Valerie D. Hipkins[1], USDA Forest Service, Pacific Southwest Research Station, National Forest Genetic Electrophoresis Laboratory, 2480 Carson Road, Placerville, California 95667

Barbara L. Wilson, Institute for Applied Ecology, 227 SW 6th Street, Corvallis, Oregon 97333

Richy J. Harrod, USDA Forest Service, Okanogan and Wenatchee National Forests, 215 Melody Lane, Wenatchee, Washington 98801

and

Carol Aubry, USDA Forest Service, Olympic National Forest, 1835 Black Lake Blvd. SW, Olympia, Washington 98512

Isozyme Variation in Showy Stickseed, a Washington Endemic Plant, and Relatives

Abstract

Hackelia venusta, a rare plant endemic to Chelan County, Washington, consists of two entities that differ most notably by flower color and geographic location. We evaluated the hypothesis that these entities are separate species using a phenotypic analysis of isozyme variation to compare both forms of *H. venusta* with sympatric and allopatric populations of the closely related *H. diffusa* var. *arida,* and with the allopatric taxa *H. diffusa* var. *cottonii* and *H. diffusa* var. *diffusa*. Enzyme band phenotypes revealed that all populations were variable, with 60% to 80% polymorphic enzyme systems. Most variation was within population, with only 32% among population variation detected. The level of variation was moderate to high in *H. venusta* populations compared to other *Hackelia* populations. Enzyme band pattern data support the separation of *H. venusta* from *H. diffusa*. However, data do not provide evidence for the taxonomic separation of the two color forms of *H. venusta* at the species level. Regardless of the taxonomic recognition of *H. venusta* color forms, they should be conserved as rare extremes of *Hackelia* morphology and habitat preference.

Introduction

Hackelia venusta (showy stickseed), of the family Boraginaceae, is a 2–4 dm, insect-pollinated, perennial plant endemic to steep rocky slopes covered with granite scree in Chelan County, Washington. The species was first described from a population in Tumwater Canyon at 488 m elevation (Piper 1924). The Tumwater Canyon population has white flowers with corolla limbs averaging 7.4 mm long, and is the only extant *H. venusta* population that has white flowers. Three additional Chelan County populations of 2–3 dm plants with smaller blue flowers (corolla limbs averaging 4.2 mm long) have been included in the *H. venusta* species concept (Carr 1974, Gentry and Carr 1976). These blue-flowered populations are located 16 km south southwest of the white-flowered population at ca. 2000 m elevation. The morphological and habitat differences between white-flowered and blue-flowered *H. venusta* support the hypothesis that the white- and blue-flowered populations represent two taxa (Gamon 1988). The taxonomic controversy is of particular current interest because *H. venusta* is considered endangered in Washington (Gamon 1988, Washington Natural Heritage Program 1991) and has been proposed for endangered species status (U.S. Fish and Wildlife Service 2000). The blue-flowered taxon is being described in another paper, but has no published name at this time. Therefore, in this paper we consistently refer to these plants as white- and blue-flowered forms of *H. venusta*.

The taxonomic status of *H. venusta* is complicated by morphological variation in the widespread taxon *H. diffusa* var. *arida* (sagebrush diffuse stickseed). *Hackelia diffusa* var. *arida* is a 4–6 dm species, and usually has small, white flowers that may be lightly washed with blue. However, three *H. diffusa* var. *arida* populations growing 5–40 km from the white-flowered *H. venusta* population are similar to white-flowered *H. venusta* in flower and fruit characteristics. Carr (1974) and Gentry and Carr (1976) suggested that the observed morphological similarities resulted from introgression from white-flowered *H. venusta* into nearby *H. diffusa* var. *arida*. *Hackelia diffusa* var. *arida* also intergrades with cream-colored *H.*

[1]Author to whom correspondence should be addressed.
E-mail: vhipkins@fs.fed.us

diffusa var. *cottonii* (Columbia stickseed) in the Yakima area of Washington. In turn, *H. diffusa* var. *cottonii* intergrades with blue-flowered *H. diffusa* var. *diffusa* (western diffuse stickseed) in the Columbia Gorge (Gentry and Carr 1976). A recent analysis of *Hackelia* morphology supports recognizing *H. diffusa* var. *arida,* and the white- and blue-flowered components of *H. venusta* as separate but closely related taxa (Harrod et al. 1999).

