Differentiation of Montane and Alpine Ecotypes of *Viola adunca* in Southwestern Colorado Using Microsatellite DNA Analysis

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Introduction

Speciation is generally viewed as a process resulting in the genetic divergence of lineages into discrete forms over time. Various factors both intrinsic to the organism or present in the environment can lead to this divergence. The most critical initial step in speciation is the formation of reproductive barriers. Once established these barriers restrict gene flow and selection or drift can occur independently in the segregate groups leading to greater levels of divergence.

Ecotypes are discrete genetic forms of species which correlate to specific environmental variables. In an evolutionary context these ecotypes can represent an early stage in the process of speciation as populations differentiate along environmental gradients (Abbott and Comes, 2007). In the Southern Rockies the common blue violet *Viola adunca* is viewed as consisting of two discrete ecotypes, a montane type inhabiting woodlands and a dwarf alpine ecotype (Figures 1 & 2). The alpine type has been variously viewed as conspecific with *V. adunca*, as a variety (*V. adunca* var. *bellidifolia*) or as a distinct species (*V. bellidifolia* or *V. labradorica* (misapplication)). This varied application of taxon status is a result of little understanding of the evolutionary history of these divergent forms.

To understand the level of genetic divergence between the two ecotypes, multiple populations were sampled representing both forms and the populations genotyped. Genotyping utilized multiple microsatellite loci. Microsatellites are repetitive DNA regions randomly dispersed throughout the genome which are particularly susceptible to copy errors as a result of chromosomal crossing-over and DNA replication slippage (Jarne and Lagoda, 1996). Genetic divergence and relatedness among the populations was then evaluated by multiple means to infer the level of segregation of the two forms.

The results could then be interpreted according to two alternative hypotheses: 1) if the two forms represented contemporary diverged ecotypes of the same taxon than genetic similarity would parallel geographic distance among populations, i.e. geographically close populations would show a greater genetic similarity, and 2) if the two forms represented distinct lineages, with a longer period of time since divergence than morphologically similar forms would show greater genetic similarity regardless of geographic proximity.

Experimental Design and Methods

Field Collection:
Eight populations representing both ecotypes were sampled from the San Juan Mountains in the summer of 2011 (Figure 1). 10 individuals were randomly sampled per population and tissue stored at -80C until DNA extraction could be performed.

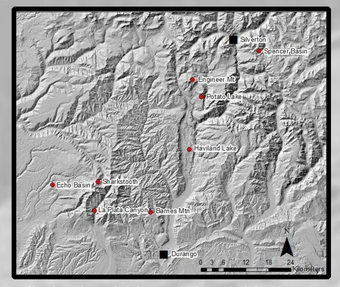

Figure 1: Sampling locations of eight populations of *Viola adunca* ecotypes in the San Juan Mountains.

Microsatellite Amplification:
Total genomic DNA was extracted from leaf tissue using the DNeasy plant mini kit (Qiagen). Following extraction, DNA extracts were stored at -20°C. PCR amplification of microsatellite fragments was performed using six fluorescent primer pairs (loci) originally designed for *Viola pubescens* (Culley, 2005). These six loci were amplified simultaneously in multiplexed reactions using the QIAGEN Multiplex Kit (QIAGEN) in 5 µl reactions. PCR products were analyzed for fragment size at the Proteomics and Metabolomics Facility at Colorado State University. Final analysis was performed using PeakScanner ver. 1.0 (Applied Biosystems).

Genetic Analysis:
Genetic differentiation among populations was calculated using θ (Weir and Cockerham, 1984), an analog of F_{st}, which takes into account small and/or unequal population sizes and was calculated in TFPGA ver. 1.3 (Tools for Population Genetic Analysis) (Miller, 1997). Confidence intervals for θ used to determine significance from zero were generated by bootstrapping across loci using 1000 replicates. To visualize genetic relationships among populations a UPGMA (Unweighted Pair Group Method with Arithmetic Mean) phenogram of populations was constructed using pair-wise calculations of θ generated in TFPGA. Further genetic structure and analysis of gene sharing was modeled using the program STRUCTURE ver. 2.3.3 (Pritchard et al., 2000; Falush et al. 2003), which implements a Bayesian clustering method using multi-locus genotype data to assign populations to clusters or gene pools (K).

Figure 1: Alpine ecotype (*Viola bellidifolia*). Sharkstooth Pass (3,600 meters), July 2011.

Figure 2: Montane ecotype (*Viola adunca*). Barnes Mountain (2900 meters), July 2011.

