PhiPT	0.518	0.001
Within Populations	123	551.729	4.486	4.486	PhiPT	0.482	0.001
Among Regions	1	247.335	247.335	2.856	PhiRT	0.293	0.001
Among Populations within Regions	4	207.013	51.753	2.411	PhiPR	0.350	0.001
Individuals Within Populations	123	551.729	4.486	4.486	PhiPT	0.540	0.001

Table 2.4. Pearson and Spearman correlations for *V. flettii*.
With pairwise deletions for elevation, aspect, population size, ϕ_{st}, P and mean leaf number for each population. Bold numbers indicate strong significant correlations and underlined numbers indicate weak significant correlations.

		Elevation	Transformed Aspect	Population Size	Mean Leaf #	Mean Reproductive #	ϕ_{st}
Transformed Aspect	Pearson	0.14					
	Spearman	0.12					
Population Size	Pearson	0.25	**0.63**				
	Spearman	0.26	**0.81**				
Mean Leaf Number	Pearson	0.16	0.15	**0.61**			
	Spearman	0.03	<u>0.41</u>	**0.60**			
Mean Reproductive Structure Number	Pearson	0.31	-0.31	0.32	**0.85**		
	Spearman	0.26	-0.06	0.31	**0.83**		
ϕ_{st}	Pearson	<u>0.44</u>	-0.25	-0.07	**0.58**	**0.67**	
	Spearman	0.14	<u>-0.43</u>	-0.12	<u>0.35</u>	**0.75**	
P	Pearson	**-0.84**	-0.13	0.16	0.06	-0.09	<u>-0.45</u>
	Spearman	**-0.70**	-0.10	0.06	-0.20	-0.35	<u>-0.31</u>

Figure 2.1. A geographic distribution map of *V. flettii* populations studied. Populations 1, 2 and 3 were collected from throughout the Olympic National Park in 1999 and the populations 4, 5 and 6 were collected from the Olympic National Forest in 2001. Topographical map provided by DeLorme Topo USA version 4.0.

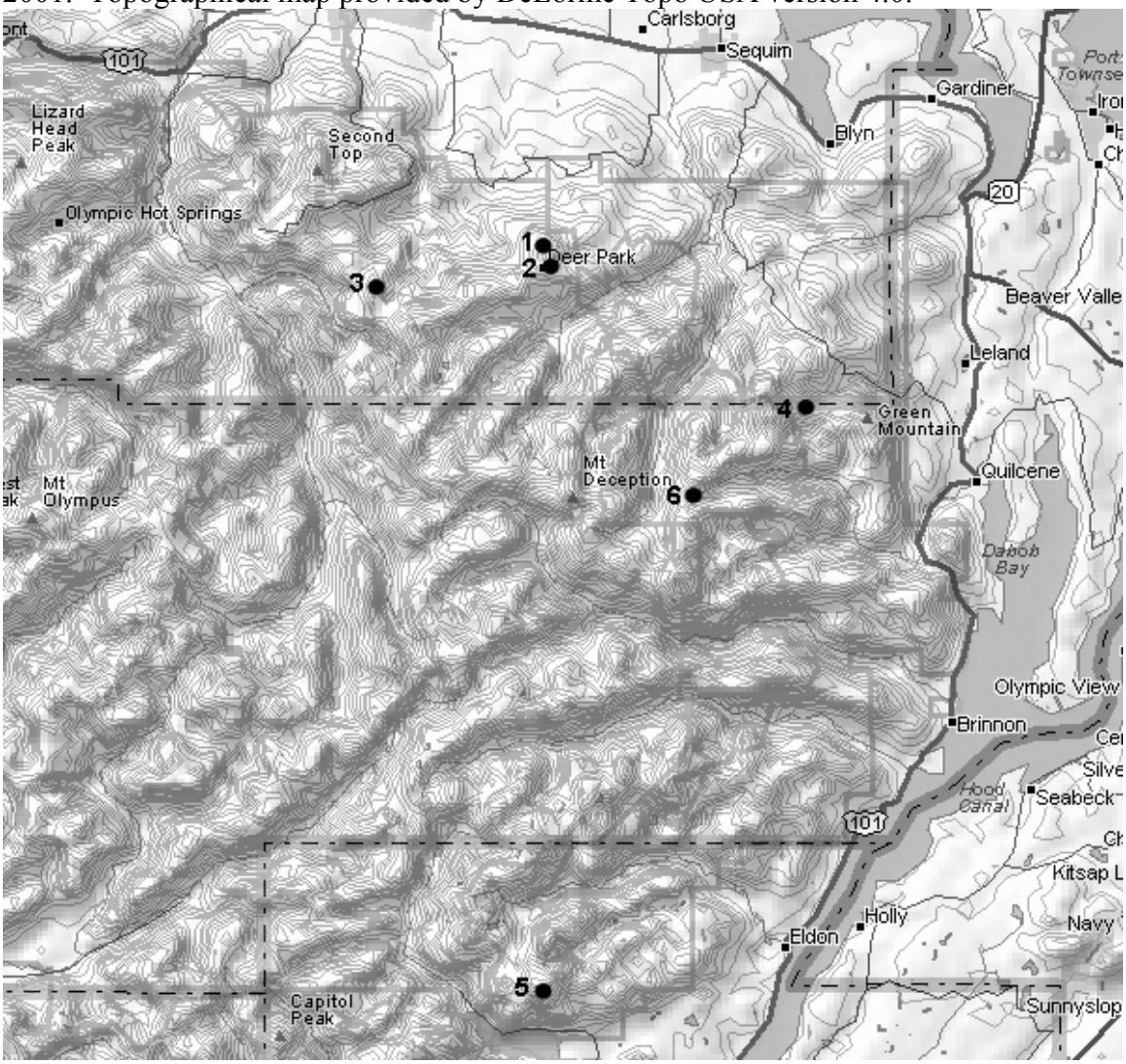


Figure 2.2. Gel showing ISSR fragments.
Fragments amplified for primer HB10 for individuals 21 through 30 of population *V. flettii* 1 from near Blue Mountain. A 250 bp ladder to score the fragment size is on the right and left of edges of gel.

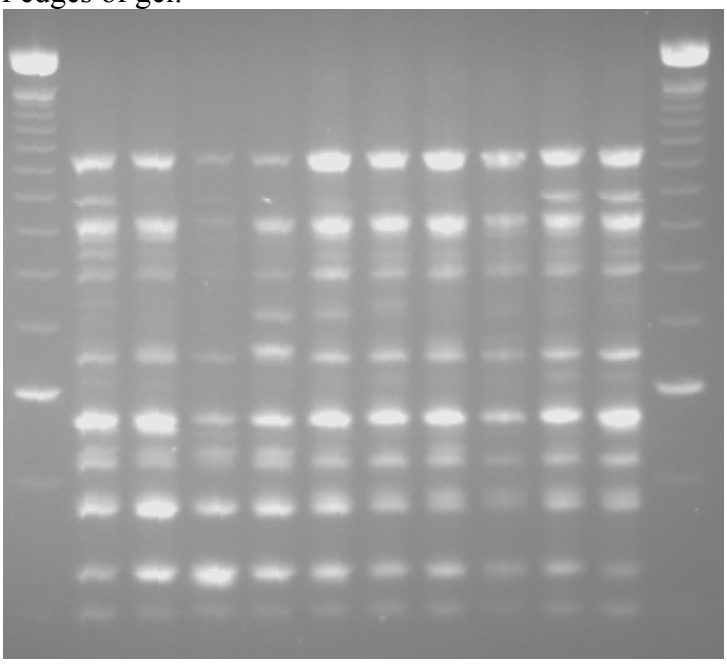


Figure 2.3. UPGMA cluster dendrogram of ISSR data for *V. flettii*.
Grey denotes population 1 (Near Blue Mountain), white denotes population 2 (Blue Mountain), black denotes population 3 (Eagle Point), striped denotes population 4 (Mount Townsend), pyramids denote population 5 (Mount Ellinor) and circled patterns denote population 6 (Marmot Pass).

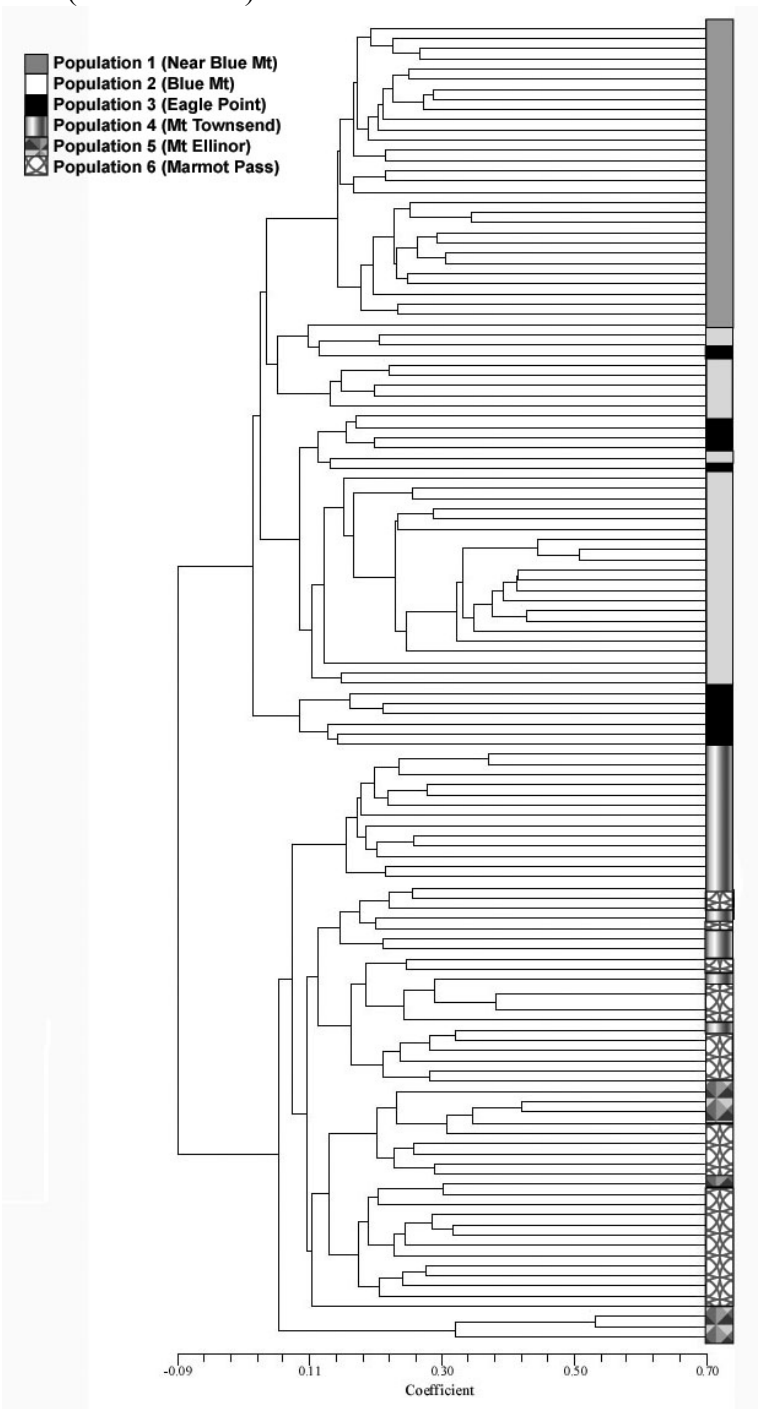


Figure 2.4. The PCoA of the first three axes from analysis of ISSR data for *V. fletti*. Open circles denote population 1 (Near Blue Mountain), stars denote population 2 (Blue Mountain), gray squares denote population 3 (Eagle Point), black squares denote population 4 (Mount Townsend), gray circles denote population 5 (Mount Ellinor) and black circles denote population 6 (Marmot Pass).

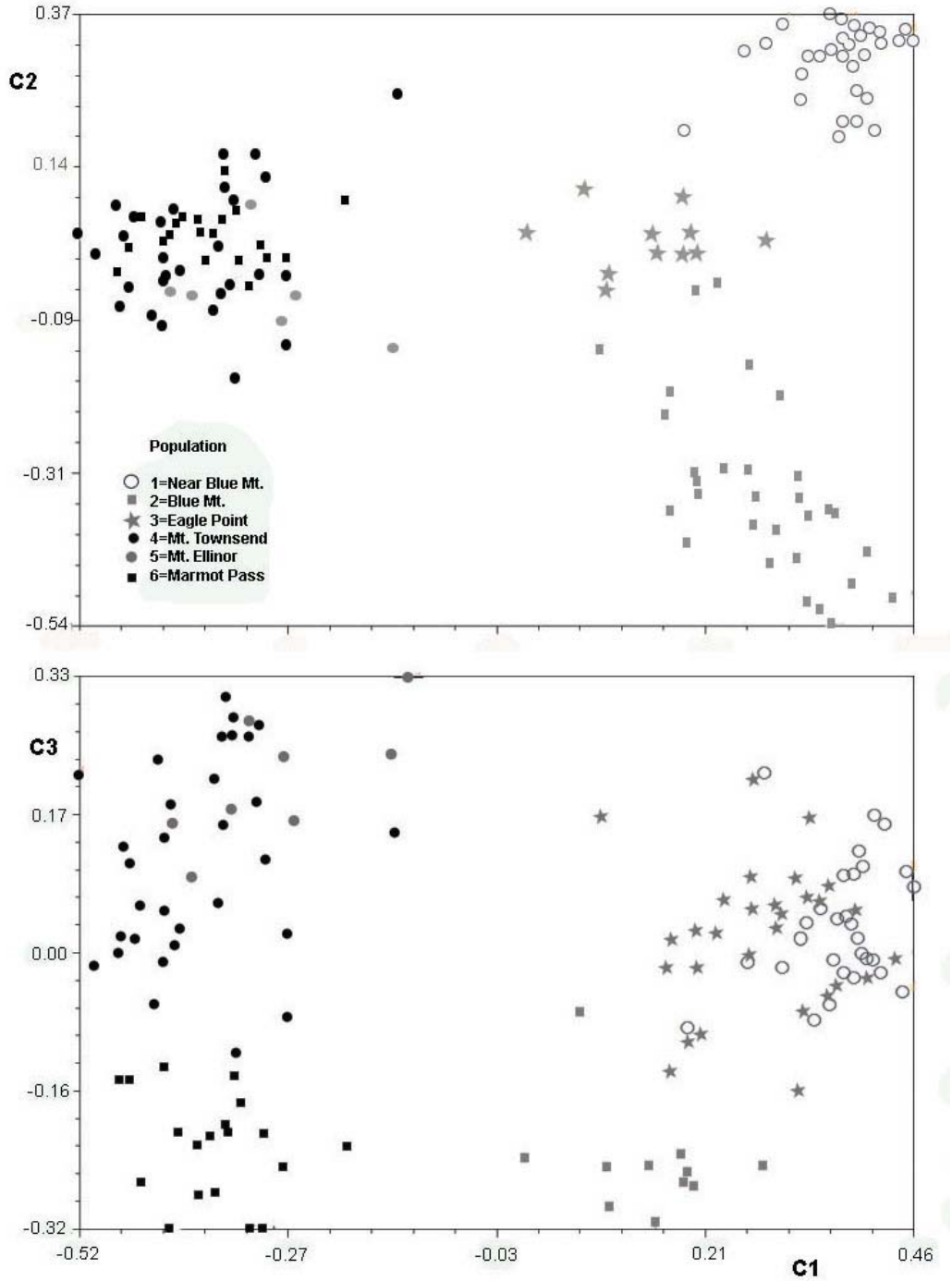
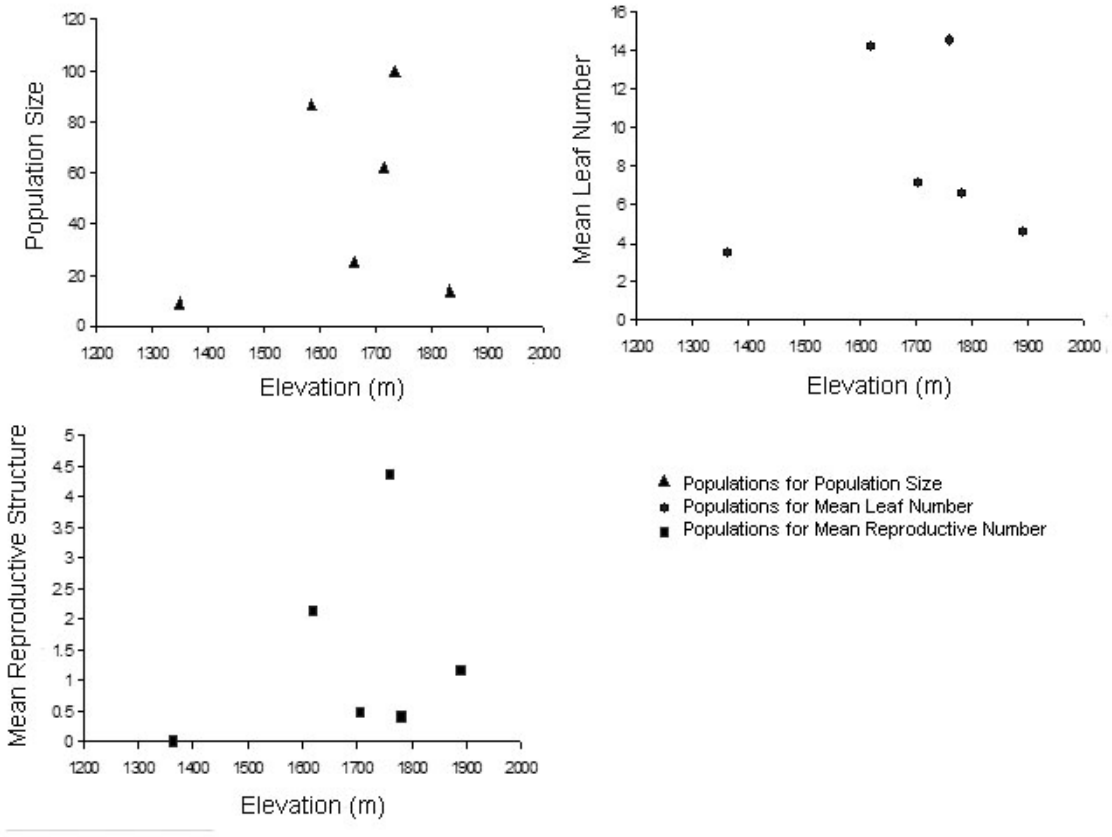


Figure 2.5: Scatter plots of elevation versus population size, mean leaf and mean reproductive structure showing middle elevational peaks.
Each point represents a population.



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Chapter 3: Interpretation of Historical Events in the Evolution of Three Closely Related Violets

Abstract

Within the *Viola canadensis* complex (Violaceae), which contains 6 distinct species, *Viola cuneata* S. Watson, *Viola fletti* Piper and *Viola ocellata* Torr. & A. Gray are demonstrably very closely related but morphologically and ecologically quite distinct species. All three are endemic to different habitats in coastal and near-coastal western North America (*V. fletti* to subalpine and alpine areas of the Olympic Mountains in Washington; *V. cuneata*, to serpentine barrens; and *V. ocellata*, to forested areas of Oregon and California). They represent different forms of rarity within their relatively narrow geographic distributions. Polymerase Chain Reaction - Restriction Fragment Length Polymorphisms (PCR-RFLP) data were generated from multiple samples of several populations for each morphologically distinct species, for chloroplast intergenic spacers and the Internal Transcribed Spacer using a diversity of restriction enzymes. Nested Clade Analysis (NCA) performed on the data suggested extensive past hybridization and subsequent chloroplast capture in both data sets, despite sharp morphological and ecological distinctions. These patterns, although extreme, echo similar results from many other Pacific Northwest species groups which maintain genetic polymorphisms from hybridization which may date back to a Pleistocene or early post-Pleistocene time period.

Introduction

Historical climatic and geological events have likely played a large role in the genetic structure and present-day distribution of species. However, details of biological and historical patterns will be unique to particular species and even populations, at least at some levels. Numerous comparisons of population genetic structure between widespread and narrowly restricted species have been made in the past (Song *et al.* 1999, Purdy *et al.* 1994, Sherman-Broyles *et al.* 1992, Karron 1987). Often, the implicit presumption has been made that they are closely related or even sister species without prior demonstration based on external genetic evidence (Sherman-Broyles *et al.* 1992, Cosner & Crawford 1994). In the case of *Viola cuneata* S. Watson, *V. flettii* Piper and *V. ocellata* Torr. & A. Gray it has already been shown that they are nearest sisters based on variation in the Internal Transcribed Spacer (ITS) region (Ballard *et al* 1997). Since these are close relatives with restricted ranges and ostensibly similar breeding systems, their population genetic structures should be similar; however, they inhabit dramatically different environments (*V. flettii* in alpine and subalpine sites, *V. cuneata* in open serpentine barrens, and *V. ocellata* in shady forests) and their divergent levels of local rarity and population isolation could presumably yield quite different genetic consequences. Comparative studies of their ecological responses to various environmental factors would also be enlightening, given their modally different habitats.

Species Studied. *Viola flettii* grows in rock crevices and talus slopes, with little to no soil development in alpine and subalpine environments in the Olympic Mountains in

Washington State. The growing season lasts from June to August but due to the elevation can still be quite cold. The species occurs mainly in the drier eastern parts of the Olympic Mountains, and populations may be more common on the south, drier sides of mountains. Summer is the dry season, but rainy and foggy days are common. This species seems to be restricted to very specific microhabitats that are strongly discontinuous throughout *V. flettii*'s small geographic range.

Viola cuneata grows in serpentine areas of the mountains of northern California and southern Oregon, also in a relatively small geographic area. The habitats that *V. cuneata* occur in seem to differ slightly geographically, being at higher elevations in moister areas near the southern end of its range and at lower elevations in drier areas near the northern end. Populations found in Josephine County, Oregon are common in areas of a sparse overstory of pines and other conifers, with a patchy understory of low sclerophyllous shrubs that transition into herbs (mainly graminoids) with increasing xeric conditions (Whittaker 1960). The populations found on Horse Mountain in California had a similar vegetation type with a *Pinus jeffreyi* overstory and a heath shrub layer with bare soil patches. This species seems to be the hardiest of the three species, being found in very large populations (sometimes estimated at or above 1,000 individuals in the area) and was common in several of the serpentine areas of Josephine County.

Viola ocellata occurs from central California to central Oregon throughout the coastal mountains in a variety of forested areas including redwoods, mainly at the forest edge or where the forest cover is less dense, or when forest coverage allows only moderate shade. It appears to be a physiological generalist, and certainly has the largest

geographic range, but it is sparsely located in small populations throughout this range. Compared with the other two species, the soil where *V. ocellata* grows is rich in organic matter and has high soil moisture levels. In forested serpentine vegetations, *V. ocellata* has been listed as an indicator species (Kruckeberg 1984).

Endemism. Endemics are organisms that have limited geographic ranges. Endemic plant species can be limited by dispersal, narrow requirements or both (Kruckeberg & Rabinowitz 1985). As classified by Rabinowitz (1981), there are several types of rarity for any species in an environment, and some plant species adhere very closely to one or a few of these particular categories. The three *Viola* species serving as the focus of this research show three distinctly different types of rarity (Figure 3.1). A species can have a small range and then have a limited distribution within that range, exemplified by *V. flettii*, which occurs in the small geographic area of the Olympic Mountains, and also only occurs in small isolated populations throughout that range. *Viola cuneata*, however, has a slightly larger geographic range, covering more than one mountain range, and its distribution is more common throughout its range. Another form of rarity is represented by species which typically grow sparsely within a given site. In this case, a species may have a large geographic range but is very infrequently found throughout that range, similar to the pattern of *V. ocellata*. The range of *V. ocellata* is a good portion of the coastal ranges, but it occurs sporadically in this range and often in small isolated populations.

All three species occur in geographic areas where endemism is common due to past historic events. The Olympic Peninsula has several endemic plant and animal species and infraspecific taxa due to the isolation of the peninsula during the latest ice age and the possible isolation of these species to mountaintops above the glacier level (Peterson *et al.* 1997). The California Floristic Province where *V. cuneata* is restricted to and *V. ocellata* is almost restricted to, is an area of high endemism in California and southern Oregon. It is an important area for survival and persistence of relict species from the north temperate arcto-tertiary forests (Raven & Axelrod, 1978). *Viola cuneata* is most common in the Klamath-Siskiyou region which is an area of high endemism within the California Floristic Province and possesses a very unique flora.

Western Floras and Pleistocene Refugia. A study on *Tiarella trifoliata* (Saxifragaceae) found this species to have northern and southern chloroplast DNA types with disjunction of the northern type in populations at high elevations of the Siskiyou-Klamath mountains and the southern type in populations of the Olympic Peninsula, both areas are thought to be glacial refugia for *Tiarella trifoliata* and possibly other Saxifragaceae species such as *Tellima grandiflora* and *Tolmiea menziesii* (Soltis & Soltis 1991, Soltis *et al.* 1992). Genetic data can resolve phenomena of past hybridization, ancient or recent, and introgression between species that may otherwise display no evidence of such. A study on *Packera* species in Alberta found low levels of phylogeny resolution and hybridization and introgression between two different phylogenetic groups, one historically from arid regions of the western United States, and the other historically from coastal habitats of

the Pacific Northwest (Golden & Bain 2000). *Metrosideris* species from New Zealand lacked species differentiation based on ITS sequences, presumably the result of hybridization and introgression between the species in glacial refugia during the last glacial period (Gardner *et al* 2004).

All three *Viola* species are thought to be paleoendemics as they, and most other narrow endemics in the Canadensis clade, are placed basal in the nuclear ribosomal phylogeny relative to the highly derived and transcontinentally distributed species, *Viola canadensis* (Ballard *et al.* 1997).

Western Serpentine Species and Evolutionary Origins. Serpentine areas often have unique floras with physiological adaptations to tolerate high heavy metal concentrations, although past historic events may also have played a role in restricting a species to these areas. The presence of both paloendemics, the original subspecies isolated on serpentine, as well as neoendemics, subsequent genetic and morphological differentiated subspecies, were postulated for the *Streptanthus glandulosus* complex, composed of mostly serpentine endemics (Mayer & Soltis 1994, Mayer *et al* 1994). Studies have found no direct evidence for a genetic separation of serpentine and non-serpentine populations, instead displaying regional genetic distributions (Westerbergh & Saura 1992, *Silene dioica*; Mayer & Soltis 1994, *Streptanthus glandulosus*). *Silene paradoxa* chloroplast microsatelites showed less variation than RAPD (nuclear) data due either inbreeding not observed with chloroplast data or increased genetic variability associated with heavy metal tolerance displayed only in nuclear data (Mengoni *et al* 2001). In the *Streptanthus*

glandulosus complex discontinuity between chloroplast and allozyme (nuclear) data was thought to relate to gene flow and past hybridization between subspecies (Mayer & Soltis 1994), however differing results for chloroplast and ITS data allowed further resolution of phylogeography both when compared and combined (Mayer & Soltis 1999). In taxa where serpentine endemism has arisen multiple times, such as the closely related genera *Caulanthus*, *Streptanthus* and *Guillenia*, a predisposition to barren and dry conditions could play a role (Pepper & Norwood 2001).

Gene Flow Between Species. Gene flow caused by pollen and seed exchange between populations is likely low in the isolated populations of all three species; this has been suggested by recent ecological and genetic studies of *Viola flettii* (McCreary *et al.* 2005). Many *Viola* species utilize limited seed dispersal by ballistic means, with dry capsules dehiscing explosively followed by ant dispersal of the scattered seeds (Beattie & Lyons 1975). All three violets investigated here have erect green capsules characteristic of this "diplochorous" seed dispersal syndrome. However, despite the "explosive" dehiscence feature, ants typically carry seeds short distances from the parent plants (Gómez & Espadaler 1998, Ohkawara & Higashi 1994), leading to low seed dispersal rates. Most *Viola* species are insect, particular bee, pollinated (Beattie 1976). Given the relatively small foraging areas followed by most bee species (Stenström & Bergman 1998, Visscher & Seeley 1982), this would be expected to limit pollen dispersal in widely geographically separated *Viola* populations.

These three species are paleoendemics (Ballard et al. 1997), thus, initial speciation and origin of species occurred possibly a million or more years ago. Since that time the species may have existed together in glacial refugia. When the species arrived at their current geographic distrubtions is yet to be discovered.

Patchy Habitat Distribution. Patchy habitat distribution of these three species may be related to narrow requirements for particular soil and other microhabitat characteristics. Patchy habitat distribution and presumably limited seed and pollen dispersal would be expected to result in high within and among population differentiation, local inbreeding, and high rates of fixation due drift in small populations. Restricted plant species have been found to have less genetic polymorphism than widespread species (Karron 1987). Endemic species have also been found to have lower levels of genetic variation than their widespread relatives, although there can be exceptions (Soltis & Soltis 1991). All of these effects might decrease the fitness and vigor of a population and could eventually lead to its extinction. How genetic variation and fitness relate is not truly known and it seems that some species may be able to overcome problems associated with small population sizes and low genetic variation (Ellstrand & Elam 1993).

Nested Contingency Analysis. Nested Clade Analysis (NCA) uses haplotype trees from non-recombinant genetic information to create a hierarchical set of nested branches. Nested phylogeographic analysis involved using nested contingency analysis to test for associations between the NCA and categorical and continuous geographic data

(Templeton 1998). Once geographic association is found further tests are able to determine which elements of population structure or past historical events, such as restricted gene flow, range expansion and fragmentation, created this association (Templeton *et al.* 1995). There have been disputes as to whether nested phylogeographic analysis predictions are correct (Masta *et al.* 2003, Printzen *et al.* 2003) and whether they should constitute the sole test for inferring historical events (Masta *et al.* 2003). More rudimentarily, an association between the clades in the NCA and any data set can be tested with Mantel Tests (Soucy *et al.* 2002). Permutation contingency analysis (PCA) can also be used to test the association between haplotypes and ecological values that possibly pertain to the separation of species or populations (Gómez-Zurita *et al.* 2000). Two *Arabis* species of North America demonstrated haplotype distributions occurring with ice sheets and other regions influenced by the last glacial period, NCA predicted restricted gene flow with isolation by distance for populations south and range expansions for haplotypes north of glacial maximum (Dobeš *et al* 2004). Based on NCA, three *Armeria* species from Europe demonstrated horizontal transfer as shared haplotypes were regionally specific, and altitudinal gene transfer was predicted between the species on the Sierra Nevada massif during migration from glaciation (Gutiérrez Larena *et al* 2002).

PCR-RFLP Technique. Polymerase Chain Reaction-Restriction Fragment Length Polymorphism is a technique that looks at the restriction fragment length polymorphisms (RFLPs) stemming from restriction enzyme digestion of a series of selected amplified

regions of the genome, most often from the chloroplast. The chloroplast genome in plants is maternally inherited, ranges from 120-217 kilobases (Kb) in size, and has a low rate of change in both structure and sequences (Palmer 1987). This chloroplast PCR-RFLP technique has worked well and yielded well-resolved phylogenies of species within families (Rieseberg *et al.* 1992, Datisaceae; Palmer 1987). Other successful studies have examined population variation as part of phylogeographic studies within the species *Tiarella trifoliata* (Soltis *et al.* 1992). In the study by El Mousadik and Petit (1996), populational variation of *Argania spinosa* was observed in 10 chloroplast and 2 mitochondria regions with only one restriction enzyme (HindFL). Several studies done with European species (*Olea europaea* and *Armeria* sp., respectfully) have shown evidence of species dispersal from glacial refugia (Besnard *et al* 2002, Gutiérrez Larena *et al* 2002). Although studies using amplified nuclear gene regions are few, a similar approach could be taken with closely related species as that using chloroplast regions. However, expectations of a reticulate pattern of inheritance through population gene flow or interspecies hybridization would need to be accommodated with appropriate distance methods of analysis.

This method is cheaper and far quicker than direct sequencing of PCR products, especially for multiple gene regions and numerous samples. More importantly, with chloroplast regions it generates a uniparental molecular data set amenable to cladistic analysis, on which Templeton's evolutionary inference approach relies for success. However, another study by Stehlik (2002) has combined NCA and PCR-RFLP and found that NCA phylogeographic analysis provided greater detail than more traditionally used

statistical analysis, mainly due to its ability to discern between restricted gene flow and range expansion.

Objectives.

1. The objective of this study was to compare the population differentiation of chloroplast regions in these closely related endemic species and possibly related species of the *Viola canadensis* complex. This study should result in a better understanding of the Pleistocene and post-Pleistocene evolutionary history of these closely related endemic species. It was assumed a priori that there would be some biological similarities between the species, even if they are ecologically highly specialized in their divergent environments.

2. Since many of the populations of all three species are geographically isolated--*V. flettii* in northwesternmost Washington is presently separated from *V. cuneata* and *V. ocellata* of southern Oregon and California by hundreds of miles--and each possibly experiences inbreeding as well as random genetic drift through small population sizes in many sites, a high level of between-population genetic differentiation and a lower- within-population genetic diversity would be expected.

3. Additional subpopulations collected in close geographic proximity to other larger populations for all three of these species were predicted to be more genetically similar than the geographically isolated populations because of potential gene flow between subpopulations and with more numerous populations near the center of the species' main range.

4. Populations that were nearest to the geographic center of the range of each species were also expected to have a higher level of within-population diversity but lower among-population differentiation.

Methods

Taxon Sampling and Materials Collection. Data were collected in the summer during the growing season from all three species in 1998 when collecting material for the *Viola canadensis* complex, in 1999 from *V. flettii*, in 2001 from a variety of new populations of all three species, and in 2003 from a last few populations to fill out the ranges of *V. cuneata* and *V. ocellata* (Table 3.1, Figure 3.1). The estimated number of plants per population, mapped locations, dominate tree species visually interpreted for each site, and detailed descriptions of habitat, were recorded for each population. Leaf material from 20 individuals per population, where available, was dried in silica gel in 1.5 ml micro-centrifuge tubes for genetic studies.