Starch-gel electrophoresis was used to determine whether genetic data support recognition of white- and blue-flowered color forms of *H. venusta* as distinct species. Enzyme electrophoresis has been successfully employed in systematic studies of plants, largely because of its ability to quantify genetic similarities (Crawford 1989, Crawford 1990, Briggs and Walters 1997). To establish standards for evaluating taxonomic significance of electrophoretic variation in *H. venusta,* we sampled seven populations of *H. diffusa* var. *arida*, and one population each of *H. diffusa* var. *cottonii* and *H. diffusa* var. *diffusa.* We scored enzyme variation in terms of phenotypic band similarity, and used these data to assess the magnitude of genetic variation within populations and the degree of similarity among populations. Variation and similarity within *H. diffusa* served as a template for judging *H. venusta* taxonomic rank. Under a phenetic species concept, species are distinct and distinguishable based on similarity (Winston 1999). Using a biological species concept, a species is defined as a reproductively isolated system of breeding populations (Winston 1999). Therefore, besides being characterized by lower genetic similarities (as in the phenetic species concept), congeneric species usually have fixed or consistent differences in isozyme alleles at some loci (biological species concept) (Avise 1994). If the *H. venusta* color forms are simply divergent populations of *H. diffusa* var. *arida,* we expect one or both forms will be more similar to the *H. diffusa* var. *arida* populations included in the study (all collected within 80 km of the *H. venusta* populations) than they are to *H. diffusa* var. *cottonii* and *H. diffusa* var. *diffusa.*

Incidental to the taxonomic goal of this study, we addressed the concern that white-flowered *H. venusta* may have extremely limited variation compared to the blue-flowered form, as well as to *H. diffusa* populations. Genetic analysis of isozyme variation reveal that endemic plants often have limited variation (Hamrick and Godt 1990). However, measures of isozyme variation in populations of rare plants have been shown to be correlated with those measures in populations of their widespread congeneric relatives, and sometimes the endemic plants are more variable (Gitzendanner and Soltis 2000).

Methods

Populations sampled included the one extant population of white-flowered *H. venusta,* one of the two extant populations of blue-flowered *H. venusta,* the same seven populations of *H. diffusa* var. *arida* examined in a morphological study (Harrod et al. 1999), and one population each of *H. diffusa* var. *cottonii* and *H. diffusa* var. *diffusa* (Table 1). The *H. diffusa* var. *cottonii* and *H. diffusa* var. *diffusa* populations were collected 190 and 320 km from

TABLE 1. *Hackelia* populations sampled in this study. *Hackelia venusta* (blue-flower) samples were micropropagated plantlets, and all other samples consisted of wild plant collections.

Taxon	Acronym	Location	# individuals sampled
H. venusta (white-flower)	WFV	Tumwater Canyon, 9.6 km west of Leavenworth, WA	22
H. venusta (blue-flower)	BFV	Crystal Lake, 19.0 km southwest of Leavenworth, WA	12
H. diffusa var. *arida*	BM	Burch Mountain, 4.8 km northwest of Wenatchee, WA	25
H. diffusa var. *arida*	DC	Douglas Creek, 26.0 km north of Quincy, WA	25
H. diffusa var. *arida*	DE	Derby Canyon, 11.3 km southeast of Leavenworth, WA	25
H. diffusa var. *arida*	MC	Moses Coulee, 24.0 km north of Quincy, WA	25
H. diffusa var. *arida*	PE	Ponderosa Estates, 17.7 km north of Leavenworth, WA	25
H. diffusa var. *arida*	SC	Swakane Canyon, 19.3 km northeast of Wenatchee, WA	25
H. diffusa var. *arida*	TW	Tumwater Canyon, 1.6 km west of Leavenworth, WA	25
H. diffusa var. *cottonii*	COT	Rattlesnake Hills, near Moxee, WA	29
H. diffusa var. *diffusa*	DIF	Oneota Gorge near Multnomah Falls, OR	30

