OREGON DEPARTMENT OF AGRICULTURE

NATIVE PLANT CONSERVATION PROGRAM

Assessing the population genetics, taxonomy, reproductive ecology, and life history traits of Humboldt milk-vetch (*Astragalus agnicidus*) in relation to conservation and management

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for

California Department of Fish and Wildlife

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PROJECT SUMMARY

1. Lab and field work was conducted between fall, 2010 and winter, 2012, to assess the population genetics, taxonomy, and natural history of Humboldt milk-vetch (*Astragalus agnicidus*), or ASAG. Information was also collected for Bald Mountain milk-vetch (*A. umbraticus*, or ASUM) as an ancillary part of the study. ASAG and ASUM are unique, forest-dwelling, seral species restricted to temporary openings in mesic, coniferous forests.

2. Population genetic study (using microsatellite analysis) revealed little differentiation among white-flowered ASAG populations, but an isolated pink-flowered form of the species in southern Mendocino County was significantly different (nearly at the species level) from the white-flowered populations. Evidence for incipient inbreeding depression was also noted.

3. Phylogenetic analysis of selected species within *Astragalus*, section *Miselli* showed that ASAG and ASUM are genetically isolated, and unique within that group and the genus.

4. Chromosome numbers for all sampled populations of ASAG were 2n=16.

5. Principle components analysis of morphological traits (using field and greenhouse-grown plants) supported the molecular data, and demonstrated several differences between the pink- and white-flowered forms of ASAG in addition to flower color.

6. Taken together, the population genetics evaluation and morphometric analyses support the segregation of the pink-flowered form of ASAG as a new taxon, probably at the varietal level.

7. Field observations during this study are consistent with previous work, which indicates that ASAG and ASUM are early seral species that evidently rely on long-lived seed banks, germinating en masse and reproducing from one to several years after habitat disturbance.

8. Observations and measurements of pollinators and reproductive effort (in the field and greenhouse) confirm that ASAG is highly fecund, and that a hypothetical population of 1,000 plants existing for five years is capable of producing over 10,000,000 seeds. Seed viability is high and seeds readily germinate, especially when briefly exposed to low temperatures.

9. Principle pollinators of ASAG and ASUM were species of *Bombus* and *Osmia* (both native bees), which were common on plants during warm, sunny days.

10. Pre-dispersal fruit and seed predation was documented for ASAG, as well as herbivory by deer or elk, both of which likely impact seed production in the species.

11. The most serious threats to ASAG are (1) exclusion of periodic site disturbance, which is needed to stimulate growth and reproduction, and (2) any action that interferes with seed production when populations are in their active, reproductive phases. Current forest management practices are generally not in conflict with the conservation of ASAG or ASUM.

Assessing the population genetics, taxonomy, reproductive ecology, and life history traits of Humboldt milk-vetch (*Astragalus agnicidus*) in relation to conservation and management[1,2]

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I. INTRODUCTION

Species and project background

Astragalus agnicidus (ASAG), commonly known as the Humboldt milk-vetch, is a rare member of the Fabaceae. It is found exclusively in lower montane redwood forest habitats of north-coastal California (Figure 1), primarily on private lands. Its limited number of occurrences, coupled with continued uncertainty about its overall conservation status, has resulted in the species being listed as endangered. A robust, early successional, short-lived perennial, ASAG was thought to have become extirpated after the single known patch of the species, located south of Miranda in Humboldt County (Decker et al. 2002), was ploughed under nearly 60 years ago (Barneby 1957, Pickart et. al. 1991). The destruction of the population in 1957, by a frustrated landowner, followed a series of sheep-poisonings in the private pasturelands where the milk-vetch grew. Thus, by the time researchers formally recognized and named ASAG that same year, based on that one population, the species was thought to be extinct. As an interesting side note, the name subsequently chosen for the new species—*agnicidus*—is derived from *agnus*, lamb, and *caedere*, to kill, or lamb-killer (Barneby 1957).

Down but not out, ASAG reappeared 30 years later in the same area where the only known population had been previously eradicated (Berg and Bittman 1988), a presumed testament to a long-lived seed bank. Since its initial rediscovery in 1987, four land managers, including Campbell Timberland Management, Humboldt Redwood Company (formerly the Pacific Lumber Company, or PALCO), Mendocino Redwood Company, and the California Department of Forestry and Fire Protection (Cal Fire), have reported irruptive occurrences of the species on lands they manage (Davis and Bittman 1999, CNDDB 2007). The currently known distribution of the species extends, irregularly, from near the Sonoma-Mendocino County line north into the southern half of Humboldt County. The species is considered endemic to northwestern California.

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[1] Completed under State of California Agreement No. P0982024

[2] Submitted to the California Department of Fish and Wildlife, Eureka Field Office, in July, 2013

[3] The Meinke lab group at OSU (email: meinker@science.oregonstate.edu) is administered through the Native Plant Conservation Program (NPCP) of the Oregon Department of Agriculture

Figure 1. ASAG flowers from plant grown from seed in the OSU greenhouses (July, 2012).

Habitats supporting ASAG are dominated by Douglas-fir (*Pseudotsuga menziesii*) and (usually second-growth) redwood (*Sequoia sempervirens*), along with other scattered hardwood and shrub species. The milk-vetch populations readily occupy unpaved road margins, landings, and other open woodland settings (Hickman 1993), principally in areas where overstory removal has taken place, frequently in combination with some level of mechanical or fire-related disturbance in the understory. Populations have been observed to thrive, at least in the short-term, under the canopy openings and within edge habitats created by timber harvest (Decker et al. 2002, Renner et al. 2009).

Previous investigations have evaluated a range of attributes of Humboldt milk-vetch (focusing on habitat and life history traits), including seed and germination biology, reproductive ecology, relationships to disturbance and succession, and monitoring of demographic trends (summarized in Bencie 1997; also see Pickart et al. 1991, Hiss and Pickart 1992, Pickart 1995, Pickart and Stauffer 1994, Decker et al. 2002, Renner et al. 2009). The information collected suggests ASAG is an early seral species that initially reproduces well and is sustained through long-lived seed banks, with plants appearing episodically in response to gap-creating disturbances within forests, such as fires, wind throw, road construction, or logging. Populations then decline and disappear within a few years, as the open habitat begins to fill in with later successional species.

Reproductive assessment and limited genetic studies, using isozyme analysis, were completed in the latter 1990's for a single ASAG population (Bencie 1997, M.A. thesis at Humboldt State University). Prior to this work, there had been concern that the life cycle

and habitat preferences of the species, which facilitate spatial and temporal isolation, may also promote inbreeding depression. Bencie's research ultimately found limited evidence for inbreeding depression, although the genetic variability measured within the study population was considered low.

So why conduct another Humboldt milk-vetch study?

Although most of the previous studies of ASAG are from the 1990's, the results and observations reported remain generally relevant, and have contributed significantly to the management approaches in practice today. A key exception deals with our understanding of genetic variation, considering that the only available information (from the Bencie thesis) had been gleaned from a single site. As the geographic distribution of the species across Mendocino and Humboldt counties became better known, a disjunct pattern was revealed that raised questions concerning the level of genetic diversity existing within and between populations.

Wide separation of populations may be detrimental to overall population fitness (McGlaughlin et. al. 2002), especially if the species depends on frequent outcrossing to sustain genetic variability. California Department of Fish and Wildlife (CDFW; Mary Ann Showers, pers. comm.) was concerned that, due to their isolation, ASAG populations might be genetically distinct at the county level, for example, or that specific individual populations may be unique, in particular, a peripheral population in extreme southern Mendocino County. Plants there exhibited a different corolla color from ASAG plants found elsewhere (pink, instead of the normal pure white), as well as other potential morphological dissimilarities that had yet to be assessed. The evolutionary relationship of this southern outlier, and (more importantly) it's possible taxonomic uniqueness, were questions worth investigating.

Some observers also wondered if the later discovered Mendocino sites might actually represent recent introductions of the species from the north, resulting from logging-related traffic along haul roads (Decker et al. 2002; Russ Shively, pers. comm.). And there was initial speculation that the species might not even be native, owing to its so-called "weedy behavior" on the property where it was first discovered (Barneby 1957). Genetic analysis could help assess these questions.

Consequently, CDFW developed a proposal in 2007 to implement a range-wide study of the genetic variability within and between ASAG populations, with the goal of answering questions regarding genetic diversity. As initially envisioned, the project was intended strictly as a molecular assessment that would provide information potentially useful in improving or updating management strategies for the species. Contact was made with researchers at the Oregon Department of Agriculture's Native Plant Conservation Program (NPCP), whose botanists (stationed at Oregon State University) had consulted previously with CDFW on other endangered *Astragalus* species, to determine their level of interest in working on the study. Conversations between CDFW and NPCP in 2008 ultimately resulted in a tentative project outline, which in additional to the genetic study, added proposed work on morphological variation among populations, cytology, reproductive biology (including pollinator studies), and life history. There is apparently no

official recovery plan for ASAG, and the study described here may provide information, covering populations in both counties, that could be useful in developing or updating recovery objectives for the species.

Although the project has expanded in scope since originally conceived by CDFW in 2007, the central theme and focus of the work has remained the same, i.e., *to determine if there are measureable and genetically significant differences between populations of ASAG*. And while we'll be re-visiting some topics addressed by previous observers, the distinction here is that we'll be integrating the natural history data (pollination, seed production, etc.) from *several* sites with the molecular genetics and morphometric studies, to hopefully come up with a more robust evaluation of the species across its populations. A focus on genetic evaluation, in combination with ecological and life history studies, may provide information that in turn could improve the conservation status of ASAG. This information could then help with revising management priorities, and potentially form the basis for initiating a recovery plan.

CDFW contract specifications

Once a contract was developed, CDFW identified (in Agreement No. P09882024) two principal tasks (with additional subtasks) to be accomplished. These were listed as follows:

Genetic evaluation:

1. Evaluate population-level genetic diversity in Humboldt milkvetch.
2. Using sequencing, estimate levels of uniqueness between populations of Humboldt milkvetch by contrasting them with themselves, as well as contrast them with Bald Mountain milkvetch, which is probably the closest relative of Humboldt milkvetch.
3. Establish chromosome numbers for both species.
4. Conduct a morphometric analysis, which will include all known populations of Humboldt milkvetch and representative series of populations of Bald Mountain milkvetch from Oregon and northern California, to complement the genetic work.

Ecology and demography:

1. Gather a demographic snapshot of the two species via long-term monitoring plots.
2. Assess plant longevity, survival, and reproduction, i.e., flower, fruit, and seed production; impacts from pre-dispersal seed predation; and seed viability for both species.
3. Evaluate the role and diversity of insect pollinators in both milkvetch species.

What was our study approach?