Nine populations of *V. cuneata* were sampled, including a good sampling from Josephine county Oregon where it is most densely populated and California populations representing the southern end of its species range. All *V. cuneata* populations, except the smaller subpopulation at Horse Mountain (VC2, 8 individuals), were large. Eight populations of *V. flettii* were sampled, including both southern and northern populations per Chapter 2 and the genetic divide of the species. All populations but Marmot Pass (VF6) were small, with those at of Eagle Point (VF3, 11 individuals), Mount Ellinor (VF5, 8 individuals) and the smallest Blue Mountain northern subpopultion (VF7, 8 individuals) having material from less than 20 individuals collected. Nine populations of *V. ocellata* were sampled. As much of the larger and more diverse range of the species was surveyed as possible, including the southern limit of the species range, south and north of San Francisco, throughout the coastal Redwoods, further east over the coastal

mountains and northern Oregon. All of these populations were smaller, but only two possessed under 20 individuals, Uvas Creek (VO1, 17 individuals) and Phillipsville (VO2, 16 individuals).

Chloroplast PCR-RFLPs. DNA was exacted from silica gel-dried leaves using a Wizard Genomic Extraction kit. The amplification of chloroplast regions was screen based on universal primers and protocols from Demesure *et al* (1995). The atpH/atpI region was selected for further study, as it was the most reliably amplified region. Restriction fragment size variation was collected for 10 randomly selected individuals per population, for the atpH/atpI region , amplified from the universal primers (CCAGCAGCAATAACGGAAGC and ATAGGTGAATCCATGGAGGG), using a RoboCycler (Stratagene RoboCycler Gradient 96 Hot Top Combo) with program data [96°C 2 min, (96°C 30 sec, 51°C 60 sec, 72°C 3 min) 25 cycles, 72°C 20 min] modified from (Demesure *et al.* 1995) in 30µl amounts with [23.4 ul water, 3ul buffer, 1.8 ul (50 mM) MgCl, 0.6 ul (2 mM) dNTP, 0.3 ul BSA, 0.375 ul (25 mM) primers, 0.15 ul Taq DNA Polymerase for each reaction]. The products were verified by gel electrophoresis with 5µl of product on a 1.3% agrose gel stained with Ethidium Bromide. Restriction enzyme digestion with EcoRI and EcoRV was performed in 15µl amounts in separate reactions using the same RoboCycler to maintain a 37°C temperature for 5 hours. Concentrations of master mix materials for the enzyme digestion were 5 ul of PCR products for both enzymes and 1 ul BSA, 1ul Promega Buffer H [1X concentrations: Tris-HCL (90 mM), $MgCl_2$ (10 mM), NaCl (50 mM), pH 7.5], 1 ul EcoRI restriction

enzyme, and 7 ul water for EcoRI; and 1.5 ul Promega Universal Buffer [1X concentrations: Tris-Acetate (25 mM), Potassium Acetate (100 mM), Magnesium Acetate (10 mM), DTT 1mM, pH 7.5] and 0.25 ul EcoRV restriction enzyme, 8.3 ul water for EcoRV.

Final digested fragments were electrophoresed on 1.8% MetaPhor Agarose gels to verify fragment size differences, with a 100bp ladder for size scoring. Gels were imaged and analyzed for fragment sizes and inferrence of different haplotypes.

Analysis. The Restriction Enzyme Analysis Package (REAP) software program (McElroy *et al.* 1992) was used to generate a nucleotide substitution matrix for restriction fragment data (D values) for maximum parsimony analysis with PAUP* (Swofford 2003) and neighbor-joining and cluster analysis with Phylip (Felsenstein 1995).

Commonly used molecular systematic methods were employed and compared for consistencies to add support to findings. Maximum parsimony attempts to minimize the steps necessary to explain a particular set of data. Data from the outgroups *Viola sempervirens* and *Viola orbiculata*, which are presumed from ITS data to be close relatives of the Canadensis complex that the three studied violets are a part of, were used to root the maximum parsimony trees. Statistical support can be generated for Maximum parsimony trees. Neighbor joining is an additive method of tree creation that focuses on the nodes of the tree. It results in an unrooted network, yet is able to display the distance between taxa. UPGMA (unweighted pair group method using arithmetic averages) is a

commonly used cluster analysis that represents the similarity between data points in a matrix.

A heuristic search of most parsimony trees was performed, using random-addition replicates and Maxtrees set at 100,000; the potential tree space was searched by varying the number of trees saved and the intensity of the search, following recommendations by Olmstead *et al* (1993). Both strict and majority rule consensus trees were produced. Neighbor-joining and UPGMA trees were generated from the data. A Principal Coordinates Analysis (PCoA) was performed using the Dice coefficient of similarity using NTSYSpc (Rohlf 2002) in order to search for trends in the broader distribution of clusters of chloroplast haplotypes across the multidimensional space. Arlequin (Schneider *et al.* 2000) was used to calculate the population genetic parameters of Tau, pairwise differentiation and polymorphic sites.

Nested Contingency Analysis. Latitude and longitude for all populations were obtained from a GPS unit in the field during the summer of 2003; MapSend and 7.5 minute maps (USGS) were used to obtain these values for other populations during the subsequent field season.

PAUP* yielded maximum parsimony scores for the REAP data file, which were then used to make a minimum spanning tree with NTSYSpc. From this tree, a Nested Clade Analysis was conducted by hand, by drawing out the clades that the minimum spanning tree described. From the NCA clade values and geographical distances between populations, a Nested Contingency Analysis was run with GeoDis 2.1 (Posada *et al*.

2000). Because of incompatibilities in combining PCR-RFLP data directly with NCA procedures in available computer resources, GeoDis analysis had to be done manually in part.

Internal Transcribed Spacer (ITS) PCR-RFLPs and analysis. After preliminary analysis of chloroplast PCR-RFLP data showed no clear distinction between the species, suggesting large-scale hybridization perhaps early in the history of the species complex, a smaller project using the PCR-RFLP approach with the nuclear ribosomal Internal Transcribed Spacer (ITS) region was performed for comparison. Using sequences obtained for the three species as a template, examinations of restriction cut sites from Sequencher (Gene Codes Corp.) software showed that the restriction enzyme HaeIII would yield several cut site differences and fragment length polymorphisms to distinguish each species. Amplification was done only for ITS2, since it was found to be more readily amplifiable, yielding only single products using the primers ITS3B (5' GCATCGATGAAGAACGTAGC) and ITS4 (5' TCCTCCGCTTATTGATATGC), using a Robocycler (Stratagene RoboCycler Gradient 96 Hot Top Combo) and amplification protocol per Ballard *et al* (1999). Amplification verification and restriction enzyme digestion were done with the same protocol as that used for chloroplast PCR-RFLPs. The restriction enzyme HaeIII was used and the Promega buffer C (1X concentration: Tris-HCl 10mM, $MgCl_2$ 10mM, NaCl 50mM, DTT 1mM, pH 7.9) accompanying this enzyme was substituted.

Since size differences between expected fragments could be interpreted on regular agarose gels would probably only resolve to within 10-20 bp, final digested fragments were electrophoresed on 2.0% Metaphor Agar, as with the chloroplast fragments, with a 25bp ladder. The gels were stained with Ethidium Bromide and fragment sizes were scored as before. Phylip (Felsenstein 1995) was used to generate a neighbor joining tree using both presence of cut sites and insertion-deletion events.

Results

V. cuneata. In the majority rule tree haplotypes from northern populations clustered, as well as haplotypes from the smaller Horse mountain subpopulation (VC2) with Eight Dollar mountain haplotypes, and southern haplotypes with *V. flettii* and *V. ocellata* (Figure 3.2). A few northern haplotypes were present in a unique cluster of all three species in the neighbor joining tree (Figure 3.3). Overall, *V. cuneata* had lower mean values than the other two species for genetic diversity variables (Φ_{ST}, P) (Table 3.3). *Viola cuneata* northern Eight Dollar Mountain versus southern Eight Dollar Mountain and Lone Mountain Road showed large variances in genetic statistics. Genetic differentiation (Φ_{ST}) was high for the smaller subpopulation at Horse Mountain and the Southwestern Eight Dollar Mountain population, and lowest for the large population at Horse Mountain.

In the NCA (Nested Clade Analysis), most of *V. cuneata* haplotypes were found in the central Clade 1-1 (Figure 3.4). Two separate Eight Dollar Mountain haplotypes nested with a coastal *V. ocellata* haplotype and with southern and northern *V. ocellata* and *V. flettii* haplotypes. One southern haplotype from northern California nested in the separate Clade 3-2 with distinct middle range *V. ocellata* haplotypes and associated *V. flettii*.

In the ITS neighbor joining tree, *V. cuneata* displayed the greatest variation. Haplotypes clustered with southern *V. ocellata* and a unique *V. flettii* haplotype, and with central *V. ocellata* (Figure 3.7).

V. cuneata was not distinct with either chloroplast or ITS data from the other species. Although associations with specific haplotypes from *V. ocellata* and *V. flettii* no clear patterns were elucidated.

V. flettii. A few northern haplotypes associated separately with distinct *V. ocellata* and northern *V. cuneata* haplotypes in both the Strict Consensus and Majority Rule trees (Figures 3.1, 3.2). Haplotypes from the southern populations of Mount Townsend (VF4) and Mount Ellinor (VF5) were clustered in the Majority Rule tree, as well, northern and Marmot Pass (VF6) haplotypes clustered with *V. ocellata* and *V. flettii*, Marmot Pass haplotypes clustered with middle range *V. ocellata* and southern *V. cuneata*, and northern haplotypes clustered with southern *V. cuneata* (Figure 3.2). A unique neighbor joining cluster of all species contained mainly southern haplotypes from Mount Townsend and Mount Ellinor with a few northern haplotypes (Figure 3.3).

The southern Mount Townsend and Mount Ellinor populations had the highest P values, while Marmot Pass had the lowest (Table 3.3). The smallest of the Blue Mountain populations (VF7) had the highest genetic differentiation (Φ_{ST}).

Most of the *V. flettii* haplotypes nested in the central Clade 1-1 (Figure 3.4). Northern haplotypes were present in the separate Clade 3-2 that also contained distinct *V. ocellata* haplotypes and one southern *V. cuneata* haplotype. Southern and northern haplotypes nested with northern *V. cuneata* and mainly northern and southern *V. ocellata* in Clade 1-4.

The ITS data contained only one unique *V. flettii* haplotype which clustered with *V. cuneata* and *V. ocellata* in the most separated branch in the neighbor joining tree (Figure 3.7).

No distinction between *V. flettii* and the other two species was seen in either chloroplast or ITS data. The strongest association within the species was between the southern populations of Mount Townsend and Mount Ellinor. There were also associations with both *V. cuneata* and *V. ocellata*, although without a great deal of clarity on underlying meaning.

V. ocellata. Haplotypes from populations in the north-central range of *V. ocellata* clustered in both the parsimony consensus trees (Figures 3.1, 3.2), and expressed long branch lengths in the neighbor-joining tree (Figure 3.3). Other strongly supported branches in the majority-rule consensus tree were the two subpopulations of *V. ocellata* in Oregon. In the majority-rule consensus tree, *V. ocellata* populations from the south-central region of its range were loosely clustered, although this did not include the southernmost population at Palo Colorado Rd. PCoA showed the *V. ocellata* individuals that clustered in the trees to be widespread and distinct (Figures 3.4, 3.5).

Oregon subpopulations of *V. ocellata* had high variation in genetic statistics. The populations in central portions of *V. ocellata*'s range had a higher number of polymorphic restriction sites, which might correspond to the longer branch lengths between these populations on the neighbor joining tree (Table 3.3). The central population from Phillipsville (VO3) and the furthest south population (VO5) possessed the highest genetic

differentiation (Φ_{ST}). Nested Clade Analysis (NCA) showed one central California *V. ocellata* individual in a large clustering of several different populations and species, including some common shared haplotypes and less common haplotypes that group with them (Figures 3.4, 3.5). Only two branches came out from this central clustering. The larger branch included mainly one of the *V. ocellata* subpopulations from Oregon, two individuals from the central California *V. ocellata*, a few *V. flettii* individuals and *V. cuneata* from Oregon. The smaller branch placed in a separate clade included mainly individuals from central California *V. ocellata*, with two individuals of *V. flettii* from northern populations and one *V. cuneata* individual from a northern California population.

In the ITS neighbor joining tree a haplotype from the south was present in the largest branch with *V. cuneata* and *V. flettii*. A central *V. ocellata* haplotype was shared and associated with northern California *V. cuneata* (Figure 3.7).

V. ocellata showed no distinction from the other species in either nuclear or chloroplast data. However, it contained the most differentiated haplotypes from the central area of the species range, and may be central to these three species.

Overall Results. None of the species were distinct in any analysis performed on the PCR-RFLP data. Similar haplotypes grouped in various analysis and displayed general trends in the clustering of populations and broad separation among species for most populations.

In all analysizes the *V. flettii* population from nearby blue mountain in the northern region of that species clustered with the larger population of *V. cuneata* at Horse

mountain and the *V. cuneata* population north of Eight Dollar mountain. In the neighbor joining tree there was a distinct clustering of populations from all three species that included mostly southern populations from *V. flettii*, three populations of *V. cuneata* from Oregon, and *V. ocellata* from the extreme southern and northern extents of its range (Figure 3.3). Haplotypes from the extreme northern and southern populations of the *V. ocellata* were associated with others from *V. flettii* and *V. cunetea* (Figure 3.6). Another common association contained several individuals from *V. flettii* and *V. cuneata* and one of the outgroups.

Polymorphic loci (P) was low, under 20%, for all species and populations (Table 3.3). Genetic differentiation between populations (Φ_{ST}), however, varied between and among species.

Geographic analysis of the NCA showed range expansion in Step 1 clades and restricted gene flow/isolation by distance in most Step 2 and 3 clades (Table 3.2). These predictions were restricted to only Clade 3-1, so predictions excluded the smaller NCA branch.

A consensus parsimony tree of PCR-RFLP data for the ITS 2 region did not show the clear distinction between populations as hoped (Figure 3.7). Only five haplotypes were found, with sixty-five of the individuals studied having only one of those, including the outgroups. Two of the other more unique haplotypes were limited to only one population and the other two were shared between a few individuals from *V. ocellata* and *V. cuneata*.

Discussion

The presence of general trends and relationships between maximum parsimony, neighbor joining and Nested Clade Analysis strengthens evidence and predictions based on each individual analysis. However, the lack of clear distinctions between regions and also between species is still troubling. Other information collected in these studies (environmental, ecological and morphological analysis in Chapter 4), as well as the traditional taxonomic distinctions between the species, provide convincing evidence that the three species are very distinct morphologically and ecologically. Presuming that this is still true, then we must account for the predominant absence of discrete groupings of individuals of each species in the independent chloroplast and nuclear data sets, and also the weak trends of population groupings within species (or, ignoring species assignments, weak geographic groupings).

A number of explanations may hold, and perhaps more than one together may eventually prove applicable. The first explanation invokes the possibility of ancient hybridization and chloroplast capture in a western coastal refugium during the Pleistocene, as revealed by phylogeographic studies of several other Pacific Northwest plant groups. If this has occurred only partially or infrequently, and hybridization has continued occasionally among different chloroplast types, some populations could possess a heterogeneous haplotype combination, and this heterogeneity could even extend across species ranges. The second explanation, not exclusive to the first, is ongoing recent hybridization. It is possible that although the species are distinct at the phenotypic level, they may still experience low-level gene flow and even introgression

from time to time. While this would not explain the current results with *V. flettii* embedded in clusters of other species, *V. cuneata* and *V. ocellata* are sympatric across a substantial extent of their range in southern Oregon and northern California, and hybridization and further breakdown of species genotypes may be taking place, providing further confusion to molecular profiles.

A third problem could be simultaneous conflicting insertion/deletion events in the chloroplast spacer and ITS spacer that confound correct interpretation of variation in some populations and species.

A fourth, mostly theoretical consideration, but one that is often invoked to explain discordance in nuclear results, is incomplete lineage sorting of divergent ITS restriction variants within and among populations, which would primarily affect populations.

A fifth issue is potential paralogy of ITS copies from duplicate loci (detected by various researchers in some violet groups), which could affect results within or among species. Without cloning and sequencing of many individual PCR products, we could not address this. Preliminary verifications of PCR products showed single bands, but if the paralogues differ by very small indels or mere restriction site differences, they would only be revealed after restriction digestion and electrophoresis on MetaPhor gels.

A sixth issue relates to problems with certain analyses, particularly with NCA, in providing reliable results for some types of data.

Finally, it is possible that more than one of these explanations may hold, or others may apply that we have not proposed; or that the single chloroplast and nuclear gene regions and single restriction enzymes used simply cannot provide sufficient

phylogenetic signal for resolving this complex scenario. Some of these are discussed in more detail, but further study using a wide range of evolutionary and molecular approaches besides traditional genetic information is clearly needed to clarify this situation and identify biologically and methodologically real issues responsible for the present patterns (and provide the "true" pattern if those presented are artifactual at some level).

Comparison of population differentiation of chloroplast regions in these closely related endemic species. It is also possible the genetic trends observed are a snapshot of past relationships between the species during past glacial periods when populations of all three species may have existed in much closer geographic relationships. Such hybridization and introgression toward one parent may in fact be ongoing as well.

Two of the species occur in geographic areas thought to be past glacial refugia (*V. cuneta* in the Siskiyou-Klamath mountains and *V. flettii* in the Olympic Mountains). Soltis *et al.* (1992) in a study on *Tiarella trifoliata* found shared chloroplast phylogenies between the Olympic Mountains and higher elevations of the Siskiyou-Klamath Mountains. Although a similar genetic trend between either of these areas and the more southern range of *V. ocellata* has not been found, the Siskiyou-Klamath Mountains share a vegetation history with areas of northern and central California where *V. ocellata* does occur (Raven & Axelrod 1978). Thus, even with the present wide separation of these three species ranges, especially that of *V. flettii*, the idea of past restriction together in glacial refugia could be a viable option.

The neighbor-joining tree offers a unique grouping of all three species sister to the divergent clustering of central California *V. ocellata*. Included are northern *V. cuneata*, northern and southern *V. ocellata*, and individuals of *V. flettii* from throughout its range. Some of these individuals also form Clade 2-3 in the NCA, which is connected to the largest Clade 1-1. It is possible that these individuals represent a middle level of divergence. It is also possible that these two clusters reflect past fragmentation between central *V. ocellata* populations and other portions of a "combined genome pool" involving all three species that were trapped together during the last glacial period.

No present geographic links were concluded in these patterns, although they may represent past hybridization between ancestral individuals of these populations. *Viola cuneata* and *V. flettii* do seem to weakly cluster more than the other species combinations, and cluster tightly with one outgroup in the PCoA. This may reflect the common evolutionary history shared by these species and their relatively more distant relationship to *V. ocellata*. Phylogenetic results of the genus *Viola* and the whole *Viola canadensis* complex based on ITS sequences supports this (Ballard *et al.* 1997). A partial explanation suggested by these data together, then, is incomplete phylogenetic differentiation of the genomes of these three species in the more distant past, perhaps further confounded by more recent hybridization of *V. cuneata* and *V. ocellata* in Oregon and California.

Evolutionary predictions from GeoDis center on range expansion, mainly of the lower clades, and restricted gene flow/isolation by distance, mainly of higher clades. Lower clades may be representing the events of interbreeding populations expanding into

their current ranges. The odd mixtures of present-day populations and species, especially in the largest Clade 1-1, would indicate past localization and hybridization previous to this range expansion. Following these range expansions, greater geographic distances and limited gene flow abilities would have caused restricted gene flow, although possibly not divergence into clear species and population lineages. Clade 3-2, which no predictions could be made on, may indicate a grouping of more diverged individuals, and the lack of such divergence in clade 3-1 and/or the presence of pre-fragmentation haplotypes may have limited the predictive ability for this clade and its nested clades.

A high level of between-population genetic differentiation and a lower within-population genetic diversity would be expected. Both of these expectations were proven in some way. Genetic diversity within populations was low, not above 20% polymorphic loci for any surveyed population. For a few populations genetic differentiation was high. Many of these were smaller populations in close proximity to larger populations [*V. cuneata* (Horse Mountain=2, SW Eight Dollar Mountain=7) and *V. flettii* (North of Blue Mt.=7)], or may have been disjunct [*V. ocellata* (Clover Creek=5)].

Additional subpopulations collected in close geographic proximity to other larger populations for all three of these species were predicted to be more genetically similar Some of the other repeated clusters were individuals of the same populations, or from close geographic populations [*V. flettii* (Mt. Townsend=4, Mt. Ellinor=5), *V. ocellata* Oregon subpopulations, *V. ocellata* southern populations (Uvas Creek=1, Memorial

Park=8 and South of San Francisco=9)]. However, many of the other trends and clusters were between individuals and/or populations of separate species.

Populations that were nearest to the geographic center of the range of each species were also expected to have a higher level of within-population diversity but lower among-population differentiation. One consistent trend was certain individuals from central California populations of *V. ocellata* (Phillipsville=2, Middle Creek=3 and Clover Creek=4) being distinct and distant in neighbor joining, consensus trees and although one of these individuals was central in the NCA the others were all peripheral, especially in the more distinct Clade 3-2. It's possible these individuals represent a past fragmentation of populations geographically separated in central California from the rest of the three species during past glaciation events. Their central locations in the NCA may instead indicate they are more diverged from the past ancestral interbreeding events. The hig diversity and divergence seen in these populations compared with the rest of the species could indicate they are a geographic center of speciation for either *V. ocellata* or all three species. Certainly, their relationship to other individuals, populations and species may allow the best hints as to past evolutionary events.

Hybridization and Chloroplast Capture. The idea of chloroplast capture relates to past hybridization followed by subsequent introgression toward one parental species, so that eventually, individuals that are indistinguishable morphologically or ecologically from one species still retain the chloroplast of another, by chance. Chloroplast capture of

maternally inherited DNA between species is a possibility in plants, and is thought to be favorable when incompatibilities between genomes causes limited male sterility leading to greater female reproductive fitness (Tsitrone *et al.* 2003). *Viola* species in many groups are known to easily hybridize and create fertile hybrids.

ITS Data. Studies have found differing genetic results for chloroplast and nuclear gene data analysis (Mayer & Soltis 1994, Gamache *et al* 2003). ITS PCR-RFLP data from this nuclear gene did not show clear species delineations as expected if this were only a case of chloroplast capture of maternally inherited DNA between species. Only half of the ITS gene was surveyed since a smaller DNA fragment was easier to consistently PCR, however, this may have led to insufficient data to differentiate these species. This is suggested by the fact that sixty-five percent of the samples shared one haplotype. Both the ITS and chloroplast data sets indicate that if hybridization has played or is playing a role (or both), then it was extensive and goes far beyond one or a few chloroplast capture events; the extremely complex pattern of species and population associations in the chloroplast PCR-RFLP data supports this notion.

Analysis Limitations. Shared haplotypes between groups or even species was found in *Acacia acuminata*, *Armeria* sp., and *Pimelia* (beetles), respectively (Byrne *et al.* 2002, Gutiérrez Larena *et al.* 2002, Contreras-Dìaz *et al*. 2003), and can aid in determining phylogeographic histories when they show clear trends or distributions. NCA seems to require the presence of new mutations in post-fragmentation haplotypes and the removal

of pre-fragmentation haplotypes due to genetic drift. When these events conditions are not present, Nested Contingency Analysis predictions can give incorrect results (Printzen *et al.* 2003).

Conclusions. These three species have an interesting (albeit currently confused) evolutionary history with possible hybridization and chloroplast capture between all three species, likely while they existed together in glacial refugia during the last ice age. Subsequent range expansion and fragmentation appears not to have created clear maternally inherited genetic divergence between these species to date. However, this same lack of divergence seems to have provided a snapshot into the species past, indicating possible links between populations of these species. Most interesting is the sharp separation of central California *V. ocellata* individuals from the remaining taxa. If the patterns of genetic intermingling (and maintenance of this gene flow) in such morphologically and ecologically distinct species is accepted as a working hypothesis, then hybridization may have taken place recurrently and over extended periods, with subsequent further differentiation and isolation. Following an initial origin from a common western North American ancestor, perhaps originally found in both open and forested drier microsites some time prior to Pleistocene glaciation, populations across the ancestral range invaded forests, serpentine barrens and subalpine areas and subsequently differentiated, with periodic bouts of hybridization bringing their genomes back into contact at various stages of their differentiation. Following the last glacial epoch they completed their ecological and morphological diversification in different areas and

assumed their present geographic ranges upon final retreat of the northern continental and more southerly alpine glaciers. The complex genetic pattern represented in the species indicates that sufficient time has not elapsed to homogenize or filter out effects of this recurrent and extended hybridization. Nevertheless, their strong morphological and ecological differentiation would suggest that the remnants of "foreign" genomes introduced by inter-taxon hybridization may have been partially or largely eliminated, leaving components (e.g., ribosomal and chloroplast genes) not directly involved with adaptive environmental responses intact.

PCR-RFLP markers, a relatively new approach, has proved useful in recovering molecular genetic variation in both the chloroplast and nucleari ribosomal spacers investigated for the multiple populations of the three species. Although such data require manual nested clade creation to use with NCA and GeoDis, PCR-RFLPs were amenable to these analytical methods and provided some trends in phylogenic history of these species which, along with other lines of evidence, deserve much further study. Many potential issues need to be resolved in order to elucidate the evidently complex and fascinating evolutionary history of these morphologically and ecologically quite divergent western violets. It is possible that more refined fingerprint methodologies such as nuclear AFLPs, more extensive sampling of the chloroplast genome using chloroplast simple sequence repeats, and traditional biosystematic methods such as controlled hybridizations and field studies of gene flow, may provide future insights.

Chapter 3 Tables and Figures

Table 3.1. List of populations of *V. cuneata*, *V. flettii* and *V. ocellata* collected for the project.

Species	Population ID code	Location	Year	# Indiv. Collected
V. cuneata	VC1	Horse Mountain, CA	2001	40
V. cuneata	VC2	Horse Mountain, CA	2001	8
V. cuneata	VC3	Near Gasquet, CA	2001	20
V. cuneata	VC4	Sanger peak Rd., OR	2001	30
V. cuneata	VC5	Lone Mountain Rd., OR	2001	30
V. cuneata	VC6	SW Eight Dollar Mountain, OR	2003	30
V. cuneata	VC7 (HB1)	North Eight Dollar Mountain, OR	1998	20
V. cuneata	VC8 (HB2)	South Eight Dollar Mountain, OR	1998	20
V cuneata	VC9 (HB3)	Little Bald Hills, CA	1998	20
V. flettii	VF1	Near Blue Mountain, WA	1999	30
V. flettii	VF2	Blue Mountain, WA	1999	30
V. flettii	VF3	Eagle Point, WA	1999	11
V. flettii	VF4	Mount Townsend, WA	2001	25
V. flettii	VF5	Mount Ellenor, WA	2001	8
V. flettii	VF6	Marmot Pass, WA	2001	30
V. flettii	VF7 (HB1)	North of Blue Mountain, WA	1998	8
V. flettii	VF8 (HB2)	Blue Mountain, WA	1998	30
V. ocellata	Vo HB5	Redwoods	1998	30
V. ocellata	VO1	Uvas Creek, CA	2001	17
V. ocellata	VO2	Phillipville, CA	2001	16
V. ocellata	VO3	Middle Creek Valley, CA	2001	20
V. ocellata	VO4	Clover Creek, CA	2001	30
V. ocellata	VO5	Palo Colorado Canyon Rd, CA	2003	30
V. ocellata	VO6 (HB1)	OR subpopulation	1998	30
V. ocellata	VO7 (HB2)	OR subpopulation	1998	30
V. ocellata	VO8 (HB3)	Memorial Park, CA	1998	30
V. ocellata	VO9	South of San Francisco	1998	30

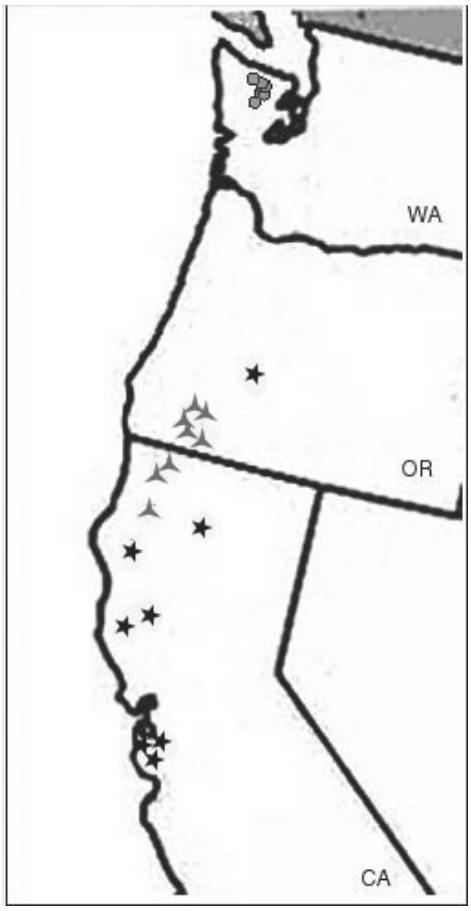
Table 3.2. Geographic analysis of the Nested Clade Analysis for all three species.
Significance below 0.05 denoted by *.

1 Step				2 Step				3 Step			
Clade	X^2	p-value	Conclusion	Clade	X^2	p-value	Conclusion	Clade	X^2	p-value	Conclusion
1-1*	721	0.000	Range Expansion	2-1*	45.3	0.078	Restricted Gene Flow	3-1*	103	0.000	Restricted Gene Flow
1-5	2.0	1.000									
1-4*	74.7	0.000	Range Expansion	2-3	12.6	0.173	Range Expansion				
1-6*	7.0	0.044	Range Expansion								
1-2	6.0	1.000		2-2	4.0	1.000		3-2	4.3	1.000	
1-3	3.0	1.000									

Table 3.3. Polymorphic sites, and Φ_{ST} values for populations of *V. cuneata*, *V. flettii* and *V. ocellata* based on PCR-RFLP data.

Population	Location	Theta st (Φ_{IS})	Polymorphic Sites (P)
VC1	Horse Mountain, CA	0.036	7.3
VC2	Horse Mountain, CA	1.149	4.28
VC3	Near Gasquet, CA	0.138	7.3
VC4	Sanger Peak Rd., OR	Did not converge	
VC5	Lone Mountain Rd., OR	0.151	3.68
VC6	SW Eight Dollar Mountain, OR	1.052	6
VC7	North Eight Dollar Mountain, OR	0.164	2.33
VC8	South Eight Dollar Mountain, OR	0.34	6.53
VC9	Little Bald Hills, CA	Did not converge	
VF1	Near Blue Mountain, WA	0.551	4.81
VF2	Blue Mountain, WA	0.204	3.74
VF3	Eagle Point, WA	0.271	6.09
VF4	Mount Townsend, WA	0.313	12.52
VF5	Mount Ellenor, WA	0.238	8.19
VF6	Marmot Pass, WA	0.011	0.34
VF7	North of Blue Mountain, WA	1.025	4.7
VF8	Blue Mountain, WA	0.085	1.44
VO1	Redwoods	0.298	3.24
VO2	Uvas Creek, CA	0.437	16.1
VO3	Phillipville, CA	1.242	9.99
VO4	Middle Creek Valley, CA	0.516	15.62
VO5	Clover Creek, CA	1.102	3.42
VO6	Palo Colorado Canyon Rd, CA	0.152	7.26
VO7	OR subpopulation	0.488	1.35
VO8	OR subpopulation	Population sample too small	
VO9	Memorial Park, CA	0.176	2.97

Figure 3.1. Distribution of collected populations.
Viola cuneata represented by black stars, *V. flettii* by grey circles and *V. ocellata* by grey triangles.



Made with ArcView

Figure 3.2. Strict consensus parsimony tree for PCR-RFLP data of all three species. VC=*V. cuneata*, VF=*V. flettii* and VO=*V. ocellata*. Number indicates population and letter individual. When groups or haplotypes include more than one individual or population letters and numbers are not present. SEM and Or represent the two outgroups used to root tree.

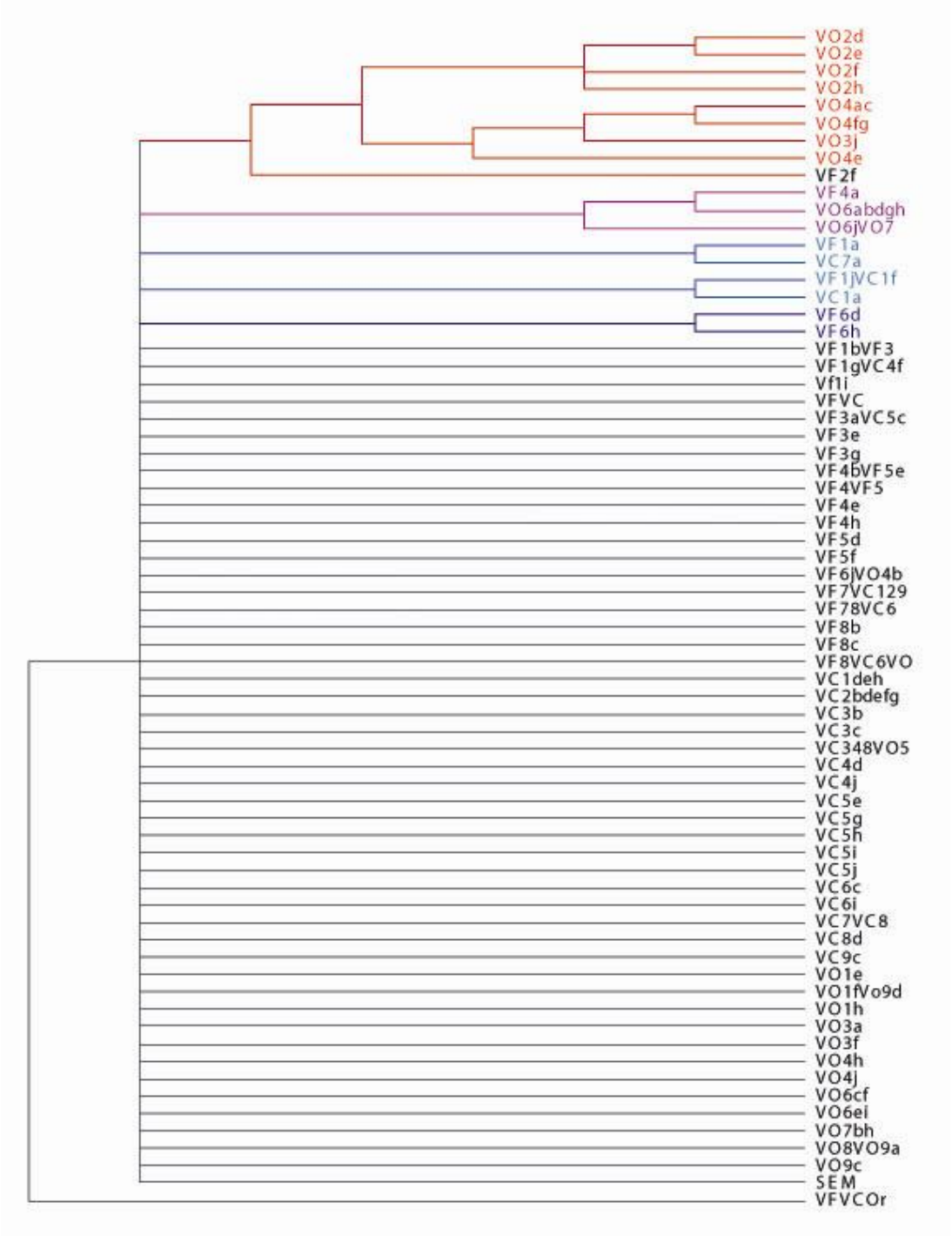


Figure 3.3. Majority rule parsimony tree for PCR-RFLP data for all three species. Numbers above branches indicates percent of trees with that branch. VC=*V. cuneata*, VF=*V. flettii* and VO=*V. ocellata*. Number indicates population and letter individual. When groups or haplotypes include more than one individual or population, letters and numbers are not present. SEM and Or represent the two outgroups used to root tree.