the white-flowered *H. venusta* population. Due to the rarity of blue-flowered *H. venusta*, small samples of plant tissue were taken from the living plants and micropropagated (Edson et al. 1996). Only 12 plants were sampled, due to site inaccessibility and a desire to minimize negative impacts to the small populations. For the other populations, three to five leaves per plant were collected in the field and transported to the laboratory on ice. Voucher specimens of the *H. venusta* populations were deposited at the University of Washington herbarium. Vouchers for the *H. diffusa* populations were deposited at the herbarium of the Leavenworth Ranger District of the Okanogan and Wenatchee National Forests.

Sample Preparation

Samples were prepared using NFGEL standard operating procedures (USDA Forest Service 1995). For each individual, 10 mm^2 of leaf tissue was ground in liquid nitrogen and proteins were extracted in a Tris buffer pH 7.5 (Gottlieb 1981). The resulting slurry was transferred to microtiter plate wells and stored at -70°C. For electrophoresis, the slurry was thawed and absorbed onto 3 mm wide wicks prepared from Whatman 3MM chromatography paper.

Electrophoresis

Methods of electrophoresis follow the general methodology of Conkle et al. (1982) except that most enzyme stains are somewhat modified as outlined in USDA Forest Service (1995). A lithium borate electrode buffer (pH 8.3) was used with a Tris citrate gel buffer, pH 8.3 (Conkle et al. 1982), to resolve malic enzyme (ME), phosphoglucomutase (PGM), and phosphoglucose isomerase (PGI). A sodium borate electrode buffer (pH 8.0) was used with a Tris citrate gel buffer, pH 8.8 (Conkle et al. 1982), to resolve glycerate-2-dehydrogenase (GLYDH), glutamate-oxaloacetate transaminase (GOT), glucose-6-phosphate dehydrogenase (G6PDH), triosephosphate isomerase (TPI), and uridine diphosphoglucose pyrophosphorylase (UGPP). A morpholine citrate electrode and gel buffer, pH 8.0, was used to resolve isocitrate dehydrogenase (IDH), malate dehydrogenase (MDH), phosphogluconate dehydrogenase (6PGD), and shikimic acid dehydrogenase (SKD) (USDA Forest Service 1995). Two zones, designated F (faster) and S (slower), were resolved for each of the enzyme stains GOT, PGI, and UGPP, for a total of 15 enzyme systems. All enzymes were resolved on 11% starch gels. Enzyme stain recipes follow USDA Forest Service (1995) except that GOT was stained using the recipe from Wendel and Weeden (1989). Two people independently scored each gel. When they disagreed, a third person resolved the conflict. For quality control, 10% of the individuals were run and scored twice.

Data Analysis

We performed a phenotypic analysis of isozyme variation. We use the term enzyme system here as a unit in a phenotypic analysis of isozymes. The enzyme system is a region of activity on an isozyme (starch) gel, which in a diploid plant would correspond to a single locus. In polyploids it corresponds to a set of loci that are homologous or homeologous (depending on the method of inheritance). All enzyme systems were scored for band patterns and band presence/absence. A band pattern consists of one to several bands. For a given enzyme system, all bands observed at a specific migration distance are treated as identical, even if they occur in different patterns. These data are suited for a phenotypic instead of genotypic analysis. Scoring starch gel electrophoresis data as enzyme band-pattern phenotypes has been successfully employed to describe how variation is partitioned within and among populations (Kahler et al. 1980, Poverene and Curvetto 1989, Vickery 1990, Chung et al. 1991, Dolan 1994, Rogers 1994, Samman et al. 2000, Wilson et al. 2000).