The project technically started in 2010, but the contract was formalized too late to begin field work that year. However, some ASAG seeds were shipped to NPCP staff by CDFW in late summer, as well as some limited leaf tissue samples. These were used to jumpstart the molecular genetics work, as they allowed us to establish primers (strands of nucleic acid that serve as starting points for DNA synthesis) necessary for amplifying simple sequence repeats, which are repeating sequences of a few base pairs of DNA useful in assessing differences between populations (see #3, below). All of the field work and most of the lab and greenhouse work associated with this project were completed in 2011 and 2012.

The following narrative provides a quick overview of the planning and approach we used to accomplish the project, and summarizes any adjustments we needed to make to the project plan, either due to unforeseen circumstances or to improve the study and our expected results. Additional details on methods are provided in the individual report sections, below, which focus on specific project tasks and subtasks.

1. *Site visits and site selection*

Site visits in Humboldt and Mendocino counties (for sample collections, natural history observations, morphometric data collection, etc.) took place June, July, and August in 2011, and June and July in 2012, in conjunction with meetings with CDFW and various landowners of ASAG sites. During our first visits, in June, 2011, we identified which populations we intended to use as primary study sites for the project.

Although the project specifications in the CDFW contract indicated that "all known" populations of ASAG would be included in the morphometric studies, it was quickly apparent that this was impractical. Populations varied tremendously in size, based on how long it had been since they had first appeared. Being a seral species tied to disturbance, most known populations (at the time of our work) were now small and winking out (only a few individuals remaining), as succession eliminated their habitat, while others in areas with more recent disturbance were larger. We chose to focus on the latter sites, to ensure that there would be (1) enough ASAG plants for both the morphological and genetic studies, and (2) reasonably broad intrapopulation variability within our samples. The only exception was the pink-flowered outlier population in extreme southern Mendocino County, which by the time the work started had fewer than 100 individuals present (most were non-flowering during our visits). Its unique status in the study made it imperative that it be included, regardless of size.

So after consideration of the above, and consultation with CDFW, Cal Fire, and private land managers (i.e., Mendocino and Humboldt Redwood Companies), we settled on five primary study sites for ASAG (Figure 2), three from Mendocino County and two from Humboldt County. There were also two study sites for ASUM (ASUM), known as Bald Mountain milk-vetch, one on private land (managed by Green Diamond Resource Company) in northern Humboldt County, California, and the other on the Umpqua National Forest in the southern Oregon Cascades (in Douglas County). Inclusion of ASUM

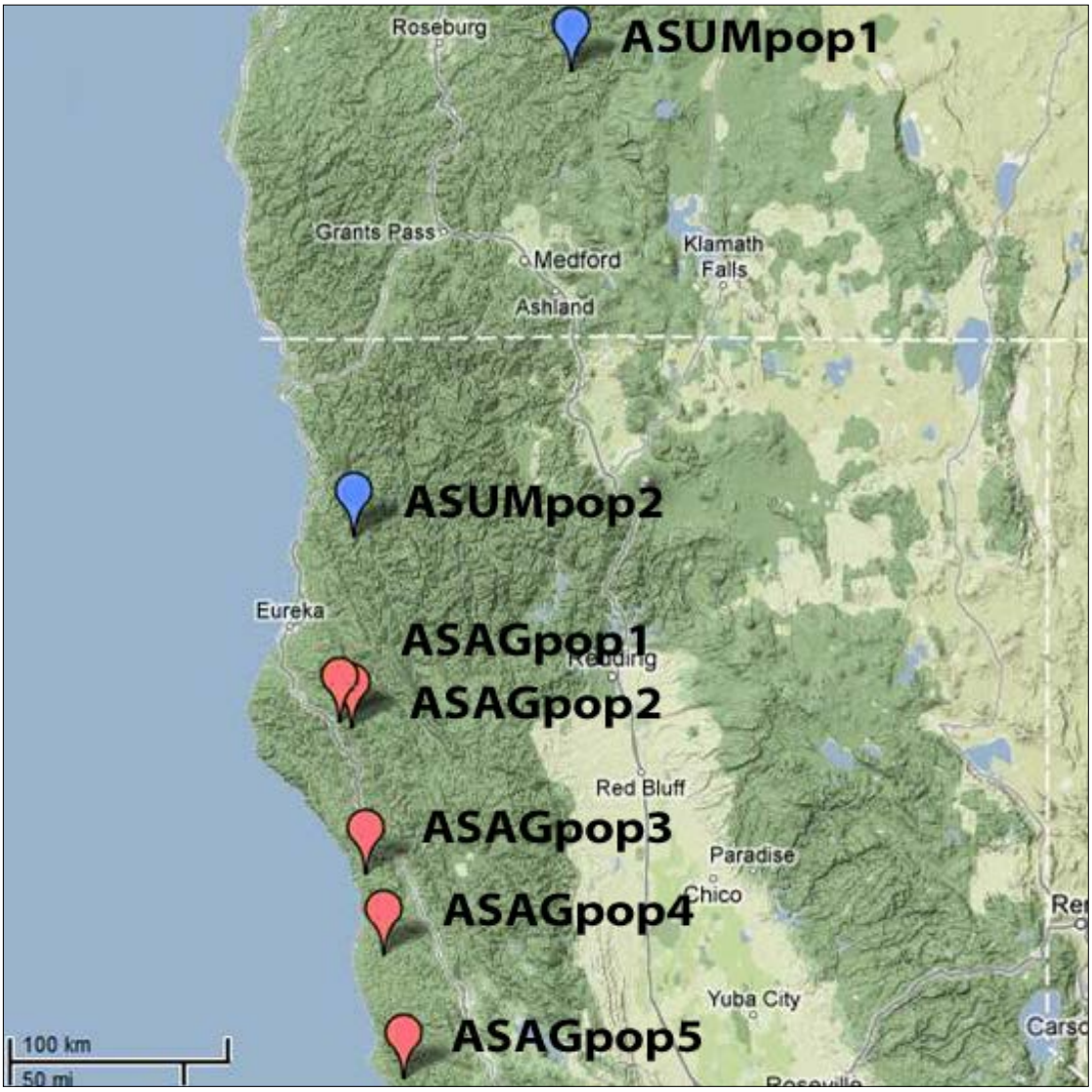

Figure 2. Locations of *A. agnicidus* (ASAG) and *A. umbraticus* (ASUM) populations we sampled.

Table 1. Coordinates of *A. agnicidus* and *A. umbraticus* populations surveyed. HRC=Humboldt Redwood Co. MRC=Mendocino Redwood Co. CF=Cal Fire. GD=Green Diamond Resource Co. UNF=Umpqua National Forest.

Population	Latitude	Longitude
ASAGpop1 (HRC)	40.388627	-123.855003
ASAGpop2 (HRC)	40.369967	-123.829262
ASAGpop3 (MRC)	39.740046	-123.75312
ASAGpop4 (CF)	39.39564	-123.649652
ASAGpop5 (MRC)	38.85311	-123.536444
ASUMpop1 (GD)	43.092667	-122.618639
ASUMpop2 (UNF)	41.172	-123.82

in the project, especially for the molecular work, was considered important due to its presumed close phylogenetic relationship with ASAG (Barneby, 1964)—see further explanation, below.

2. *The role of* ASUM *(Bald Mountain milk-vetch) in the project*

ASUM is generally considered the closest relative of ASAG, and the two species may form an evolutionary alliance (see Barneby 1964) with *Astragalus congdoni* of the Sierra Nevada, *A. paysonii* (an unusual forest species from the Rocky Mountains), and a handful of other poorly-known taxa from the western U.S. and Mexico. Barneby (1964) considered this a "primitive" group of species, largely based on their fruit characters and a preference for cooler, mesic habitats (unusual in the genus, which is well known for its highly xerophytic taxa).

So due to its presumed relatedness to and geographic affinity with ASAG, we considered it important to include ASUM in the molecular part of the study. Speculation by landowners at the time ASAG was originally discovered and described (Barneby 1957) implied that the species might not even be native, based on its observed ruderal tendencies at the sheep ranch it was first recorded from. Although the potential for ASAG to be exotic seemed remote, even at the time, a molecular comparison with the undisputedly native ASUM would conclusively settle the matter.

Additionally, as a close relative with many similarities to ASAG, ASUM was an initial logical choice as an "outgroup" for the molecular work. The level of within-population variability in ASAG (i.e., how different are the Mendocino versus Humboldt populations, are the pink-flowered plants at the south end of the range really unique, etc.?) can then be matched against the differences that exist between ASAG and ASUM, and (depending on time and budget) other related milk-vetch species.

3. *Population genetics: tissue collections*

We collected young leaf material from at least 20 plants from five ASAG population complexes across the range of the species, including the outlying occurrence near the Sonoma County line purported to be ASAG (but which has the unusual pinkish corolla coloration). We collected all the necessary materials for our lab work in June and July, 2011. Leaves were kept separate (by plant) in double zip-lock baggies, and placed in ice chests prior to transport to OSU in Corvallis. They were then stored in the lab at -30°C until needed for DNA extractions and sequencing work.

4. *Population genetics: use of microsatellites*

The primary goal of this project has been the evaluation of genetic diversity within and among principal populations of ASAG, as well as between ASAG and the closely related species ASUM. To accomplish this we had originally suggested using *amplified fragment length polymorphisms* (or AFLPs) to assess genetic differences.

However, since then we've elected to use a more robust technique referred to as *microsatellite analysis*. Short of whole genome sequencing, this is currently considered a more accurate approach in assessing population anomalies—we'll be comparing short (or

simple) sequence repeats (or SSRs, often called microsatellites) obtained from randomly selected individual plants sampled from target populations. The leaves collected in June, 2011 were used to extract DNA at OSU for the evaluations. Voucher specimens from collection sites were pressed, if enough reproductive material was available on site to justify this, for deposit at the herbaria at Humboldt State University and OSU.

We also changed the molecular work by expanding the number of related species covered, in order to generate a partial phylogeny of the Section *Miselli*, in which both ASAG and ASUM are taxonomically aligned (Barneby 1964). This provides us with a better understanding of the relationship between ASAG populations and sister taxa. We used herbarium specimens from OSU to extract DNA for species we could not visit in the field.

Multivariate analyses were used to portray genetic patterns and statistically evaluate population differentiation, and genetic data were interpreted within the context of morphological observations to provide a biologically-meaningful analysis.

5. *Chromosome counts*

We felt the best approach here was to germinate and grow milkvetch plants in the greenhouse at OSU, and then try to obtain chromosome numbers from one or more populations in cultivation. We attempted chromosome counts from cells in actively growing root tips extruding from the bottom of well-watered pots. We successfully obtained counts for ASAG (the more important of the two study species), but not ASUM.

6. *Morphometric population comparisons*

Utilizing morphological analyses to compare the study populations added an important element to compliment the molecular work. We attempted to measure traits for at least 20 plants per study population, using a set of morphological markers (described later in the report) that we considered important for differentiating ASAG and related species.