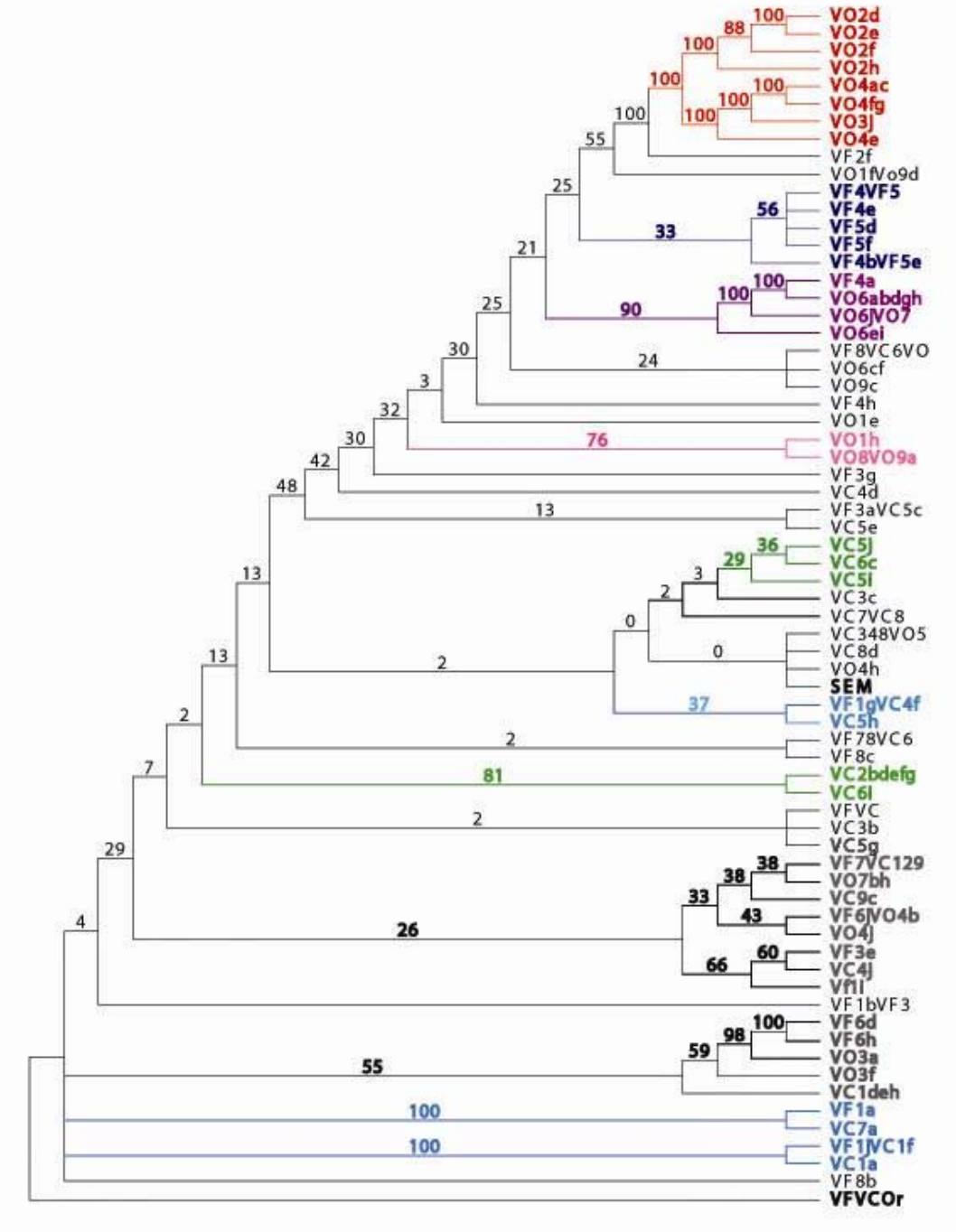


Figure 3.4. Neighbor Joining tree for PCR-RFLP data for all three species. VC=*V. cuneata*, VF=*V. flettii* and VO=*V. ocellata*. Number indicates population and letter, individual. When groups or haplotypes include more than one individual or population, letters and numbers are not present. SEM and Or represent the two outgroups used to root tree outgroups used to root tree.

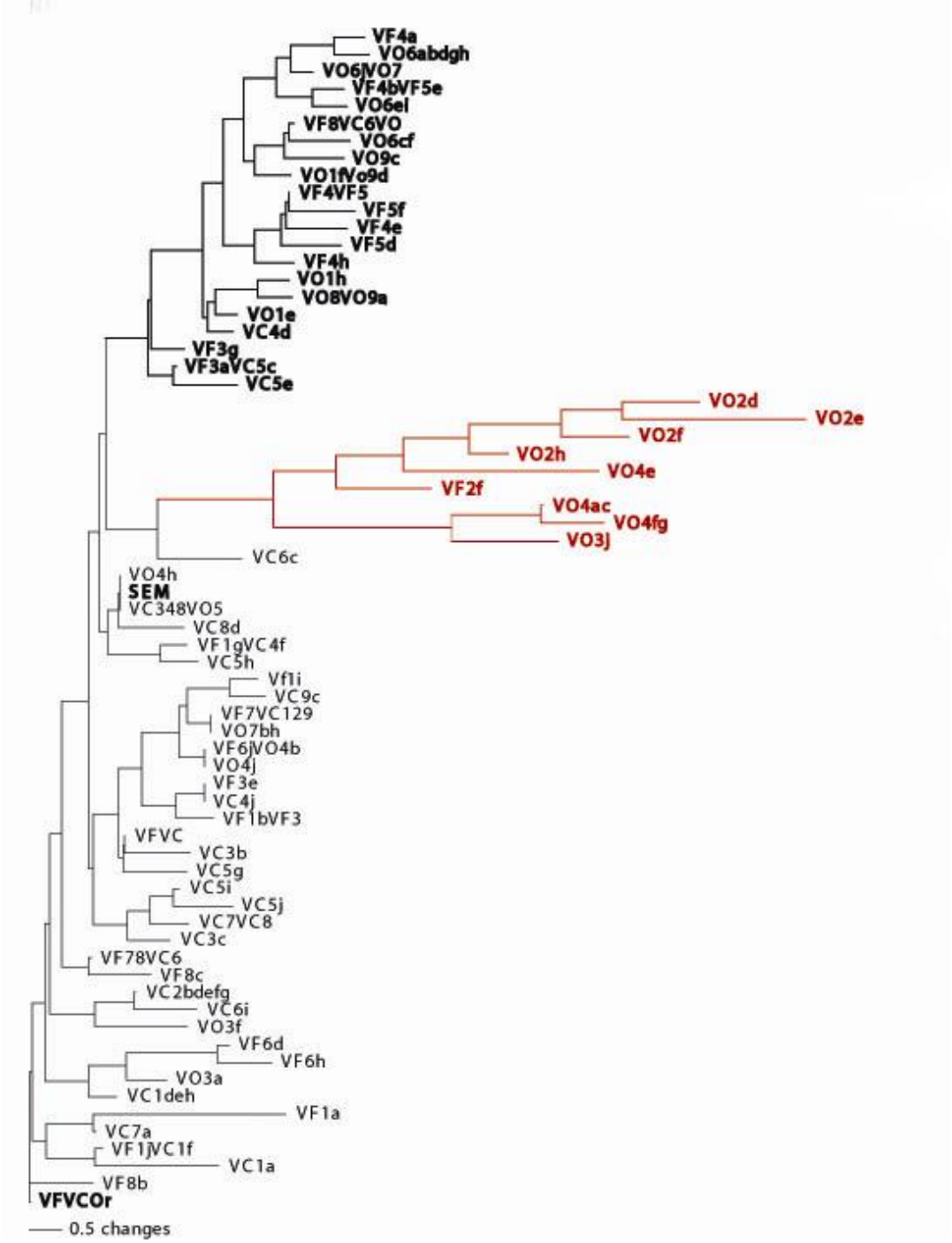


Figure 3.5. Nested Clade Analysis (NCA) of PCR-RFLP data for all these species. VC=*V. cuneata*, VF=*V. flettii* and VO=*V. ocellata*. Number indicates population and letter, individual. When groups or haplotypes include more than two individuals or populations, letters and numbers are not present.

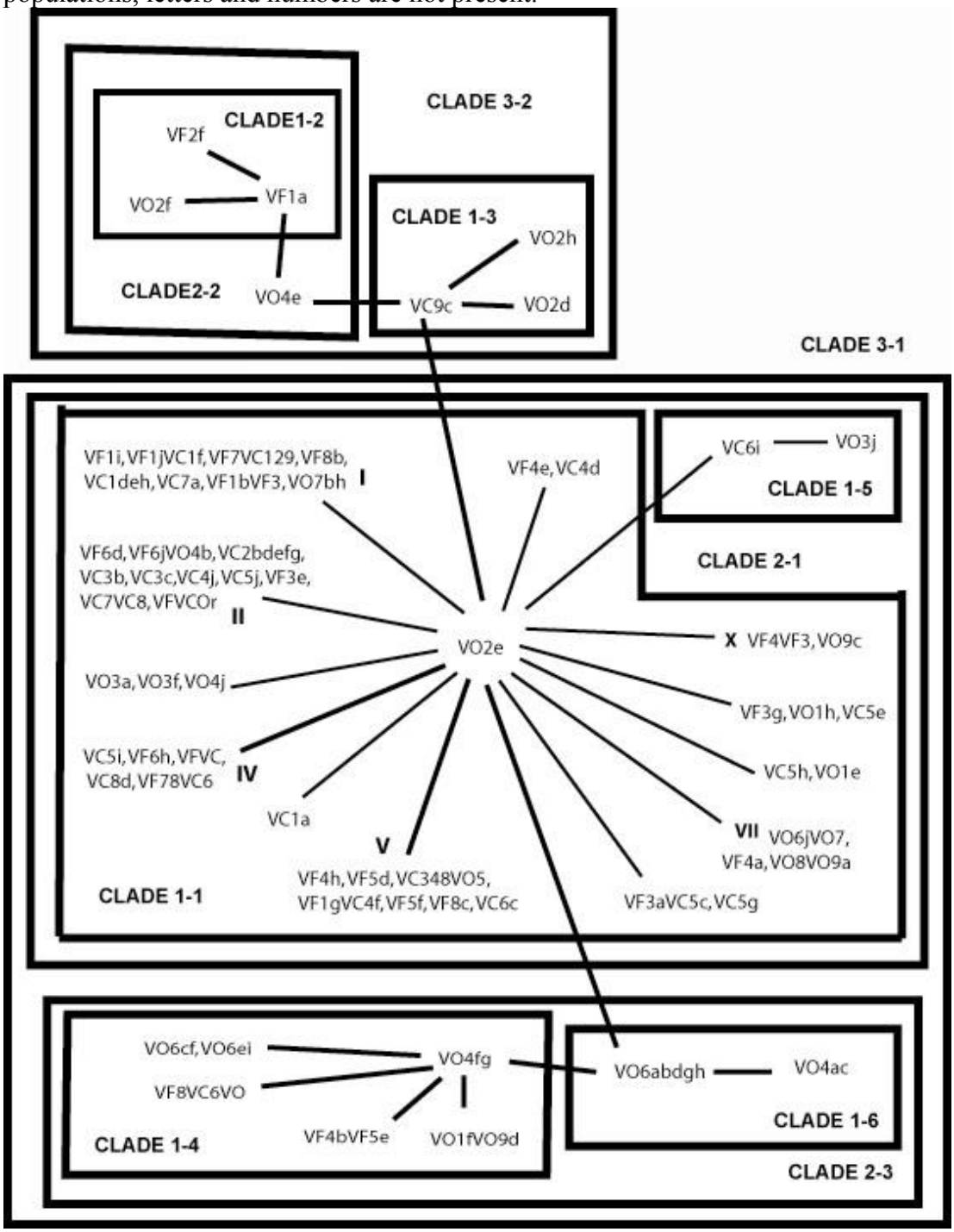


Figure 3.6. A scaled representation of the NCA from PCR-RFLP data for all three species.
The length of branches represents the evolutionary distance between groupings, and the diameter of circles represents the amount of individuals in the groupings and clades. Roman numerals denote original haplotype groupings made with REAP. Colors represent species in groupings, green=*V. cuneata*, blue=*V. flettii*, red=*V. ocelllata*, light blue=*V. cuneata* and *V. flettii*, purple=*V. flettii* and *V. ocellata*, and grey=all three species.

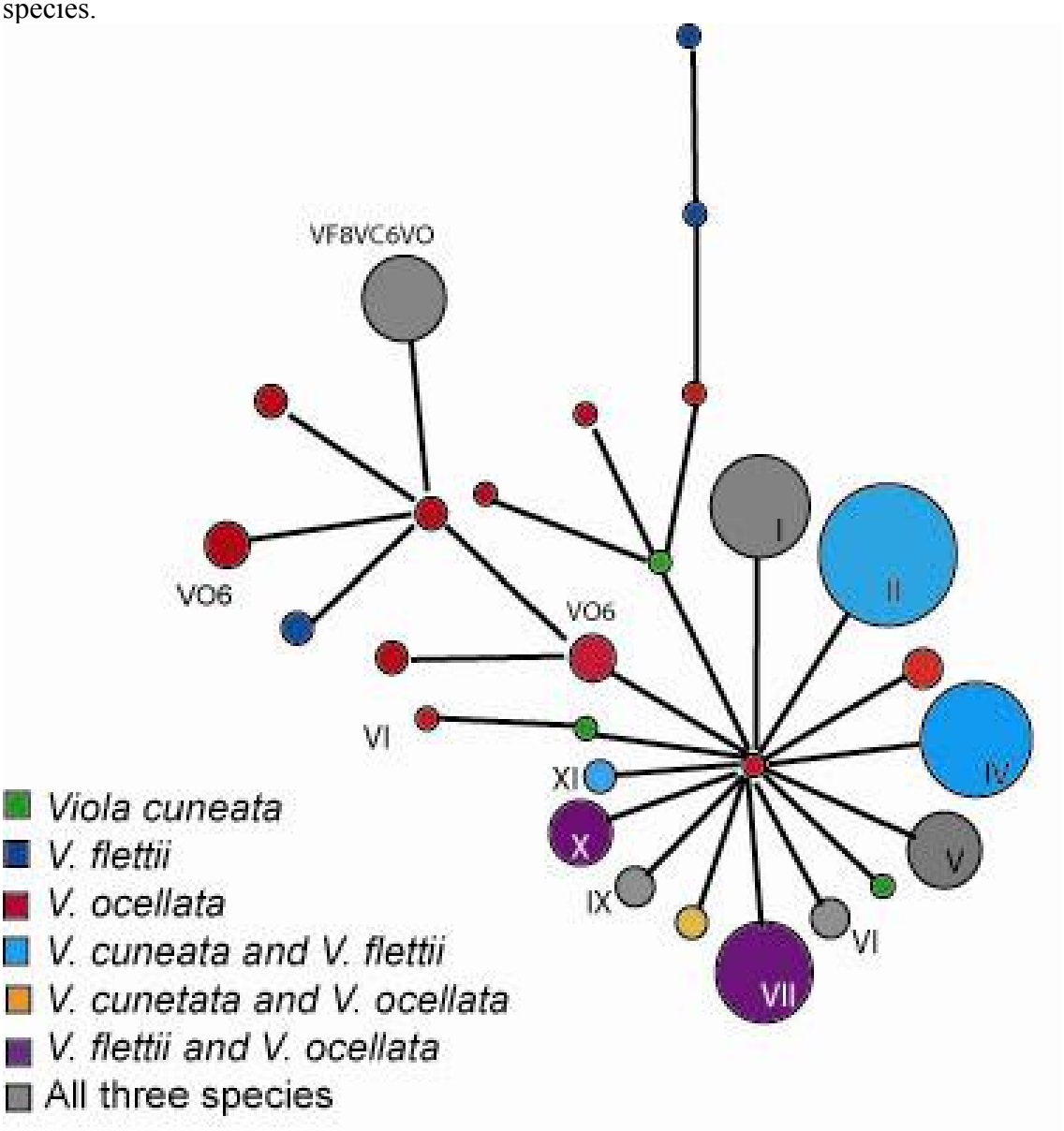


Figure 3.7. First and second axes of PCoA of PCR-RFLP data for *V. cuneata*, *V. flettii* and *V. ocellata*.
Black stars represent the two outgroups used to root tree. Colors represent species in groupings, yellow=*V. cuneata*, blue=*V. flettii*, red=*V. ocelllata*, green=*V. cuneata* and *V. flettii*, purple=*V. flettii* and *V. ocellata*, and grey=all three species.

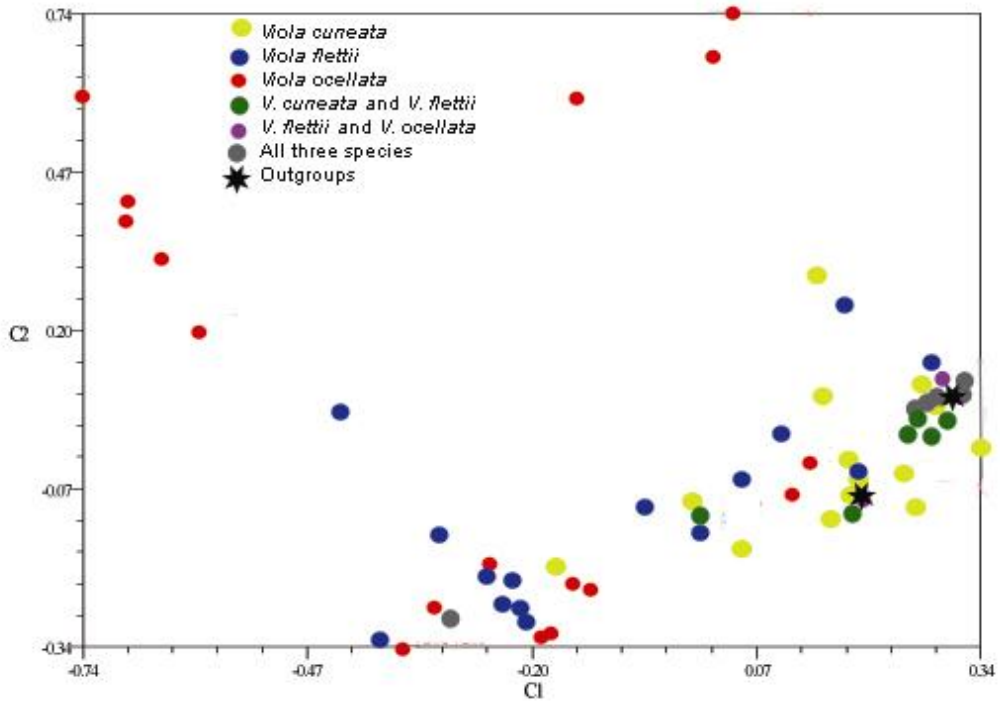
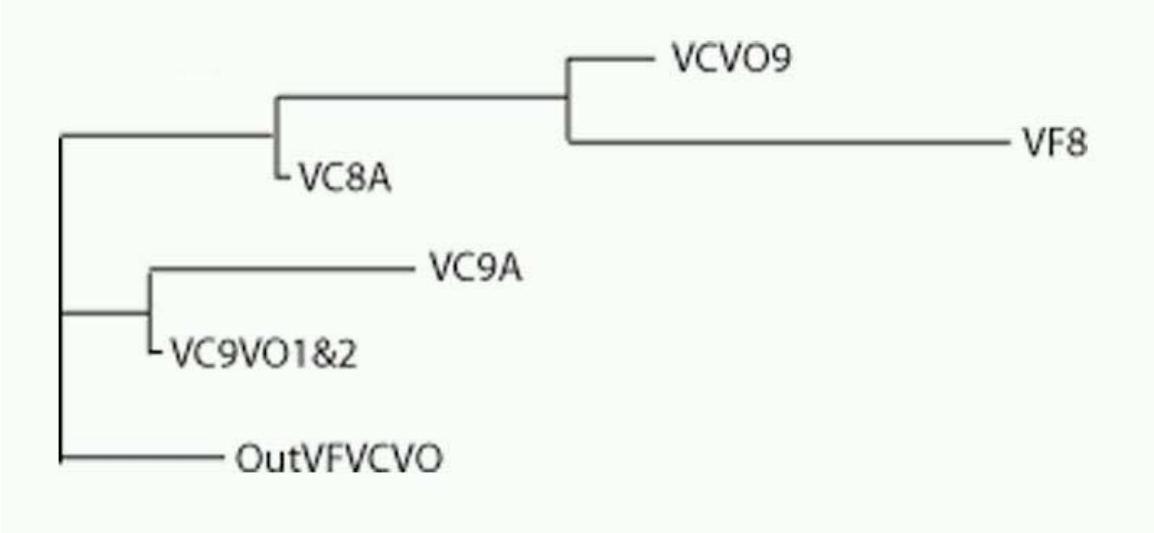


Figure 3.8 Neighbor Joining tree of ITS PCR-RFLP data for V. cuneata, V. flettii and V. ocellata.
Haplotypes are represented by species and populations they include.



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Chapter 4: Ecological Differentiation between Three *Viola* Species and its Relationship to Evolution and Speciation

Abstract

Although *Viola cuneata* S. Watson, *Viola fletti* Piper and *Viola ocellata* Torr. & A. Gray are closely related based on independent molecular phylogenetic evidence, they grow in very different environments. These three species have evolved divergent physiological, morphological, and anatomical mechanisms to deal with the different ecological conditions they face. The question was how these three species differed in morphological and ecological details and how this differentiation might relate to their evolutionary diversification. Methods characterized consistent differences in leaf morphology and environmental conditions, and statistical analyses tested correlations between them to find evidence of adaptive traits. Mantel tests examined potential associations between phylogeographic (Nested Clade Analysis) groupings and environmental conditions. These three *Viola* species occurred in significantly different environments (with respect to elevation, canopy openness, and soil cations). They differed as well in their leaf morphology (leaf angle and leaf shape). Although leaf angles different from those find in living plants were hypothesized to increase light and temperature stress, manipulations of leaf angle had no effect on measured leaf temperature in any of the three species. Mantel tests showed relationships between Nested Clade Analysis (NCA) structure versus elevation and limited climate data, and molecular diversity versus elevation, geographic distance and climate data.

Introduction

Viola cuneata S. Watson, *Viola fletti* Piper and *Viola ocellata* Torr. & A. Gray are closely related members of the *Viola canadensis* complex (Ballard *et al.* 1997) and are endemic to different areas of western North America (Figure 3.1). *Viola cuneata* grows in droughty serpentine barrens at mid-slope elevations in a small area of northern California and southern Oregon. *Viola flettii* grows in rocky crevices and talus slopes at subalpine and alpine elevations of the Olympic Mountains in extreme northwestern Washington. *Viola ocellata* occurs in shaded mesic to dry mesic forest, including Coastal Redwoods, at somewhat lower elevations than the other two species, from central California to southern Oregon. The species are morphologically very distinct and ecologically differentiated. Besides the great degree in vegetative and floral morphology differences with no recognizable transitions between species, *V. cuneata* (droughty serpentine barrens) and *V. flettii* (alpine screes and rock crevices) are limited to specific microhabitats, refuting the likelihood these taxa are mere ecotypes of a broadly distributed species.

Preadaption via the presence of genotypes able to survive under unusual or divergent edaphic conditions, such as serpentine substrates, could lead to distinct tolerant ecotypes and races in the early stages of speciation, with subsequent acquisition of adaptive morphological characteristics; however, isolation that limits or prohibits gene flow between the differentiating taxa would be a prerequisite for speciation to proceed further (Kruckeberg 2002). A speciation model similar to a traditional geographical

isolation model of evolution may have operated in these species, with the added role of adaptation to or narrow tolerance of particular environmental conditions. It is likely that all three species have a predisposition to harsh and droughty environments, as *V. cuneata* occurs in barrens with heavy metal content, *V. flettii* occurs in rock crevices in exposed subalpine and alpine areas, and even *V. ocellata* occurs abundantly in drier and rockier forest sites. A plausible scenario for the origin of the three species, based on results of chloroplast and nuclear phylogeographic studies (see Chapter 3), is that they arose from a common western North American ancestor of dry microsites some time prior to Pleistocene glaciation, began differentiation into forests, serpentine barrens and subalpine areas in situ, and during glacial advances experienced recurrent bouts of hybridization as they differentiated further both ecologically and morphologically. Cluster of semi-differentiated species showing high degrees of hybridization were likely isolated geographically and then via selection to the various substrates and environmental conditions resulting differentiated ecologically. Over time the isolation and ecological selection drove speciation limiting them to their respective habitats, particularly *V. cuneata* and *V. flettii*.

It is proposed that these *Viola* species in the *Viola canadensis* complex are the result (at least partially) of ecological speciation. To test this idea, soil concentrations of calcium, magnesium and heavy metals (chromium, nickel and iron); leaf morphology (particularly leaf angle and leaf base angle); other environmental characteristics (elevation and canopy density); and associations between phylogenetic data and ecological characteristics, were examined in these species. If ecological differentiation is

a primary driver of speciation, it would be expected to show clear statistical differences among species and would also correlate with phylogeographic or phylogenetic evidence on relationships.

Ecological Differentiation. *Viola flettii* occurs in the rain shadow in the eastern half of the Olympic Mountains on the Olympic Peninsula in northwestern Washington at elevations of 1,340 to 1,980 meters, growing in little to no substrate in rock crevices and on talus slopes. Mountainous alpine and subalpine environments have short growing seasons where temperatures often remain extremely low, especially at night. The Olympic Mountains contain steep moisture gradients, from greater than 600 cm precipitation/year on average at the crest of the mountains to less than 40 cm precipitation/year at the northeastern coast of the peninsula, and have sharp elevational gradients that produce very dissimilar microhabitats in close proximity to one another (Peterson *et al.* 1997). The summer growing season is dry and cool but fog can be common. *Viola flettii* is rare throughout its limited range, seeming to have specific microhabitat requirements and very limited means of seed dispersal to new areas. Although it might have a preference to certain rock substrate, there is no evidence that it is adapted to or tolerant of high heavy metal concentrations.

Viola cuneata is endemic to openings in mixed conifer woodlands on serpentine soils or exposed rock in the mountains of northern California from Mendocino and Trinity counties to southwestern Oregon in Curry and Josephine counties (Abrams 1951). This landscape is often dry with bare soil, exposed rock and only a thin forest overstory

consisting mainly of serpentine-restricted conifers, especially *Pinus jeffreyii*, and an understory of shrubs, forbs and herbs. *Viola cuneata* has an elevational range of 365 to 1,525 meters, seeming to occur at higher elevations in the southern portions of its range (specimens from Jepson Herbarium and San Jose State University Herbarium accessed via CALFLORA). There also appears to be a trend towards ecological differentiation of the northern and southern ends of the species' range. Southern populations of *V. cuneata* occur in areas with higher substrate moisture and a greater cover of shrubs as opposed to forbs. Northern populations from Josephine Co. Oregon are on drier sites with more forbs present. Large populations were seen in both the northern and southern extents of this species' range, and populations from both ends of the range were studied.

Viola ocellata occurs in partially shady sites on mesic organic loam in forests along the coastal mountains of California and Oregon from Monterey County in central California to Douglas County in central Oregon (Abrams & Ferris 1951, Hitchcock & Cronquist 1973). It occurs in a variety of locations from dense shade to open woodland, at times along waterways, and is found in Redwood forest as well as other conifer forest types. In Shasta County it occurs on limestone outcrops (specimens from Dean W. Taylor Herbarium accessed via CALFLORA) and has been listed as occurring on serpentine soil (Kruckeberg 1984). It seems to occur more commonly at forest edges or where forest cover is not as dense, possibly due to a higher light requirement than other forest herbs. These forests have well-developed organic soils and higher moisture in comparison to habitats of *V. flettii* and *V. cuneata*. This species is sparsely distributed throughout its range and appears to be the least restricted to specific environments. It is

known from quite a few sites across its ranges, although populations themselves are commonly represented by a small number of individuals. Either phenotypic plasticity is present to deal with differing environments, or ecotypes have differentiated in response to the different environmental conditions found throughout *V. ocellata*'s range. The summer season is the dry season for California and Oregon, but along the coast morning fog is very common. *Viola ocellata* occurs primarily at low elevations from 0 to 1,067 meters (Munz 1959). It is patchily distributed, and small isolated populations may be related to very limited seed dispersal, but could also be caused by the limited distribution of sites with environmental conditions suitable to seedling establishment and seedling environmental requirements. Habitat fragmentation and loss of intervening populations during the warmer Hypsithermal period 5,000 to 6,000 years ago may well explain its spotty distribution (Raven & Axelrod 1978).

Both *V. cuneata* and *V. flettii* occur in high light environments, while *V. ocellata* grows in the partial to complete shade of forests (Table 4.1). Air temperature is greater for *V. cuneata*, moderate for *V. ocellata*, but quite low overall for *V. flettii* (Table 4.1). It is expected that these three species will partition out based on these environmental conditions. By virtue of its tolerance in a California serpentine community, *V. cuneata* must deal with heavy metal toxicity, water stress, high light, high temperatures and low nutrients. The alpine *V. flettii*, on the other hand, encounters freezing stress, low temperatures and high light and UV, as well as possibly low nutrients and water stress in the gravelly locations it can be found. *Viola ocellata* tolerates shading, and high temperatures and water stress towards the end of its growing season.

Heavy Metal Substrates. The serpentine soils where *V. cuneata* thrive, and are likely limited to, are characterized by moderate pH, moderate to high cation exchange capacity (CEC), high magnesium, iron, nickel, and chromium levels, and often low calcium, potassium and phosphorous levels (Brooks 1987) and rapid and massive erosion (Kruckeberg 1992). These harsh conditions limit or stunt flora present on nearby substrates, while likely the combination of many means have driven the evolution of a unique and endemic flora. The characteristics of physical and chemical soil properties, water and temperature variations, season growth, community structure, recycling of biomass, herbivory and disturbance unique to serpentine environments may cause a feedback loop that drives the adaptation of plants to the environment (Kruckeberg 2002). *Viola cuneata* has been shown to accumulate nickel in its leaves with a geometric mean of 257 μg/g drymass, while neither *V. flettii* nor *V. ocellata* do so (Reeves *et al.*1983), even *V. ocellata* sometimes tolerates serpentine soil (Kruckeberg 1984).

The serpentine factor that limits the growth of many plants on such substrates likely involves an abundance of magnesium, and heavy metals in combination with nutrient deficiencies and limited calcium levels, which help emolliate the effects of heavy metal toxicity (Brooks 1987). Iron (Fe) ameliorated the effects of nickel toxicity in *Cochlearia pyrenaica* subsp. *alpina*, a serpentine endemic, particularly by increasing dry shoot weight, as well as by limiting the damage to photosystem II (Nagy & Proctor 2001). Serpentine endemics may be limited to this substrate by a requirement for higher soil concentrations of cations present in excess in these area, (such as cobalt, chromium, iron, magnesium and nickel) or they may have adaptations to tolerate otherwise toxic

effects of serpentine soils and are not competitive with plants that occur on other soil substrates (Kruckeberg 1992). It is likely that serpentine endemics are restricted due to the combination of a number of these factors working together.

It has been shown that root ectomycorrhizae can be utilized by plants to alleviate immediate impacts of heavy metal toxicity (Jentschke & Godbold 2000, Hilderbrandt *et al.* 1999). Rhizobacterial interactions have been shown to increase Nickel uptake in the hyperaccumulator *Alyssum murale* when the plants are inoculated with them (Abou-Shanab *et al.*2003). *Glomus* sp., an arbuscular fungi isolated from *Viola calaminaria* which is specific to Zinc-laden soils, allowed greater uptake of Zinc and Cadmium to the roots of heavy metal-intolerant *Trifolium subterraneum* when inoculated into the soil of the latter (Tonin *et al*. 2001). Rhizobacterial interactions have been shown to increase Nickel uptake in the hyperaccumulator *Alyssum murale* when the plants are inoculated with rhizobacteria (Abou-Shanab *et al.*2003). Thus ectomycorrhizal associations may play an important role in heavy metal tolerance and toxicity remediation, especially in species such as *Viola*.

Leaf Morphology and Leaf Angle. Leaf morphology of all three species differs greatly. Morphology terms were based on the Manual of Leaf Architecture (Leaf Architecture Working Group 1999). *Viola flettii* is a small plant with small (microphyllous), thick, obovate, horizontally oriented leaves with cordate bases and long petioles, sometimes revolute (curled downward) at the margins. Reddish-purple anthocyanin pigmentation in petals and leaves may be an adaptation to alpine conditions and deleterious high UV light

levels, which could decrease photosynthetic efficiency and elevate mutation rates during gamete formation. *Viola ocellata* has larger (notophyllous), thin, horizontally oriented, ovate-elliptic leaves with cordate bases and long petioles. The ovate/obovate leaf shape and cordate base in the first two species are common traits in mesic forest herbs with horizontally oriented blades; Givnish (1986) has argued that such traits are biomechanically required in long-petiolate leaves in order to maintain the optimal (horizontal) angle for maximal photosynthetic efficiency in forest herbs, or in those with depressed light conditions such as those in the commonly overcast alpine habitat of *V. flettii*. *Viola cuneata* is different from the first two species in having intermediate sized (notophyllous), very thick, ovate vertical leaves with truncate to broadly rounded bases and long petioles, and often revolute margins. The vertically oriented leaves are presumably an adaptation to reduce photoinhibition and loss of moisture under the intense sunlight and high temperatures of the species' barrens environment. Givnish's 1986) biomechanical prediction that the vertical leaf-blade orientation does not require a cordate base seems to be upheld in this species.