Phenotypic diversity measures were calculated from both band presence/absence and multi-banded patterns. For presence/absence data, phenotypic diversity was measured by a polymorphic index (PI), based on the frequency of occurrence of each band among individuals in a population (Chung et al. 1991). For multi-band patterns, phenotypic diversity measures include: (1) the number of band patterns found in each sampled population, (2) percent of enzyme systems that yield more than one band pattern, (3) the average number of band patterns per enzyme system in each population, and (4) Shannon-Weaver Diversity Index values (Shannon and Weaver 1949). The frequency of each enzyme band pattern was used to calculate the Shannon-Weaver Diversity Index. Larger Shannon-Weaver indices indicate more diverse populations. The distribution of the total variation within

and among populations was determined by partitioning the total Shannon-Weaver Diversity Index (Chung et al. 1991). The phenotypic relationships among populations were determined by calculating Hedrick's identities (Hedrick 1971) for multi-band pattern data, and cluster (UPGMA) analysis (Chung et al. 1991). Band pattern frequencies and the Shannon-Weaver Diversity Index were calculated using Popgene (Yeh et al. 1997), using the haploid codominant marker settings.

Results

The *Hackelia* populations sampled were all variable, with 60-80% of the enzyme systems polymorphic, 38 – 50 bands present, and 2.00 – 3.40 multibanded patterns per enzyme system (Table 2). At the population level, isozyme variation in white-flowered *H. venusta* was moderate to high compared to other *Hackelia* populations. The blue-flowered *H. venusta* population was moderately variable. At the species level, *H. diffusa* var. *arida* was more variable than *H. venusta* at three diversity indicators (# bands, # patterns per enzyme system, and Shannon-Weaver diversity index). The isolated populations of *H. diffusa* var. *cottonii* and *H. diffusa* var. *diffusa* had the lowest levels of enzyme variability at all diversity measures. Over the entire study, most variation was within populations, with only 32% of the variation among populations. Of the total variation measured within each species, 20.3% of the variation was among populations in *H. diffusa* var. *arida*, and 14.2% was shared between the blue- and white-flowered populations of *H. venusta* (Table 2).

Most band patterns were shared among populations, but some were restricted to single taxa. Of the 100 enzyme patterns observed, 86 occurred in *H. diffusa* var. *arida,* and 78 (91%) of the patterns that occurred in *H. diffusa* var. *arida* also occurred in at least one other taxon. The remaining 14 of the 100 total band patterns occurred only in taxa other than *H. diffusa* var. *arida*. However, the individual bands making up eight of these patterns did occur in *H. diffusa* var. *arida.* The other six of the 14 patterns included a total of six bands not observed in *H. diffusa* var. *arida.* One of these six unusual patterns, occurring only in white-flowered *H. venusta,* lacked a TPI-F band observed in all other samples.

TABLE 2. Variation in *Hackelia* populations, revealed by starch-gel electrophoresis. N = mean sample size/enzyme system. %P = percent polymorphic enzyme systems. P.I. = polymorphic index. S.W. = Shannon-Weaver diversity index.

Taxon: population	N	# bands	# unique bands	% P	# patterns/enzyme system (s.e.)	P.I.	S.W.	% S.W. among populations
H. venusta	30.9	54	4	86.7	3.80 (0.51)	0.380	0.747	14.2
white	19.1	50	4	80.0	3.40 (0.58)	0.364	0.697	
blue	11.7	47	0	66.7	2.33 (0.41)	0.337	0.577	
mean	15.4	48.5	2	73.4	2.87	0.351	0.637	
H. diffusa var. *arida*	134.5	65	12	80.0	5.73 (0.38)	0.377	0.843	20.3
BM	22.9	44	1	73.3	3.07 (0.41)	0.268	0.626	
DC	21.6	44	0	73.3	3.13 (0.47)	0.308	0.652	
DE	16.3	48	3	66.7	3.00 (0.60)	0.307	0.585	
MC	22.4	43	0	60.0	2.87 (0.48)	0.231	0.537	
PE	15.1	50	0	80.0	3.13 (0.55)	0.371	0.752	
SC	19.3	46	0	80.0	3.20 (0.48)	0.349	0.752	
TW	18.0	38	0	60.0	2.64 (0.54)	0.227	0.487	
mean	19.4	44.7	0.6	70.5	3.06	0.294	0.627	
H. diffusa var. *cottonii*	21.4	39	1	60.0	2.43 (0.38)	0.237	0.460	–
H. diffusa var. *diffusa*	19.9	38	0	66.7	2.00 (0.24)	0.217	0.410	–
Total	205.3	73	–	93.3	6.67 (0.38)	0.414	0.926	31.8