We initially hoped to flag and then measure (in the field) the same plants that we used for collecting leaf tissue (for the molecular work) for the morphometric studies. However, this proved impractical due to herbivory and other issues, so we measured plants in the immediate vicinity at the same study sites. Morphological data was collected in the field to the degree possible. We also grew ASAG plants from the study sites in the greenhouse at OSU in 2011 and 2012, and these cultivated plants proved to be an important complement to our morphological measurements from wild plants. Morphological data were evaluated using principle components analysis.

7. *Natural history and population ecology*

The following comments relate primarily to data collection and observations for ASAG, the focus species of this study. Our original goal was to collect the same level of life history data for ASUM that we were collecting for ASAG. As it turned out, viable populations of ASUM were more difficult to locate than anticipated, both in California and on the Umpqua National Forest in Oregon, and those we did find were small and unsuitable for extensive comparative study. ASUM populations clearly succumb to the

same successional habitat shifts that permit ASAG to flourish and then rapidly decline after disturbance. Overall, we considered this a minor issue, since the principle reason for including ASUM in the study was to contrast its molecular profile with that of ASAG. There were enough plants at both ASUM study sites to accomplish this important task.

- *Flower and fruit production:* Average values for the number of flowers and fruit per plant were calculated for a subset of randomly selected plants from each study site.
- *Seed production, and ovule and seed predation***:** Average per-fruit values for mature seeds and the number of ovules/seeds aborted or damaged by insect predation were calculated for a subset of randomly selected. Samples of observed seed predators were collected and preserved for potential identification at OSU.
- *Seed production by population:* Direct counts of the number of fruits and mature seeds produced per plant was used to calculate estimates for the number of seeds potentially available for dispersal into the seed bank.
- *Seed viability:* Representative seed samples were evaluated for germinability and germination requirements. Previous work identified a combination of physical and physiological seed dormancy in ASAG.
- *Breeding system and pollinators:* Previous work demonstrated that ASAG was likely self-compatible, but that the species required insect pollinators to effect significant seed set. We tested for autogamy in the greenhouse, using cultivated plants. A species of *Bombus* was identified in 1997 as being the dominant floral visitor at a single ASAG population. We measured pollinator abundance, diversity, and visitation rates at our study sites. Specimens of observed pollinators were collected for identification at OSU.

8. Population censusing.

Initially, our project plans called for setting up traditional monitoring plots in populations suitable for *population viability analysis* (PVA), which would have eventually (over many years) produced the "demographic snapshot" of the species, as described in the contract specifications.

The value of this approach was soon questioned, however, considering that there is considerable observed and anecdotal evidence that links the appearances of both ASAG and ASUM populations to forest disturbance. This means that the relatively rapid increases and declines in plant numbers observed for these species are not necessarily, in and of themselves, indicative of threats to a population. The long-term, annual monitoring of plots associated with PVA is designed to identify (or predict) population trends (see review by Menges 2000), which in turn can be used to suggest threat level and management direction for a given site. An example of a milk-vetch species where this approach is justified is described by Lesica (1995).

However, when ASAG and ASUM populations appear, it's now apparent that unless a site is artificially maintained in an actively disturbed or open state, the size and vigor of the resident milk-vetch population is inherently designed to go in one direction

within a few years: back to the seed bank to await the next fire or logging operation. So it is our opinion that PVA would probably be of little significance as a risk assessment tool for either species.

II. METHODS AND RESULTS

Genetic evaluations

The molecular evaluation of ASAG was the focal point of the project, with the primary goals being (1) to evaluate population-level genetic diversity within the species, including the potential for inbreeding depression, as well as (2) to contrast the species with its close relative, ASUM. The data would hopefully provide insights as to whether ASAG was in fact, a native species (this question is discussed in Barneby 1957), and then whether or not the anomalous, pink-flowered form of the species occurring in extreme southern Mendocino County (i.e., ASAGpop5 in Fig. 1 and Table 1) was genetically (and potentially taxonomically) more distinct than other populations.

We elected to use microsatellites in our investigation, considered to be a more specific and informative technique than allozymes (Freville et al. 2001), to measure genetic variability between populations of ASAG. In addition, and for comparison, two populations of the presumed sister species of ASAG, namely ASUM (Barneby 1964), were also sampled. Along with its putative relatedness to ASAG, ASUM has a life cycle similar to ASAG, in which long periods of dormancy (in the form of seed banks) are followed by comparatively short periods of vegetative growth and reproduction following disturbance. This similarity offers an opportunity to contrast the genetic variation found in ASAG with another species having this particular life history.

Finally, no phylogenetic study focusing on section *Miselli* species has been previously completed, which we addressed here by sampling ASAG, ASUM, and selected related species available in the OSU herbarium. The goal was to evaluate the phylogenetic position of ASAG and ASUM within the section, using both nuclear and chloroplast markers.

Methods

Microsatellite analysis

In order to obtain DNA for study, milk-vetch foliar samples were collected during the 2011 growing season in northern California and southern Oregon. In total, five populations of ASAG and two populations of ASUM were visited (see Figure 1 and Table 1 for locations), with leaves collected from up to 20 mature individuals. The leaves were labeled by individual and population, quickly placed in plastic collections bags, and stored on ice. The samples were then returned to Corvallis (OSU), and re-bagged and stored in a -80°C freezer. Samples remained in the freezer until DNA extractions were performed at a later date.

In addition to leaf samples, one 'voucher' specimen, consisting of several leaves and flowers (if available), was collected from each sampled population. The 'voucher' specimens were pressed either on site, or shortly after the visit, in standard herbarium plant presses. After drying the specimens were to be deposited at either OSU or Humboldt State University.

DNA extractions were initially obtained from four randomly selected ASAG specimens. These extractions were screened for their level of DNA quantity using a spectrophotometer. Extractions with high quantities of DNA were also screened for quality by amplifying micro-quantities of the extractions with one set of nuclear primers (nuclear ribosomal internal transcribed spacer) and one set of chloroplast primers (*trn*T-L). These amplifications were Sanger sequenced, and the resulting electropherograms analyzed for accuracy and quality.

From among the four initial ASAG extractions, the extraction with the highest quantity and quality DNA was sequenced, in a single lane, on an Illumina HiSeq 2000 high throughput sequence sequencing machine. This sample, as a result of the sequencing run, yielded (after Illumina quality filtering) 148,224,146 one hundred base pair reads, with 3,218,464 reads (~2%) that corresponded to adapters being discarded. We next chose to filter for tri-nucleotide repeats, rather than di-nucleotide repeats, following the method of Jennings et al. (2011), for the former's less ambiguous ability to be scored accurately with future analyses. This filtering resulted in 4,898 reads. Further filtering for reads, which were identical to one another as well as those that matched known chloroplast and mitochondrial reads (within Fabaceae), reduced the read pool to 3,276.

Primers were next designed using BatchPrimer3 (You et al. 2008) with a setting for six or more repeat units, product sizes from 150 – 400 bp, and an optimal annealing temperature of 57°C. This resulted in the generation of 3,264 primer pairs for the amplification of 2,239 unique microsatellite loci. From among the primer pairs, 40 candidate loci were chosen for testing. Fluorescently tagged primers, specific for these loci, were used to screen for potential variability in samples of ASAG and ASUM. Based on variability (Tables 2 and 3), the length of the repeats, and success of amplification, 10 sets of primers, which amplified triplet repeats with a product between 200-300 base pairs, were chosen for the final analysis (Table 4).

Table 2. Number of distinct alleles amplified, by locus, within all ASAG populations.

Locus	Number of distinct alleles
1	5
2	3
3	6
4	5
5	6
6	5
7	3

8	5
9	4
10	5

Table 3. Number of distinct alleles amplified, by locus, within all ASUM populations.

Locus	Number of distinct alleles
1	3
2	2
3	3
4	3
5	3
6	3
7	2
8	3
9	3
10	3

Table 4. Microsatellite primers used for genetic diversity analysis.

Primer Name	Sequence (5' - 3')
1F	TGTAAAACGACGGCCAGTTCAAAGAAGAAGAAGAAGAAGAA
1R	GTTAACCCTCAGCCACAAAAT
2F	TGTAAAACGACGGCCAGTAAAAAGAAGAACACCGAGAGG
2R	TAAACGCAAAATGAGCACTAA
3F	TGTAAAACGACGGCCAGTGAGGAAAATGAAGGAAATGCT
3R	TGAGTTAGAAGAGGGGTTTGA
4F	TGTAAAACGACGGCCAGTCTCTTGCTTGCTAATGGTTGT
4R	TTGTTACACGTACCTGGGAAT
5F	TGTAAAACGACGGCCAGTGGACAACAACAACAACAACAA
5R	CATGCTTTCTTCACCATCTTT
6F	TGTAAAACGACGGCCAGTTCTGCTTACTCTTCCACTTCC
6R	GTTTCTGAATCATCTGGCATC
7F	TGTAAAACGACGGCCAGTAGAGGGGAGAAGAAGAACAAA
7R	AACAGCAACAGCAACAGAAG
8F	TGTAAAACGACGGCCAGTCCTCTCTCTCTCTCTCTCACG
8R	CTCTTGAAATCAAACAAAAACCT
9F	TGTAAAACGACGGCCAGTAATGAAAAGTAAGAGGGGAAAA
9R	CCAGAATCTAGAACCAGCTCA
10F	TGTAAAACGACGGCCAGTACACTCCTTTGCTCACACCTA
10R	TCGTCACTGACACTGTCCAC

All polymerase chain reactions (670 total) were fluorescently labeled and amplified using the methods of Schuelke (2000). Products were resolved in an ABI 3730 capillary DNA sequencer. Electropherograms were analyzed using ABI GeneMapper software and incorporated into a data matrix for final analysis of genetic diversity (Table 5). All calculations of genetic diversity were carried out in the program GenalEX V. 6.5b2 (Peakall and Smouse 2012).

Table 5. Length of products derived from microsatellite amplifications.