Small leaf size decreases the boundary layer around a leaf, which aids conductance, making the leaf temperature nearer ambient air temperature (Gutschick 1999). The convection leaf coupling factor of leaf temperature calculation is related to leaf boundary layer where a lower air density, as found at high elevations, would slightly decrease convection, the reddish leaf pigment would also affect solar energy absorption (Ehleringer 1991). Thus, *V.flettii* leaves should show fewer temperature extremes than the leaves of *V. cuneata* and *V. ocellata*, which are larger.

Cordate leaves provide the architectural stability needed to maintain the leaf blade horizontally on a long petiole (Givnish 1986), which would increase intercepted irradiance and thus limit lower temperatures at night via radiative cooling. This may be an important factor, especially with *V. flettii* where growing season nightly temperatures can dip below freezing, but also with *V. ocellata* where in coastal locations evening fog can create cool conditions. Strong selection pressures on leaves may be uncommon, especially in combination as they would lead to 'genetic deaths', however, hundreds of selection pressures may exist for comparable traits, and there appears to be multiple solutions for similar environmental challenges (Gutschick 1999).

Leaf angle is important in the photosynthetic capture of adequate light energy in a shaded forest, as well as the limitation of photosystem decoupling in high temperature and drought stress in dry, open canopy environments. Leaf angle is often close to vertical in dry environments and is thought to reduce deleteriously high leaf temperature s and improve plant water economy (King 1997). High light (sun) leaves have higher photosynthetic capacity (Givnish 1988) as well as altered leaf anatomy to add thickness to epidermis, hypodermis and overall leaf thickness (Arens1997). Shade and slow growing sun plants however may have photosynthetic efficiency which improves with moderately high and fluctuating light (Ögen & Sundin 1996). Similar phenotypic leaf characteristics may be due to close phylogenetic relations instead of ecological distributions (Nicotra *et al.* 1997). High and low light adapted species may differ in how photosynthetic capacity affects leaf mass with light availability (Chazdon & Kaufmann 1993). Leaf angle and self-shading appears to aid in achieving an efficient balance

between carbon gain and minimization of conditions leading to photoinhibition (Valladores & Pearcy 1999). Leaf angle was significantly steeper in light as opposed to shaded conditions for several woody deciduous species, and leaf angle was not thought to be a primary function of protection against photoinhibition (McMillen & McClendon 1979).

Evolutionary Trends in Ecological Differentiation. Studies directly linking ecological characteristics and genetic structure between populations or closely related species have been rarely done, especially with phylogentic data and evolutionary questions. However, it is a good place to begin to determine what the interaction between specific environmental variables and populations/species is in an evolutionary context and then to use this data to better direct further garden, lab and molecular biology studies. The phylogentic structure produced by NCA (Nested Clade Analysis) for leaf beetles was tested with permutational contingency analysis for associations with the ecological variables of tropic selection and habitat altitude to further characterize these species ecologically as well as elucidate how these variables related to evolution of the taxa (Gómez-Zurita *et al.* 2000). More directly, the evolutionary significance of sociality, a behavioral characteristic, in the evolution of sweat bees (*Halictus rubicundus*) was determined by using both NCA structure and genetic maximum likelihood distances in mantel tests versus several environmental factors, determining that the number of days with inch deep snow had a great impact on sociality and presumably its evolution (Soucy & Danforth 2002).

Objectives.

1. The goal of this study was to characterize the morphology, habitat, and evolutionary ecology of three closely related species, and attempt to determine which environmental factors have spurred diversification and evolution of these *Viola* species.

2. Analysis of soil material from *V. cuneata* should demonstrate lower calcium levels and higher magnesium and heavy metal concentration levels than either of the other two species which do not occur (*V. flettii*) or only occasionally occur (*V. ocellata*) on serpentine substrates.

3. All of these species were predicted to differ in leaf morphology from each other, an indicator of specification in the *Viola* genus. *Viola ocellata* and *V. flettii* should be shown to have similar cordate leaf base morphology and lower leaf angles, since neither occur on as droughty or light-intensive conditions as *V. cuneata*. The leaf angle manipulation experiment was predicted to cause a rise in leaf temperature for *V. cuneata*'s leaves manipulated to vertical and greater irradiance, and lower or no effect for *V. ocellata* and *V. flettii*'s leaves.

5. These species were predicted to show differing elevational ranges, with *V. flettii* having the highest and *V. ocellata* with the lowest range, and percent overstory coverage levels, with *V. ocellata* having the highest and *V. flettii* having the lowest levels.

6. Associations based on Mantel tests were expected between phylogeographic (NCA) data and ecological and climatic data.

Methods

Field Data and Material Collection. Materials and data were collected from California, Oregon and Washington in the summer of 2003. Data were collected from 3 sites for each species, with 15 quadrat plots per site. Pressed leaves for leaf base morphological characteristics were collected from one plant in each plot. At each plot a soil core (or small chip of rock for *V. flettii* sites) was taken for subsequent heavy metal concentration analyses (Elzinga 2001). The number of violets present in each plot was recorded. For each plot the percent overstory cover was determined using a mirrored concave densiometer. Holding the densiometer at torso and approximately one foot away from body, the open grid blocks were recorded for the exact location facing in the four main compass directions (north, south, east and west). These were averaged for total percent overstory coverage. Morning, mid-day and evening temperatures were measured at each site. Leaf angle was recorded based on the horizontal for two plants in each plot using a leveled protector with a level of precision of five degrees (Figure 4.1). Location and elevation of each plot was recorded using a handheld Magellan SportTrak Map GPS unit.

Soil Analyses. Soil samples were collected from each plot, and dried in the field. Samples were later sieved through a 10mm sieve (using a hammer when needed for rocky samples). Magnesium and calcium were extracted from 5g of sieved soil with 50ml of Mehlich III extracting solution [0.01mM NH_4F, 0.1mM EDTA (Ethylenedinitrilotetraacetic acid), 250mM NH_4NO_3, 0.08% HNO_3 and 1.15% Acetic Acid]. Heavy metals were extracted from 10g of sieved soil using 20 ml of DTPA

solution [0.005M DTPA (diethylenetriaminepentaacetic acid), 0.1M TEA (triethanolamine), 0.01M $CaCl_2$, adjusted to pH 7.3 with HCl] per Jones (2001). A Varian SpectrAA Model 20 atomic absorption machine with multi-element bulbs for Mg, Ca and Al, and Co, Cr, Cu, Ni, Fe, and Mn was used to measure concentrations based on standards provided by Fisher Scientific. Dilutions of extractions made with deionized water were used when needed to read the samples within the standards' ranges. Raw data were converted to parts per million (ppm) concentrations and then normalized by soil mass.

Manipulation of Leaf Angle. Metal wire was used to carefully mechanically manipulate leaf angle for 15 similarly sized plants (from the natural near 90 degrees of horizontal to zero degrees for *V. cuneata*, and from the natural near zero degrees to 90 degrees for *V. flettii* and *V. ocellata*), one in each of the 15 plots per site. An initial leaf temperature was recorded for each plant to be manipulated, using a Traceable Noncontact infrared thermometer gun with a +/- 2% accuracy (Model # 060664-38, Fisher Scientific) held at the appropriate 8:1 scale distance to encase only the leaf measured. The same variable was also measured on a "control" plant in each of the 15 plots. Manipulation and initial measurements were recorded at approximately 9 AM, and the experiment was continued throughout the day, taking leaf and air temperature readings every two hours for *V. cuneata* and *V. ocellata*, and 3 times throughout the day (9 AM, 1 PM and 5 PM) for *V. flettii* to reduce damage caused by traveling through the sub-alpine site. All measured plants were temporarily tagged with paper tags.

All days were clear to partially cloudy. *Viola cuneata* air temperatures ranged from 16°C (morning) to 38°C (late afternoon) with collection dates of July 14, 17 and 19 (Table 4.3). *Viola ocellata* air temperatures ranged from 16°C (morning) to 32°C (late afternoon) with collection dates of July 5, 8 and 11. For *V. flettii* air temperatures ranged from 15°C (morning) to 39°C (late afternoon) with collection dates of July 26, 31 and August 1.

Leaf Morphology. Leaf base morphology was characterized using a radiometer by lining up the midrib and with widest area of a leaf from the center. Length values at 30 degree intervals were taken around the right side of the leaf; and leaf apex angle (from midrib out) and leaf base angle (from midrib out) were measured (Figure 4.2).

Weather Data. Temperature and precipitation data of the National Weather Service-National Climatic Data Center from the nearest weather collection station for each population was gathered. Winter average data between the months December to February, and summer average data between the June to August were further averaged for overall winter temperature and precipitation and summer temperature and precipitation for each population. Concentration on only winter and summer seasons was done assuming these are the most stressful times for these species.

Statistical Analysis. Data were analyzed with NCSS. In the cases of soil analysis, elevational, leaf angle and leaf base angle (both in radians) data non-normal distributions,

uneven variances and small sample sizes caused the rejection of Analysis of Variance (ANOVA) assumptions, so data were analyzed via the Kruskal-Wallis One Way ANOVA on ranks. Leaf Temperature data were analyzed with repeated measures ANOVA. Leaf morphology data were normalized to eliminate size effects, but was then also analyzed with raw data. Canonical variate analysis, using NCSS, was generated for both raw data and ratio data.

Mantel tests were preformed versus clade structure and standard molecular diversity similar to Soucy & Danforth (2002). Populations were separated out from haplotypes. Differences in geographic distance and climate data between populations were calculated to create dissimilarity matrixes. Clade structure was represented with numbers signifying distance clade distance, such that 0 equaled in the same clade, 1 equaled one clade step away, and so on. All matrixes were decentered to normalize data, and then geographic distance and climate dissimilarity matrixes were tested for association with the clade structure and molecular diversity matrixes via mantel tests with NTSYS (Rohlf 2002).

Results

Soils Analysis. The results of soil analysis were all as expected. Calcium in soils collected from *V. ocellata* was significantly higher than the low levels of *V. cuneata* soils (Figure 4.4). Magnesium was significantly higher in *V. cuneata* soils than *V. ocellata* and *V. flettii* soils (Figure 4.4). Nickel and Chromium were significantly higher in *V. cuneata* than the other species (Figure 4.5). Iron levels in *V. ocellata* soils, although lower were not significantly different than *V. cuneata* levels, however, *V. flettii* had significantly lower Iron levels compared to both other species.

When looking at individual populations (Table 4.4), chromium levels differed between *V. cuneata* populations, being highest at Sanger Peak and lowest at Eight Dollar Mountain. Iron levels were high or intermediate for all of *V. cuneata* populations, but were also high, medium and low for *V. ocellata* populations, suggesting that perhaps *V. ocellata* can also tolerate higher heavy metal concentrations.

Manipulation of Leaf Angle. It was expected that manipulated leaf temperature would differ from control, un-manipulated temperature, with *V. cuneata* leaves manipulated to horizontal having higher temperatures and *V. flettii* and *V. ocellata* leaves manipulated to vertical having lower temperatures. There were no overall significant temperature difference between control and manipulated leaves for any species (Table 4.6). Although, manipulated leaf temperature in *V. cuneata* at times 1 PM and 3 PM were higher as expected, and in *V. flettii* at times 1 PM and 5 PM were lower as expected, though none of these differences were significant (Figure 4.3). However, there were

significant temperature differences between times for *V. cuneata* and *V. ocellata* (Table 4.6 and Figure 4.3), as well as between species.

Leaf Morphology and Leaf Angle. The raw and ratio, where data was normalized to reduce size effects, data for leaf morphology showed all three species distinctly clustering apart from each other, with *V. flettii* and *V. ocellata* clustering closest, and most of the variation was represented by the first axis (Figure 4.6). The first axis was correlated with measurements near the leaf base for the raw data and that and leaf base angle for the ratio data, while the second axis was correlated with the leaf base and apex angles for raw data and middle leaf measurements for ratio data (Table 4.7). When leaf morphology was analyzed within species there was a great deal of overlap between sites, especially with *V. flettii* (Figure 4.7), thus showing no noticeable clines in leaf morphology with any of these species.

The leaf angle from field calculations transformed into radians was also significant for all three species, with *V. ocellata* being lowest, most horizontally held, and *V. cuneata* being highest, most vertically held, and *V. flettii* being intermediate (Figure 4.6). The leaf base angle for *V. cuneata* was significantly different from both *V. flettii* and *V. ocellata*, having truncate to broadly rounded leaf bases and consequently greater leaf base angles.

Other Ecological Data. There was no significant difference in violet number present in each plot for the three species (Table 4.8). As expected, all three species had

significantly different Percent Overstory Coverage measured with a densiometer for each plot, with *V. flettii* having the lowest cover and *V. ocellata* having the highest (Table 4.8).

Evolutionary Trends of Ecological Differentiation. In mantel tests, clade structure showed a significant correlation with elevation, mean winter temperatures and molecular diversity (Table 4.10). Molecular diversity had significant correlations with respect to geographic distance (the typical genetic mantel test) and all climate data (summer and winter mean temperature and mean precipitation). Molecular diversity for individual species was then compared against climate data using a Mantel test. *Viola cuneata* and *V. ocellata*, showed significant associations except for mean winter precipitation (Table 4.10). *Viola flettii* only showed an association between molecular diversity and mean summer temperature.

Discussion

The goal of this study was to characterize the morphology, habitat, and evolutionary ecology of three closely related species, and attempt to determine which environmental factors have spurred diversification and evolution of these *Viola* species.

V. cuneata should demonstrate lower calcium levels and higher magnesium and heavy metal concentration levels than either of the other two species. Soil metal concentrations levels were the greatest characterized ecological differences between these *Viola* species. *Viola cuneata* soils contained the characteristics common to serpentine soil: low Calcium, high Magnesium and high heavy metals (Ni, Fe and Cr). *Viola ocellata* soils, excluding iron levels, and *V. flettii* soils were the opposite of *V. cuneata*. The high iron levels of *V. ocellata* soils, statistically indistinguishable from *V. cuneata* soil, indicates the species is tolerate of iron. Whether it would also be tolerate of the harsher heavy metals nickel and chromium, which *V. cuneata* is tolerate of, is still unknown. This was as expected, showing differing metal tolerance levels certainly has an ecological role for these species, but may also have an evolutionary one.

Species were predicted to differ in leaf morphology. Leaf Angle and Leaf Base Angle were statistically different between the *Viola* species. A leaf angle near 90 degrees has been predicted to relate to lower leaf base angle due to physical constraints of holding a leaf at such an angle (Givish 1986). Both *V. flettii* and *V. ocellata* had leaf angles near 90 degrees and low leaf base angles, while *V. cuneata* had higher leaf angles (from holding

their leaves upright) and higher leaf base angles, thus the results for these species indicate that such an associate may exist.

Canical-Variate scores from leaf morphology data, with and without a size factor, showed all three species to have morphogically distinct leaf patterns, as assumed. Distinct leaf morphology is often a good characteristic of species distinction in the *Viola* genus. Leaf shape was also proven as a good taxonomic character in *Begonia*, although allozymes were not correlated with leaf shape (McLellan 2000). RAPD molecular marker has detected similar diversity as leaf morphology (Persson & Gustovsson 2001). Leaf shape traits are under genetic control, possibly by only a few genetic regions, and thus are questioned as an indicator of introgression (Wu *et al* 1997)

Leaf angle manipulation experiment was predicted to cause a rise in leaf temperature for *V. cuneata*'s leaves manipulated to vertical, and lower leaf temperature for *V. ocellata* and *V. flettii*'s leaves. The leaf angle manipulation study showed little to no difference in leaf temperature upon angle manipulation. Certainly there was variation through the day as air temperature increased. A study on *Heteromeles arbutifolia* showed that the light environment is heterogeneous and that the relations between light level, carbon gain, leaf orientation, leaf position, leaf age and physiological acclimation is not obvious (Valladores & Pearcy 1999). Thus, a lack of significant differences in leaf temperatures after leaf manipulation may be related to this heterogeneous light environment being too variable, even on such a small scale, to recognize a difference. The complete inversion of leaves of *Heteromeles arbutifolia* took 24 to 48 hours for a difference in the

photochemical efficiency of Photosystem II to be observed (Valladores & Pearcy 1999). So the limited amount manipulation time may not have been insignificant to observe differences in leaf temperature.

As well as leaf angle, solar angle, leaf azimuth (compass direction a leaf blade faces), and the slope and azimuth of a site can all factor into the radiate energy a leaf absorbs and thus have an effect on leaf temperature (Ehleringer 1991). Thus, not measuring and accounting for these environmental variables could have heightened site and microsite effects. Coupled with instrumentation error this may have limited the statistical ability of finding significant differences between control and manipulated leaves.

Species were predicted to show differing elevational ranges and percent overstory coverage levels. All three species had significantly different elevational ranges and percent overstory coverage, based on surveyed populations. *V. cuneata* possessed a middle elevational range and the lowest percent overstory coverage, *V. flettii* possessed the highest elevational range and middle percent overstory coverage, and *V. ocellata* possessed the lowest elevational range and highest percent overstory coverage. This was all as expected for the species, and adds support to the differing ecological requirements the species may possess.

Associations based on Mantel tests were expected between phylogeographic (NCA) data and ecological and climatic data. Clade structure showing a correlation with elevation

may indicate residual population and species associations lingering in similar elevations even if geographic regions have changed. The correlations between molecular diversity and clade structure and geographic distance are most likely due to past isolation and/or separation via range expansion, both are mentioned in predictions from Chapter 3.

Winter temperatures being related to the clade structure may best show not only latitudinal separations but also clusters of populations and species where similar low temperatures exist or existed previously. Molecular diversity was highly correlated with temperature and precipitation data. When individual species were looked at for correlations between climate data and molecular diversity, both *V. cuneata* and *V. ocellata* showed significant associations. This could be caused by optimum environmental conditions resulting in greater molecular diversity through higher reproductive fitness in these species. The inclusion of climate data from only averages for the entire summer and winter, as well as having to use the nearest collection station instead of the true sites, may have caused incorrect results in what the relationships between populations and species with climate characteristics.

Conclusions. Although chloroplast PCR-RFLP data displayed no distinct separation between these three species (Chapter 3), morphologically and ecologically they are very distinct. Leaf shape is related to leaf base angles and leaf angles in all three species. The manipulation of leaf angle, however, resulted in no significant differences in leaf surface temperature. It is possible that the sampling error with this method was too great to interpret finer-scale microhabitat influences, or perhaps the instrument or method used to

obtain leaf temperature was too coarse for the study. More refined techniques examining different small areas of the leaf blade over the entire surface might detect significant differences, whereas the present method may have "homogenized" the temperature over a larger surface area. Secondly, the surface temperature is perhaps not reflective of the internal leaf temperature (which might exhibit a significant difference), in which case the use of internal leaf thermocouples may yield different results. Direct or indirect measurements on physiological processes themselves, such as photosynthetic rate and efficiency, were not conducted, and these may provide further evidence for the "adaptive" nature of leaf angle for each species under varying ecological conditions.

Precipitation and temperature may affect the fitness of at least *V. cuneata* and *V. ocellata*. The limited microsite characteristics available when looking at *V. flettii* weather data may have limited the ability to see a statistically significant relationship between *V. flettii* fitness and environmental conditions. Ecological differentiation withmorphological change has clearly taken place in the process of speciation. The relations of ecological and morphological characteristics that separate these species to their phylogeographic relationships were too complicated to elucidate using the methods applied here. The phylogeographic pattern in both the nuclear and chloroplast genomes is possibly complicated by recurrent past hybridization and maintenance of polymorphism in these more "selectively neutral" gene regions, hindering the establishment of clear associations between phylogeographic patterns with and morphological or ecophysiological traits.

Chapter 4 Tables and Figures

Table 4.1. Expected ecological factor levels for all three *Viola* species.

Species	Light	Temperature	Soil Moisture	Mg soil conc.	Ni soil conc.	Mg/Ca ratio
Viola cuneata	High	High (27 °C)	Low	High	High	High
Viola flettii	High	Low (10 °C)	Low	Low	Low	Low
Viola ocelllata	Low to Medium	Medium to high (20-27 °C)	High	Low to High	Low to High	Low to High

Table 4.2. Expected leaf characteristics for all three *Viola* species.

Species	Leaf Size	Leaf Base Morphology	Normal Leaf Angle	Manipulated Leaf Temperature
Viola cuneata	Medium	Truncate	Close to 90°	Higher than normal
Viola flettii	Small	Cordate	Close to 0°	Lower than normal
Viola ocellata	Large	Cordate	Close to 0°	Lower than normal

Table 4.3. Air temperature values for days of leaf manipulation studies for all three *Viola* species.

Species	Population	Time	Temperature (C)	Date
V. ocellata	**Memorial Park**	9:00 AM	16.0	5-Jul
		11:00 AM	18.0	
		1:00 PM	19.0	
		3:00 PM	19.0	
		5:00 PM	18.0	
Daily Average			18.0	
	Middle Creek	9:00 AM	20.0	8-Jul
		11:00 AM	27.0	
		1:00 PM	29.0	
		3:00 PM	30.0	
		5:00 PM	28.0	
Daily Average			26.8	
	Clover Creek	9:00 AM	24.0	11-Jul
		11:00 AM	28.0	
		1:00 PM	31.0	
		3:00 PM	31.0	
		5:00 PM	32.0	
Daily Average			29.2	
V. cuneata	**Horse Mt**	9:00 AM	19.0	
		11:00 AM	20.0	14-Jul
		1:00 PM	24.0	
		3:00 PM	27.5	
		5:00 PM	21.0	
Daily Average			22.3	
	Sanger Peak	9:00 AM	16.0	17-Jul
		11:00 AM	21.0	
		1:00 PM	24.0	
		3:00 PM	31.0	
		5:00 PM	36.0	
Daily Average			25.6	
	Eight Dollar Mt	9:00 AM	26.5	19-Jul
		11:00 AM	30.0	
		1:00 PM	36.0	
		3:00 PM	38.0	
		5:00 PM	38.0	
Daily Average			33.7	

Table 4.3 (cont.). Air temperature values for days of leaf manipulation studies for all three *Viola* species.

Species	Population	Time	Temperature (C)	Date
V. flettii	**Marmot Pass**	9:00 AM	15.0	26-Jul
		1:00 PM	30.0	
		5:00 PM	19.0	
Daily Average			21.3	
	Near Blue Mt	9:00 AM	14.0	31-Jul
		1:00 PM	32.0	
		5:00 PM	39.0	
Daily Average			28.3	
	Blue Mountain	9:00 AM	19.0	1-Aug
		1:00 PM	25.0	
		5:00 PM	24.0	
Daily Average			22.7	

Table 4.4. Means for Calcium and Magnesium in the collected soil samples of *V. cuneata*, *V. ocellata* and *V. flettii* separated by species and population.
Letters after means denote significant differentiation Kruskal-Wallis One-Way ANOVA on Ranks by multiple comparison tests.

Species	Populations	mg/g soil			
		Mean Ca	SE	Mean Mg	SE
V. cuneata	Horse Mountain	0.60 a	0.28	2.62 bc	0.21
	Eight Dollar Mt.	0.68 a	0.27	1.54 b	0.21
	Sanger Peak	0.44 a	0.27	3.43 c	0.21
V. ocellata	Memorial Park	5.12 c	0.27	1.15 ab	0.21
	Middle Creek	1.82 b	0.27	0.55 a	0.21
	Clover Creek	2.55 b	0.27	0.92 ab	0.21
V. flettii	Near Blue Mt			0.93 ab	0.21
	Blue Mt			0.59 a	0.27
	Marmot Pass			0.75 a	0.47

Table 4.5. Means for Nickel, Chromium and Iron in the collected soil samples of *V. cuneata*, *V. ocellata* and *V. flettii* separated by species and population.
Letters after means denote significant differentiation Kruskal-Wallis One-Way ANOVA on Ranks by multiple comparison tests.

Species	**Populations**	**mg/g soil**					
		Mean Ni	SE	Mean Cr	SE	Mean Fe	SE
V. cuneata	Horse Mountain	1.30E-01 b	2.24E-03	1.84E-02 bc	2.87E-03	1.45E-01 bc	2.33E-02
	Eight Dollar Mt.	3.80E-01 b	2.42E-02	2.17E-03 b	2.77E-03	7.20E-02 b	2.33E-02
	Sanger Peak	1.42E-01 b	2.24E-03	3.95E-03 c	2.68E-03	1.99E-01 c	2.33E-02
V. ocellata	Memorial Park	2.59E-03 a	2.24E-03	9.10E-04 ab	2.68E-03	1.77E-01 c	2.33E-02
	Middle Creek	4.27E-04 a	2.24E-02	4.18E-04 a	2.77E-03	1.61E-02 a	2.33E-02
	Clover Creek	1.70E-04 a	2.62E-02	8.69E-04 b	2.87E-03	1.25E-01 b	2.42E-02
V. flettii	Near Blue Mt	1.00E-04 a	4.34E-02	1.87E-04 a	3.66E-03	5.56E-03 a	2.33E-02
	Blue Mt	7.00E-05 a	2.51E-02	2.05E-04 a	5.98E-03	6.63E-03 a	2.57E-02
	Marmot Pass	8.00E-05	2.62E-02	3.25E-04 a	3.12E-03	2.91E-02 a	2.42E-02

Table 4.6. Analysis of Variance table for repeated measures analysis of manipulated leaf temperature data for all three *Viola* species.

Analysis of Variance Table				
V. cuneata				
Source of Variation	SS	DF	MS	p-value
Total (Adjusted)	62497	449		
Population	9670	2	4735	
Plot	0	12	0	
Time	16094	4	4023	0.0297
Treatment	112	1	112.5	0.1556
V. flettii				
Source of Variation	SS	DF	MS	p-value
Total (Adjusted)	22357	179		
Population	1.6	1	1.6	0.9078
Plot	1498	13	115	0.4436
Time	6181	2	3090	0.2689
Treatment	66	1	66	0.2786
V. ocellata				
Source of Variation	SS	DF	MS	p-value
Total (Adjusted)	21440	449		
Population	10044	2	5022	
Plot	0	12	0	
Time	3340	4	835	0.0182
Treatment	7.3	1	7.34	0.3380

Table 4.7 Variable-Variate Correlations for Canical Variates Analysis (CVA) for raw and ratio leaf morphology data. Bold is significant correlations.

	Raw Data		Ratio Data	
Variable	Variate1	Variate2	Variate1	Variate2
Apex	-0.156393	**0.805844**	0.030390	-0.098762
Base	-0.142501	**0.347758**	**0.728353**	-0.072480
0º	-0.139732	0.185667	-0.472065	-0.061475
30º	-0.032933	0.066457	**-0.312365**	**0.640777**
60º	-0.128900	0.198167	**-0.291500**	**0.868259**
90º	0.042633	0.034542	-0.077677	**0.309957**
120º	**0.382541**	0.117896	-0.229832	**0.650842**
150º	0.031831	0.105093	0.016196	0.217055
180º	**0.742544**	0.053400	**0.472065**	0.061475

Table 4.8. Kruskal-Wallis rank test for Percent Overstory Coverage, number of violets per plot, leaf base in radians and leaf angle in radians comparing all three species. Letters following numbers denote significant differences.

	% Overstory Coverage		# Violets/Plot		Leaf Base (Radians)		Leaf Angle (Radians)	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
V. cuneata	35.585 [b]	1.47	6.533	0.81	2.263 [b]	0.025	1.058 [c]	0.027
V. flettii	21.528 [a]	2.47	6.222	0.45	0.533 [a]	0.041	0.361 [b]	0.046
V. ocellata	71.743 [c]	0.84	5.489	0.54	0.552 [a]	0.033	0.160 [a]	0.037
	p-value<0.001				p-value<0.001		p-value<0.001	

Table 4.9. Mantel tests for clade structure and molecular diversity versus elevation, geographic distance and climate data with all three species combined.

	Clade Structure		**Molecular Diversity**	
	r	**p value**	**R**	**p value**
Elevation	0.04687	0.0125	0.08531	0.0009
Geographic Distance	0.02689	0.0558	-0.05972	0.0020
Summer Precipitation	0.02047	0.0789	-0.03580	0.0070
Summer Temperature	-0.01696	0.0749	-0.03763	0.0001
Winter Precipitation	0.00250	0.3333	-0.02378	0.0310
Winter Temperature	0.03334	0.0314	-0.08859	0.0010
Molecular Diversity	0.26914	0.0001		

Table 4.10. Mantel tests for clade structure and molecular diversity versus elevation, geographic distance and climate data with all three species separated.

		Molecular Diversity of Individual Species	
		Normalized Z (r)	P-value
V. cuneata			
	Summer Precipitation	-0.112	0.0055
	Summer Temperature	0.216	0.0003
	Winter Precipitation	0.216	0.0006
	Winter Temperature	-0.113	0.023
V. flettii			
	Summer Precipitation	0.007	0.3985
	Summer Temperature	0.111	0.0052
	Winter Precipitation	0.049	0.1248
	Winter Temperature	Calculation not possible	
V. ocellata			
	Summer Precipitation	0.129	0.0238
	Summer Temperature	0.091	0.0177
	Winter Precipitation	0.045	0.1012
	Winter Temperature	0.206	0.0038

Figure 4.1. Leaf angle calculations were taken by determining horizontal with a level and then measuring the distance from horizontal to the plane of the leaf.

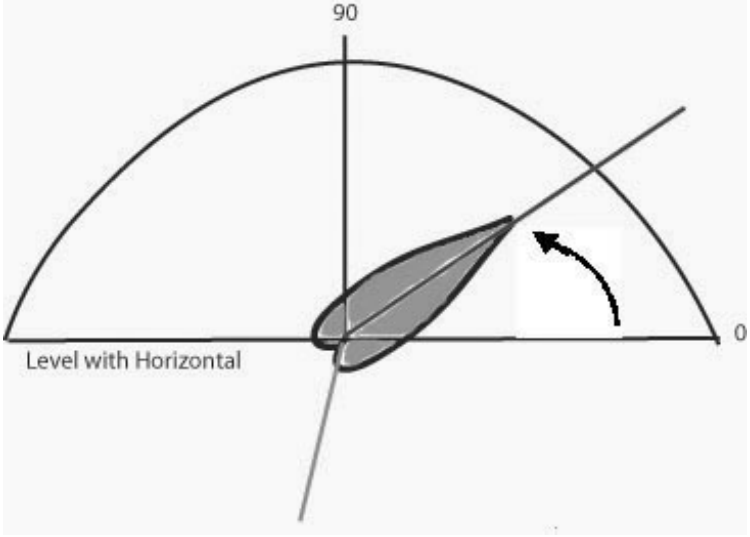


Figure 4.2. Leaf morphology measurements were taken with a radiometer laid over the leaf.
Distances from the leaf petiole to leaf margin on the right side at 0, 30, 60, 90, 120, 150 and 180 degrees were recorded. Leaf base angle was measured from the 180 degree line to the first portion of leaf.

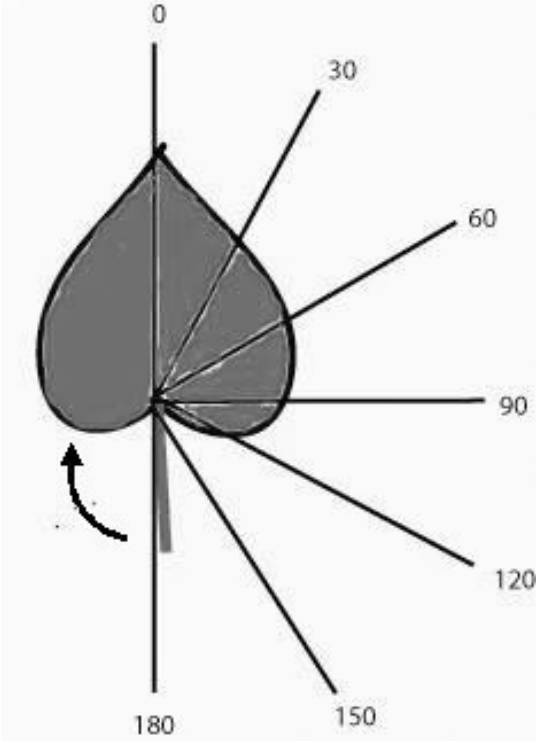


Figure 4.3. Mean control (black) and manipulated (diagonal striped) temperature values with error bars for each time value [9am, 11am, 1pm (13), 3pm (15) and 5pm (17)]. Letters denote significant differences in time values determined with Repeated Measures ANOVA and Turkey-Kramer Multiple Comparison tests. Right side of graph displays mean leaf angle in degrees of *V. cuneata* (dark gray), *V. flettii* (hashing) and *V. ocellata* (horizontal stripes) with manipulated leaf angles (*V. cuneata* leaf angle = 0 degrees), letters denote significant differences determined by Kruskal-Wallis One-Way ANOVA on ranks with multiple comparison test.