We observed 73 bands in the 100 enzyme patterns, and 65 (89%) of these occurred in *H. diffusa* var. *arida*. One PGI-S band was unique to *H. diffusa* var. *cottonii*. One PGM band was unique to the *H. diffusa* var. *cottonii/H. diffusa* var. *diffusa* pair and occurred in all sampled individuals in those taxa. The white-flowered *H. venusta* population had four unique bands plus the unique absence of one band, however, all patterns unique to this population occurred at < 11% frequency. In contrast, only two of the seven sampled populations of *H. diffusa* var. *arida* had unique bands (one in BM and three in one pattern in DE). All the bands observed in blue-flowered *H. venusta* also occurred in *H. diffusa* var. *arida*, but not necessarily in the same combinations; three patterns (in PGI-S, TPI, and UGPP-F) occurred in blue-flowered *H. venusta* and not in *H. diffusa* var. *arida*.

Eight enzyme band patterns were shared only by *H. venusta* and those sampled *H. diffusa* var. *arida* populations (PE, SC, and TW) which had been hypothesized to show signs of introgression (Carr 1974). Three of these patterns (two in PGI-S and one in UGPP-S) occurred in moderate frequency (0.2 – 0.5) in white-flowered *H. venusta* and were rare or uncommon (frequency 0.05 – 0.11) in the *H. diffusa* var. *arida* populations in which they occurred.

Hedrick's similarities among populations of *H. diffusa* var. *arida* averaged 0.786 (range 0.667 to 0.934). Hedrick's similarities between *H. diffusa* var. *arida* and the other varieties of *H. diffusa* averaged less. Similarities to *H. diffusa* var. *cottonii* averaged 0.694 and those to *H. diffusa* var. *diffusa* averaged 0.706; none of the pairwise similarities exceeded 0.733. Hedrick's similarities between *H. diffusa* var. *arida* populations and *H. venusta* were lower yet, averaging 0.660 (range 0.561 – 0. 746) for white-flowered *H. venusta* and 0.634 (range 0.520 – 0.764) for blue-flowered *H. venusta*. Hedrick's similarities between *H. venusta* and the *H. diffusa* var. *arida* populations hypothesized to show introgression with *H. venusta* (Gentry and Carr 1976) were not statistically different from the similarities between *H. venusta* and other *H. diffusa* var. *arida* populations. These three *H. diffusa* var. *arida* populations appear no more similar to *H. venusta* than do the remaining four *H. diffusa* var. *arida* populations. Hedrick's similarity between the *H. diffusa* var. *cottonii* and *H. diffusa* var. *diffusa* populations sampled was 0.662. The similarity between white-flowered *H. venusta* and the blue-flowered *H. venusta* population sampled was 0.877 (Figure 1).