Pop	Locus1		Locus2		Locus3		Locus4		Locus5	
ASAGpop1	249	249	240	243	231	234	243	243	231	234
ASAGpop1	249	249	240	240	234	234	243	243	234	234
ASAGpop1	246	249	240	240	231	234	240	243	231	234
ASAGpop1	246	249	240	240	231	234	240	243	231	234
ASAGpop1	246	249	240	243	231	234	240	243	231	234
ASAGpop1	249	249	240	240	228	234	243	243	234	234
ASAGpop1	249	249	240	240	234	234	243	243	234	234
ASAGpop1	246	249	240	240	231	234	240	243	231	234
ASAGpop1	246	246	243	243	231	231	240	240	231	231
ASAGpop1	246	246	240	243	231	231	240	240	231	231
ASAGpop2	246	246	240	240	234	234	240	240	234	234
ASAGpop2	246	246	240	240	228	228	240	240	231	231
ASAGpop2	246	249	240	240	231	234	240	243	231	234
ASAGpop2	246	249	240	240	231	234	240	243	231	234
ASAGpop2	246	249	240	240	231	234	240	243	231	234
ASAGpop2	249	249	240	240	234	234	243	243	234	234
ASAGpop2	249	249	240	240	234	234	243	243	234	234
ASAGpop2	246	246	240	240	231	231	240	243	231	231
ASAGpop2	246	249	240	243	231	234	240	243	231	234
ASAGpop2	249	249	243	243	234	234	243	243	234	234
ASAGpop3	240	246	240	240	222	231	234	240	225	231
ASAGpop3	249	249	240	240	234	234	243	243	234	234
ASAGpop3	246	246	240	240	231	234	240	240	231	231
ASAGpop3	249	249	240	240	234	234	243	243	234	234
ASAGpop3	246	249	240	240	231	234	240	243	231	234
ASAGpop3	249	249	240	240	234	234	243	243	234	234
ASAGpop3	249	249	240	243	234	234	243	243	234	234
ASAGpop3	246	249	243	243	231	234	240	243	231	234
ASAGpop3	246	246	243	243	231	234	240	240	231	234
ASAGpop3	249	249	243	243	234	234	243	243	234	234
ASAGpop4	246	246	240	240	231	234	240	240	231	234
ASAGpop4	249	249	240	240	234	234	243	243	234	234

ASAGpop4	246	249	240	240	231	234	240	243	231	234
ASAGpop4	249	249	240	240	228	234	243	243	234	234
ASAGpop4	246	249	240	240	231	234	240	243	231	234
ASAGpop4	249	249	243	243	234	234	243	243	234	234
ASAGpop4	249	249	243	243	234	234	243	243	234	234
ASAGpop4	246	249	243	243	231	234	240	243	231	234
ASAGpop4	237	246	243	243	222	231	231	240	222	231
ASAGpop4	249	249	243	243	234	234	243	243	234	234
ASAGpop5	231	231	240	240	216	216	225	225	216	216
ASAGpop5	231	246	237	240	216	231	225	240	216	231
ASAGpop5	246	246	243	243	231	231	240	240	231	231
ASAGpop5	246	246	243	243	231	231	240	240	231	231
ASAGpop5	246	246	243	243	231	231	240	240	231	231
ASAGpop5	231	246	237	240	231	231	225	240	231	231
ASAGpop5	231	246	243	243	249	231	225	240	249	231
Pop	**Locus6**		**Locus7**		**Locus8**		**Locus9**		**Locus10**	
ASAGpop1	258	261	264	267	243	243	252	255	252	252
ASAGpop1	261	261	264	264	243	243	252	252	252	252
ASAGpop1	258	261	264	264	240	243	252	252	249	252
ASAGpop1	258	261	264	264	240	243	252	252	249	252
ASAGpop1	258	261	264	267	240	243	252	255	249	252
ASAGpop1	261	261	264	264	243	243	252	252	252	252
ASAGpop1	261	261	264	264	243	243	252	252	252	252
ASAGpop1	258	261	264	264	240	243	252	252	249	252
ASAGpop1	258	258	267	267	240	240	255	255	249	249
ASAGpop1	258	258	264	267	240	240	240	255	249	249
ASAGpop2	258	258	264	264	240	240	252	252	249	249
ASAGpop2	258	258	264	264	240	243	252	252	249	249
ASAGpop2	258	261	264	264	240	243	252	252	249	252
ASAGpop2	258	261	264	264	240	243	252	252	249	252
ASAGpop2	258	261	264	264	240	243	252	252	249	252
ASAGpop2	261	261	264	264	243	243	252	252	252	252
ASAGpop2	261	261	264	264	243	243	252	252	252	252
ASAGpop2	258	261	264	264	240	243	252	252	249	252
ASAGpop2	258	261	264	267	240	243	252	255	249	252
ASAGpop2	261	261	267	267	240	243	255	255	252	252
ASAGpop3	252	258	264	264	234	240	252	252	243	249
ASAGpop3	261	261	264	264	243	243	252	252	243	252
ASAGpop3	258	258	264	264	240	240	252	252	249	249
ASAGpop3	261	261	264	264	243	243	252	252	252	252

ASAGpop3	258	261	264	264	240	243	252	252	249	252
ASAGpop3	261	261	264	264	243	243	252	252	252	252
ASAGpop3	261	261	264	267	243	243	252	255	252	252
ASAGpop3	258	261	267	267	240	243	255	255	249	252
ASAGpop3	258	258	267	267	240	240	255	255	249	249
ASAGpop3	261	261	267	267	243	243	255	255	252	252
ASAGpop4	258	258	264	264	240	240	252	252	249	249
ASAGpop4	261	261	264	264	243	243	252	252	252	252
ASAGpop4	258	261	264	264	240	243	252	252	249	252
ASAGpop4	261	261	264	264	243	243	252	252	252	252
ASAGpop4	258	261	264	264	240	243	252	252	249	252
ASAGpop4	261	261	267	267	243	243	255	255	252	252
ASAGpop4	261	261	267	267	243	243	255	255	252	252
ASAGpop4	258	261	267	267	240	243	255	255	249	252
ASAGpop4	249	258	267	267	231	240	255	255	240	249
ASAGpop4	261	261	267	267	243	243	255	255	252	252
ASAGpop5	243	243	264	264	225	225	252	252	234	234
ASAGpop5	243	258	264	264	225	240	252	252	234	249
ASAGpop5	258	258	267	267	240	240	255	255	249	249
ASAGpop5	258	258	267	267	240	240	255	255	249	249
ASAGpop5	258	258	267	267	240	240	255	255	249	249
ASAGpop5	243	258	261	264	225	240	249	252	234	249
ASAGpop5	243	258	267	267	225	240	255	255	234	249
Pop	**Locus1**		**Locus2**		**Locus3**		**Locus4**		**Locus5**	
ASUMpop1	255	255	243	246	240	243	249	249	240	243
ASUMpop1	258	258	243	246	243	243	252	252	243	243
ASUMpop1	255	255	246	246	240	240	249	249	240	240
ASUMpop1	255	255	243	246	240	240	249	249	240	240
ASUMpop1	255	258	246	246	240	243	249	252	240	243
ASUMpop1	255	258	243	243	240	243	249	252	240	243
ASUMpop1	249	255	243	243	234	240	243	249	234	240
ASUMpop1	255	258	243	243	240	243	249	252	240	243
ASUMpop1	255	258	243	246	240	243	249	252	240	243
ASUMpop1	258	258	246	246	243	243	252	252	243	243
ASUMpop2	258	258	243	246	243	243	252	252	243	243
ASUMpop2	258	258	246	246	243	243	252	252	243	243
ASUMpop2	255	255	243	246	240	243	249	249	240	243
ASUMpop2	255	255	246	246	240	240	249	249	240	240
ASUMpop2	255	258	246	246	240	243	249	252	240	243
ASUMpop2	255	258	243	243	240	243	249	252	240	243

ASUMpop2	249	255	243	243	240	240	243	249	240	240
ASUMpop2	255	258	243	243	240	243	249	252	240	243
ASUMpop2	255	258	243	243	240	243	249	252	240	243
ASUMpop2	255	255	246	246	240	240	249	249	240	240
Pop	**Locus6**		**Locus7**		**Locus8**		**Locus9**		**Locus10**	
ASUMpop1	267	267	267	270	249	249	255	258	258	258
ASUMpop1	270	270	267	270	252	252	255	258	261	261
ASUMpop1	267	267	270	270	249	249	258	258	258	258
ASUMpop1	267	267	267	270	249	249	255	258	258	258
ASUMpop1	267	270	270	270	249	252	258	258	258	261
ASUMpop1	267	270	267	267	249	252	255	255	258	261
ASUMpop1	261	267	267	267	243	249	252	252	252	258
ASUMpop1	267	270	267	267	249	252	255	252	258	261
ASUMpop1	267	270	267	267	249	252	255	252	258	261
ASUMpop1	270	270	270	270	252	252	258	258	261	261
ASUMpop2	270	270	267	270	252	252	255	258	261	261
ASUMpop2	270	270	270	270	252	252	258	258	261	261
ASUMpop2	267	267	270	270	249	249	258	258	258	258
ASUMpop2	267	267	270	270	249	249	258	258	258	258
ASUMpop2	267	270	270	270	249	252	258	258	258	261
ASUMpop2	267	270	267	267	249	252	255	255	258	261
ASUMpop2	261	267	267	267	243	249	255	255	252	258
ASUMpop2	267	270	267	267	249	252	255	255	258	261
ASUMpop2	267	270	267	270	249	252	255	255	258	261
ASUMpop2	267	267	270	270	249	249	258	258	258	258

Phylogenetic analysis

To estimate a phylogeny, 1,829 base pair (bp) of DNA (nrITS, 653 bp; *psbA-trnH*, 363 bp; *trnL-trnF* 137 bp; *trnS-trnG*, 676 bp) were sequenced from 14 specimens. DNA was obtained from randomly chosen samples of ASAG and ASUM used in the microsatellite study, from all populations surveyed, and then dried herbarium specimens were used for the remaining taxa (*A. arthurii*, OSC216377, Asotin Co., WA *Urban 91-0006*; *A. congdonii*, OSC205963, Tulare Co., CA, *Sipe 393*; *A. howellii*, OSC162795, Wasco Co., OR, *Wright 1650*; *A. misellus*, OSC220817, Wheeler Co., OR, *Otting 1355*; *A. oniciformis*, OSC197852, Blaine Co., ID, *Popovich 6669*; *A. paysonii*, OSC220817, Idaho Co., ID, *Lorain 2093*; *A. toquimanus* OSC207955, Nye Co., NV, *Tiehm 14506*).

Rarer species within section *Miselli*, for which adequate plant material was not available, were not included in the analysis (these included *A. ervoides, A. carminis, A. straturensis,* and *A. sinaloae*). We decided to use an herbarium specimen of *A. peckii* (a central Oregon pumice endemic) as the outgroup (OSC174767, Deschutes Co., OR, *Kaye 1233*).

All primers were obtained from and amplified following the protocols of Shaw et al. (2005), Taberlet et al. (1991), and Liston et al. (1996). Following PCR, all products were purified using QIAquick PCR purification kits (Qiagen, Valencia, CA). Sequencing was performed by the Center for Genome Research and Biocomputing at OSU. Sequences were aligned "by eye" and analyzed using BioEdit for Windows 95/98. The resulting data matrix was trimmed to ensure no cells were scored as missing data. The maximum likelihood phylogenetic analysis was conducted using PhyML 3.0 (Guindon et al. 2010) with standard settings, with the exception of branch support assessed using 1,000 bootstrap replicates.