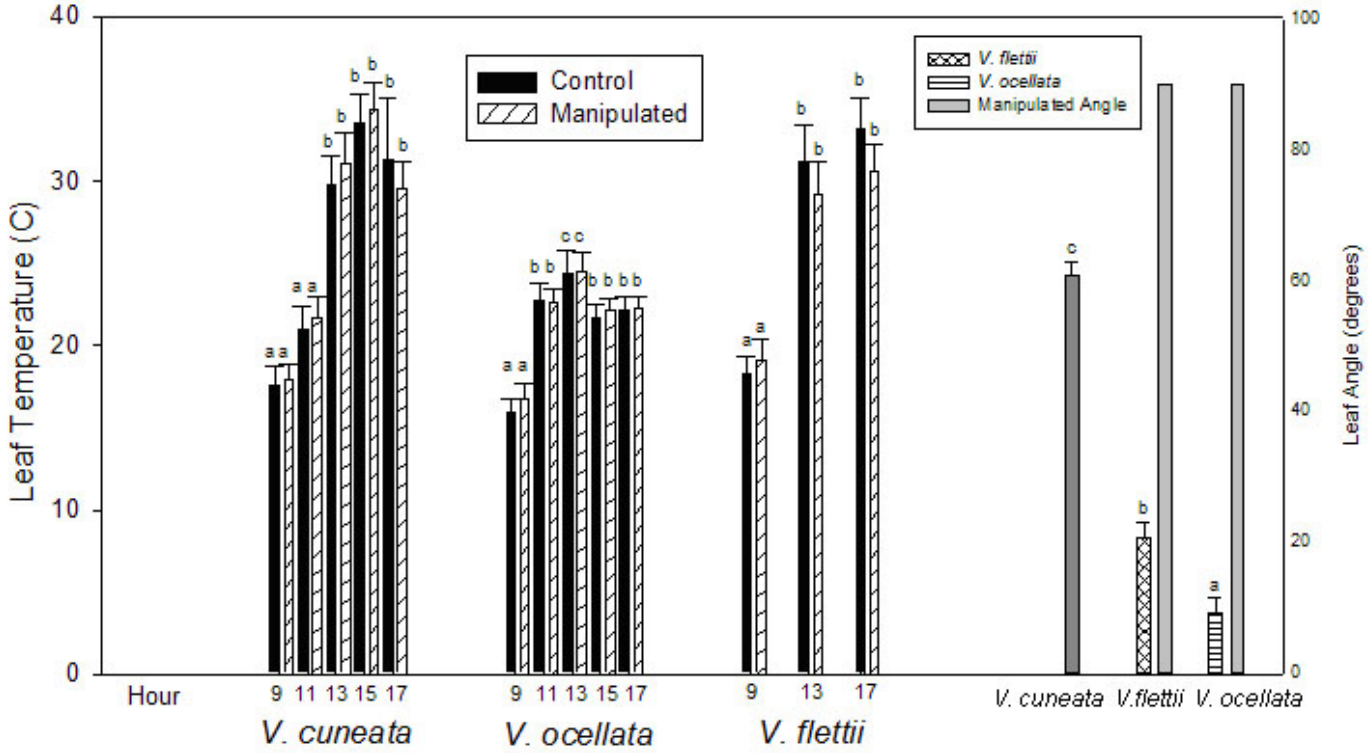


Figure 4.4. Mean values for Calcium and Magnesium with standard error bars, from soil collected around three *Viola* species.
Letters denote significant differences determined with Kruskal-Wallis One-Way ANOVA on Ranks and Multiple Comparison Z-value tests.

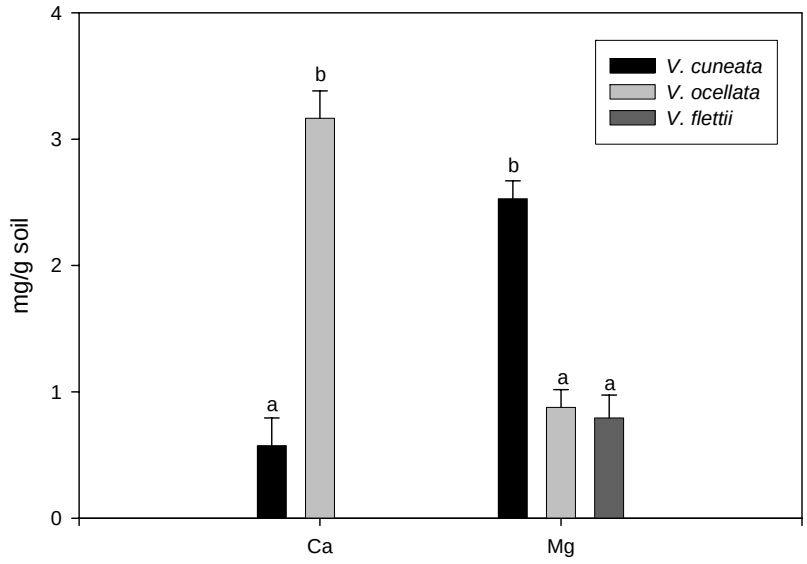


Figure 4.5. Mean values for heavy metals Nickel, Chromium and Iron with standard error bars, from soil collected around three *Viola* species.
Letters denote significant differences determined with Kruskal-Wallis One-Way ANOVA on Ranks and Multiple Comparison Z-value tests.

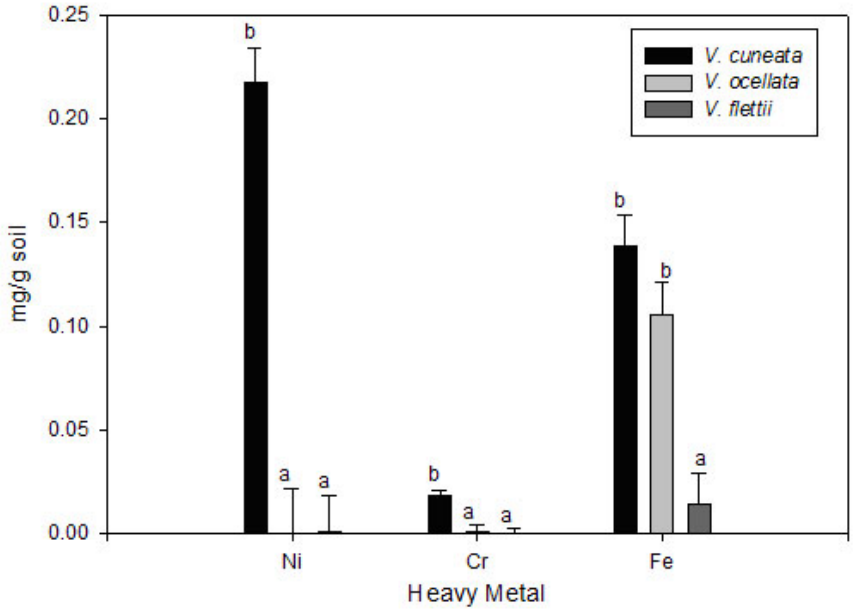


Figure 4.6. Canonical Variates plots for leaf shape of all three *Viola* species with percent variation explained by the axes.
Ratio data on the left and raw data on the right. Circles = *Viola cuneata*; triangles = *V. flettii*; squares = *V. ocellata*.

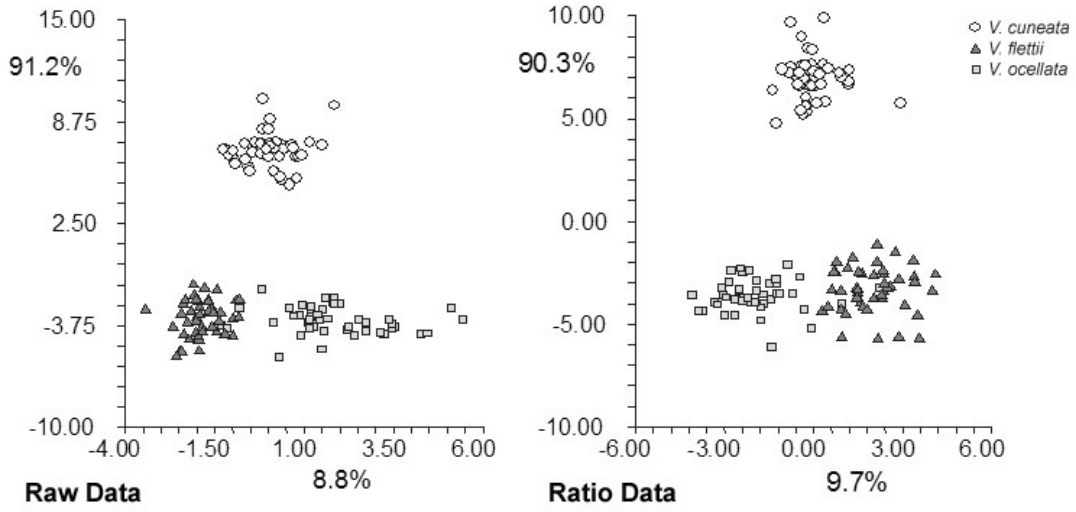
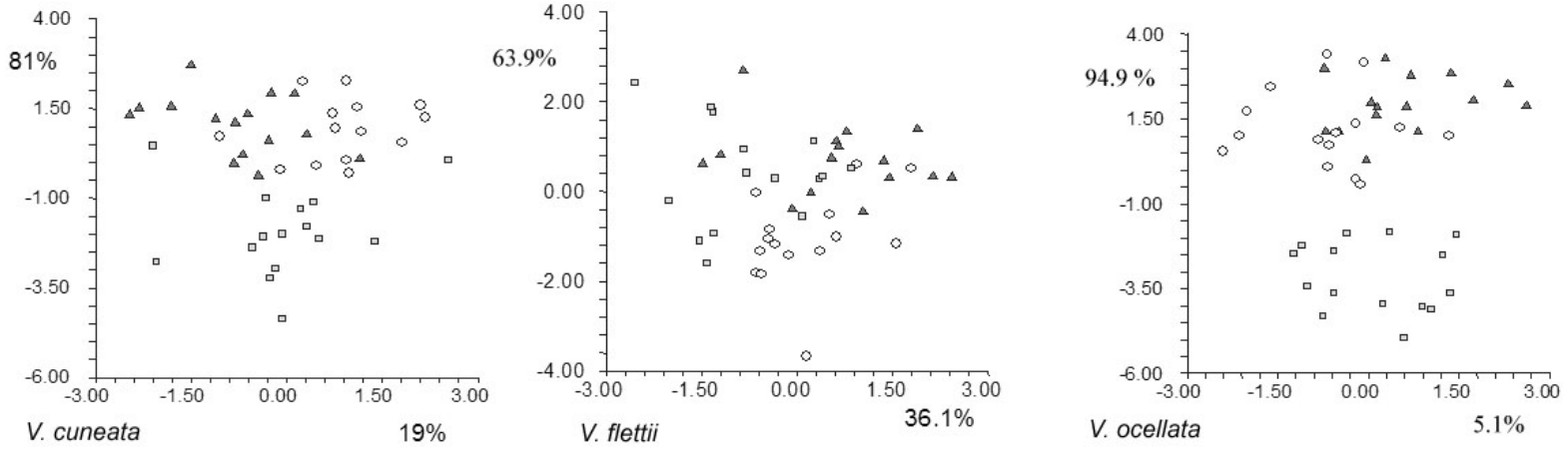


Figure 4.7. Canonical Variates plots of raw leaf shape raw data
for populations of *Viola cuneata* (right), *V. flettii* (center), and *V. ocellata* (right).



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Chapter 5: Overall Conclusions

Chapter 2 Objectives and Conclusions. Smaller isolated (peripheral) populations of *Viola flettii* were predicted to possess less genetic diversity than larger populations. Peripheral populations of *V. flettii* were predicted to have a significantly greater proportion of unique alleles. Populations of *V. flettii* on the summits (i.e., at the highest elevations) were predicted to be phenotypically and genetically different from populations in the mountain passes (at lower elevations), expressing reduced plant vigor and size, reduced fitness as expressed in reproductive output, reduced genetic diversity within populations, and greater differentiation among populations. Vigor, reproductive success and genetic diversity were predicted to correlate with population and community ecological characters along an elevational gradient.

Viola flettii, despite its limited distribution and isolated populations, displayed a great deal of diversity based on the Intersimple-Sequence-Repeat marker system (ISSR). The species also showed a clear differentiation between northern and southern populations. Smaller populations did not possess less genetic diversity and peripheral populations did not possess great amounts of different and unique alleles. The present genetic diversity appears to display an ancient past and a possible history of isolation and fragmentation during the spread of Pleistocene alpine glaciers via variable genetic drift, environmental selective pressures, high selfing rates and small populations.

Microhabitat effects were seen to be important, even along the limited elevational range of *V. flettii*. Higher elevation populations possessed less genetic diversity, partly supporting the expectations of harsher environmental negatively affecting the species.

Populations with more southernly aspect were shown to have greater genetic differentiation, showing that elevation alone is not the only limiting environmental conditions affecting the species. Both measures of fitness, leaf number and reproductive structure number, were correlated with increasing genetic differentiation (Φ_{IS}) indicating the differentiation of the species and populations may affect the overall fitness of the species, and preserving that differentiation by protecting populations should be considered an importance for the future.

Populations of *V. flettii* in both northwestern and southeasterrn regions should be considered for protection as they contain different genetic resources. Elevations at the middle of the species range had greater genetic diversity and populations with more southernly aspects had genetic differentiation, so these ecological characters should be a priority in the protection of populations. *V. flettii* presently contains high genetic diversity and genetic structuring, but its limited distribution and narrow environmental requirements puts it in danger because of climate change and its effects on the Olympic Mountains.

Chapter 3 Objectives and Conclusions. The objective of this study was to compare the population differentiation of chloroplast regions in the closely related endemic species, *Viola cuneata*, *Viola flettii* and *Viola ocellata*, and possibly related species of the *Viola canadensis* complex. A high level of between-population genetic differentiation and a lower within-population genetic diversity was expected. Additionally subpopulations collected in close geographic proximity to larger populations were predicted to be more

genetically similar than the geographically isolated populations because of potential gene flow. Populations that were nearest to the geographic center of the range of each species were also expected to have a higher level of within-population diversity but lower among-population differentiation.

These three species have a complex and confusing genetic history that may reflect past recurrent hybridization between species with chloroplast capture in some populations, likely while they existed together in glacial refugia and perhaps also earlier during the speciation and diversification process. Populations and species held limited genetic diversity. There were few geographic associations or patterns to populations and species which held haplotype similarities. Most interesting is the divergence of central range *V. ocellata* haplotypes and populations from the rest of the surveyed individuals, as well as their central location within the Nested Clade Analysis (NCA). This grouping of haplotypes may represent a geographic center of these species diversification and speciation, or it may instead show a clustering of populations that were segregated from other populations during Pleistocene glaciation.

Subsequent range expansions and fragmentations have yet to lead to clear genetic differentiation, at least in these genomic regions. However, it is possible that these genetic components of the species' genomes, especially of the chloroplast genome, have remained largely "selectively neutral" and retain the historical polymorphisms, whereas other nuclear genes being more directly involved with morphological and ecological differentiation are now predominately distinct. This hypothesis would explain the discrepancy between the complex evolutionary history resulting from hybridization on

the one hand, with the strong morphological and ecological distinctness of the taxa on the other. This also provides a look at what genetic interactions present widely divergent geographically populations within and among species had with each other in the past.

A more in depth study of nuclear genes, especially if those that may be related to ecological adaptation were to be surveyed may well find the species to be nuclearly distinct. Similarlly, more extensive chloroplast DNA survey with a molecular marker that allows less perception of differences and presumably noise would be useful, such as (Amplified Fragment Length Polymorphisms) AFLPs or chloroplast microsatillites. More surveying of the species and collection of greater populations not only in the main portions but also extremes of the species ranges would also aid in collecting further data points.

Chapter 4 Objectives and Conclusions. The goal of this chapter was to characterize the morphology, habitat, and evolutionary ecology of three closely related *Viola* species, and attempt to determine which environmental factors have their spurred diversification and evolution. Analysis of soil material from *V. cuneata*, which occurs on serpentine barrens, was expected to demonstrate the characteristic serpentine lower calcium levels and higher magnesium and heavy metal concentration levels in comparison to the other two species. All of these species were predicted to differ in leaf morphology from each other, an indicator of specification in the *Viola* genus. The leaf angle manipulation experiment was predicted to show relationships between leaf angle and leaf temperature, leaf angle and leaf base angle were also expected to be associated. These species were predicted to

show differing elevational ranges and percent overstory coverage levels. Associations based on Mantel tests were expected between phylogeographic (NCA) data and ecological and climatic data.

As expected, *Viola cuneata* showed significantly lower calcium levels and significantly higher magnesium and heavy metal (chromium and nickel) levels than the other two species, iron levels, however, were not different for *V. cuneata* and *V. ocellata*. These three species were significantly distinct in leaf morphology, leaf base angle, leaf angle. The manipulation of leaf angle caused no significant differences in leaf temperature possibly because sampling error was too great to compensate for microhabitat influences. Greater leaf base angle was associated with greater leaf angle, as expected. All three species were significantly different with respect to elevation and percent overstory coverage, showing they occur in environmentally diverse locations, as already suspected. Both precipitation and temperature could affect the fitness of *V. cuneata* and *V. ocellata*. Although the exact characteristics are still unknown, ecological differentiation appears to have played a part in speciation for these *Viola* species.

Summary. In all three species, genetic data indicate past history, although the historic pattern is not easy to interpret, more convincing that it is not partially an artifact of low variation in molecular markers used or a confounding biological phenomenon obscuring the story. *Viola flettii* showed a distinct separation between northern and southern populations, likely the result of past isolation and subsequent genetic drift and environmental selection. Microsite and microhabitat selection is present for *V. flettii*.

However, the combined genetic data displayed a lack of differentiation between populations and species, even though populations are often small and isolated and the species are greatly separated geographically. Ecologically and morphologically the species are clearly distinct giving rise to the conclusion that the genetic intermixing seen in the chloroplast DNA is due to historical interbreeding leading to chloroplast capture possibly both anciently before or during speciation as well as in Plesticene glacial refugia. Ecological differentiation may have played an important role in the speciation of these three Viola species. They share a complex history, and further studies are needed to clarify their genetic diversity and phylogenetic relationships, and to identify more specifically the ecological and environmental factors which may have driven their ecological differentiation.

Appendices

1. Ecological Data for *Viola flettii*

Date	Population	Individual	# Leaves	# Flowers	#Fruits	# Fertile Structures	Juvenile
8/4/1999	1	1	10	0	3	3	
8/4/1999	1	2	8	1	5	6	
8/4/1999	1	3	8	0	3	3	
8/4/1999	1	4	20	3	5	8	
8/4/1999	1	5	11	0	6	6	
8/4/1999	1	6	7	0	3	3	
8/4/1999	1	7	6	0	1	1	
8/4/1999	1	8	26	1	4	5	
8/4/1999	1	9	3	0	4	4	
8/4/1999	1	10	20	3	4	7	
8/4/1999	1	11	9	0	2	2	
8/4/1999	1	12	5	0	4	4	
8/4/1999	1	13	4	0	1	1	
8/4/1999	1	14	17	4	2	6	
8/4/1999	1	15	40	6	1	7	
8/5/1999	1	16	28	2	8	10	
8/5/1999	1	17	9	0	2	2	
8/5/1999	1	18	12	0	6	6	
8/5/1999	1	19	13	0	4	4	
8/5/1999	1	20	8	1	0	1	
8/5/1999	1	21	8	0	1	1	
8/5/1999	1	22	29	0	5	5	
8/5/1999	1	23	15	0	8	8	
8/5/1999	1	24	14	0	1	1	
8/5/1999	1	25	7	0	2	2	
8/5/1999	1	26	12	1	4	5	
8/5/1999	1	27	20	1	2	3	
8/5/1999	1	28	17	1	2	3	
8/5/1999	1	29	14	0	3	3	
8/5/1999	1	30	36	3	8	11	
8/17/1999	2	1	11	1	0	1	
8/17/1999	2	2	7	0	5	5	
8/17/1999	2	3	4	0	1	1	
8/17/1999	2	4	31	1	0	1	
8/17/1999	2	5	12	0	1	1	
8/17/1999	2	6	3	0	1	1	
8/17/1999	2	7	16	0	4	4	
8/17/1999	2	8	14	0	2	2	

Date	Population	Individual	# Leaves	# Flowers	#Fruits	# Fertile Structures	Juvenile
8/17/1999	2	9	54	2	1	3	
8/17/1999	2	10	7	0	2	2	
8/17/1999	2	11	21	2	2	4	
8/17/1999	2	12	8	0	1	1	
8/17/1999	2	13	11	0	1	1	
8/17/1999	2	14	49	0	4	4	
8/17/1999	2	15	28	0	3	3	
8/17/1999	2	16	12	0	1	1	
8/17/1999	2	17	9	1	1	2	
8/17/1999	2	18	16	2	0	2	
8/17/1999	2	19	11	0	2	2	
8/17/1999	2	20	13	2	0	2	
8/17/1999	2	21	6	0	1	1	
8/17/1999	2	22	13	0	5	5	
8/17/1999	2	23	5	0	2	2	
8/17/1999	2	24	9	0	5	5	
8/17/1999	2	25	5	1	0	1	
8/17/1999	2	26	15	0	1	1	
8/17/1999	2	27	6	2	0	2	
8/17/1999	2	28	6	0	1	1	
8/17/1999	2	29	8	0	1	1	
8/17/1999	2	30	7	1	1	2	
8/21/1999	3	1	7	0	1	1	
8/21/1999	3	2	2	0	1	1	
8/21/1999	3	3	5	0	1	1	
8/21/1999	3	4	4	1	0	1	
8/21/1999	3	5	5	1	0	1	
8/21/1999	3	6	4	2	0	2	
7/9/2001	4	1	4				yes
7/9/2001	4	2	3				yes
7/9/2001	4	3	3				yes
7/9/2001	4	4	4				yes
7/9/2001	4	5	3				yes
7/9/2001	4	6	2				yes
7/9/2001	4	7	7	0	0	0	
7/9/2001	4	8	7	0	0	0	
7/9/2001	4	9	9	2	0	2	
7/9/2001	4	10	3				yes
7/9/2001	4	11	6	0	0	0	
7/9/2001	4	12	9	0	0	0	
7/9/2001	4	13	8	0	0	0	

Date	Population	Individual	# Leaves	# Flowers	#Fruits	# Fertile Structures	Juvenile
7/9/2001	4	14	3				yes
7/9/2001	4	15	6	0	0	0	
7/9/2001	4	16	6	0	0	0	
7/9/2001	4	17	6	0	0	0	
7/9/2001	4	18	4	0	0	0	
7/9/2001	4	19	6	0	0	0	
7/9/2001	4	20	7	0	0	0	
7/9/2001	4	21	6	0	0	0	
7/9/2001	4	22	6	0	0	0	
7/9/2001	4	23	4	0	0	0	
7/9/2001	4	24	8	1	0	1	
7/9/2001	4	25	15	1	4	5	
7/11/2001	5	1	4				yes
7/11/2001	5	2	3				yes
7/11/2001	5	3	3				yes
7/11/2001	5	4	3				yes
7/11/2001	5	5	3				yes
7/11/2001	5	6	3				yes
7/11/2001	5	7	3				yes
7/11/2001	5	8	5				yes
7/11/2001	5	9	4				yes
7/17/2001	6	1	4	0	3	3	
7/17/2001	6	2	5	0	0	0	
7/17/2001	6	3	8	0	2	2	
7/17/2001	6	4	5	0	2	2	
7/17/2001	6	5	6	0	0	0	
7/17/2001	6	6	7	0	0	0	
7/17/2001	6	7	6	0	0	0	
7/17/2001	6	8	6	0	0	0	
7/17/2001	6	9	6	0	0	0	
7/17/2001	6	10	5	0	0	0	
7/17/2001	6	11	4	0	2	2	
7/17/2001	6	12	5	0	0	0	
7/17/2001	6	13	10	0	0	0	
7/17/2001	6	14	9	0	0	0	
7/17/2001	6	15	11	0	0	0	
7/17/2001	6	16	12	0	0	0	
7/17/2001	6	17	10	0	0	0	
7/17/2001	6	18	6	0	0	0	
7/17/2001	6	19	5	0	0	0	
7/17/2001	6	20	8	0	0	0	

Date	Population	Individual	# Leaves	# Flowers	#Fruits	# Fertile Structures	Juvenile
7/17/2001	6	21	8	0	0	0	
7/17/2001	6	22	7	0	0	0	
7/17/2001	6	23	4	0	0	0	
7/17/2001	6	24	4	0	0	0	
7/17/2001	6	25	8	0	0	0	
7/17/2001	6	26	9	0	0	0	
7/17/2001	6	27	5	0	0	0	
7/17/2001	6	28	4	1	0	1	
7/17/2001	6	29	5	1	0	1	
7/17/2001	6	30	6	1	0	1	

2. Genetic (ISSR) Data for *Viola flettii*

Primer 10 Presence (1) and Absence (0) Data

Population	Individual	A	B	C	D	E	F	G	H	I	J	L	M	N	O	P	Q	R	S	T	U	V
1	1	1	1	1	1	1	1	1	1	0	0	1	0	0	1	0	0	0	0	0	0	0
1	2	1	1	1	1	1	1	1	1	0	0	0	1	0	1	0	0	0	0	0	0	0
1	3	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
1	4	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
1	5	1	1	1	1	1	1	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0
1	6	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
1	7	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
1	8	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
1	9	1	1	1	1	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0
1	10	1	1	1	1	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0
1	11	1	1	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
1	12	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
1	13	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
1	14	1	1	1	1	1	1	1	1	0	0	1	0	0	0	0	1	0	0	0	0	0
1	15	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0
1	16	1	1	1	1	1	1	1	1	0	0	0	0	1	0	0	1	0	0	0	0	0
1	17	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
1	18	1	1	1	1	1	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0
1	19	1	1	1	1	1	1	1	1	0	0	1	0	0	0	0	0	1	0	0	0	0
1	20	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
1	21	1	1	1	1	1	1	1	1	0	0	1	1	0	1	1	0	0	0	0	0	0
1	22	1	1	1	1	1	1	1	1	0	0	0	0	0	1	1	0	0	0	0	0	0
1	23	1	1	0	1	1	1	1	1	0	0	1	1	0	1	0	0	0	0	0	0	0
1	24	1	1	1	1	1	1	1	1	0	0	0	0	1	1	1	0	0	0	0	0	0

Primer 10 data cont.

Population	Individual	A	B	C	D	E	F	G	H	I	J	L	M	N	O	P	Q	R	S	T	U	V
1	25	1	1	1	1	1	1	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0
1	26	1	1	1	1	1	1	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0
1	27	1	1	1	1	1	1	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0
1	28	1	1	1	1	1	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0
1	29	1	1	1	1	1	1	1	1	0	0	1	0	0	1	0	0	0	0	0	0	0
1	30	1	1	1	1	1	1	1	1	0	0	1	1	0	1	0	0	0	0	0	0	0
2	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	2	1	1	1	1	1	1	1	1	0	0	0	0	1	0	0	0	0	1	0	0	0
2	3	1	1	1	1	1	1	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0
2	4	1	1	1	1	1	1	1	0	0	0	0	1	1	0	0	0	0	1	0	0	0
2	5	1	1	1	1	1	1	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0
2	6	1	1	1	1	1	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
2	7	1	1	0	1	1	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0
2	8	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	9	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
2	10	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
2	11	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	12	1	1	1	1	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0
2	13	1	1	1	1	1	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0
2	14	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	15	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
2	16	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	17	1	1	1	1	1	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0
2	18	1	1	1	1	1	1	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0
2	19	1	1	1	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
2	20	1	1	1	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0

Primer 10 data cont.

Population	Individual	A	B	C	D	E	F	G	H	I	J	L	M	N	O	P	Q	R	S	T	U	V
2	21	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
2	22	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	23	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	24	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	25	1	1	1	1	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0
2	26	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	27	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	28	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	29	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	30	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	1a	1	1	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	2a	1	1	0	1	1	1	1	1	0	0	1	0	0	0	1	1	0	0	0	0	0
3	3a	1	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	4a	1	1	1	1	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0
3	5a	1	1	1	1	1	1	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0
3	6a	1	1	1	1	1	1	1	0	0	0	0	0	0	0	1	1	0	0	0	0	0
3	1j	1	1	1	1	1	1	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0
3	2j	1	1	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	3j	1	1	1	1	1	1	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0
3	4j	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	5j	1	1	1	1	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0
4	1	0	1	0	0	0	1	1	0	1	1	0	0	0	0	0	0	1	0	1	0	0
4	2	0	0	1	0	0	1	1	0	1	1	0	0	1	0	0	0	0	0	1	0	0
4	3	0	1	1	0	0	1	1	0	1	0	0	1	0	0	0	0	0	0	1	1	0
4	4	0	1	1	0	1	1	1	1	1	0	0	1	0	0	0	0	0	0	1	0	1
4	5	0	1	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	1	1	0

Primer 10 data cont.

Population	Individual	A	B	C	D	E	F	G	H	I	J	L	M	N	O	P	Q	R	S	T	U	V
4	6	0	1	0	1	1	1	1	0	1	1	0	1	0	0	0	0	0	0	1	0	0
4	7	0	1	1	1	0	1	1	0	1	1	0	1	0	0	0	0	0	0	1	0	1
4	8	0	1	0	1	1	1	1	0	1	1	0	0	0	0	0	0	0	0	1	1	0
4	9	0	1	0	0	1	1	1	0	1	1	0	1	0	0	0	0	0	0	1	1	0
4	10	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	11	0	1	0	0	1	1	1	0	1	1	0	1	0	1	0	0	0	0	1	1	1
4	12	0	1	0	0	1	1	1	0	1	1	0	0	0	0	0	0	0	0	1	0	0
4	13	0	1	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0
4	14	0	1	1	0	0	1	1	0	1	1	0	1	0	0	0	0	0	0	1	0	1
4	15	0	1	0	1	0	1	1	0	1	0	0	0	0	0	0	0	0	0	1	1	0
4	16	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0
4	17	0	1	1	0	1	1	1	0	1	1	0	0	0	0	0	0	0	0	1	0	1
4	18	0	1	0	1	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	1	1
4	19	0	1	0	0	0	1	1	0	1	1	0	0	0	0	0	0	1	0	1	0	0
4	20	0	1	0	0	1	1	1	0	1	0	0	1	0	0	0	0	0	0	1	0	1
5	1	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	1	0	1	0	1
5	2	0	1	0	1	0	0	0	0	1	0	1	0	0	0	0	0	1	0	1	0	1
5	3	0	1	0	0	1	0	1	0	1	0	0	1	0	0	0	0	0	0	0	1	0
5	4	0	1	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	1	0
5	5	0	1	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	1	0
5	6	0	1	0	1	1	0	1	0	1	0	0	0	0	0	0	0	1	0	1	0	0
5	7	0	1	0	0	1	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0
5	8	1	1	0	0	1	0	1	0	1	0	0	1	0	0	0	0	0	0	0	1	0
6	1	1	1	0	0	1	0	1	0	1	0	0	1	0	0	0	0	0	0	1	1	0
6	2	1	1	0	0	1	0	1	0	1	0	1	1	0	0	0	0	0	0	1	1	0
6	3	0	1	0	0	1	0	1	0	1	0	0	1	0	0	0	0	0	0	1	1	0

Primer 10 data cont.

Population	Individual	A	B	C	D	E	F	G	H	I	J	L	M	N	O	P	Q	R	S	T	U	V
6	4	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0
6	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	6	1	1	0	0	1	0	0	0	1	0	0	1	0	0	0	0	0	0	1	1	0
6	7	1	1	0	0	1	0	0	1	1	0	1	1	0	0	0	0	0	0	1	0	0
6	8	1	1	0	0	1	0	1	0	1	0	0	1	0	0	0	0	0	0	1	0	0
6	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	10	1	1	0	0	1	0	1	0	1	0	0	1	0	0	0	0	0	0	1	0	0
6	11	0	1	0	0	1	0	1	0	1	0	0	1	0	0	0	0	0	0	1	0	0
6	12	1	1	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0
6	13	0	1	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	1	0
6	14	0	1	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0
6	15	0	1	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0
6	16	1	1	0	0	1	0	1	0	1	0	0	1	0	0	0	0	0	0	1	0	0
6	17	0	1	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0
6	18	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0
6	19	0	0	0	0	0	1	1	0	1	0	1	0	0	0	0	0	0	0	0	1	0
6	20	1	1	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	1	1	0
6	21	1	1	0	0	1	0	1	1	1	0	0	1	0	0	0	0	0	0	1	0	0
6	22	1	1	0	0	1	0	1	1	1	0	0	0	0	0	0	0	0	0	1	0	0
6	23	0	1	0	0	1	1	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0
6	24	0	1	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0
6	25	1	1	0	0	1	0	1	1	1	0	0	1	0	0	0	0	0	0	1	0	0
6	26	0	1	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0
6	27	0	1	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0
6	28	1	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	1	0	0
6	29	0	1	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0

Primer 10 data cont.

Population	Individual	A	B	C	D	E	F	G	H	I	J	L	M	N	O	P	Q	R	S	T	U	V
6	30	0	1	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0

Primer 17899B Presence (1) and Absence (0) Data

Population	Individual	1	2	3	4	5	6	7	a	b	c	d	e	f	g	h	i	j
1	1	0	1	1	0	1	1	1	0	0	0	0	0	0	0	0	1	0
1	2	0	1	1	0	1	0	1	0	0	0	0	0	0	0	0	1	0
1	3	0	1	1	0	1	1	1	0	0	0	0	0	0	0	0	1	0
1	4	0	1	1	0	1	0	1	0	0	0	0	0	0	0	0	1	0
1	5	0	1	1	0	1	0	1	0	0	0	0	0	0	0	0	1	0
1	6	0	1	1	0	1	0	1	0	0	0	0	0	0	0	0	1	0
1	7	0	1	1	0	1	0	1	0	0	0	0	0	0	0	1	1	0
1	8	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
1	9	0	1	1	0	1	0	1	0	0	0	0	0	0	0	0	1	0
1	10	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0
1	11	0	1	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0
1	12	0	1	1	0	1	0	1	0	0	0	0	0	0	0	1	1	0
1	13	0	1	1	1	1	0	1	0	0	0	0	0	0	0	1	1	1
1	14	1	1	1	0	1	0	1	0	0	0	0	0	0	0	1	1	0
1	15	0	1	1	0	1	0	1	0	0	0	0	0	0	0	1	1	0
1	16	0	1	1	0	1	0	1	0	0	0	0	0	0	0	0	1	1
1	17	0	1	1	0	0	1	1	0	0	0	0	0	0	0	0	1	0
1	18	0	1	1	0	1	0	1	0	0	0	0	0	0	0	0	1	0
1	19	0	1	1	0	1	0	1	0	0	0	0	0	0	0	0	1	0
1	20	0	1	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0
1	21	1	1	1	0	1	0	1	1	0	0	0	0	0	0	1	1	0

Primer 17899B data cont.