Discussion

Interpretation of Enzyme Band Patterns

Normally, an initial step in analyzing isozyme bands is to infer the alleles that produce the bands and thus determine genotypes of the individuals studied. We were unable to infer alleles from the *Hackelia* isozyme patterns for several reasons, including complicated band patterns, overlapping loci, and difficulty germinating seeds for crossing studies (Harrod 1999). Both *H. diffusa* and *H. venusta* are tetraploid (2n = 48) (Carr 1974). We are unaware of published information about the origin of that polyploidy (allopolyploid or autopolyploid) or the mode of inheritance of alleles (disomic or tetrasomic), and the evidence from isozyme phenotypes was inconsistent. In enzyme systems for which genetic interpretations could be provided (Weeden and Wendel 1989), genotypes fell into two categories. The combination of two different homozygous states (one-banded patterns) and at least one heterozygous state (suggestive of tetrasomic inheritance and autopolyploidy) was observed in GOT-F (2 populations), GOT-S (5 populations), SKD (5 populations of *H. diffusa*), and UGPP-F (3 populations). Fixed or excessive heterozygosity (suggestive of disomic inheritance and allopolyploidy) was observed for 6PGD and TPI-F in all populations and for SKD and UGPP-F in white-flowered *H. venusta*.

Genotypic interpretations were not readily inferred from dimeric PGI-S and TPI-F, which had complicated patterns often involving five to seven bands, and monomeric UGPP-F, which often had three or more bands. Additionally, presumed loci overlapped in PGM (2 – 4 bands in a restricted area of the gel) and MDH (one band in 90% of individuals). In theory, genetics of *Hackelia* isozymes might have been clarified by examining progeny arrays of wild plants or by examining isozymes in seedlings produced by controlled crosses. However, seed set is low in wild plants and no seed has been produced in controlled crosses (Harrod 1999).

Enzyme Variation

Conserving biodiversity involves preserving within-taxon diversity. All the *Hackelia* populations

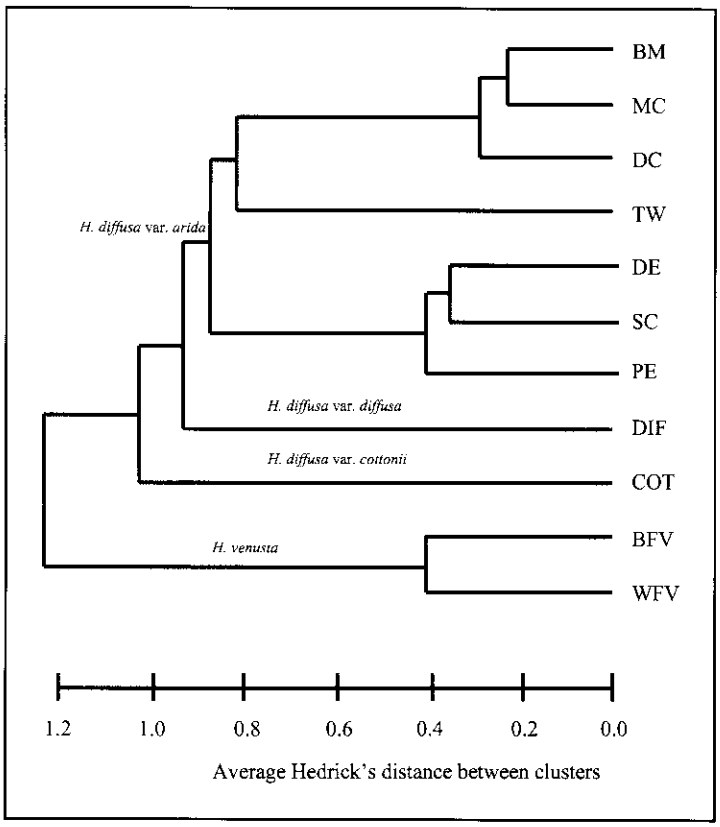

Figure 1. UPGMA dendrogram of *Hackelia* populations, based on Hedrick's distances among populations, which were calculated from band frequency data. WFV and BFV = white- and blue-flowered *H. venusta*, respectively. See Table 1 for acronyms.

studied were variable (Table 2). Although endemic plants often have limited isozyme variation (Hamrick and Godt 1990), white-flowered *H. venusta* and the nearby PE and SC populations of *H. diffusa* var. *arida* had the highest measures of variability. At the population level, all measures of variation were higher in white-flowered *H. venusta* than in the average population of *H. diffusa* var. *arida*. The high level of variation in white-flowered *H. venusta* is consistent with the observation that endemic plants are sometimes more variable than their widespread congeneric relatives (Gitzendanner and Soltis 2000). Phenotypic analysis of enzyme band patterns provides no reason to believe that lack of overall genetic variation limits survival of white-flowered *H. venusta*. The population may be specialized in some way, however, perhaps requiring high magnesium substrates (St. John 1929).