Results and Discussion

Microsatellite analysis

Among the chief concerns when evaluating species characterized by small, fragmented populations, such as ASAG and ASUM*,* is whether high levels of genetic inbreeding resulting in the fixation of deleterious genes has occurred, or may be a future threat. Genetic variation potentially related to this threat can be measured by several indicators, including the comparison of observed heterozygosity (Ho) versus expected heterozygosity (He), fixation coefficients (F), chi-square and probability values, inbreeding coefficients (Fis), and fixation indices (Fst).

In populations that have experienced a bottleneck and/or recent isolation from other populations, the observed heterozygosity (Ho) will generally be lower within populations than the expected heterozygosity (He). This, in turn, is also reflected in the calculation of fixation coefficients [F=(He-Ho)/He], which can range from -1 to 1. An F value of greater than zero indicates that a population has heterozygosity lower than expected, and that it may be experiencing a degree of inbreeding depression (Cornuet and Luikart 1996; Severns et al. 2011).

Within the sampled populations of ASAG, the observed heterozygosity was lower than expected. Additionally, fixation coefficient values ranged from 0.127-0.435, with an average of 0.284 (Table 6). This number is far greater than the 0.031 calculated by Bencie (1997). However, given the more precise nature of microsatellite data versus allozyme data, and the additional samples and populations available for our study, this was not unexpected. While the average fixation coefficient value is not yet at a critical level (higher than 0.5), it does indicate that reduced fitness of ASAG populations, over the long-term, may be a legitimate concern.

Table 6. Observed heterozygosity (Ho), expected heterozygosity (He) and fixation coefficients (F) of ASAG populations.

Pop		**Ho**	**He**	**F**
ASAGpop1	Mean	0.410	0.465	0.127
	SE	0.031	0.017	0.040
ASAGpop2	Mean	0.370	0.427	0.201

	SE	0.065	0.038	0.106
ASAGpop3	Mean	0.280	0.492	0.435
	SE	0.044	0.012	0.092
ASAGpop4	Mean	0.310	0.488	0.358
	SE	0.071	0.006	0.146
ASAGpop5	Mean	0.329	0.483	0.300
	SE	0.037	0.015	0.088
Total	Mean	0.340	0.471	0.284
	SE	0.023	0.009	0.046

The previous finding was further substantiated by the calculation of chi-square and probability values, which test whether recent bottlenecks and potential inbreeding depression has occurred. If probability values are calculated as less than 0.05, this indicates the chance of a recent bottleneck is significant. Within ASAG, four of the five populations indicated a significant probability value of less than 0.05 at one or more loci (Table 7). Only two populations (ASAGpop3 and ASAGpop4), however, had low probability values at more than one locus, suggesting that bottlenecks may have occurred at differing periods of time, and the amount of future, potential inbreeding depression may vary between populations.

Table 7. Chi-square and probability values of ASAG populations by locus. (ns indicates no significance, * indicates a probability value <0.05, ** indicates a probability value <0.01)

Pop	Locus	ChiSquare	Probability	Significance
ASAGpop1	Locus1	0.278	0.598	ns
ASAGpop1	Locus2	0.400	0.527	ns
ASAGpop1	Locus3	1.131	0.770	ns
ASAGpop1	Locus4	0.278	0.598	ns
ASAGpop1	Locus5	0.001	0.975	ns
ASAGpop1	Locus6	0.001	0.975	ns
ASAGpop1	Locus7	0.400	0.527	ns
ASAGpop1	Locus8	0.278	0.598	ns
ASAGpop1	Locus9	4.090	0.252	ns
ASAGpop1	Locus10	0.278	0.598	ns
ASAGpop2	Locus1	0.400	0.527	ns
ASAGpop2	Locus2	3.695	0.055	ns
ASAGpop2	Locus3	10.000	0.019	*
ASAGpop2	Locus4	0.001	0.975	ns
ASAGpop2	Locus5	0.278	0.598	ns

ASAGpop2	Locus6	0.001	0.975	ns
ASAGpop2	Locus7	3.695	0.055	ns
ASAGpop2	Locus8	1.715	0.190	ns
ASAGpop2	Locus9	3.695	0.055	ns
ASAGpop2	Locus10	0.001	0.975	ns
ASAGpop3	Locus1	4.019	0.259	ns
ASAGpop3	Locus2	6.087	0.014	*
ASAGpop3	Locus3	3.673	0.299	ns
ASAGpop3	Locus4	4.019	0.259	ns
ASAGpop3	Locus5	2.669	0.445	ns
ASAGpop3	Locus6	4.019	0.259	ns
ASAGpop3	Locus7	6.087	0.014	*
ASAGpop3	Locus8	4.019	0.259	ns
ASAGpop3	Locus9	6.087	0.014	*
ASAGpop3	Locus10	1.931	0.587	ns
ASAGpop4	Locus1	2.669	0.445	ns
ASAGpop4	Locus2	10.000	0.002	**
ASAGpop4	Locus3	4.249	0.643	ns
ASAGpop4	Locus4	2.669	0.445	ns
ASAGpop4	Locus5	3.673	0.299	ns
ASAGpop4	Locus6	2.669	0.445	ns
ASAGpop4	Locus7	10.000	0.002	**
ASAGpop4	Locus8	2.669	0.445	ns
ASAGpop4	Locus9	10.000	0.002	**
ASAGpop4	Locus10	2.669	0.445	ns
ASAGpop5	Locus1	0.031	0.860	ns
ASAGpop5	Locus2	8.750	0.033	*
ASAGpop5	Locus3	2.458	0.483	ns
ASAGpop5	Locus4	0.031	0.860	ns
ASAGpop5	Locus5	2.458	0.483	ns
ASAGpop5	Locus6	0.031	0.860	ns
ASAGpop5	Locus7	7.280	0.063	ns
ASAGpop5	Locus8	0.031	0.860	ns
ASAGpop5	Locus9	7.280	0.063	ns
ASAGpop5	Locus10	0.031	0.860	ns

Observed heterozygosity, expected heterozygosity, and fixation coefficient values of the ASUM populations surveyed had values similar to ASAG (Table 8). These values (average F=0.161), however, were lower in comparison and indicate that future inbreeding depression, within the species, is likely not as great a threat. In addition, the chi-square and probability values indicate that only one population, at one locus, has a

probability value of less than 0.05 (Table 9), suggesting few or no recent bottlenecks, and a lower probability of inbreeding depression than observed for ASAG.

Table 8. Observed heterozygosity (Ho), expected heterozygosity (He) and fixation coefficients (F) of ASUM populations.

Pop		**Ho**	**He**	**F**
ASUMpop1	Mean	0.490	0.540	0.093
	SE	0.028	0.012	0.047
ASUMpop2	Mean	0.400	0.514	0.229
	SE	0.052	0.007	0.096
		Ho	**He**	**F**
Total	Mean	0.445	0.527	0.161
	SE	0.030	0.007	0.054

Table 9. Chi-square and probability values of ASUM populations by locus. (ns indicates no significance, * indicates a probability value <0.05)

Pop	**Locus**	**DF**	**ChiSq**	**Prob**	**Signif**
Pop1	Locus1	3	0.930	0.818	ns
Pop1	Locus2	1	0.400	0.527	ns
Pop1	Locus3	3	1.131	0.770	ns
Pop1	Locus4	3	0.930	0.818	ns
Pop1	Locus5	3	1.131	0.770	ns
Pop1	Locus6	3	0.930	0.818	ns
Pop1	Locus7	1	1.552	0.213	ns
Pop1	Locus8	3	0.930	0.818	ns
Pop1	Locus9	3	3.475	0.324	ns
Pop1	Locus10	3	0.930	0.818	ns
Pop2	Locus1	3	0.930	0.818	ns
Pop2	Locus2	1	3.600	0.058	ns
Pop2	Locus3	1	0.001	0.975	ns
Pop2	Locus4	3	0.930	0.818	ns
Pop2	Locus5	1	0.001	0.975	ns
Pop2	Locus6	3	0.930	0.818	ns
Pop2	Locus7	1	3.403	0.065	ns
Pop2	Locus8	3	0.930	0.818	ns
Pop2	Locus9	1	6.368	0.012	*
Pop2	Locus10	3	0.930	0.818	ns

A further exploration of current and potential inbreeding can be analyzed through a measurement of inbreeding coefficients (Fis). Similar to fixation coefficients, inbreeding coefficients range in number from -1 to 1 and measure the extent of inbreeding within populations, with a positive number indicating a degree of inbreeding. Additionally, the degree of genetic differentiation between populations is measured with fixation indices (Fst). A value of 0 indicates no differentiation between populations (strong levels of gene exchange between populations), while a value of 1 indicates complete genetic differentiation between populations (great isolation).

Within the populations of ASAG surveyed, Fis values averaged 0.292 (Table 10), mirroring the fixation coefficient values and indicating some potential for future inbreeding depression. Fst values averaged 0.151, indicating a small to moderate amount of genetic differentiation between the ASAG populations surveyed. While this value does not indicate an extreme lack of variation between populations it may, similar to the chi-square and probability values, suggest recent bottlenecks or range fragmentation within the species. Graphically, both the amount of genetic differentiation within and between the populations is illustrated by Figure 3.

Table 10. Inbreeding coefficient (Fis) and fixation indices (Fst) for ASAG.

Locus	**Fis**	**Fst**
Locus1	0.209	0.161
Locus2	0.636	0.134
Locus3	0.038	0.176
Locus4	0.167	0.162
Locus5	0.110	0.176
Locus6	0.131	0.158
Locus7	0.698	0.116
Locus8	0.084	0.162
Locus9	0.707	0.109
Locus10	0.143	0.158
Mean	0.292	0.151
SE	0.086	0.007

Inbreeding coefficients, within ASUM, were of both negative and positive values, and averaged 0.157 (Table 11). This average value implies that inbreeding, at least within the two populations of ASUM surveyed, is not yet a serious threat, but may indicate a slight trend toward future inbreeding depression. Fst values averaged 0.005, or very little genetic variation between populations (Figure 4). While this average may appear of great concern, the two populations of ASUM surveyed are located near the northern and southern extremes of the species' range (see Figure 1). As a result, it is plausible that the large physical distance between the populations (approximately 220 km), and the lack of data from intervening populations, explains this result. If additional data from several

other populations is obtained, a more precise measure of genetic variation between ASUM populations will be possible.