Population	Individual	1	2	3	4	5	6	7	a	b	c	d	e	f	g	h	i	j
1	22	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0
1	23	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0
1	24	0	1	1	0	1	1	1	0	0	0	0	0	0	0	1	1	0
1	25	0	1	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0
1	26	0	1	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0
1	27	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0
1	28	0	1	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0
1	29	0	1	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0
1	30	1	1	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0
2	1	1	1	1	1	1	1	1	0	0	0	1	1	0	0	0	1	0
2	2	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
2	3	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
2	4	1	1	1	1	1	1	1	0	0	0	0	1	0	0	0	0	0
2	5	1	1	1	1	1	1	1	0	0	0	1	1	0	0	0	0	0
2	6	1	1	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0
2	7	0	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
2	8	1	1	1	0	1	1	1	0	0	0	1	1	0	1	0	0	0
2	9	1	1	1	1	1	1	1	0	0	0	1	1	0	0	0	0	0
2	10	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0
2	11	1	0	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0
2	12	1	1	1	1	1	0	1	0	0	0	1	1	0	0	0	0	0
2	13	1	1	1	1	1	0	0	0	0	0	1	0	0	0	0	0	0
2	14	1	1	1	1	1	0	1	0	0	0	1	0	0	0	0	1	0
2	15	1	1	1	0	1	0	0	0	0	0	1	1	0	0	0	1	0
2	16	1	1	1	0	1	0	0	0	0	0	1	1	0	0	0	1	0
2	17	1	1	1	1	1	0	0	0	0	0	1	0	0	0	0	1	0

Primer 17899B data cont.

Population	Individual	1	2	3	4	5	6	7	a	b	c	d	e	f	g	h	i	j
2	18	1	1	1	0	1	0	0	0	0	0	1	1	0	1	1	0	0
2	19	1	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0
2	20	1	0	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0
2	21	1	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0
2	22	1	1	1	0	1	0	0	0	0	0	1	1	0	0	0	0	0
2	23	1	1	1	0	1	0	0	0	0	0	1	1	0	1	0	0	0
2	24	1	1	1	0	1	0	0	0	0	0	1	1	0	0	0	0	0
2	25	1	1	1	0	1	0	0	0	0	0	1	1	0	0	0	0	0
2	26	1	1	1	0	1	0	0	0	0	0	1	1	0	0	1	0	0
2	27	1	1	1	0	1	0	0	0	0	0	1	1	0	0	1	0	0
2	28	1	0	1	0	1	0	0	0	0	0	1	1	0	0	0	0	0
2	29	1	1	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0
2	30	1	0	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0
3	1a	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	1	0
3	2a	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
3	3a	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	1	0
3	4a	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	1	0
3	5a	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	1	0
3	6a	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	1	0
3	1j	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	1	0
3	2j	1	1	1	1	1	1	1	0	0	0	1	1	0	0	0	1	0
3	3j	1	1	1	1	1	1	1	0	0	0	1	1	0	0	0	1	0
3	4j	1	1	1	1	1	1	1	0	0	0	1	0	0	0	0	1	0
3	5j	1	1	1	1	1	1	1	0	0	0	1	1	0	0	0	1	0
4	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	1	0
4	2	1	1	1	1	1	1	1	0	0	0	1	0	0	0	0	1	0

Primer 17899B data cont.

Population	Individual	1	2	3	4	5	6	7	a	b	c	d	e	f	g	h	i	j
4	3	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	1	0
4	4	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	1	0
4	5	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	1	0
4	6	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	1	0
4	7	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
4	8	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	1	0
4	9	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
4	10	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
4	11	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	1	0
4	12	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
4	13	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
4	14	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	1	0
4	15	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	1	0
4	16	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
4	17	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	1	0
4	18	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	1	0
4	19	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	1	0
4	20	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	1	0
5	1	0	1	1	1	0	0	0	0	0	1	0	1	0	1	1	0	0
5	2	0	1	1	1	1	0	1	0	0	0	1	0	1	1	1	0	0
5	3	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
5	4	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0
5	5	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0
5	6	0	1	1	1	1	1	1	0	1	0	0	0	0	1	1	0	0
5	7	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0
5	8	0	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0

Primer 17899B data cont.

Population	Individual	1	2	3	4	5	6	7	a	b	c	d	e	f	g	h	i	j
6	1	1	1	1	1	1	1	1	0	0	1	0	0	0	0	0	0	0
6	2	1	1	1	1	1	1	1	0	1	1	0	0	0	0	0	1	0
6	3	1	1	1	1	1	1	1	0	1	1	0	0	0	0	0	0	0
6	4	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0
6	5	0	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
6	6	1	1	1	1	1	1	1	0	1	0	0	0	0	0	0	0	0
6	7	1	1	1	1	1	1	1	0	1	0	0	0	0	0	0	0	0
6	8	0	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
6	9	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0
6	10	1	1	1	1	1	1	1	0	0	0	1	1	0	0	0	1	0
6	11	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
6	12	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
6	13	1	1	1	1	1	1	1	0	0	0	1	1	0	0	0	1	0
6	14	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	1	0
6	15	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
6	16	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
6	17	1	1	1	1	1	1	1	0	0	0	1	0	1	0	0	1	0
6	18	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	1	0
6	19	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	1	0
6	20	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	1	0
6	21	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
6	22	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
6	23	0	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
6	24	0	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
6	25	0	1	1	1	1	1	1	0	1	0	0	0	0	0	0	0	0
6	26	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0

Primer 17899B data cont.

Population	Individual	1	2	3	4	5	6	7	a	b	c	d	e	f	g	h	i	j
6	27	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
6	28	0	1	1	1	1	1	1	0	1	0	0	0	0	0	0	0	0
6	29	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0	1	0
6	30	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0

Primer 844 Presence (1) and Absence (0) Data

Population	Individual	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1	1	0	0	1	1	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0
1	2	0	0	1	1	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
1	3	0	0	1	1	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
1	4	1	0	1	1	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
1	5	1	0	1	1	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
1	6	1	0	1	1	1	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
1	7	0	0	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
1	8	0	0	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
1	9	1	0	1	1	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
1	10	1	0	1	1	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
1	11	1	0	1	1	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
1	12	1	0	1	1	1	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0
1	13	1	0	1	0	1	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
1	14	1	1	1	0	1	0	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0
1	15	0	0	1	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0
1	16	1	0	1	0	1	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
1	17	1	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	18	1	0	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
1	19	1	0	1	0	1	0	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0

Primer 844 data cont.

Population	Individual	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1	20	1	0	1	1	0	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
1	21	1	0	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
1	22	1	0	1	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
1	23	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
1	24	1	1	1	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
1	25	1	1	1	1	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
1	26	1	0	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
1	27	1	0	1	0	1	0	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0
1	28	1	0	1	0	0	0	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0
1	29	1	0	1	1	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
1	30	0	1	1	1	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
2	1	0	1	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	1	0
2	2	0	1	0	1	0	0	1	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0
2	3	1	1	0	1	1	0	1	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0
2	4	1	1	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	1	1	0	0
2	5	1	1	0	1	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0
2	6	0	0	0	1	0	0	1	0	0	1	1	0	0	0	0	0	1	0	0	1	0	0
2	7	0	1	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	0	1
2	8	0	1	1	1	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	1	0	0
2	9	0	1	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	1	0
2	10	1	1	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0
2	11	0	1	0	1	0	0	1	0	0	1	1	0	0	0	0	0	1	0	0	1	0	0
2	12	0	0	0	1	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	1	0	0
2	13	1	1	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0
2	14	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0	1	1	0
2	15	0	1	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	0	0

Primer 844 data cont.

Population	Individual	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
2	16	0	1	1	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	0	0
2	17	1	1	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0
2	18	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	19	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0
2	20	0	1	1	1	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0
2	21	0	0	1	1	1	1	0	0	0	1	1	0	0	0	0	0	0	0	0	1	0	0
2	22	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	1	1	0	0	1	0	0
2	24	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	0	0
2	25	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0
2	26	1	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	1	0
2	27	1	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0
2	28	1	1	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	1	1	0
2	29	0	0	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0
2	30	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
3	1	0	1	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1
3	2	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	3	1	1	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1	0	0
3	4	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	5	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1	0
3	6	0	1	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	1	1	1	0
3	1j	0	0	0	1	0	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0
3	2j	1	1	1	1	0	0	1	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0
3	3j	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
3	4j	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
3	5j	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
4	1	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0

Primer 844 data cont.

Population	Individual	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
4	2	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
4	3	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
4	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	5	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
4	6	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0
4	7	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
4	8	0	0	0	1	1	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0
4	9	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
4	10	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0
4	11	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0
4	12	0	0	0	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0
4	13	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
4	14	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0
4	15	0	0	0	0	1	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0
4	16	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
4	17	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
4	18	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
4	19	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
4	20	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0
5	1	0	0	1	0	1	0	1	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0
5	2	0	0	1	0	0	0	0	0	0	1	1	0	1	0	1	0	0	0	1	0	0	0
5	3	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
5	4	0	1	1	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0
5	5	0	0	1	0	0	0	1	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0
5	6	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	1	0	0	0	0	0
5	7	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0

Primer 844 data cont.

Population	Individual	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
5	8	0	0	0	0	0	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0
6	1	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0
6	2	0	0	0	0	1	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0
6	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	4	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0
6	5	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0
6	6	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
6	7	0	0	0	0	1	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0
6	8	0	0	0	0	1	0	1	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0
6	9	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
6	10	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
6	11	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
6	12	0	0	0	0	1	0	1	0	0	1	1	0	1	1	1	0	0	0	0	0	0	0
6	13	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
6	14	0	0	0	0	1	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0
6	15	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0
6	16	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0
6	17	0	0	0	0	1	0	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0
6	18	0	0	0	0	1	0	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0
6	19	0	0	0	1	1	0	0	0	1	1	1	0	1	1	1	0	0	0	0	0	0	0
6	20	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0
6	21	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
6	22	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
6	23	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
6	24	0	0	0	0	1	0	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0
6	25	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0

Primer 844 data cont.

Population	Individual	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
6	26	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0
6	27	0	0	0	0	1	0	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0
6	28	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
6	29	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0
6	30	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0

3. Phylogeographic (Chloroplast PCR-RFLP) Data

Haplotypes for PCR-RFLP data for individuals *Viola flettii*, *V. cuneata*, *V. ocellata* and outgroups

Indiv	Haplotype	Indiv	Haplotype	Indiv	Haplotype	Indiv	Haplotype
VF1a	Of	VF7d	AI	VC5i	BT	VO4g	DH
VF1b	EA	VF8b	AJ	VC5j	BV	VO4h	GI
VF1c	AA	VF8d	AR	VC6c	ZK	VO4j	GH
VF1d	AA	VF8f	AI	VC6f	AI	VO5a	AR
Vf1e	AA	VC1a	QA	VC6j	AR	VO5b	AR
VF1f	AA	VC1b	AA	VC7a	AU	VO5c	AR
VF1g	JA	VC1c	AA	VC7b	AT	VO6a	AE
VF1h	AA	VC1d	AL	VC7c	AT	VO6b	AE
Vf1i	SH	VC1e	AL	VC7d	AT	VO6c	AY
VF1j	IA	VC1f	IA	VC7e	AT	VO6d	AE
VF2a	BA	VC1g	AA	VC7f	AT	VO6e	AC
VF2b	AA	VC1h	AL	VC7g	AT	VO6f	AY
VF2f	RR	VC1j	AH	VC7h	AT	VO6g	AE
VF2i	BA	VC2a	AH	VC7i	AT	VO6h	AE
VF2j	BA	VC2b	AN	VC7j	AT	VO6i	AC
VF3a	BB	VC2c	AA	VC8a	AT	VO6j	AF
VF3b	BA	VC2d	AN	VC8b	AT	VO7a	AF
VF3c	EA	VC2e	AN	VC8c	AT	VO7b	AG
VF3d	EA	VC2f	AN	VC8d	Bd	VO7c	AF
VF3e	SA	VC2g	AN	VC8e	BI	VO7d	AF
VF3f	EA	VC2h	AA	VC9a	AH	VO7e	AF
VF3g	AB	VC3a	BA	VC9b	AH	VO7f	AF
VF3h	BA	VC3b	BO	VC9c	EH	VO7g	AF
VF3i	BA	VC3c	BP	VC9d	AH	VO7h	AG
VF3j	BA	VC3d	BI	VC9e	AH	VO8a	AQ
VF4a	BE	VC3e	BA	VO1d	AR	VO8b	AQ
VF4b	BC	VC3f	BA	VO1e	AX	VO8c	AQ
VF4c	BD	VC3g	BA	VO1f	BR	VO8d	AQ
VF4d	BD	VC3h	BA	VO1h	BQ	VO8e	AQ
VF4e	HD	VC3i	BA	VO1i	AR	VO8f	AQ
VF4f	BA	VC3j	BA	VO1j	AR	VO8g	AQ
VF4g	BD	VC4a	BI	VO2b	AR	VO8h	AQ
VF4h	AD	VC4b	BA	VO2c	AR	VO8i	AQ
VF4j	BD	VC4d	BX	VO2d	VR	VO8j	AQ
VF5c	BD	VC4f	JA	VO2e	WR	VO9a	AQ
VF5d	FD	VC4g	AA	VO2f	NR	VO9b	AR
VF5e	BC	VC4j	FA	VO2h	XR	VO9c	AS
VF5f	TD	VC5a	BA	VO3a	Ah	VO9d	BR
VF5g	BD	VC5b	BA	VO3f	Ai	VO9e	AR
VF5h	BD	VC5c	BB	VO3j	Ch	VO9f	AR
VF6d	Bb	VC5d	AA	VO4a	CH	VO9g	AR
VF6h	Ba	VC5e	MB	VO4b	BH	ORB	AA
VF6j	BH	VC5f	BA	VO4c	CH	SEM	BI
VF7b	AA	VC5g	MA	VO4e	CC		
VF7c	AH	VC5h	JI	VO4f	DH		

Banding pattern based on fragment size for Haplotypes of EcoRI digested PCR-RFLP data of all three species

Haplotypes	1250	1150	1080	930	840	700	540	440	410	360	300	250	210	170	150	110
A	0	0	0	0	0	1	1	0	0	1	0	1	0	1	0	0
B	0	0	0	0	0	0	1	0	0	1	0	1	0	1	0	0
C	0	0	0	0	0	0	1	1	0	1	0	0	1	0	1	0
D	0	0	0	0	0	0	0	1	0	1	0	0	1	0	1	0
E	0	0	0	0	0	1	1	0	0	1	0	1	0	1	0	1
F	0	0	0	0	0	0	1	0	0	1	0	1	0	1	0	1
G	0	0	0	0	0	0	1	0	0	1	0	1	0	1	0	0
H	0	0	0	0	0	0	1	0	0	1	0	1	0	0	0	0
I	0	1	0	0	0	1	1	0	0	1	0	1	0	1	0	0
J	0	0	0	0	0	0	0	0	0	1	0	1	0	1	0	0
K	0	0	0	1	0	1	1	0	0	1	0	1	0	1	0	0
L	0	0	0	0	0	1	1	1	0	1	0	1	0	1	0	0
M	0	0	1	0	0	0	1	0	0	1	0	1	0	1	0	0
N	0	0	0	0	0	1	1	1	0	0	1	0	1	0	0	0
O	0	0	0	1	0	1	1	0	0	1	1	1	0	0	0	0
P	0	0	0	1	0	0	0	1	1	0	1	0	1	0	0	0
Q	0	1	0	0	0	1	1	1	0	1	0	1	0	0	0	0
R	0	0	0	0	0	0	1	1	0	1	0	0	0	1	0	1
S	0	0	0	0	0	0	1	0	0	1	0	1	0	1	0	1
T	0	0	0	0	1	0	1	0	0	1	0	1	0	1	0	0
U	1	0	0	0	0	0	1	0	0	1	0	1	0	1	0	0
V	0	0	0	0	1	0	1	0	1	0	1	0	1	0	0	0
W	0	0	0	1	0	0	0	1	1	0	1	0	1	0	0	0
X	0	0	0	0	0	0	1	1	0	0	1	1	0	0	0	0
Y	0	0	0	0	0	1	1	1	0	0	1	1	0	0	0	0
Z	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	0

Banding pattern based on fragment size for haplotypes of EcoRV digested PCR-RFLP data for all three species

Haplotype	1600	1500	1415	1250	1130	1040	910	425	340	245	200
A	0	0	0	1	0	0	1	0	0	1	1
B	0	0	0	0	1	0	1	0	0	1	1
C	0	0	0	0	1	1	0	0	0	0	1
D	0	0	0	0	1	1	0	0	0	1	1
E	0	0	1	0	0	1	0	0	0	0	1
F	0	0	0	0	0	1	0	0	0	0	1
G	0	0	0	1	0	0	1	1	0	1	1
H	0	0	0	1	0	0	1	1	0	1	1
I	0	0	0	0	0	0	1	0	0	1	1
J	0	0	0	1	0	1	1	0	0	1	1
K	0	0	0	0	0	0	1	0	1	1	1
L	0	0	0	1	0	0	1	0	1	1	1
M	1	0	0	1	0	0	1	0	1	1	1
N	0	0	1	0	0	0	1	0	0	1	1
O	0	1	0	1	0	0	1	0	0	1	1
P	1	0	0	1	0	0	1	0	0	1	1
Q	0	0	0	0	1	0	0	0	0	1	0
R	0	0	0	0	1	0	0	0	0	0	1
S	0	0	0	0	1	0	0	1	0	0	1
T	1	0	0	0	0	0	1	0	0	1	1
U	0	0	0	1	0	0	1	0	0	1	0
V	1	0	0	0	0	0	1	0	1	1	1
X	0	0	0	0	1	0	0	0	0	1	1
Y	0	0	1	0	1	0	0	0	0	0	1
Z	0	0	0	0	0	1	1	0	0	1	1
A	0	0	0	1	0	1	0	0	1	0	1
B	0	0	0	1	0	1	0	0	1	1	1
C	0	0	1	0	0	0	1	0	0	0	1
D	0	0	0	0	0	0	1	0	0	1	0
E	0	0	0	0	0	0	1	0	0	0	1
F	0	0	0	1	0	0	1	0	0	1	0
G	0	0	0	1	0	0	1	1	1	0	0
H	0	0	0	1	0	0	0	0	1	1	1
I	0	0	1	1	0	0	0	0	0	1	1
J	0	0	0	0	0	0	1	0	0	1	1

ITS PCR-RFLP haplotypes from HaeIII digestion prediction for individuals

Individual	Haplotype
GLA	A
ORB	A
VC1A	A
VC1B	B
VC2	B
VC3B	B
VC3A	A
VC4B	A
VC4A	B
VC5B	B
VC5A	A
VC6A	A
VC6B	A
VC7B	A
VC7A	B
VC8A	B
VC8B	D
VC9A	E
VC9B	F
VF1B	A
VF1A	A
VF2B	A
VF2A	A
VF3	A
VF4	A
VF8	C
VO1A	A
VO1B	F
VO2A	F
VO2B	A
VO3B	A
VO3A	A
VO4A	A
VO4B	A
VO5	A
VO6	A
VO7	A
VO8	B
VO9A	A
VO9B	A

Fragment Pattern for haplotypes of ITS HaeIII digestion for PCR-RFLP data

Haplotype						
A	0	0	1	1	0	1
B	1	0	1	1	0	1
C	1	0	1	0	0	1
D	1	0	1	1	1	1
E	0	1	1	1	1	1
F	0	0	1	1	1	1

4. Ecological Factors Recorded for Three Species

Ecological site and leaf angle data for all three species

Date	Species	Site	Plot #	# of Violets	% Overstory	GPS co-ordinates	Elevation (m)	Leaf Angle 1 (degrees)	Leaf Angle 2 (degree)
7/4/2003	*V. ocellata*	MP	1	5	60.58	37 deg 16.506 N 122 deg 17.305 W	94	20	20
7/4/2003	*V. ocellata*	MP	2	5	68.38	37 deg 16.510 N 122 deg 17.312 W	93	11	11
7/4/2003	*V. ocellata*	MP	3	3	73.84	37 deg 16.512 N 122 deg 17.312 W	92	14	6
7/4/2003	*V. ocellata*	MP	4	5	76.96	37 deg 16.516 N 122 deg 17.307 W	95	11	9
7/4/2003	*V. ocellata*	MP	5	3	78.26	37 deg 16.512 N 122 deg 17.307 W	68	9	4
7/4/2003	*V. ocellata*	MP	6	4	76.44	37 deg 16.504 N 122 deg 17.324 W	75	-4	7
7/4/2003	*V. ocellata*	MP	7	2	71.76	37 deg 16.500 N 122 deg 17.332 W	75	-1	14
7/4/2003	*V. ocellata*	MP	8	2	87.62	37 deg 16.510 N 122 deg 17.331 W	69	12	1
7/4/2003	*V. ocellata*	MP	9	7	80.08	37 deg 16.503 N 122 deg 17.341 W	82	12	20
7/4/2003	*V. ocellata*	MP	10	5	74.1	37 deg 16.503 N 122 deg 17.347 W	67	2	2
7/4/2003	*V. ocellata*	MP	11	2	78.26	37 deg 16.503 N 122 deg 17.336 W	104	-7	0
7/4/2003	*V. ocellata*	MP	12	2	74.1	37 deg 16.501 N 122 deg 17.343 W	73	16	8
7/4/2003	*V. ocellata*	MP	13	3	71.24	37 deg 16.497 N 122 deg 17.339 W	73	15	5
7/4/2003	*V. ocellata*	MP	14	4	70.72	37 deg 16.494 N 122 deg 17.360 W	73	-1	7
7/4/2003	*V. ocellata*	MP	15	6	67.08	37 deg 16.502 N 122 deg 17.358 W	73	2	2
7/8/2003	*V. ocellata*	MC	1	4	60.32	39 deg 15.323 N 122 deg 55.833 W	469	-7	-3
7/8/2003	*V. ocellata*	MC	2	20	65.78	39 deg 15.317 N 122 deg 55.840 W	467	10	8
7/8/2003	*V. ocellata*	MC	3	9	74.88	39 deg 15.316 N 122 deg 55.838 W	469	-3	0
7/8/2003	*V. ocellata*	MC	4	6	63.18	39 deg 15.316 N 122 deg 55.838 W	478	0	10
7/8/2003	*V. ocellata*	MC	5	16	71.5	39 deg 15.322 N 122 deg 55.841 W	473	57	24
7/8/2003	*V. ocellata*	MC	6	8	71.24	39 deg 15.320 N 122 deg 55.840 W	476	30	-8
7/8/2003	*V. ocellata*	MC	7	8	74.36	39 deg 15.315 N 122 deg 55.842 W	477	68	-10
7/8/2003	*V. ocellata*	MC	8	2	69.16	39 deg 15.312 N 122 deg 55.843 W	475	28	20
7/8/2003	*V. ocellata*	MC	9	3	67.6	39 deg 15.307 N 122 deg 55.849 W	473	-10	14

Ecological site data cont.

Date	Species	Site	Plot #	# of Violets	% Overstory	GPS co-ordinates	Elevation (m)	Leaf Angle 1 (degrees)	Leaf Angle 2 (degree)
7/8/2003	*V. ocellata*	MC	10	3	73.32	39 deg 15.308 N 122 deg 55.845 W	469	3	10
7/8/2003	*V. ocellata*	MC	11	9	66.3	39 deg 15.312 N 122 deg 55.840 W	461	-15	-15
7/8/2003	*V. ocellata*	MC	12	6	66.82	39 deg 15.317 N 122 deg 55.834 W	464	0	-15
7/8/2003	*V. ocellata*	MC	13	8	69.16	39 deg 15.317 N 122 deg 55.383 W	474	34	30
7/8/2003	*V. ocellata*	MC	14	10	68.12	39 deg 15.318 N 122 deg 55.839 W	475	8	0
7/8/2003	*V. ocellata*	MC	15	12	76.18	39 deg 15.319 N 122 deg 55.840 W	485	10	-15
7/10/2003	*V. ocellata*	CC	1	3	70.72	40 deg 42.000 N 121 deg 55.280 W	840	-1	10
7/10/2003	*V. ocellata*	CC	2	3	67.08	40 deg 42.087 N 121 deg 55.221 W	830	12	2
7/10/2003	*V. ocellata*	CC	3	3	74.62	40 deg 42.131 N 121 deg 55.182 W	838	-2	10
7/10/2003	*V. ocellata*	CC	4	4	76.96	40 deg 42.132 N 121 deg 55.187 W	819	-1	15
7/10/2003	*V. ocellata*	CC	5	5	78	40 deg 42.129 N 121 deg 55.177 W	785	20	12
7/10/2003	*V. ocellata*	CC	6	2	69.68	40 deg 42.136 N 121 deg 55.181 W	811	14	44
7/10/2003	*V. ocellata*	CC	7	7	70.46	40 deg 42.136 N 121 deg 55.171 W	823	-4	0
7/10/2003	*V. ocellata*	CC	8	5	64.48	40 deg 42.136 N 121 deg 55.174 W	825	-10	-15
7/10/2003	*V. ocellata*	CC	9	4	74.88	40 deg 42.118 N 121 deg 55.183 W	834	0	11
7/10/2003	*V. ocellata*	CC	10	6	80.08	40 deg 42.137 N 121 deg 55.174 W	820	15	15
7/10/2003	*V. ocellata*	CC	11	4	70.2	40 deg 42.135 N 121 deg 55.171 W	834	22	18
7/10/2003	*V. ocellata*	CC	12	3	75.4	40 deg 42.120 N 121 deg 55.168 W	805	9	12
7/10/2003	*V. ocellata*	CC	13	4	77.48	40 deg 42.129 N 121 deg 55.179 W	822	15	10
7/10/2003	*V. ocellata*	CC	14	7	62.66	40 deg 42.122 N 121 deg 55.171 W	862	-2	46
7/10/2003	*V. ocellata*	CC	15	5	68.38	40 deg 42.124 N 121 deg 55.176 W	856	24	32
7/13/2003	*V. cuneata*	HM	1	6	35.62	40 deg 51.340 N 123 deg 43.580 W	1470	90	55
7/13/2003	*V. cuneata*	HM	2	2	51.74	40 deg 51.351 N 123 deg 43.585 W	1479	30	43
7/13/2003	*V. cuneata*	HM	3	1	20.54	40 deg 51.353 N 123 deg 43.572 W	1484	86	84
7/13/2003	*V. cuneata*	HM	4	9	48.62	40 deg 51.352 N 123 deg 43.564 W	1497	25	35
7/13/2003	*V. cuneata*	HM	5	2	49.4	40 deg 51.357 N 123 deg 43.557 W	1484	34	50

Ecological site data cont.

Date	Species	Site	Plot #	# of Violets	% Overstory	GPS co-ordinates	Elevation (m)	Leaf Angle 1 (degrees)	Leaf Angle 2 (degree)
7/13/2003	*V. cuneata*	HM	6	2	25.74	40 deg 51.363 N 123 deg 43.552 W	1482	87	75
7/13/2003	*V. cuneata*	HM	7	4	33.28	40 deg 51.372 N 123 deg 43.549 W	1485	85	70
7/13/2003	*V. cuneata*	HM	8	10	28.86	40 deg 51.367 N 123 deg 43.545 W	1471	63	86
7/13/2003	*V. cuneata*	HM	9	5	51.48	40 deg 51.359 N 123 deg 43.542 W	1476	65	74
7/13/2003	*V. cuneata*	HM	10	3	28.86	40 deg 51.358 N 123 deg 43.532 W	1470	80	90
7/13/2003	*V. cuneata*	HM	11	4	27.82	40 deg 51.348 N 123 deg 43.534 W	1483	93	61
7/13/2003	*V. cuneata*	HM	12	7	40.3	40 deg 51.345 N 123 deg 43.544 W	1482	66	78
7/13/2003	*V. cuneata*	HM	13	2	46.8	40 deg 51.343 W 123 deg 43.553 W	1479	70	25
7/13/2003	*V. cuneata*	HM	14	18	44.46	40 deg 51.346 W 123 deg 43.555 W	1472	40	20
7/13/2003	*V. cuneata*	HM	15	8	33.02	40 deg 51.351 N 123 deg 43.564 W	1476	101	60
7/16/2003	*V. cuneata*	SP	1	2	33.28	42 deg 0.864 N 123 deg 39.394W	951	44	15
7/16/2003	*V. cuneata*	SP	2	2	28.34	42 deg 0.866 N 123 deg 39.396 W	945	80	70
7/16/2003	*V. cuneata*	SP	3	2	25.48	42 deg 0.864 N 123 deg 39.399 W	948	80	50
7/16/2003	*V. cuneata*	SP	4	19	26.26	42 deg 0.864 N 123 deg 39.401 W	954	90	70
7/16/2003	*V. cuneata*	SP	5	4	31.2	42 deg 0.864 N 123 deg 39.404 W	952	83	107
7/16/2003	*V. cuneata*	SP	6	7	39.26	42 deg 0.863 N 123 deg 39.405 W	948	85	50
7/16/2003	*V. cuneata*	SP	7	2	24.96	42 deg 0.864 N 123 deg 39.407 W	944	68	58
7/16/2003	*V. cuneata*	SP	8	6	22.88	42 deg 0.862 N 123 deg 39.403 W	946	32	56
7/16/2003	*V. cuneata*	SP	9	2	19.76	42 deg 0.863 N 123 deg 39.411 W	946	32	80
7/16/2003	*V. cuneata*	SP	10	3	25.74	42 deg 0.862 N 123 deg 39.412 W	946	65	40
7/16/2003	*V. cuneata*	SP	11	9	37.96	42 deg 0.861 N 123 deg 39.415 W	944	35	20
7/16/2003	*V. cuneata*	SP	12	13	37.44	42 deg 0.864 N 123 deg 39.418 W	948	68	90
7/16/2003	*V. cuneata*	SP	13	31	34.32	42 deg 0.864 N 123 deg 39.421 W	947	82	48
7/16/2003	*V. cuneata*	SP	14	5	32.76	42 deg 0.864 N 123 deg 39.421 W	948	82	79
7/16/2003	*V. cuneata*	SP	15	3	39.52	42 deg 0.863 N 123 deg 39.425 W	945	95	94
7/18/2003	*V. cuneata*	8$	1	9	21.06	42 deg 14.441 N 123 deg 40.542 W	420	68	34

Ecological site data cont.

Date	Species	Site	Plot #	# of Violets	% Overstory	GPS co-ordinates	Elevation (m)	Leaf Angle 1 (degrees)	Leaf Angle 2 (degree)
7/18/2003	*V. cuneata*	8$	2	7	30.94	42 deg 14.439 N 123 deg 40.542 W	424	42	18
7/18/2003	*V. cuneata*	8$	3	11	28.6	42 deg 14.437 N 123 deg 40.539 W	429	5	35
7/18/2003	*V. cuneata*	8$	4	6	42.9	42 deg 14.438 N 123 deg 40.540 W	431	56	34
7/18/2003	*V. cuneata*	8$	5	4	23.66	42 deg 14.436 N 123 deg 40.545 W	417	54	53
7/18/2003	*V. cuneata*	8$	6	7	43.16	42 deg 14.434 N 123 deg 40.544 W	420	15	20
7/18/2003	*V. cuneata*	8$	7	7	25.74	42 deg 14.428 N 123 deg 40.542 W	422	55	75
7/18/2003	*V. cuneata*	8$	8	6	31.2	42 deg 14.427 N 123 deg 40.548 W	427	110	74
7/18/2003	*V. cuneata*	8$	9	8	44.46	42 deg 14.429 N 123 deg 40.546 W	426	84	86
7/18/2003	*V. cuneata*	8$	10	5	41.08	42 deg 14.424 N 123 deg 40.548 W	425	15	63
7/18/2003	*V. cuneata*	8$	11	11	40.82	42 deg 14.422 N 123 deg 40.552 W	424	86	64
7/18/2003	*V. cuneata*	8$	12	7	47.84	42 deg 14.420 N 123 deg 40.552 W	424	35	35
7/18/2003	*V. cuneata*	8$	13	2	47.06	42 deg 14.425 N 123 deg 40.556 W	426	92	60
7/18/2003	*V. cuneata*	8$	14	6	50.44	42 deg 14.424 N 123 deg 40.552 W	425	44	35
7/18/2003	*V. cuneata*	8$	15	5	56.68	42 deg 14.423 N 123 deg 40.555 W	424	75	65
7/26/2003	*V. flettii*	MA	1	8	29.38	47 deg 49.054 N 123 deg 07.783 W	1718	15	10
7/26/2003	*V. flettii*	MA	2	12	44.2	47 deg 49.057 N 123 deg 07.780 W	1691	21	19
7/26/2003	*V. flettii*	MA	3	9	28.34	47 deg 49.070 N 123 deg 07.797 W	1691	14	24
7/26/2003	*V. flettii*	MA	4	7	14.82	47 deg 49.069 N 123 deg 07.800 W	1691	24	33
7/26/2003	*V. flettii*	MA	5	6	15.6	47 deg 49.075 N 123 deg 07.805 W	1691	55	30
7/26/2003	*V. flettii*	MA	6	5	30.16	47 deg 49.077 N 123 deg 07.800 W	1691	56	70
7/26/2003	*V. flettii*	MA	7	6	14.82	47 deg 49.085 N 123 deg 07.795 W	1691	15	15
7/26/2003	*V. flettii*	MA	8	10	21.84	47 deg 49.089 N 123 deg 07.803 W	1691	35	26
7/26/2003	*V. flettii*	MA	9	17	23.66	47 deg 49.112 N 123 deg 07.787 W	1691	17	8
7/26/2003	*V. flettii*	MA	10	6	15.86	47 deg 49.112 N 123 deg 07.787 W	1691	90	12
7/26/2003	*V. flettii*	MA	11	5	32.5	47 deg 49.094 N 123 deg 07.782 W	1699	15	7
7/26/2003	*V. flettii*	MA	12	5	26.78	47 deg 49.091 N 123 deg 07.747 W	1699	30	38

Ecological site data cont.