Blue-flowered *H. venusta* was also variable, but most measures of enzyme variation were lower than the average for *H. diffusa* var. *arida* populations (Table 2). This may be an artifact of sampling because only 12 micropropagated blue-flowered plantlets were available for this study.

Taxonomic Rank of White- and Blue-flowered *Hackelia venusta*

In general, populations of different congeneric species are distinguished by low genetic identities (Nei 1978). Genetic identities among congeneric species average 0.68, while those between populations of the same species are usually > 0.9 (Crawford 1989). However, not all pairs of related species meet this expectation. Some pairs of congeneric species have genetic identities > 0.99 and as low as 0.25 (Crawford 1989). We use Hedrick's similarities (Hedrick 1971) as a phenetic

measure of population similarity to assess the taxonomic rank of *H. venusta* color forms using a phenetic species concept (Winston 1999).

Hedrick's similarities clearly separate *H. venusta* from *H. diffusa* (Figure 1). Similarities average highest among *H. diffusa* var. *arida* populations and lowest between populations of *H. venusta* and *H. diffusa* var. *arida*. Therefore, *H. venusta* populations cluster farther from *H. diffusa* var. *arida* than the taxonomically recognized *H. diffusa* var. *cottonii* and *H. diffusa* var. *diffusa* (Figure 1). These enzyme data support the morphological data that recognize *H. venusta* and *H. diffusa* as closely related taxa (Carr 1974, Gentry and Carr 1976, Harrod et al. 1999), despite the intermediate appearance of a few *H. diffusa* var. *arida* populations (Carr 1974, Gentry and Carr 1976). Blue- and white-flowered *H. venusta* are more similar (Hedrick's similarity = 0.877) than most pairs of *H. diffusa* var. *arida* populations. Also, the *H. venusta* color forms are less similar to *H. diffusa* var. *arida* than are the named varieties *H. diffusa* var. *cottonii* and *H. diffusa* var. *diffusa* (Figure 1). Enzyme phenotype data alone do not provide evidence for a taxonomic separation of the forms using a phenetic species concept (Winston 1999).

When comparing band presence/absence between *H. venusta* color forms, the white-flowered form had four unique bands and one unique absence of a band. However, all these unique bands occurred at low frequencies. In fact, no fixed band difference distinguished *H. diffusa* var. *cottonnii* from *H. diffusa* var *diffusa*, or *H. diffusa* var. *arida* from *H. venusta*, providing no evidence for taxonomic separation of the color forms using a biological species concept (Winston 1999). Although the enzyme phenotypic data do not support species level recognition of the *H. venusta* color forms, blue- and white-flowered *H. venusta* are readily and consistently differentiated from each other by flower color, flower size, and habitat (Harrod et al. 1999).

Implications for Management

Preserving the large levels of diversity found among *Hackelia* populations will require preserving the genetic extremes represented by white- and blue-flowered *H. venusta*. It is necessary to preserve both color forms whether we label white- and blue-flowered *H. venusta* as conspecific populations, as distinct varieties, or as distinct species. To preserve genetic variation and prevent loss of the rarer alleles, large populations should be maintained in the wild. Off-site germplasm banks should include collections from as many different individuals as practical.

Although we hope this enzyme study contributes to the conservation of *Hackelia* biodiversity, immediate decisions crucial to *Hackelia* conservation must be based on entirely different criteria. If preserving white- and blue-flowered *H. venusta* is a priority, management should be driven by concerns about demographics. The only extant population of white-flowered *H. venusta* has severely declined in number over the last two decades. Since 1992, one population of blue-flowered *H. venusta* has been extirpated by a landslide and the two remaining populations are small. Questions of taxonomy and genetic diversity will be moot if these plants become extinct.

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