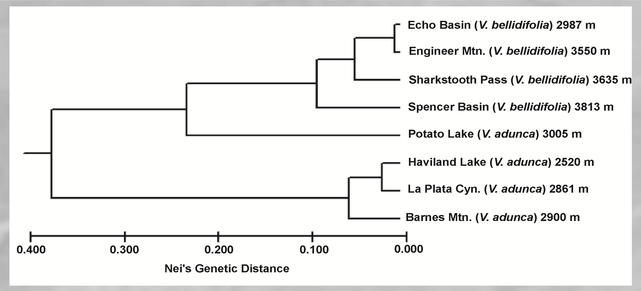

Figure 4: UPGMA phenogram illustrating the lineage relationships between the eight different populations of *V. adunca* and *V. bellidifolia*.

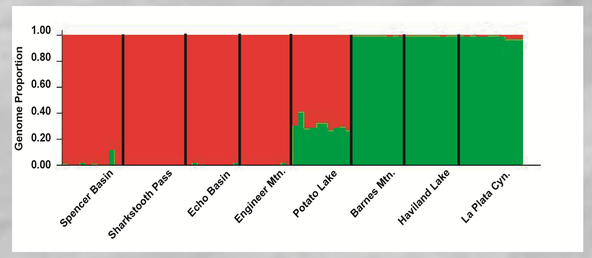

Figure 5: Results of STRUCTURE analysis indicating two distinct gene pools representing distinct ecotypes. Admixture in the form of gene sharing is portrayed in the Potato Lake population.

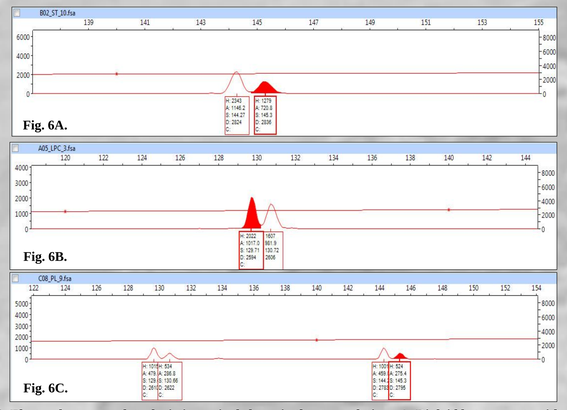

Figure 6: Electrophenogram data depicting a single locus in three populations. A. Diploid heterozygote with alleles at 144 -145 b.p. from a Sharkstooth Pass individual. B. Diploid heterozygote alleles at 129-130 b.p. from a LaPlata Canyon individual. C. Hypothesized tetraploid heterozygote in an individual from Potato Lake depicting constituent alleles of alpine and montane populations.

Results

Microsatellite Amplification:
Of the six tested microsatellite loci from *Viola pubescens*, four were successful in PCR amplification**.** Electrophenograms showed distinct differences in microsatellite allele expression across both ecotypes.

Population Relatedness and Differentiation:
Total genetic differentiation among all populations gave a value of θ = 0.74 (s.d. = 0.053) inferring a highly significant level of differentiation between the two ecotypes. The UPGMA phenogram (Figure 4) showed that populations within specific ecotypes showed a closer relationship than with other more geographically abutting populations. The exception to this was the lower montane ecotype population at Potato Lake which clustered with the alpine form. The STRUCTURE analysis (Figure 5) indicated the presence of two distinct gene pools, each corresponding to separate ecotypes which exhibited little admixture. Evidence of gene sharing however was evident in the Potato Lake population suggesting that hybridization was possible between forms in this area.

The occurrence of gene sharing in the Potato Lake population was further investigated by direct examination of the microsatellite fragment patterns. Multiple loci in the Potato Lake population exhibited the expression of four independent alleles indicating that these individuals were tetraploid rather than the expected diploid (Figure 6).

Discussion and Conclusion

These results suggest that very little gene flow is occurring between the two ecotypes of *V. adunca* in the San Juan Mountains. Violets present at the highest elevations displayed very similar allele frequencies from population to population. This same result was clear among the populations of *V. adunca* growing at lower elevations. Genetic differentiation (θ = 0.74) indicated very high levels of isolation and points to two segregated groups that are in general not linked by gene flow. This suggests that geographical isolation of habitat has greatly affected the interaction of the two ecotypes to a point of significant differentiation. This differentiation is likely sufficient to warrant taxonomic recognition for both ecotypes.

The occurrence however of gene sharing via likely hybridization at Potato Lake indicated that complete reproductive barriers are likely not present between the two forms. These hypothesized hybrids appear to be tetraploid while all other populations appear to be diploid. Hybridization then is likely resulting in the production of allopolyploids which express both constituent genomes and are fertile (Figure 6). The Potato Lake area while being fully forested is in close proximity to the tree line and the surrounding area rapidly climbs to elevations conducive to the habitat of the alpine form. Thus, close geographic proximity of the two variants of *V. adunca* allows for gene flow to occur.

Future studies should involve further investigation into the hypothesized allopolypolidy and further morphological investigation to establish phenotypic differences associated with differences in chromosome number.

References

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Background: View East of Sharkstooth Pass, LaPlata Mountains