Date	Species	Site	Plot #	# of Violets	% Overstory	GPS co-ordinates	Elevation (m)	Leaf Angle 1 (degrees)	Leaf Angle 2 (degree)
7/26/2003	*V. flettii*	MA	13	5	35.62	47 deg 49.103 N 123 deg 07.732 W	1699	10	5
7/26/2003	*V. flettii*	MA	14	5	17.94	47 deg 49.090 N 123 deg 07.721 W	1699	20	15
7/26/2003	*V. flettii*	MA	15	9	23.66	47 deg 49.088N 123 deg 07.724 W	1705	20	20
7/30/2003	*V. flettii*	BM	1	5	7.02	47 deg 57.064 N 123 deg 15.358 W	1691	10	10
7/30/2003	*V. flettii*	BM	2	7	17.42	47 deg 57.058 N 123 deg 15.355 W	1689	0	10
7/30/2003	*V. flettii*	BM	3	10	0	47 deg 57.064 N 123 deg 15.373 W	1695	10	95
7/30/2003	*V. flettii*	BM	4	4	0	47 deg 57.068 N 123 deg 15.384 W	1691	0	0
7/30/2003	*V. flettii*	BM	5	5	0.52	47 deg 57.069 N 123 deg 15.387 W	1695	10	30
7/30/2003	*V. flettii*	BM	6	5	0	47 deg 57.083 N 123 deg 15.389 W	1701	10	25
7/30/2003	*V. flettii*	BM	7	4	0	47 deg 57.083 N 123 deg 15.393 W	1702	0	10
7/30/2003	*V. flettii*	BM	8	2	0	47 deg 57.091 N 123 deg 15.392 W	1702	35	50
7/30/2003	*V. flettii*	BM	9	2	15.08	47 deg 57.093 N 123 deg 15.401 W	1706	5	30
7/30/2003	*V. flettii*	BM	10	7	40.56	47 deg 57.096 N 123 deg 15.404 W	1699	15	45
7/30/2003	*V. flettii*	BM	11	11	0.26	47 deg 57.100 N 123 deg 15.397 W	1703	25	30
7/30/2003	*V. flettii*	BM	12	8	3.9	47 deg 57.095 N 123 deg 15.395 W	1710	40	25
7/30/2003	*V. flettii*	BM	13	5	3.12	47 deg 57.096 N 123 deg 15.398 W	1713	-10	5
7/30/2003	*V. flettii*	BM	14	8	0	47 deg 57.084 N 123 deg 15.397 W	1700	35	25
7/30/2003	*V. flettii*	BM	15	7	0	47 deg 57.083 N 123 deg 15.405 W	1702	50	5
7/31/2003	*V. flettii*	NB	1	8	24.18	47 deg 57.668 N 123 deg 15.806 W	1735	20	30
7/31/2003	*V. flettii*	NB	2	7	18.46	47 deg 57.667 N 123 deg 15.806 W	1749	-10	0
7/31/2003	*V. flettii*	NB	3	3	6.76	47 deg 57.670 N 123 deg 15.809 W	1751	0	0
7/31/2003	*V. flettii*	NB	4	2	41.34	47 deg 57.672 N 123 deg 15.808 W	1745	15	20
7/31/2003	*V. flettii*	NB	5	2	36.14	47 deg 57.673 N 123 deg 15.813 W	1752	15	5
7/31/2003	*V. flettii*	NB	6	4	53.3	47 deg 57.680 N 123 deg 15.805 W	1754	35	20
7/31/2003	*V. flettii*	NB	7	5	49.92	47 deg 57.675 N 123 deg 15.808 W	1758	35	10
7/31/2003	*V. flettii*	NB	8	4	45.24	47 deg 57.672 N 123 deg 15.816 W	1753	20	5

Ecological site data cont.

Date	Species	Site	Plot #	# of Violets	% Overstory	GPS co-ordinates	Elevation (m)	Leaf Angle 1 (degrees)	Leaf Angle 2 (degree)
7/31/2003	V. flettii	NB	9	3	12.22	47 deg 57.673 N 123 deg 15.814 W	1747	25	35
7/31/2003	V. flettii	NB	10	10	14.3	47 deg 57.673 N 123 deg 15.815 W	1745	42	55
7/31/2003	V. flettii	NB	11	3	36.4	47 deg 57.675 N 123 deg 15.817 W	1749	35	40
7/31/2003	V. flettii	NB	12	3	28.86	47 deg 57.677 N 123 deg 15.819 W	1745	15	20
7/31/2003	V. flettii	NB	13	9	43.16	47 deg 57.675 N 123 deg 15.866 W	1749	20	15
7/31/2003	V. flettii	NB	14	5	50.44	47 deg 57.666 N 123 deg 15.825 W	1750	-10	-10
7/31/2003	V. flettii	NB	15	7	44.98	47 deg 57.676 N 123 deg 15.826 W	1750	-30	-10

Concentrations in ppm of Ca, Mg, Ni, Cr and Fe in soils for all three species (VC=*Viola cuneata*, VO=*V. ocellata*, and VF= *V. flettii*). Five grams of soil used for extracting Ca and Mg from samples. Ten grams of soil used for extraction of Ni, Cr and Fe.

Species	Population	Individual	Ca	Mg	Ni	Cr	Fe
VC	HM	1	57.25	344.05	63.26		71.2
VC	HM	2	22.05	297.85	9.52	0.093	24
VC	HM	3	0	46.17	25.84	0.063	27.1
VC	HM	4	38.75	182.1	94.86	0	81.9
VC	HM	5	256.55	444.7	2.02	0.234	0.5
VC	HM	6	5.75	272.2	154.74	11.8	123.75
VC	HM	7	11.85		30.16	5.9	26.1
VC	HM	8	24.2	224.25	129.24	4.45	111.95
VC	HM	9	107.75	272.65	160.16	4.22	80.15
VC	HM	10	37.85	125.65	45.62	14.85	198.2
VC	HM	11	30.15	322.5	95.54	4.35	139.7
VC	HM	12	90.9	563	16.58	10.35	14.55
VC	HM	13	91.4	140.95	61.8	14.8	126.2
VC	HM	14	50.95	394.6	33.34	4.6	0.62
VC	HM	15	13.75	223.1	53.9	1.27	59.15
VC	SP	1	19.65	242.95	109.9	25.4	61.1
VC	SP	2	23.1	599	62.1	10.7	71.6
VC	SP	3	56.25	452.45	45	29.3	74.6
VC	SP	4	60.6	455.55	25.3	25	89.2
VC	SP	5	60.95	596.5	26.6	29.8	167.3
VC	SP	6	131.45	264.4	219.2	20.2	176.25
VC	SP	7	111.2	302.25	36.2	34.9	106.5
VC	SP	8	38.6	309.3	47.6	42.5	114.5
VC	SP	9	4.9	189.25	53.6	29.1	68.3
VC	SP	10	21.9	266.3	61.5	16.7	83.5
VC	SP	11	19.2	321.5	27.8	0.9	45
VC	SP	12	48.45	241.35	15.7	3.15	180.1
VC	SP	13	5.8	253.2	181.5	17.8	77.3
VC	SP	14	55.5	438.05	10.3	9.7	100.3
VC	SP	15	9.1	206.2	144.45	1.09	73.05
VC	ED	1	5.1	268.1	118.25	0.26	21.55
VC	ED	2	4.7	357.6	223.95	4.4	27.95
VC	ED	3	85.6	73.45	203.8	1.12	47.2
VC	ED	4	68.9	121.8	127.6	3	25.5
VC	ED	5	43	69.78	82.7	0.272	24.6
VC	ED	6	110.3	74.13	119.75	0.19	31.25
VC	ED	7	61.18	78.34	110.85	1.14	50.5
VC	ED	8	47.1	43.31	106.05	0.255	32.85
VC	ED	9	16.2	59.82	108.75	0.249	20.8

Soil analysis data cont.

Species	Population	Individual	Ca	Mg	Ni	Cr	Fe
VC	ED	10	65.8	139.3	283.85	2.5	37.5
VC	ED	11	44.1	132.7	260.55	0.212	33.8
VC	ED	12	43.8	205.8	328	1.05	23.9
VC	ED	13	48.1	265.4	316.55	0.216	40.6
VC	ED	14	274.8	150.7	186.15	0.298	52.5
VC	ED	15	100.5	275	274.3		69.2
VO	MP	1	282.25	141.0	1.29	0.267	198.7
VO	MP	2	441.55	72.15	0.955	0.161	140.55
VO	MP	3	271.95	70.55	0.963	0.188	174.3
VO	MP	4	303.2	123.4	0.877	0.226	87.05
VO	MP	5	717.4	85.18	1.384	0.225	47.25
VO	MP	6	471.05	147.6	0.7	0.2	13.55
VO	MP	7	249.2	92	1.312	0.106	55.4
VO	MP	8	520.85	154.15	0.815	0.265	28.4
VO	MP	9	509.4	107.35	0.216	0.546	60.6
VO	MP	10	896.7	92.3	1.216	0.17	114.4
VO	MP	11	625.7		1.668	0.992	86.85
VO	MP	12	610.4	118.05	1.54	0.754	62.25
VO	MP	13	501	177.3	2.955	1.245	148.8
VO	MP	14	493.7	118.25	1.684	0.67	54.6
VO	MP	15	789	96.05	1.87	0.812	52.7
VO	MC	1	260.3	19.45	0.466	0.275	12.87
VO	MC	2	231.1	0	0.128	0.256	11.988
VO	MC	3	141.2	0	0.113	0.18	11.352
VO	MC	4	289.3	0	0.109	0.15	6.354
VO	MC	5	179.5	0	0.13	0.157	5.682
VO	MC	6	292.5	0	0.137	0.091	4.233
VO	MC	7	198.1	0	0.161	0.234	3.438
VO	MC	8	113.6	0	0.117	0.168	3.855
VO	MC	9	135.9	0	0.148	0.226	10.764
VO	MC	10	240.7	0	0.17	0.28	5.622
VO	MC	11	149.4	0	0.151	0.132	5.439
VO	MC	12	165.8	0	0.14	0.3	8.01
VO	MC	13	136.4	0	0.17	0.389	11.277
VO	MC	14	105.8	0	0.91	0.266	12.084
VO	MC	15	96.1	0	0.154	0.035	7.416
VO	CC	1	139.4	149.4	0		36.8
VO	CC	2	417.45	80.56	0	0.342	47.85
VO	CC	3	110.4	0	0.048	0.384	33.05
VO	CC	4	185.3	0	0.11	0.581	19.85
VO	CC	5	260.3	53.29	0.135	0.39	55.45

Soil analysis data cont.

Species	Population	Individual	Ca	Mg	Ni	Cr	Fe
VO	CC	6	379.6	0	0.102	0.695	34.85
VO	CC	7	253	0	0.03	0.225	14.71
VO	CC	8	79.9	0	0.246	0.573	107.7
VO	CC	9	37.5	0	0	0	0
VO	CC	10	383.6	0	0.128	0.29	19.45
VO	CC	11	251.1	0	0.02	0.397	36.5
VO	CC	12	267.9	0	0.001	0.183	21.25
VO	CC	13	377.2	0	0	0.323	16.5
VO	CC	14	317	0	0.018	0.782	38.4
VO	CC	15	363.3	0	0.073	0.483	395.95
VF	NB	1		65.75	0.009	0	2.457
VF	NB	2		107.095	0.042	0	5.236
VF	NB	3		55.685	0.039	0.172	5.178
VF	NB	4		51.565	0	0	3.635
VF	NB	5		87.11	0	0.174	3.27
VF	NB	6		164	0	0.137	2.536
VF	NB	7		80.33	0	0	1.711
VF	NB	8		138.665	0	0	2.542
VF	NB	9		19.74	0.104	0.011	1.149
VF	NB	10		111.13	0	0.142	2.144
VF	NB	11		60.425	0	0	2.466
VF	NB	12		103.05	0	0.048	1.866
VF	NB	13		137.525	0	0	2.87
VF	NB	14		134.535	0	0.012	2.31
VF	NB	15		70.87	0	0.055	2.362
VF	BM	1		67.675	0.055	0	5.85
VF	BM	2		113.92	0.037	0	2.084
VF	BM	3		54.66	0.021	0	1.39
VF	BM	4			0.008	0	6.819
VF	BM	5			0.073	0	2.101
VF	BM	6			0.109		0
VF	BM	7			0.031	0	0.56
VF	BM	8			0.033	0	2.278
VF	BM	9			0		
VF	BM	10		63.41	0.038	0.094	4.742
VF	BM	11		37.395	0.002	0.071	4.072
VF	BM	12		25.155	0.01	0	2.719
VF	BM	13		20.355	0	0	4.603
VF	BM	14		63.185	0	0.143	3.879
VF	BM	15		81.55	0.032	0	1.978
VF	MA	1			0.136	0.486	18.08

Soil analysis cont.

Species	Population	Individual	Ca	Mg	Ni	Cr	Fe
VF	MA	2			0.021	0.206	13.73
VF	MA	3			0.038	0.175	11.64
VF	MA	4			0.04	0.098	6.935
VF	MA	5			0	0	3.265
VF	MA	6			0.002	0.014	5.95
VF	MA	7			0.078	0.202	18.36
VF	MA	8			0	0.176	10.725
VF	MA	9			0.031	0.154	14.655
VF	MA	10		85.125	0.009	0	2.2
VF	MA	11		90.63	0	0.006	4.445
VF	MA	12		48.25	0.003	0.043	8.085
VF	MA	13			0.002	0	66.65
VF	MA	14					
VF	MA	15			0.069	0.269	19.235

Temperature data for leaf angle manipulation of all three species (VO=*Viola ocellata*, VC=*V. cuneata* and VF=*V. flettii*). Time= time of day on 24 hour scale.

Species	Population	Plot	Time	Treatment	Leaf Temp.
VO	MP	1	9	Control	12
VO	MP	1	9	Manipulated	12
VO	MP	1	11	Control	30
VO	MP	1	11	Manipulated	26
VO	MP	1	13	Control	36
VO	MP	1	13	Manipulated	32
VO	MP	1	15	Control	16
VO	MP	1	15	Manipulated	16
VO	MP	1	17	Control	15
VO	MP	1	17	Manipulated	15
VO	MP	2	9	Control	17
VO	MP	2	9	Manipulated	17
VO	MP	2	11	Control	35
VO	MP	2	11	Manipulated	32
VO	MP	2	13	Control	19
VO	MP	2	13	Manipulated	18
VO	MP	2	15	Control	16
VO	MP	2	15	Manipulated	16
VO	MP	2	17	Control	15
VO	MP	2	17	Manipulated	15
VO	MP	3	9	Control	9
VO	MP	3	9	Manipulated	9.5
VO	MP	3	11	Control	16
VO	MP	3	11	Manipulated	16
VO	MP	3	13	Control	16
VO	MP	3	13	Manipulated	16
VO	MP	3	15	Control	13
VO	MP	3	15	Manipulated	13
VO	MP	3	17	Control	15
VO	MP	3	17	Manipulated	15
VO	MP	4	9	Control	8
VO	MP	4	9	Manipulated	8
VO	MP	4	11	Control	16
VO	MP	4	11	Manipulated	17
VO	MP	4	13	Control	16
VO	MP	4	13	Manipulated	16
VO	MP	4	15	Control	15
VO	MP	4	15	Manipulated	15
VO	MP	4	17	Control	14
VO	MP	4	17	Manipulated	15

Temperature data cont.

Species	Population	Plot	Time	Treatment	Leaf Temp.
VO	MP	5	9	Control	7
VO	MP	5	9	Manipulated	8
VO	MP	5	11	Control	18
VO	MP	5	11	Manipulated	17
VO	MP	5	13	Control	17
VO	MP	5	13	Manipulated	16
VO	MP	5	15	Control	16
VO	MP	5	15	Manipulated	17
VO	MP	5	17	Control	15
VO	MP	5	17	Manipulated	15
VO	MP	6	9	Control	9
VO	MP	6	9	Manipulated	9
VO	MP	6	11	Control	16
VO	MP	6	11	Manipulated	19
VO	MP	6	13	Control	16
VO	MP	6	13	Manipulated	16
VO	MP	6	15	Control	16
VO	MP	6	15	Manipulated	17
VO	MP	6	17	Control	28
VO	MP	6	17	Manipulated	23
VO	MP	7	9	Control	11
VO	MP	7	9	Manipulated	11
VO	MP	7	11	Control	15
VO	MP	7	11	Manipulated	16
VO	MP	7	13	Control	16
VO	MP	7	13	Manipulated	16
VO	MP	7	15	Control	17
VO	MP	7	15	Manipulated	17
VO	MP	7	17	Control	16
VO	MP	7	17	Manipulated	16
VO	MP	8	9	Control	9
VO	MP	8	9	Manipulated	10
VO	MP	8	11	Control	15
VO	MP	8	11	Manipulated	15
VO	MP	8	13	Control	14
VO	MP	8	13	Manipulated	16
VO	MP	8	15	Control	14
VO	MP	8	15	Manipulated	14
VO	MP	8	17	Control	14
VO	MP	8	17	Manipulated	16
VO	MP	9	9	Control	12

Temperature data cont.

Species	Population	Plot	Time	Treatment	Leaf Temp.
VO	MP	9	9	Manipulated	12
VO	MP	9	11	Control	14
VO	MP	9	11	Manipulated	15
VO	MP	9	13	Control	14
VO	MP	9	13	Manipulated	14
VO	MP	9	15	Control	14
VO	MP	9	15	Manipulated	15
VO	MP	9	17	Control	16
VO	MP	9	17	Manipulated	17
VO	MP	10	9	Control	9
VO	MP	10	9	Manipulated	10
VO	MP	10	11	Control	15
VO	MP	10	11	Manipulated	15
VO	MP	10	13	Control	13
VO	MP	10	13	Manipulated	14
VO	MP	10	15	Control	15
VO	MP	10	15	Manipulated	17
VO	MP	10	17	Control	14
VO	MP	10	17	Manipulated	15
VO	MP	11	9	Control	8
VO	MP	11	9	Manipulated	7
VO	MP	11	11	Control	15
VO	MP	11	11	Manipulated	15
VO	MP	11	13	Control	13
VO	MP	11	13	Manipulated	13
VO	MP	11	15	Control	16
VO	MP	11	15	Manipulated	16
VO	MP	11	17	Control	14
VO	MP	11	17	Manipulated	14
VO	MP	12	9	Control	9
VO	MP	12	9	Manipulated	10
VO	MP	12	11	Control	15
VO	MP	12	11	Manipulated	15
VO	MP	12	13	Control	14
VO	MP	12	13	Manipulated	14
VO	MP	12	15	Control	16
VO	MP	12	15	Manipulated	19
VO	MP	12	17	Control	14
VO	MP	12	17	Manipulated	15
VO	MP	13	9	Control	8
VO	MP	13	9	Manipulated	8

Temperature data cont.

Species	Population	Plot	Time	Treatment	Leaf Temp.
VO	MP	13	11	Control	15
VO	MP	13	11	Manipulated	15
VO	MP	13	13	Control	16
VO	MP	13	13	Manipulated	14
VO	MP	13	15	Control	15
VO	MP	13	15	Manipulated	15
VO	MP	13	17	Control	15
VO	MP	13	17	Manipulated	14
VO	MP	14	9	Control	8
VO	MP	14	9	Manipulated	8
VO	MP	14	11	Control	15
VO	MP	14	11	Manipulated	16
VO	MP	14	13	Control	13
VO	MP	14	13	Manipulated	13
VO	MP	14	15	Control	13
VO	MP	14	15	Manipulated	13
VO	MP	14	17	Control	14
VO	MP	14	17	Manipulated	15
VO	MP	15	9	Control	10
VO	MP	15	9	Manipulated	10
VO	MP	15	11	Control	15
VO	MP	15	11	Manipulated	16
VO	MP	15	13	Control	13
VO	MP	15	13	Manipulated	14
VO	MP	15	15	Control	13
VO	MP	15	15	Manipulated	14
VO	MP	15	17	Control	14
VO	MP	15	17	Manipulated	15
VO	MC	1	9	Control	19
VO	MC	1	9	Manipulated	18
VO	MC	1	11	Control	19
VO	MC	1	11	Manipulated	16
VO	MC	1	13	Control	25
VO	MC	1	13	Manipulated	25
VO	MC	1	15	Control	26
VO	MC	1	15	Manipulated	23
VO	MC	1	17	Control	27
VO	MC	1	17	Manipulated	23
VO	MC	2	9	Control	26
VO	MC	2	9	Manipulated	28
VO	MC	2	11	Control	22

Temperature data cont.

Species	Population	Plot	Time	Treatment	Leaf Temp.
VO	MC	2	11	Manipulated	29
VO	MC	2	13	Control	25
VO	MC	2	13	Manipulated	30
VO	MC	2	15	Control	25
VO	MC	2	15	Manipulated	26
VO	MC	2	17	Control	25
VO	MC	2	17	Manipulated	26
VO	MC	3	9	Control	26
VO	MC	3	9	Manipulated	39
VO	MC	3	11	Control	23
VO	MC	3	11	Manipulated	23
VO	MC	3	13	Control	29
VO	MC	3	13	Manipulated	28
VO	MC	3	15	Control	26
VO	MC	3	15	Manipulated	25
VO	MC	3	17	Control	23
VO	MC	3	17	Manipulated	26
VO	MC	4	9	Control	25
VO	MC	4	9	Manipulated	25
VO	MC	4	11	Control	25
VO	MC	4	11	Manipulated	22
VO	MC	4	13	Control	25
VO	MC	4	13	Manipulated	23
VO	MC	4	15	Control	25
VO	MC	4	15	Manipulated	24
VO	MC	4	17	Control	25
VO	MC	4	17	Manipulated	25
VO	MC	5	9	Control	16
VO	MC	5	9	Manipulated	18
VO	MC	5	11	Control	21
VO	MC	5	11	Manipulated	21
VO	MC	5	13	Control	24
VO	MC	5	13	Manipulated	25
VO	MC	5	15	Control	24
VO	MC	5	15	Manipulated	24
VO	MC	5	17	Control	27
VO	MC	5	17	Manipulated	26
VO	MC	6	9	Control	15
VO	MC	6	9	Manipulated	15
VO	MC	6	11	Control	22
VO	MC	6	11	Manipulated	22

Temperature data cont.

Species	Population	Plot	Time	Treatment	Leaf Temp.
VO	MC	6	13	Control	26
VO	MC	6	13	Manipulated	26
VO	MC	6	15	Control	25
VO	MC	6	15	Manipulated	24
VO	MC	6	17	Control	25
VO	MC	6	17	Manipulated	26
VO	MC	7	9	Control	17
VO	MC	7	9	Manipulated	18
VO	MC	7	11	Control	21
VO	MC	7	11	Manipulated	23
VO	MC	7	13	Control	25
VO	MC	7	13	Manipulated	25
VO	MC	7	15	Control	25
VO	MC	7	15	Manipulated	25
VO	MC	7	17	Control	24
VO	MC	7	17	Manipulated	27
VO	MC	8	9	Control	15
VO	MC	8	9	Manipulated	15
VO	MC	8	11	Control	22
VO	MC	8	11	Manipulated	22
VO	MC	8	13	Control	24
VO	MC	8	13	Manipulated	25
VO	MC	8	15	Control	24
VO	MC	8	15	Manipulated	25
VO	MC	8	17	Control	24
VO	MC	8	17	Manipulated	26
VO	MC	9	9	Control	25
VO	MC	9	9	Manipulated	22
VO	MC	9	11	Control	21
VO	MC	9	11	Manipulated	22
VO	MC	9	13	Control	25
VO	MC	9	13	Manipulated	26
VO	MC	9	15	Control	25
VO	MC	9	15	Manipulated	25
VO	MC	9	17	Control	24
VO	MC	9	17	Manipulated	25
VO	MC	10	9	Control	21
VO	MC	10	9	Manipulated	28
VO	MC	10	11	Control	21
VO	MC	10	11	Manipulated	22
VO	MC	10	13	Control	25

Temperature data cont.

Species	Population	Plot	Time	Treatment	Leaf Temp.
VO	MC	10	13	Manipulated	25
VO	MC	10	15	Control	25
VO	MC	10	15	Manipulated	25
VO	MC	10	17	Control	25
VO	MC	10	17	Manipulated	25
VO	MC	11	9	Control	24
VO	MC	11	9	Manipulated	25
VO	MC	11	11	Control	21
VO	MC	11	11	Manipulated	21
VO	MC	11	13	Control	25
VO	MC	11	13	Manipulated	25
VO	MC	11	15	Control	25
VO	MC	11	15	Manipulated	25
VO	MC	11	17	Control	24
VO	MC	11	17	Manipulated	25
VO	MC	12	9	Control	17
VO	MC	12	9	Manipulated	17
VO	MC	12	11	Control	21
VO	MC	12	11	Manipulated	22
VO	MC	12	13	Control	24
VO	MC	12	13	Manipulated	24
VO	MC	12	15	Control	24
VO	MC	12	15	Manipulated	25
VO	MC	12	17	Control	24
VO	MC	12	17	Manipulated	24
VO	MC	13	9	Control	20
VO	MC	13	9	Manipulated	23
VO	MC	13	11	Control	22
VO	MC	13	11	Manipulated	24
VO	MC	13	13	Control	28
VO	MC	13	13	Manipulated	28
VO	MC	13	15	Control	32
VO	MC	13	15	Manipulated	31
VO	MC	13	17	Control	25
VO	MC	13	17	Manipulated	25
VO	MC	14	9	Control	20
VO	MC	14	9	Manipulated	19
VO	MC	14	11	Control	20
VO	MC	14	11	Manipulated	23
VO	MC	14	13	Control	26
VO	MC	14	13	Manipulated	30

Temperature data cont.

Species	Population	Plot	Time	Treatment	Leaf Temp.
VO	MC	14	15	Control	24
VO	MC	14	15	Manipulated	27
VO	MC	14	17	Control	24
VO	MC	14	17	Manipulated	24
VO	MC	15	9	Control	19
VO	MC	15	9	Manipulated	20
VO	MC	15	11	Control	22
VO	MC	15	11	Manipulated	21
VO	MC	15	13	Control	29
VO	MC	15	13	Manipulated	28
VO	MC	15	15	Control	26
VO	MC	15	15	Manipulated	26
VO	MC	15	17	Control	24
VO	MC	15	17	Manipulated	24
VO	CC	1	9	Control	17
VO	CC	1	9	Manipulated	17
VO	CC	1	11	Control	30
VO	CC	1	11	Manipulated	34
VO	CC	1	13	Control	30
VO	CC	1	13	Manipulated	34
VO	CC	1	15	Control	29
VO	CC	1	15	Manipulated	29
VO	CC	1	17	Control	30
VO	CC	1	17	Manipulated	30
VO	CC	2	9	Control	25
VO	CC	2	9	Manipulated	22
VO	CC	2	11	Control	23
VO	CC	2	11	Manipulated	25
VO	CC	2	13	Control	22
VO	CC	2	13	Manipulated	23
VO	CC	2	15	Control	21
VO	CC	2	15	Manipulated	22
VO	CC	2	17	Control	23
VO	CC	2	17	Manipulated	25
VO	CC	3	9	Control	19
VO	CC	3	9	Manipulated	19
VO	CC	3	11	Control	34
VO	CC	3	11	Manipulated	29
VO	CC	3	13	Control	25
VO	CC	3	13	Manipulated	25
VO	CC	3	15	Control	24

Temperature data cont.

Species	Population	Plot	Time	Treatment	Leaf Temp.
VO	CC	3	15	Manipulated	25
VO	CC	3	17	Control	26
VO	CC	3	17	Manipulated	26
VO	CC	4	9	Control	19
VO	CC	4	9	Manipulated	19
VO	CC	4	11	Control	34
VO	CC	4	11	Manipulated	29
VO	CC	4	13	Control	25
VO	CC	4	13	Manipulated	25
VO	CC	4	15	Control	24
VO	CC	4	15	Manipulated	25
VO	CC	4	17	Control	26
VO	CC	4	17	Manipulated	26
VO	CC	5	9	Control	17
VO	CC	5	9	Manipulated	18
VO	CC	5	11	Control	24
VO	CC	5	11	Manipulated	24
VO	CC	5	13	Control	24
VO	CC	5	13	Manipulated	24
VO	CC	5	15	Control	24
VO	CC	5	15	Manipulated	26
VO	CC	5	17	Control	25
VO	CC	5	17	Manipulated	24
VO	CC	6	9	Control	17
VO	CC	6	9	Manipulated	19
VO	CC	6	11	Control	24
VO	CC	6	11	Manipulated	24
VO	CC	6	13	Control	26
VO	CC	6	13	Manipulated	30
VO	CC	6	15	Control	23
VO	CC	6	15	Manipulated	24
VO	CC	6	17	Control	37
VO	CC	6	17	Manipulated	27
VO	CC	7	9	Control	17
VO	CC	7	9	Manipulated	17
VO	CC	7	11	Control	23
VO	CC	7	11	Manipulated	26
VO	CC	7	13	Control	28
VO	CC	7	13	Manipulated	30
VO	CC	7	15	Control	25
VO	CC	7	15	Manipulated	25

Temperature data cont.

Species	Population	Plot	Time	Treatment	Leaf Temp.
VO	CC	7	17	Control	27
VO	CC	7	17	Manipulated	27
VO	CC	8	9	Control	16
VO	CC	8	9	Manipulated	17
VO	CC	8	11	Control	25
VO	CC	8	11	Manipulated	25
VO	CC	8	13	Control	31
VO	CC	8	13	Manipulated	28
VO	CC	8	15	Control	25
VO	CC	8	15	Manipulated	24
VO	CC	8	17	Control	26
VO	CC	8	17	Manipulated	25
VO	CC	9	9	Control	16
VO	CC	9	9	Manipulated	18
VO	CC	9	11	Control	26
VO	CC	9	11	Manipulated	29
VO	CC	9	13	Control	26
VO	CC	9	13	Manipulated	31
VO	CC	9	15	Control	22
VO	CC	9	15	Manipulated	24
VO	CC	9	17	Control	25
VO	CC	9	17	Manipulated	27
VO	CC	10	9	Control	17
VO	CC	10	9	Manipulated	18
VO	CC	10	11	Control	47
VO	CC	10	11	Manipulated	27
VO	CC	10	13	Control	62
VO	CC	10	13	Manipulated	47
VO	CC	10	15	Control	25
VO	CC	10	15	Manipulated	28
VO	CC	10	17	Control	23
VO	CC	10	17	Manipulated	25
VO	CC	11	9	Control	18
VO	CC	11	9	Manipulated	19
VO	CC	11	11	Control	27
VO	CC	11	11	Manipulated	34
VO	CC	11	13	Control	49
VO	CC	11	13	Manipulated	40
VO	CC	11	15	Control	28
VO	CC	11	15	Manipulated	28
VO	CC	11	17	Control	25

Temperature data cont.

Species	Population	Plot	Time	Treatment	Leaf Temp.
VO	CC	11	17	Manipulated	27
VO	CC	12	9	Control	18
VO	CC	12	9	Manipulated	18
VO	CC	12	11	Control	31
VO	CC	12	11	Manipulated	30
VO	CC	12	13	Control	28
VO	CC	12	13	Manipulated	28
VO	CC	12	15	Control	26
VO	CC	12	15	Manipulated	26
VO	CC	12	17	Control	26
VO	CC	12	17	Manipulated	26
VO	CC	13	9	Control	18
VO	CC	13	9	Manipulated	18
VO	CC	13	11	Control	25
VO	CC	13	11	Manipulated	25
VO	CC	13	13	Control	27
VO	CC	13	13	Manipulated	29
VO	CC	13	15	Control	25
VO	CC	13	15	Manipulated	26
VO	CC	13	17	Control	26
VO	CC	13	17	Manipulated	25
VO	CC	14	9	Control	16
VO	CC	14	9	Manipulated	18
VO	CC	14	11	Control	24
VO	CC	14	11	Manipulated	26
VO	CC	14	13	Control	37
VO	CC	14	13	Manipulated	44
VO	CC	14	15	Control	25
VO	CC	14	15	Manipulated	26
VO	CC	14	17	Control	24
VO	CC	14	17	Manipulated	25
VO	CC	15	9	Control	17
VO	CC	15	9	Manipulated	18
VO	CC	15	11	Control	40
VO	CC	15	11	Manipulated	32
VO	CC	15	13	Control	27
VO	CC	15	13	Manipulated	28
VO	CC	15	15	Control	24
VO	CC	15	15	Manipulated	25
VO	CC	15	17	Control	25
VO	CC	15	17	Manipulated	25

Temperature data cont.

Species	Population	Plot	Time	Treatment	Leaf Temp.
VC	HM	1	9	Control	14
VC	HM	1	9	Manipulated	18
VC	HM	1	11	Control	11
VC	HM	1	11	Manipulated	11
VC	HM	1	13	Control	14
VC	HM	1	13	Manipulated	14
VC	HM	1	15	Control	16
VC	HM	1	15	Manipulated	26
VC	HM	1	17	Control	7
VC	HM	1	17	Manipulated	10
VC	HM	2	9	Control	21
VC	HM	2	9	Manipulated	25
VC	HM	2	11	Control	1
VC	HM	2	11	Manipulated	3
VC	HM	2	13	Control	14
VC	HM	2	13	Manipulated	14
VC	HM	2	15	Control	14
VC	HM	2	15	Manipulated	12
VC	HM	2	17	Control	12
VC	HM	2	17	Manipulated	12
VC	HM	3	9	Control	14
VC	HM	3	9	Manipulated	17
VC	HM	3	11	Control	3
VC	HM	3	11	Manipulated	11
VC	HM	3	13	Control	29
VC	HM	3	13	Manipulated	40
VC	HM	3	15	Control	38
VC	HM	3	15	Manipulated	37
VC	HM	3	17	Control	18
VC	HM	3	17	Manipulated	29
VC	HM	4	9	Control	16
VC	HM	4	9	Manipulated	17
VC	HM	4	11	Control	15
VC	HM	4	11	Manipulated	10
VC	HM	4	13	Control	13
VC	HM	4	13	Manipulated	14
VC	HM	4	15	Control	38
VC	HM	4	15	Manipulated	30
VC	HM	4	17	Control	23
VC	HM	4	17	Manipulated	20
VC	HM	5	9	Control	15

Temperature data cont.