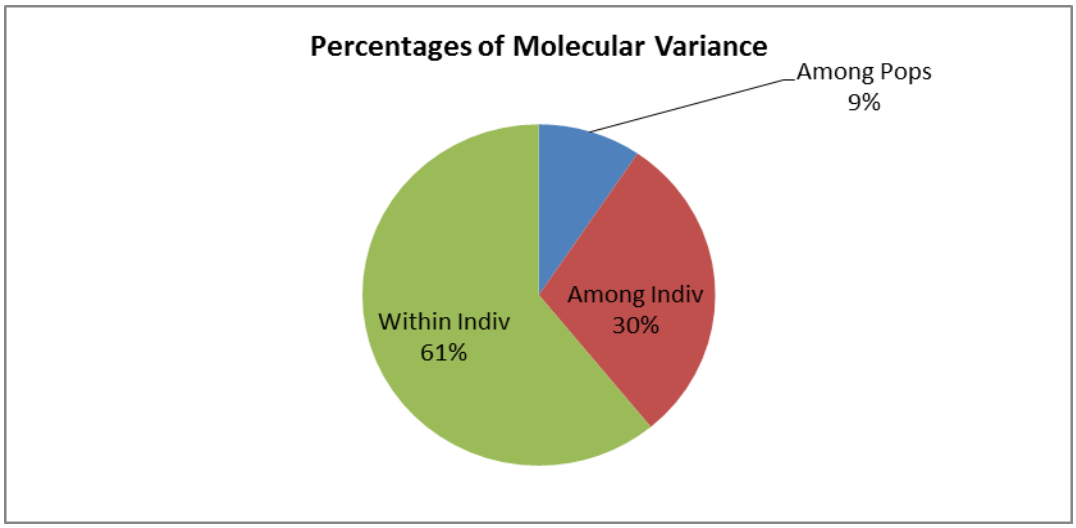

Figure 3. Genetic variance within and between populations of ASAG.

Table 11. Inbreeding coefficient (Fis) and fixation indices (Fst) for ASUM.

Locus	Fis	Fst
Locus1	0.065	0.000
Locus2	0.400	0.000
Locus3	-0.058	0.002
Locus4	0.065	0.000
Locus5	-0.058	0.002
Locus6	0.065	0.000
Locus7	0.487	0.023
Locus8	0.065	0.000
Locus9	0.469	0.026
Locus10	0.065	0.000
Mean	0.157	0.005
SE	0.067	0.003

Genetic evidence of an undescribed taxon

Expanding on the comparison of fixation index values between populations, a pairwise population matrix of Fst values for all populations of ASAG and ASUM populations surveyed was constructed (Table 12). From this table, a comparison of genetic differentiation between ASAG and ASUM, as distinct taxa, is available (higher numbers indicate a stronger differentiation). Interestingly, the values between ASAGpop5, the population with pink flowers, are highly differentiated from both ASUM as well as all other populations of "normal" (i.e., white-flowered) ASAG.

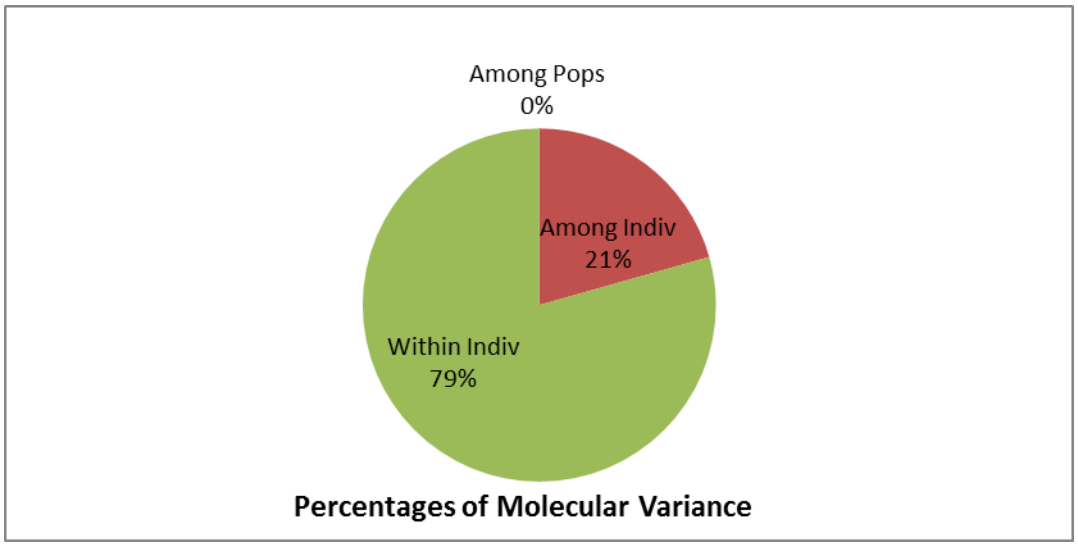

Figure 4. Genetic variance within and between populations of ASUM.

Table 12. Pairwise population matrix of fixation index (Fst) values for all populations of ASAG and ASUM populations surveyed. The population containing "pink" flowered ASAG plants (ASAGpop5) is indicated in bold. See text for discussion.

ASAGpop 1	ASAGpop 2	ASAGpop 3	ASAGpop 4	**ASAGpop 5**	ASUMpop 1	ASUMpop 2	
0.000							ASAGpop 1
0.008	0.000						ASAGpop 2
0.011	0.020	0.000					ASAGpop 3
0.030	0.051	0.011	0.000				ASAGpop 4
0.184	**0.207**	**0.195**	**0.203**	**0.000**			**ASAGpop 5**
0.299	0.322	0.280	0.272	**0.282**	0.000		ASUMpop 1
0.321	0.345	0.302	0.293	**0.299**	0.005	0.000	ASUMpop 2

Exploring this finding more specifically, pairwise matrix values of "normal" ASAG populations (ASAGpop1-ASAGpop4) versus all ASUM populations ranged between 0.272-0.345. These are relatively high values, as would be expected *between distinct species*. As a comparison, pairwise matrix values of all "normal" ASAG populations versus one another ranged between 0.008-0.051, and 0.005 between the two ASUM populations. These low values are expected *within* distinct species.

Pairwise matrix values of the pink-flowered population (ASAGpop5) versus the other ASAG (white-flowered) populations we sampled ranged from 0.184-0.207, with the ASAGpop5 versus the two ASUM populations at 0.282 and 0.299. Although the amount of

differentiation between the “pink” form population and the “normal” ASAG populations is slightly less than that between “normal” ASAG populations and the ASUM populations, the difference is markedly high. These results indicate that while “pink” form plants are closely related to “normal” ASAG plants, the genetic difference between the two forms may be an indication of a speciation event that has, or is currently, occurring.

Phylogenetic analysis

The results of the phylogenetic analysis place all populations of ASAG within one distinct and well supported clade (Figure 5). Although the sequences obtained from the ASAGpop4 and ASAGpop5 populations differed slightly from those in the other ASAG populations, the divergence was not significant enough to differentiate one or both populations into a separate clade. Additionally, as hypothesized by Barneby (1964), ASUM is firmly resolved as the sister species of ASAG. This also puts to rest the early speculation by landowners (see Barneby 1957, 1964) that ASAG might have been an exotic species, introduced (from an unspecified location) by itinerant forest workers.

A surprising result of the phylogeny is the elongated length of the branch resulting in the ASAG/ASUM clade. This branch indicates a relatively large evolutionary divergence of ASAG and ASUM from the other species in section *Miselli* that we sampled. Based on this limited phylogeny alone, it’s presumptive to conclude that ASAG and ASUM could be considered part of a new, unnamed section of *Astragalus*. However, a more inclusive phylogeny of *Miselli*, including the missing taxa we did not have access to, would be needed to lend full support for such a reclassification. Nonetheless, the strong evolutionary divergence of ASAG and ASUM showed here suggest that these two mesic, forest species occupy a notable and unique genetic position and ecological niche within the genus *Astragalus*.

Conclusions

The size and degree of fragmentation of ASAG populations as this relates to the overall historic range of the species is unknown. So it’s difficult to conclude whether the indications of inbreeding depression found in this study represent a natural phenomenon, or if this is the result of recent range fragmentation due to forest management. In any case, it is highly recommended that on-going monitoring for inbreeding depression in the species be continued in the future. Unless periodic inspections of extant populations indicate a rapid decline in plant health, a reevaluation of inbreeding depression across major populations should be considered every 10 to 15 years.

Finally, the use of molecular data alone is not generally used to verify the existence of an undescribed taxon. However, the use of such data in conjunction with morphometric data, detailing exclusive morphological differences between populations, is appropriate in differentiating taxa (Meyers and Liston 2008). Preliminary evidence of morphological differences between the ASAG forms (including flower color and other floral traits), will be described in the next section. Based on a combination of the microsatellite and phylogenetic results, a naming of the pink-flowered variant (Figure 6), at the subspecific level, is recommended.

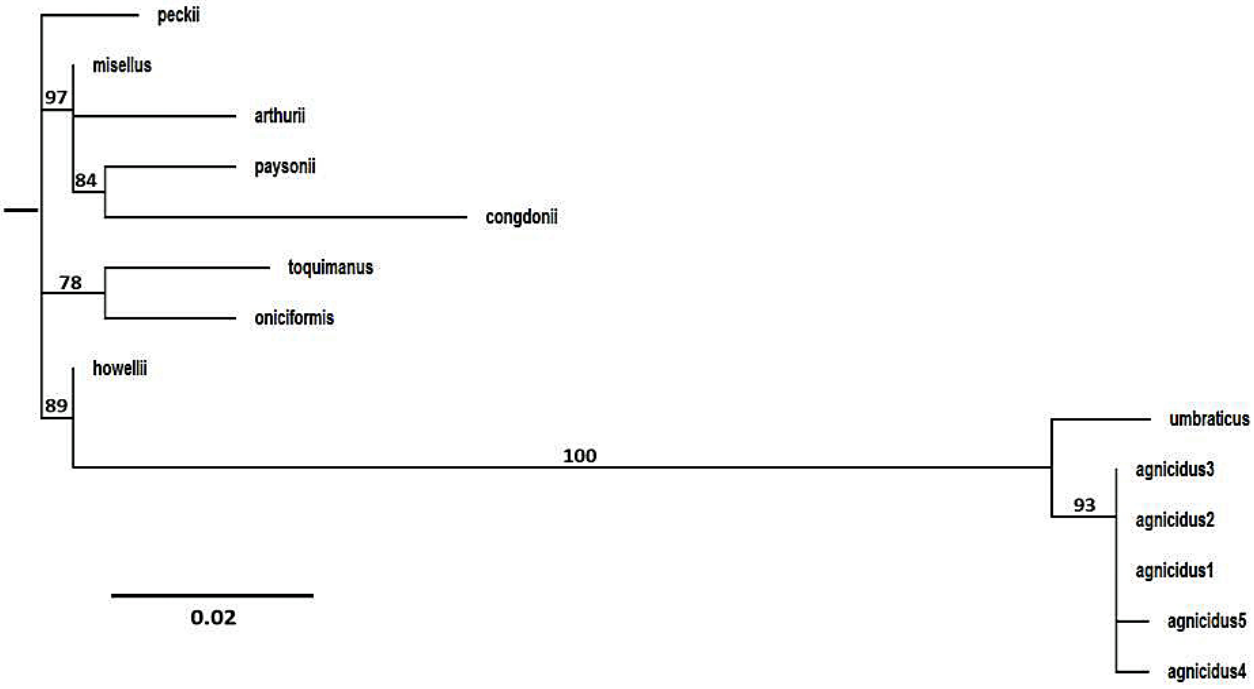

Figure 5. Maximum likelihood tree resulting from the phylogenetic analysis. Bootstrap values are shown above the branches. The scale bar is a reference for the number of changes along branches.

Figure 6. Flowers of the "pink" (left) and "normal" (right) forms of ASAG.

Morphometric comparisons

Our molecular studies of ASAG (presented in the previous section) suggest that a single population (ASAGpop5) from southern Mendocino County, located at the extreme southern edge of the species' range, is genetically different from plants in four other large populations that are distributed further north. The latter populations are characterized by plants with pure white corollas, while the anomalous population immediately stood out as "different" when discovered, based on the pinkish to purplish pigmentation in the flowers. Early questions about the relationship of these plants to "normal" ASAG populations incentivized, at least in part, the decision in 2007 to move forward with the current study.

Although molecular systematics and genetic evaluations have made tremendous contributions towards clarifying the treatments and understanding the phylogeny of many plant groups, morphological assessment remains the traditional mainstay of most taxonomic studies. Morphological studies generally form the basis for field classifications, and are necessary components if the taxonomy being developed is to be put to any practical use. Rarely do genetic or molecular evaluations result in the description of new taxa without at least some morphological corroboration. Moreover, for unique plant populations to be protected under state or federal endangered species laws, they must actually be described and published in the literature as distinct taxa, so morphologically-based taxonomic studies will continue to play an important role in conservation biology. Simple demonstration of genetic diversity is generally not enough for formal protection of plants under current legislation, unlike the allowances made for certain fish and wildlife species.

We planned to conduct a morphometric study of both ASAG and ASUM populations to lend support to the molecular data. However, since the main focus of the work was to evaluate ASAG interpopulation variability, and we had limited access to ASUM study sites, we decided not to include the latter species in the PCA (we could have used ASUM herbarium specimens, but elected not to mix fresh plants and pressed, dried plants in the analyses). Our evaluations of ASUM are limited to the molecular work. We used *principle components analysis* (PCA) to evaluate key morphological traits from ASAG plants in the field and greenhouse.

Principal components analysis

PCA is a multivariate statistical method available in the R statistical software package (R Development Core Team, 2007), and was used here to evaluate a selected group of quantitative and qualitative traits that were considered of potential taxonomic value, based on information from Barneby (1964) as well as observations made during this study. In this procedure, the investigator selects a range of characters to analyze, generally including those believed important to the taxonomy of the target group by earlier workers, as well as any important new traits that come to light during the course of exploratory research.

Usually 15-20 characters are measured at a minimum, typically including reproductive as well as vegetative features, depending on the focus species. PCA is useful in studies attempting to distinguish different taxa, because it avoids the need for preconceived assumptions about which populations are thought to represent which species. The procedure is good for teasing apart dissimilarities between populations, based on a suite of traits, even when the differences may not be readily apparent.

Statistically, PCA reduces the number of variables in the overall data set by forming linear combinations that explain most of the variability. That is, PCA is a factor analysis technique that reduces the dimensions of a set of variables by reconstructing them into uncorrelated combinations. The analysis combines the variables that account for the largest amount of variance to form the first principal component. The second principal component accounts for the next largest amount of variance, and so on, until the total sample variance is combined into variance in the total sample. All of the components are uncorrelated with each other. Often a few components will account for 75 to 90 percent of the variance in an analysis, and these components are the ones used to plot the data. In general terms, the analysis is designed to identify those morphological traits most helpful in distinguishing potential taxonomic differences, and will group measured units as data points on a scatterplot. Plotted data points may represent populations or individual plants, depending on the nature of the study and how the data is recorded.

Sampling

For this study, individual milk-vetch plants were measured for a series of traits from each of the study sites, following an approach similar to that used by Knauss (2010). During field sampling, we attempted to measure 20 characters from at least 20 plants per population, using a mm ruler or ocular micrometer, if needed (see Figure 7 for list of traits). However, it was not always possible to locate 20 suitable and representative plants for each population, largely due to herbivory and phenological considerations. To off-set this problem, we supplemented the field work with plants that we grew to maturity in the greenhouse at OSU. For these cultivated plants, we ended up measuring 22 traits for use in the PCA (see Figure 8), with the change in number (from the field sampling) a result of our reevaluating the analytic value of specific morphological features. We limited our measurements to field and greenhouse plants that were in good condition and exhibited all sample traits.

Three measurements per morphological character were made on each specimen whenever possible. Measurements for both field and greenhouse plants were made from different parts of the plant (i.e., different stems or racemes), with the goal being to focus on the most mature structures available (i.e., using fully flowering inflorescences, completely open flowers, mature fruit, etc., and avoiding plants in bud, flowers with evidence of herbivory or disease, and so forth). Although the sampling was not technically random, an effort was made to select structures for measurement on the different plants in a consistent yet arbitrary and unbiased manner. The measurements for each plant were then averaged, resulting in an arithmetic mean for each of the traits. Accordingly, each of the graphically depicted data points (in Figures 7 and 8) represent an individual plant.

PCA: field–measured plants

Figure 7 shows the results for the PCA run for ASAG plants measured in the field at the five study populations. We had very limited access to reproductive field plants for the pink-flowered plants from ASAGpop5, and only 2 individuals (after searching in both 2011 and 2012) were suitable for inclusion in this analysis. There were a few dozen plants at the site in both 2011 and 2012, but most were non-reproductive (the site was in a latter seral stage by the time of our work, and the population was quickly declining). ASAGpop5 plants are morphologically closest to ASAGpop3, although the two sites are not closest geographically. Overall, the data in Figure 7 indicate little, if any, morphological segregation among the five study populations.

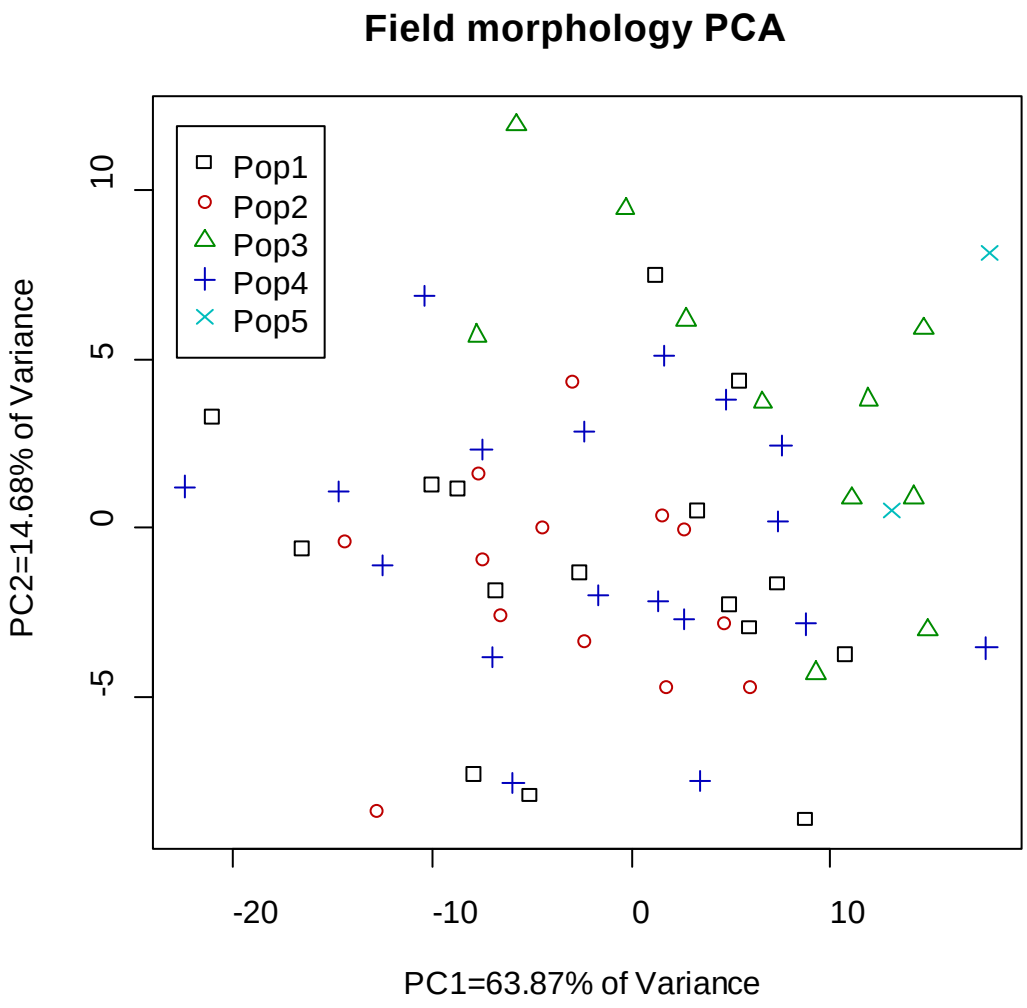

Figure 7. Scatter diagram showing PCA results (principle components 1 and 2, accounting for 78.55% of the total variation) for field samples from five ASAG study populations (see Figure 1 and Table 1). Each point represents a sampled plant that had all 20 traits available for measurement (including mature fruit). Morphological traits measured for each plant were: (1) stem pubescence (glabrous/villous/hirsute); (2) stem hair length (mm); (3) no. leaflets/leaf; (4) overall leaf length (cm); (5) stipule width at base (mm); (6) stipule length (mm); (7) terminal leaflet

length (mm); (8) terminal leaflet width (mm); (9) no. of flowers/raceme; (10) overall raceme length (cm); (11) pedicel length/mature flower (mm); (12) overall calyx length (mm); (13) longest calyx segment (=sepal) length (mm); (14) petal color (white vs. pink); (15) banner limb length (mm); (16) banner width (mm); (17) banner claw length (mm); (18) keel length (mm); (19) mature fruit length (mm); (20) mature fruit width (mm). See text for discussion.

PCA: greenhouse plants

The PCA analysis based on field-measured specimens (Figure 7) was disappointing on two fronts. First, it was difficult to get enough measurements of individual plants that included all 20 morphological traits that we wanted to use in our analysis. Phenological issues, as well as widespread ungulate herbivory, were serious obstacles. Secondly, our primary "target" population (ASAGpop5) was probably in the most advanced state of decline of all the study sites, having first appeared after substrate disturbance several years prior to our study, and by 2011 being reduced to just a few "hangers-on" as the population retreated back into the seed bank in response to succession.