Species	Population	Plot	Time	Treatment	Leaf Temp.
VC	HM	5	9	Manipulated	16
VC	HM	5	11	Control	5
VC	HM	5	11	Manipulated	7
VC	HM	5	13	Control	21
VC	HM	5	13	Manipulated	28
VC	HM	5	15	Control	21
VC	HM	5	15	Manipulated	33
VC	HM	5	17	Control	12
VC	HM	5	17	Manipulated	20
VC	HM	6	9	Control	29
VC	HM	6	9	Manipulated	37
VC	HM	6	11	Control	11
VC	HM	6	11	Manipulated	14
VC	HM	6	13	Control	41
VC	HM	6	13	Manipulated	52
VC	HM	6	15	Control	45
VC	HM	6	15	Manipulated	43
VC	HM	6	17	Control	16
VC	HM	6	17	Manipulated	14
VC	HM	7	9	Control	16
VC	HM	7	9	Manipulated	17
VC	HM	7	11	Control	35
VC	HM	7	11	Manipulated	33
VC	HM	7	13	Control	46
VC	HM	7	13	Manipulated	40
VC	HM	7	15	Control	19
VC	HM	7	15	Manipulated	19
VC	HM	7	17	Control	11
VC	HM	7	17	Manipulated	11
VC	HM	8	9	Control	16
VC	HM	8	9	Manipulated	17
VC	HM	8	11	Control	12
VC	HM	8	11	Manipulated	12
VC	HM	8	13	Control	25
VC	HM	8	13	Manipulated	45
VC	HM	8	15	Control	38
VC	HM	8	15	Manipulated	42
VC	HM	8	17	Control	22
VC	HM	8	17	Manipulated	35
VC	HM	9	9	Control	14
VC	HM	9	9	Manipulated	14

Temperature data cont.

Species	Population	Plot	Time	Treatment	Leaf Temp.
VC	HM	9	11	Control	14
VC	HM	9	11	Manipulated	13
VC	HM	9	13	Control	17
VC	HM	9	13	Manipulated	14
VC	HM	9	15	Control	26
VC	HM	9	15	Manipulated	20
VC	HM	9	17	Control	15
VC	HM	9	17	Manipulated	16
VC	HM	10	9	Control	43
VC	HM	10	9	Manipulated	43
VC	HM	10	11	Control	34
VC	HM	10	11	Manipulated	29
VC	HM	10	13	Control	48
VC	HM	10	13	Manipulated	53
VC	HM	10	15	Control	32
VC	HM	10	15	Manipulated	31
VC	HM	10	17	Control	21
VC	HM	10	17	Manipulated	25
VC	HM	11	9	Control	43
VC	HM	11	9	Manipulated	33
VC	HM	11	11	Control	29
VC	HM	11	11	Manipulated	36
VC	HM	11	13	Control	32
VC	HM	11	13	Manipulated	30
VC	HM	11	15	Control	22
VC	HM	11	15	Manipulated	27
VC	HM	11	17	Control	15
VC	HM	11	17	Manipulated	16
VC	HM	12	9	Control	24
VC	HM	12	9	Manipulated	25
VC	HM	12	11	Control	26
VC	HM	12	11	Manipulated	27
VC	HM	12	13	Control	28
VC	HM	12	13	Manipulated	38
VC	HM	12	15	Control	28
VC	HM	12	15	Manipulated	35
VC	HM	12	17	Control	15
VC	HM	12	17	Manipulated	16
VC	HM	13	9	Control	25
VC	HM	13	9	Manipulated	18
VC	HM	13	11	Control	21

Temperature data cont.

Species	Population	Plot	Time	Treatment	Leaf Temp.
VC	HM	13	11	Manipulated	17
VC	HM	13	13	Control	18
VC	HM	13	13	Manipulated	20
VC	HM	13	15	Control	16
VC	HM	13	15	Manipulated	15
VC	HM	13	17	Control	17
VC	HM	13	17	Manipulated	19
VC	HM	14	9	Control	33
VC	HM	14	9	Manipulated	23
VC	HM	14	11	Control	23
VC	HM	14	11	Manipulated	20
VC	HM	14	13	Control	17
VC	HM	14	13	Manipulated	16
VC	HM	14	15	Control	16
VC	HM	14	15	Manipulated	19
VC	HM	14	17	Control	15
VC	HM	14	17	Manipulated	16
VC	HM	15	9	Control	12
VC	HM	15	9	Manipulated	22
VC	HM	15	11	Control	32
VC	HM	15	11	Manipulated	23
VC	HM	15	13	Control	28
VC	HM	15	13	Manipulated	23
VC	HM	15	15	Control	25
VC	HM	15	15	Manipulated	23
VC	HM	15	17	Control	29
VC	HM	15	17	Manipulated	34
VC	SP	1	9	Control	15
VC	SP	1	9	Manipulated	20
VC	SP	1	11	Control	16
VC	SP	1	11	Manipulated	18
VC	SP	1	13	Control	33
VC	SP	1	13	Manipulated	42
VC	SP	1	15	Control	21
VC	SP	1	15	Manipulated	39
VC	SP	1	17	Control	22
VC	SP	1	17	Manipulated	20
VC	SP	2	9	Control	10
VC	SP	2	9	Manipulated	9
VC	SP	2	11	Control	12
VC	SP	2	11	Manipulated	19

Temperature data cont.

Species	Population	Plot	Time	Treatment	Leaf Temp.
VC	SP	2	13	Control	15
VC	SP	2	13	Manipulated	19
VC	SP	2	15	Control	33
VC	SP	2	15	Manipulated	39
VC	SP	2	17	Control	181
VC	SP	2	17	Manipulated	21
VC	SP	3	9	Control	9
VC	SP	3	9	Manipulated	9
VC	SP	3	11	Control	16
VC	SP	3	11	Manipulated	16
VC	SP	3	13	Control	28
VC	SP	3	13	Manipulated	34
VC	SP	3	15	Control	28
VC	SP	3	15	Manipulated	32
VC	SP	3	17	Control	22
VC	SP	3	17	Manipulated	21
VC	SP	4	9	Control	11
VC	SP	4	9	Manipulated	10
VC	SP	4	11	Control	11
VC	SP	4	11	Manipulated	14
VC	SP	4	13	Control	28
VC	SP	4	13	Manipulated	31
VC	SP	4	15	Control	17
VC	SP	4	15	Manipulated	19
VC	SP	4	17	Control	36
VC	SP	4	17	Manipulated	38
VC	SP	5	9	Control	11
VC	SP	5	9	Manipulated	11
VC	SP	5	11	Control	15
VC	SP	5	11	Manipulated	25
VC	SP	5	13	Control	18
VC	SP	5	13	Manipulated	17
VC	SP	5	15	Control	37
VC	SP	5	15	Manipulated	47
VC	SP	5	17	Control	28
VC	SP	5	17	Manipulated	43
VC	SP	6	9	Control	13
VC	SP	6	9	Manipulated	14
VC	SP	6	11	Control	32
VC	SP	6	11	Manipulated	19
VC	SP	6	13	Control	23

Temperature data cont.

Species	Population	Plot	Time	Treatment	Leaf Temp.
VC	SP	6	13	Manipulated	22
VC	SP	6	15	Control	24
VC	SP	6	15	Manipulated	23
VC	SP	6	17	Control	26
VC	SP	6	17	Manipulated	30
VC	SP	7	9	Control	12
VC	SP	7	9	Manipulated	13
VC	SP	7	11	Control	16
VC	SP	7	11	Manipulated	16
VC	SP	7	13	Control	28
VC	SP	7	13	Manipulated	25
VC	SP	7	15	Control	52
VC	SP	7	15	Manipulated	53
VC	SP	7	17	Control	33
VC	SP	7	17	Manipulated	44
VC	SP	8	9	Control	13
VC	SP	8	9	Manipulated	13
VC	SP	8	11	Control	23
VC	SP	8	11	Manipulated	20
VC	SP	8	13	Control	31
VC	SP	8	13	Manipulated	29
VC	SP	8	15	Control	31
VC	SP	8	15	Manipulated	31
VC	SP	8	17	Control	44
VC	SP	8	17	Manipulated	41
VC	SP	9	9	Control	10
VC	SP	9	9	Manipulated	11
VC	SP	9	11	Control	14
VC	SP	9	11	Manipulated	14
VC	SP	9	13	Control	25
VC	SP	9	13	Manipulated	19
VC	SP	9	15	Control	24
VC	SP	9	15	Manipulated	24
VC	SP	9	17	Control	31
VC	SP	9	17	Manipulated	29
VC	SP	10	9	Control	13
VC	SP	10	9	Manipulated	14
VC	SP	10	11	Control	11
VC	SP	10	11	Manipulated	12
VC	SP	10	13	Control	13
VC	SP	10	13	Manipulated	12

Temperature data cont.

Species	Population	Plot	Time	Treatment	Leaf Temp.
VC	SP	10	15	Control	23
VC	SP	10	15	Manipulated	23
VC	SP	10	17	Control	38
VC	SP	10	17	Manipulated	40
VC	SP	11	9	Control	12
VC	SP	11	9	Manipulated	14
VC	SP	11	11	Control	26
VC	SP	11	11	Manipulated	29
VC	SP	11	13	Control	17
VC	SP	11	13	Manipulated	14
VC	SP	11	15	Control	21
VC	SP	11	15	Manipulated	21
VC	SP	11	17	Control	30
VC	SP	11	17	Manipulated	24
VC	SP	12	9	Control	12
VC	SP	12	9	Manipulated	13
VC	SP	12	11	Control	14
VC	SP	12	11	Manipulated	26
VC	SP	12	13	Control	20
VC	SP	12	13	Manipulated	28
VC	SP	12	15	Control	23
VC	SP	12	15	Manipulated	27
VC	SP	12	17	Control	32
VC	SP	12	17	Manipulated	36
VC	SP	13	9	Control	24
VC	SP	13	9	Manipulated	19
VC	SP	13	11	Control	12
VC	SP	13	11	Manipulated	13
VC	SP	13	13	Control	28
VC	SP	13	13	Manipulated	29
VC	SP	13	15	Control	56
VC	SP	13	15	Manipulated	42
VC	SP	13	17	Control	36
VC	SP	13	17	Manipulated	37
VC	SP	14	9	Control	18
VC	SP	14	9	Manipulated	16
VC	SP	14	11	Control	15
VC	SP	14	11	Manipulated	15
VC	SP	14	13	Control	19
VC	SP	14	13	Manipulated	18
VC	SP	14	15	Control	43

Temperature data cont.

Species	Population	Plot	Time	Treatment	Leaf Temp.
VC	SP	14	15	Manipulated	47
VC	SP	14	17	Control	26
VC	SP	14	17	Manipulated	25
VC	SP	15	9	Control	15
VC	SP	15	9	Manipulated	14
VC	SP	15	11	Control	14
VC	SP	15	11	Manipulated	15
VC	SP	15	13	Control	41
VC	SP	15	13	Manipulated	41
VC	SP	15	15	Control	40
VC	SP	15	15	Manipulated	43
VC	SP	15	17	Control	22
VC	SP	15	17	Manipulated	22
VC	ED	1	9	Control	14
VC	ED	1	9	Manipulated	16
VC	ED	1	11	Control	30
VC	ED	1	11	Manipulated	34
VC	ED	1	13	Control	34
VC	ED	1	13	Manipulated	36
VC	ED	1	15	Control	42
VC	ED	1	15	Manipulated	47
VC	ED	1	17	Control	45
VC	ED	1	17	Manipulated	48
VC	ED	2	9	Control	14
VC	ED	2	9	Manipulated	15
VC	ED	2	11	Control	29
VC	ED	2	11	Manipulated	30
VC	ED	2	13	Control	39
VC	ED	2	13	Manipulated	51
VC	ED	2	15	Control	49
VC	ED	2	15	Manipulated	44
VC	ED	2	17	Control	36
VC	ED	2	17	Manipulated	34
VC	ED	3	9	Control	15
VC	ED	3	9	Manipulated	15
VC	ED	3	11	Control	39
VC	ED	3	11	Manipulated	37
VC	ED	3	13	Control	31
VC	ED	3	13	Manipulated	31
VC	ED	3	15	Control	47
VC	ED	3	15	Manipulated	43

Temperature data cont.

Species	Population	Plot	Time	Treatment	Leaf Temp.
VC	ED	3	17	Control	43
VC	ED	3	17	Manipulated	40
VC	ED	4	9	Control	15
VC	ED	4	9	Manipulated	17
VC	ED	4	11	Control	22
VC	ED	4	11	Manipulated	24
VC	ED	4	13	Control	33
VC	ED	4	13	Manipulated	29
VC	ED	4	15	Control	49
VC	ED	4	15	Manipulated	47
VC	ED	4	17	Control	45
VC	ED	4	17	Manipulated	40
VC	ED	5	9	Control	16
VC	ED	5	9	Manipulated	14
VC	ED	5	11	Control	24
VC	ED	5	11	Manipulated	36
VC	ED	5	13	Control	59
VC	ED	5	13	Manipulated	47
VC	ED	5	15	Control	55
VC	ED	5	15	Manipulated	57
VC	ED	5	17	Control	45
VC	ED	5	17	Manipulated	38
VC	ED	6	9	Control	16
VC	ED	6	9	Manipulated	15
VC	ED	6	11	Control	23
VC	ED	6	11	Manipulated	21
VC	ED	6	13	Control	51
VC	ED	6	13	Manipulated	48
VC	ED	6	15	Control	48
VC	ED	6	15	Manipulated	40
VC	ED	6	17	Control	34
VC	ED	6	17	Manipulated	33
VC	ED	7	9	Control	17
VC	ED	7	9	Manipulated	17
VC	ED	7	11	Control	24
VC	ED	7	11	Manipulated	26
VC	ED	7	13	Control	54
VC	ED	7	13	Manipulated	43
VC	ED	7	15	Control	43
VC	ED	7	15	Manipulated	44
VC	ED	7	17	Control	32

Temperature data cont.

Species	Population	Plot	Time	Treatment	Leaf Temp.
VC	ED	7	17	Manipulated	32
VC	ED	8	9	Control	18
VC	ED	8	9	Manipulated	18
VC	ED	8	11	Control	22
VC	ED	8	11	Manipulated	21
VC	ED	8	13	Control	45
VC	ED	8	13	Manipulated	49
VC	ED	8	15	Control	50
VC	ED	8	15	Manipulated	55
VC	ED	8	17	Control	35
VC	ED	8	17	Manipulated	38
VC	ED	9	9	Control	17
VC	ED	9	9	Manipulated	17
VC	ED	9	11	Control	29
VC	ED	9	11	Manipulated	28
VC	ED	9	13	Control	30
VC	ED	9	13	Manipulated	35
VC	ED	9	15	Control	35
VC	ED	9	15	Manipulated	35
VC	ED	9	17	Control	32
VC	ED	9	17	Manipulated	32
VC	ED	10	9	Control	17
VC	ED	10	9	Manipulated	18
VC	ED	10	11	Control	25
VC	ED	10	11	Manipulated	28
VC	ED	10	13	Control	33
VC	ED	10	13	Manipulated	32
VC	ED	10	15	Control	34
VC	ED	10	15	Manipulated	33
VC	ED	10	17	Control	45
VC	ED	10	17	Manipulated	48
VC	ED	11	9	Control	22
VC	ED	11	9	Manipulated	21
VC	ED	11	11	Control	46
VC	ED	11	11	Manipulated	43
VC	ED	11	13	Control	31
VC	ED	11	13	Manipulated	31
VC	ED	11	15	Control	52
VC	ED	11	15	Manipulated	36
VC	ED	11	17	Control	35
VC	ED	11	17	Manipulated	37

Temperature data cont.

Species	Population	Plot	Time	Treatment	Leaf Temp.
VC	ED	12	9	Control	20
VC	ED	12	9	Manipulated	20
VC	ED	12	11	Control	31
VC	ED	12	11	Manipulated	30
VC	ED	12	13	Control	43
VC	ED	12	13	Manipulated	53
VC	ED	12	15	Control	42
VC	ED	12	15	Manipulated	48
VC	ED	12	17	Control	33
VC	ED	12	17	Manipulated	52
VC	ED	13	9	Control	22
VC	ED	13	9	Manipulated	23
VC	ED	13	11	Control	31
VC	ED	13	11	Manipulated	30
VC	ED	13	13	Control	29
VC	ED	13	13	Manipulated	28
VC	ED	13	15	Control	31
VC	ED	13	15	Manipulated	32
VC	ED	13	17	Control	33
VC	ED	13	17	Manipulated	33
VC	ED	14	9	Control	19
VC	ED	14	9	Manipulated	19
VC	ED	14	11	Control	24
VC	ED	14	11	Manipulated	25
VC	ED	14	13	Control	42
VC	ED	14	13	Manipulated	33
VC	ED	14	15	Control	32
VC	ED	14	15	Manipulated	33
VC	ED	14	17	Control	31
VC	ED	14	17	Manipulated	30
VC	ED	15	9	Control	18
VC	ED	15	9	Manipulated	18
VC	ED	15	11	Control	24
VC	ED	15	11	Manipulated	25
VC	ED	15	13	Control	30
VC	ED	15	13	Manipulated	32
VC	ED	15	15	Control	31
VC	ED	15	15	Manipulated	31
VC	ED	15	17	Control	30
VC	ED	15	17	Manipulated	30
VF	BM	13	9	Control	16
VF	BM	13	9	Manipulated	15

Temperature data cont.

Species	Population	Plot	Time	Treatment	Leaf Temp.
VF	BM	13	13	Control	18
VF	BM	13	13	Manipulated	14
VF	BM	13	17	Control	49
VF	BM	13	17	Manipulated	35
VF	BM	2	9	Control	25
VF	BM	2	9	Manipulated	28
VF	BM	2	13	Control	19
VF	BM	2	13	Manipulated	19
VF	BM	2	17	Control	22
VF	BM	2	17	Manipulated	21
VF	BM	15	9	Control	31
VF	BM	15	9	Manipulated	27
VF	BM	15	13	Control	32
VF	BM	15	13	Manipulated	32
VF	BM	15	17	Control	19
VF	BM	15	17	Manipulated	19
VF	BM	4	9	Control	20
VF	BM	4	9	Manipulated	23
VF	BM	4	13	Control	34
VF	BM	4	13	Manipulated	35
VF	BM	4	17	Control	38
VF	BM	4	17	Manipulated	43
VF	BM	17	9	Control	30
VF	BM	17	9	Manipulated	26
VF	BM	17	13	Control	43
VF	BM	17	13	Manipulated	33
VF	BM	17	17	Control	33
VF	BM	17	17	Manipulated	30
VF	BM	6	9	Control	22
VF	BM	6	9	Manipulated	31
VF	BM	6	13	Control	31
VF	BM	6	13	Manipulated	51
VF	BM	6	17	Control	31
VF	BM	6	17	Manipulated	32
VF	BM	7	9	Control	32
VF	BM	7	9	Manipulated	39
VF	BM	7	13	Control	33
VF	BM	7	13	Manipulated	38
VF	BM	7	17	Control	28
VF	BM	7	17	Manipulated	25
VF	BM	8	9	Control	21

Temperature data cont.

Species	Population	Plot	Time	Treatment	Leaf Temp.
VF	BM	8	9	Manipulated	23
VF	BM	8	13	Control	47
VF	BM	8	13	Manipulated	50
VF	BM	8	17	Control	23
VF	BM	8	17	Manipulated	26
VF	BM	9	9	Control	27
VF	BM	9	9	Manipulated	31
VF	BM	9	13	Control	19
VF	BM	9	13	Manipulated	18
VF	BM	9	17	Control	16
VF	BM	9	17	Manipulated	17
VF	BM	10	9	Control	27
VF	BM	10	9	Manipulated	26
VF	BM	10	13	Control	28
VF	BM	10	13	Manipulated	23
VF	BM	10	17	Control	15
VF	BM	10	17	Manipulated	15
VF	BM	11	9	Control	19
VF	BM	11	9	Manipulated	18
VF	BM	11	13	Control	37
VF	BM	11	13	Manipulated	28
VF	BM	11	17	Control	16
VF	BM	11	17	Manipulated	15
VF	BM	12	9	Control	14
VF	BM	12	9	Manipulated	14
VF	BM	12	13	Control	7
VF	BM	12	13	Manipulated	8
VF	BM	12	17	Control	24
VF	BM	12	17	Manipulated	23
VF	BM	13	9	Control	15
VF	BM	13	9	Manipulated	15
VF	BM	13	13	Control	10
VF	BM	13	13	Manipulated	8
VF	BM	13	17	Control	46
VF	BM	13	17	Manipulated	27
VF	BM	14	9	Control	20
VF	BM	14	9	Manipulated	19
VF	BM	14	13	Control	43
VF	BM	14	13	Manipulated	33
VF	BM	14	17	Control	35
VF	BM	14	17	Manipulated	30

Temperature data cont.

Species	Population	Plot	Time	Treatment	Leaf Temp.
VF	BM	15	9	Control	25
VF	BM	15	9	Manipulated	21
VF	BM	15	13	Control	53
VF	BM	15	13	Manipulated	49
VF	BM	15	17	Control	36
VF	BM	15	17	Manipulated	47
VF	NB	13	9	Control	12
VF	NB	13	9	Manipulated	12
VF	NB	13	13	Control	38
VF	NB	13	13	Manipulated	34
VF	NB	13	17	Control	41
VF	NB	13	17	Manipulated	36
VF	NB	2	9	Control	14
VF	NB	2	9	Manipulated	15
VF	NB	2	13	Control	34
VF	NB	2	13	Manipulated	27
VF	NB	2	17	Control	46
VF	NB	2	17	Manipulated	35
VF	NB	15	9	Control	13
VF	NB	15	9	Manipulated	14
VF	NB	15	13	Control	28
VF	NB	15	13	Manipulated	30
VF	NB	15	17	Control	36
VF	NB	15	17	Manipulated	36
VF	NB	4	9	Control	15
VF	NB	4	9	Manipulated	15
VF	NB	4	13	Control	18
VF	NB	4	13	Manipulated	17
VF	NB	4	17	Control	47
VF	NB	4	17	Manipulated	34
VF	NB	17	9	Control	14
VF	NB	17	9	Manipulated	16
VF	NB	17	13	Control	27
VF	NB	17	13	Manipulated	26
VF	NB	17	17	Control	33
VF	NB	17	17	Manipulated	42
VF	NB	6	9	Control	15
VF	NB	6	9	Manipulated	15
VF	NB	6	13	Control	19
VF	NB	6	13	Manipulated	21
VF	NB	6	17	Control	47

Temperature data cont.

Species	Population	Plot	Time	Treatment	Leaf Temp.
VF	NB	6	17	Manipulated	50
VF	NB	7	9	Control	14
VF	NB	7	9	Manipulated	15
VF	NB	7	13	Control	43
VF	NB	7	13	Manipulated	34
VF	NB	7	17	Control	32
VF	NB	7	17	Manipulated	23
VF	NB	8	9	Control	15
VF	NB	8	9	Manipulated	15
VF	NB	8	13	Control	22
VF	NB	8	13	Manipulated	20
VF	NB	8	17	Control	33
VF	NB	8	17	Manipulated	37
VF	NB	9	9	Control	12
VF	NB	9	9	Manipulated	14
VF	NB	9	13	Control	39
VF	NB	9	13	Manipulated	23
VF	NB	9	17	Control	46
VF	NB	9	17	Manipulated	35
VF	NB	10	9	Control	15
VF	NB	10	9	Manipulated	17
VF	NB	10	13	Control	63
VF	NB	10	13	Manipulated	46
VF	NB	10	17	Control	42
VF	NB	10	17	Manipulated	33
VF	NB	11	9	Control	11
VF	NB	11	9	Manipulated	12
VF	NB	11	13	Control	39
VF	NB	11	13	Manipulated	31
VF	NB	11	17	Control	43
VF	NB	11	17	Manipulated	35
VF	NB	12	9	Control	14
VF	NB	12	9	Manipulated	14
VF	NB	12	13	Control	21
VF	NB	12	13	Manipulated	36
VF	NB	12	17	Control	37
VF	NB	12	17	Manipulated	40
VF	NB	13	9	Control	11
VF	NB	13	9	Manipulated	13
VF	NB	13	13	Control	28
VF	NB	13	13	Manipulated	26

VF	NB	13	17	Control	32
VF	NB	13	17	Manipulated	27
VF	NB	14	9	Control	14
VF	NB	14	9	Manipulated	15
VF	NB	14	13	Control	32
VF	NB	14	13	Manipulated	33
VF	NB	14	17	Control	23
VF	NB	14	17	Manipulated	23
VF	NB	15	9	Control	14
VF	NB	15	9	Manipulated	15
VF	NB	15	13	Control	29
VF	NB	15	13	Manipulated	32
VF	NB	15	17	Control	25
VF	NB	15	17	Manipulated	27

5. Morphological variables measured on three species

Leaf morphology variables for all three species. Measurements at each degree on radiometer in mm.

Species	Population	Individual	0°	30°	60°	90°	120°	150°	180°	Apex (degrees)	Base (degrees)
V. ocellata	MP	1	31	23	22	21	18	9	4	134	26
V. ocellata	MP	2	19	15	14	14	10	5	1	131	29
V. ocellata	MP	3	29	28	26	25	23	16	4	112	28
V. ocellata	MP	4	21	16	15	15	14	12	3	132	20
V. ocellata	MP	5	25	26	29	30	29	22	5	112	18
V. ocellata	MP	6	29	25	24	24	22	20	4	122	-11
V. ocellata	MP	7	25	21	20	20	19	15	5	127	19
V. ocellata	MP	8	25	21	21	20	17	14	4	123	22
V. ocellata	MP	9	12	10	10	10	9	1	1	125	32
V. ocellata	MP	10	27	24	24	23	22	3	2	121	21
V. ocellata	MP	11	46	47	40	40	34	5	5	115	32
V. ocellata	MP	12	22	17	16	17	16	13	3	134	6
V. ocellata	MP	13	24	19	18	18	16	11	3	130	30
V. ocellata	MP	14	18	18	18	18	14	1	1	111	36
V. ocellata	MP	15	26	24	22	21	17	4	5	123	47
V. ocellata	MC	1	31	19	17	17	16	13	2	149	15
V. ocellata	MC	2	28	18	14	14	13	2	2	150	27
V. ocellata	MC	3	23	15	14	15	14	3	2	146	44
V. ocellata	MC	4	22	13	12	13	11	2	1	158	38
V. ocellata	MC	5	30	20	16	15	14	1	1	156	34
V. ocellata	MC	6	32	19	18	19	19	9	3	155	29
V. ocellata	MC	7	23	17	15	15	13	1	1	139	35
V. ocellata	MC	8	29	17	16	16	15	4	4	155	39
V. ocellata	MC	9	42	25	23	22	22	4	5	152	49
V. ocellata	MC	10	37	21	19	14	14	3	3	156	38
V. ocellata	MC	11	20	13	12	20	19	4	4	149	35
V. ocellata	MC	12	22	14	13	11	9	1	1	148	39
V. ocellata	MC	13	30	18	17	18	15	3	2	150	36
V. ocellata	MC	14	24	15	15	16	15	2	2	151	46
V. ocellata	MC	15	24	14	13	14	13	3	3	148	48
V. ocellata	CC	1	23	16	14	13	11	8	0	146	17
V. ocellata	CC	2	26	17	15	15	15	3	3	148	33
V. ocellata	CC	3	12	9	7	8	6	0	0	140	51
V. ocellata	CC	4	23	15	15	15	15	9	3	142	22
V. ocellata	CC	5	24	15	14	15	14	4	4	149	47
V. ocellata	CC	6	27	15	12	12	11	1	1	160	31
V. ocellata	CC	7	30	20	19	19	18	3	3	145	34
V. ocellata	CC	8	33	20	15	14	14	11	2	157	25

Leaf morphology data cont.

Species	Population	Individual	0°	30°	60°	90°	120°	150°	180°	Apex (degrees)	Base (degrees)
V. ocellata	CC	9	24	16	15	15	14	10	3	146	24
V. ocellata	CC	10	20	13	11	11	9	1	1	146	37
V. ocellata	CC	11	30	17	15	15	14	4	4	152	39
V. ocellata	CC	12	23	13	12	11	10	1	1	154	55
V. ocellata	CC	13	21	16	15	14	11	1	1	133	48
V. ocellata	CC	14	24	14	12	12	14	10	2	152	20
V. ocellata	CC	15	22	16	13	12	11	7	1	143	33
V. cuneata	HM	1	12	10	12	12	8	8	10	113	129
V. cuneata	HM	2	15	14	15	15	13	11	20	110	130
V. cuneata	HM	3	10	9	9	9	6	6	9	122	126
V. cuneata	HM	4	12	10	11	12	10	10	14	115	134
V. cuneata	HM	5	10	10	10	10	9	9	15	110	131
V. cuneata	HM	6	12	11	12	13	19	10	12	116	125
V. cuneata	HM	7	8	8	8	9	8	7	10	106	120
V. cuneata	HM	8	12	10	9	9	6	5	9	1265	127
V. cuneata	HM	9	9	8	10	10	10	10	12	119	129
V. cuneata	HM	10	9	7	7	7	9	8	11	124	131
V. cuneata	HM	11	9	7	9	9	9	8	11	115	129
V. cuneata	HM	12	9	7	7	6	5	4	6	131	124
V. cuneata	HM	13	18	14	14	15	10	9	12	127	129
V. cuneata	HM	14	12	10	10	11	11	10	16	123	127
V. cuneata	HM	15	10	8	8	8	8	7	11	129	134
V. cuneata	SP	1	13	9	9	10	9	8	12	139	131
V. cuneata	SP	2	10	8	8	9	7	8	11	130	141
V. cuneata	SP	3	11	7	7	7	6	6	10	161	137
V. cuneata	SP	4	9	5	5	5	5	5	8	156	136
V. cuneata	SP	5	18	12	11	11	10	10	20	142	158
V. cuneata	SP	6	6	5	6	7	6	5	9	123	133
V. cuneata	SP	7	9	6	6	6	6	6	9	132	150
V. cuneata	SP	8	16	7	9	9	8	8	11	165	133
V. cuneata	SP	9	13	8	8	9	8	8	11	150	133
V. cuneata	SP	10	16	14	13	14	10	10	12	127	128
V. cuneata	SP	11	15	11	10	110	9	8	12	141	138
V. cuneata	SP	12	11	9	9	9	9	8	12	131	142
V. cuneata	SP	13	13	8	7	7	6	5	6	156	129
V. cuneata	SP	14	15	10	9	9	8	6	11	140	138
V. cuneata	SP	15	20	14	14	14	12	11	16	143	134
V. cuneata	ED	1	20	16	16	16	11	9	12	131	117
V. cuneata	ED	2	9	10	10	10	8	6	9	78	131

Leaf morphology data cont.

Species	Population	Individual	0°	30°	60°	90°	120°	150°	180°	Apex (degrees)	Base (degrees)
V. cuneata	ED	3	14	11	11	12	10	8	10	123	117
V. cuneata	ED	4	12	10	10	10	10	9	11	126	130
V. cuneata	ED	5	11	10	10	10	8	6	7	125	119
V. cuneata	ED	6	7	7	7	6	5	5		118	114
V. cuneata	ED	7	11	8	8	8	8	7	10	143	126
V. cuneata	ED	8	12	10	10	9	9	6	9	128	134
V. cuneata	ED	9	14	11	11	11	10	8	10	138	127
V. cuneata	ED	10	14	10	9	9	7	6	7	139	110
V. cuneata	ED	11	15	11	11	10	9	6	8	138	117
V. cuneata	ED	12	15	13	12	11	8	6	8	128	117
V. cuneata	ED	13	10	10	10	11	10	9	11	116	120
V. cuneata	ED	14	13	14	14	15	14	15	24	104	152
V. cuneata	ED	15	15	13	13	14	12	9	11	129	117
V. flettii	MA	1	11	12	13	14	13	2	2	104	41
V. flettii	MA	2	15	14	14	14	13	10	1	113	9
V. flettii	MA	3	12	13	15	16	15	10	1	93	20
V. flettii	MA	4	8	9	10	10	9	6	1	97	28
V. flettii	MA	5	13	14	14	15	10	1	1	101	50
V. flettii	MA	6	12	14	15	15	14	11	1	101	5
V. flettii	MA	7	9	10	11	12	11	10	2	93	8
V. flettii	MA	8	10	10	11	12	12	9	1	95	11
V. flettii	MA	9	13	15	16	16	14	1	1	84	20
V. flettii	MA	10	14	15	17	19	18	1	1	96	23
V. flettii	MA	11	14	13	14	13	10	1	1	111	21
V. flettii	MA	12	15	14	14	15	12	5	1	122	42
V. flettii	MA	13	11	11	12	14	14	13	3	109	16
V. flettii	MA	14	14	14	14	14	14	11	2	105	7
V. flettii	MA	15	12	12	13	13	12	10	3	103	7
V. flettii	BM	1	8	8	9	10	7	1	1	94	45
V. flettii	BM	2	13	13	15	15	12	2	2	112	18
V. flettii	BM	3	10	10	10	9	5	1	1	95	55
V. flettii	BM	4	17	16	17	16	16	10	1	105	24
V. flettii	BM	5	15	14	14	14	14	9	1	106	23
V. flettii	BM	6	11	13	14	14	12	4	4	88	45
V. flettii	BM	7	10	9	10	10	9	1	1	117	38
V. flettii	BM	8	17	16	20	21	20	6	6	106	56
V. flettii	BM	9	10	10	12	12	10	1	1	105	35
V. flettii	BM	10	14	14	14	16	14	1	1	103	30
V. flettii	BM	11	15	15	17	18	15	4	3	105	46
V. flettii	BM	12	12	12	13	13	12	1	1	106	42

Leaf morphology data cont.

Species	Population	Individual	0°	30°	60°	90°	120°	150°	180°	Apex (degrees)	Base (degrees)
V. flettii	BM	13	13	14	15	15	12	2	2	91	50
V. flettii	BM	14	16	15	16	17	17	14	3	113	27
V. flettii	BM	15	15	14	15	14	11	2	1	106	44
V. flettii	NB	1	16	16	18	17	16	12	1	106	27
V. flettii	NB	2	15	15	16	15	11	1	1	100	53
V. flettii	NB	3	14	15	15	16	15	1	1	102	33
V. flettii	NB	4	14	13	14	15	10	1	1	120	46
V. flettii	NB	5	12	14	15	15	15	12	1	98	14
V. flettii	NB	6	12	12	13	13	12	8	1	104	19
V. flettii	NB	7	10	11	12	13	12	8	1	97	34
V. flettii	NB	8	17	18	19	20	17	11	1	98	26
V. flettii	NB	9	12	12	13	12	11	8	1	116	13
V. flettii	NB	10	10	10	10	11	11	9	2	106	21
V. flettii	NB	11	12	12	13	13	12	11	2	108	10
V. flettii	NB	12	11	15	17	17	8	3	3	80	45
V. flettii	NB	13	14	13	14	14	10	4	2	113	53
V. flettii	NB	14	13	13	15	16	11	4	4	106	59
V. flettii	NB	15	16	17	17	17	15	3	3	94	35