To get around this, we collected seed in 2011 from as wide a range of parent plants as possible from the different sites, and completed test cultivation runs that year at OSU. Initial results were less than promising, although this was not altogether unexpected, since milk-vetch species are known to often grow best with soil symbionts such as *Rhizobium* or mycorrhizae (Barroetavena et al. 1998), and we had used sterile potting mix. We next grew plants from the various study sites in both uninoculated and inoculated soils (the latter created by simply adding native soil collected from around the roots of wild ASAG plants in California, with the hope that symbiont propagule would be present). The survival and performance of inoculated plants improved significantly, and subsequent examination of roots showed that these plants were heavily colonized with *Rhizobium* bacteria.

With a protocol established, we initiated cultivation efforts in late 2011 (in heated greenhouses with 12 hour photoperiod) to support our PCA work, which we had originally planned to be based on field-collected material. Although perennial, as a gap follower ASAG grows quickly and starts flowering within a few months. By mid-summer, 2012, we had many flowering and fruiting plants in cultivation that could be used to supplement the morphometric data taken from wild plants (Figure 9). A particular plus was being able to grow more plants of the pink-flowered ASAGpop5 study population, which was scarce in nature. Although only two wild plants were suitable for inclusion during field sampling (Figure 7), several plants at the site that were overall unsuitable for measurement did produce limited seeds, and we were able to grow plants from six field parents.

Growing plants in the greenhouse allowed easy access for weekly or even daily measurements of sample plants (contrasted with 10 hour drives from Corvallis to get to the California field sites), and also permitted extended, up-close study of the plants throughout their flowering phenology. The latter resulted in a slightly amended suite of traits for use in the new PCA (see Figure 8), with added focus on certain floral characters and the number of fruits produced in the absence of pollinators.

The data in Figure 8 are markedly different than that depicted in Figure 7. While the greenhouse plants still lacked any segregation among the white-flowered populations

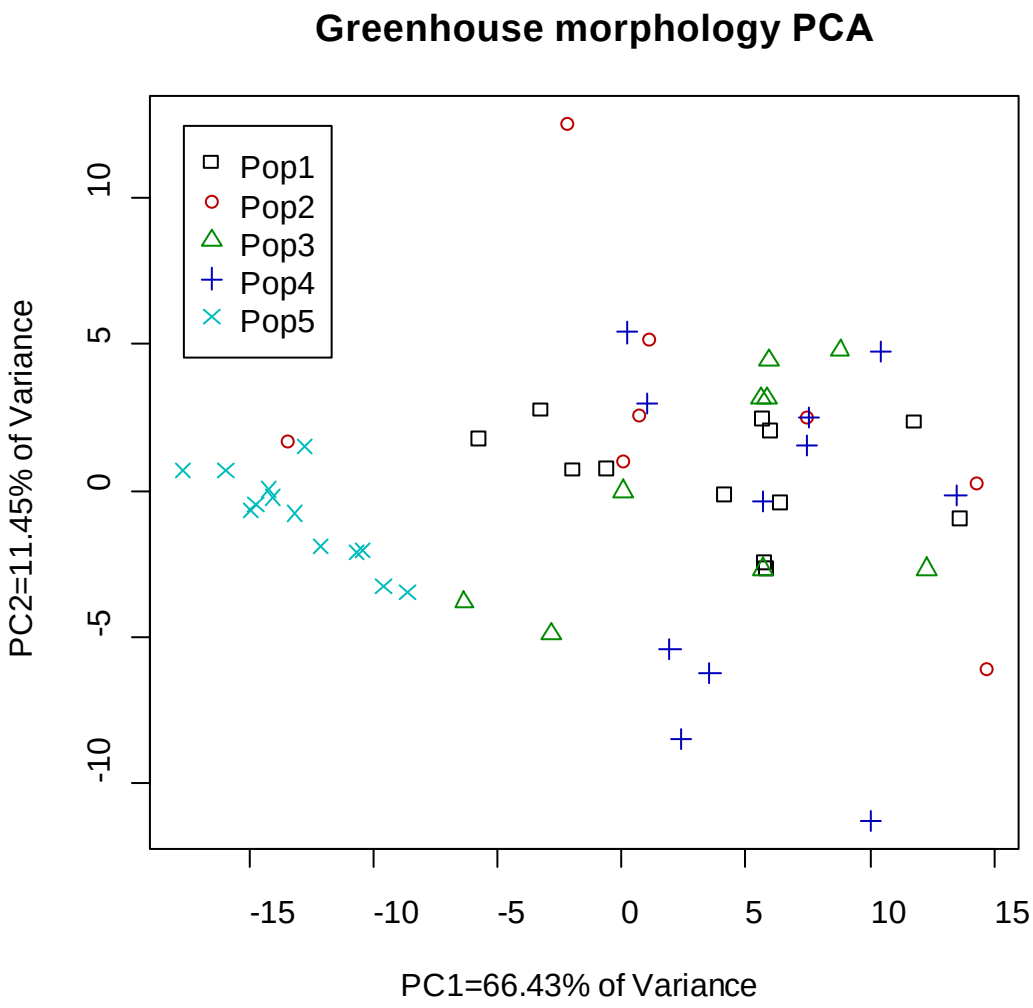

Figure 8. Scatter diagram showing PCA results (principle components 1 and 2, accounting for 77.88% of the total variation) for greenhouse samples from five ASAG study populations (see Figure 1 and Table 1). Each point represents a sampled plant that had all 22 traits available for measurement. Morphological traits measured for each plant were: (1) stem hair length (mm); (2) no. leaflets/leaf; (3) overall leaf length (cm); (4) stipule width at base (mm); (5) stipule length (mm); (6) terminal leaflet length (mm); (7) terminal leaflet width (mm); (8) no. of flowers/raceme; (9) overall raceme length (cm); (10) flowering portion of raceme length (cm) (11) pedicel length/mature flower (mm); (12) calyx tube length (mm); (13) longest calyx segment (=sepal) length (mm); (14) petal color (white vs. pink); (15) banner limb length (mm); (16) banner width (mm); (17) banner claw length (mm); (18) keel length (mm); (19) wing length (mm); (20) mature fruit length (mm); (21) mature fruit length; (22) no. of mature fruits per raceme. See text for discussion.

(ASAGpop1 through -4), the pink-flowered plants from ASAGpop5 are clearly set apart morphologically. This change probably has to do with both the additional plants we had to work with in the greenhouse, as well some distinctive new traits used in the greenhouse PCA, including added raceme characters, fruit production, calyx measurement modifications, and wing petal measurements.

Figure 9. ASAG plants under cultivation in the OSU greenhouses (August, 2012).

Taxonomic conclusions

The morphometric data presented in Figure 7 support the population genetic conclusions, which suggest that the unusual pink-flowered form of ASAG merits taxonomic recognition, probably at the subspecific level. The new variety can be distinguished from related California taxa by the following key.

Suggested *key* to the forest species of *Astragalu*s, section *Miselli*, from cismontane northern California (from our observations, and Barneby 1964):

1. Stems and foliage glabrous or seemingly so, with any hairs strongly appressed and short; ovary and pod glabrous; Klamath region in California, north sporadically into the Cascades and Coast Range of western Oregon……..……..*A. umbraticus*
1. Stems, foliage, and calyces villous to pilose, hairs spreading and not appressed; ovary and pod clearly pubescent

2. Inflorescences lax, open, tending towards secund in fruit; raceme axis clearly visible between flowers; calyx teeth subulate to triangular, <3 mm long; pod densely

villous and shaggy, >2.5 cm long, obviously short-stipitate; ovules 23-29; limited to lower elevation Sierra Nevada foothills, California……*A. congdonii*

2. Inflorescences compact, even in fruit; raceme axis scarcely visibly between most flowers; calyx teeth linear to narrowly acuminate, typically 3.0-6.0 mm long; pods thinly villous-pilose, <1.5 cm long, stipe almost imperceptible; ovules <15

3. Corolla pure white (Figure 10); calyx green, tube ca. ⅓ shorter than the longest calyx segment; racemes dense, flowers heavily overlapping, the most floriferous 20-50 flowered; flowers moderately to strongly autogamous in cultivation; limited to Mendocino and Humboldt counties, California……*A. agnicidus* var. *agnicidus*

3. Corolla pink (Figure 11), especially the lower banner; calyx green with red pigmentation, tube the same length as the longest calyx segment (or up to 0.8 mm shorter); racemes moderately dense, the most floriferous usually <20 flowered; flowers rarely produce fruit autogamously; known only from southern Mendocino County, California (northeast of Gualala)……*A. agnicidus* var. nov.

Figure 10. Typical ASAG (below, left), showing pure white flowers and crowded raceme.
Figure 11. Variant ASAG plant (right), showing pink flowers and less congested raceme.

At a glance, *A. agnicidus* var. nov. is readily distinguished by its pinkish-tinged corollas (often with bright pink visible on the lower dorsal surface of banner petals), as well as its calyces suffused with red (Figures 6, 11, and 13). The floral coloration is unique in its otherwise white-flowered species group within section *Miselli*, which also includes *A. paysonii* (Rocky Mountains), and the Mexican oak-pine woodland species *A. ervoides* and *A. sinaloae*. Barneby (1964) indicates that *A. carminis* (isolated in the Mexican state of Coahuila), the next closest species to this group in his taxonomic scheme, is "technically similar (to the above species) except that the flowers are pink." *A. agnicidus* var. nov. now represents the second taxon in this species complex with pink flowers.

Figure 12 (top). Typical ASAG floral form, showing elongate calyx teeth that equal or exceed the keel length (visible through the wing petals), and exceed the length of the calyx tube.
Figure 13 (bottom). Pink-flowered variant ASAG, showing calyx segments that are shorter than the keel length, and approximately **t**he same length as the calyx tube.

The new variety is further distinguished from typical ASAG by slightly larger floral dimensions, including a longer, narrower banner, and longer wings and keels (the latter equal to the longest calyx segment in white-flowered plants, compared to 1-3 mm longer than the calyx in the new variety). The best key characters include the relative length of the calyx teeth to the calyx tube length (about equal in the new variety, averaging roughly 30-40% longer in typical ASAG populations), as well as raceme structure and floral production.

Cultivating pink- and white-flowered plants side by side in the greenhouse enabled us to not only evaluate them under identical growth conditions, but it also allowed us to grow them to maximum size and vigor. Under cultivation, we noted that typical ASAG always outproduced pink-flowered plants in terms of flowers and fruits per inflorescence. The largest racemes of the latter taxon produced markedly fewer flowers than white-flowered plants from any of the study sites (see Figures 10 and 11), typically 20 or fewer for the new variety (mean = 17.1 ±2.9, n=39), and usually 25-50 for typical ASAG (mean=31.8 ±6.8, n=120). It's worth noting that mature ASAG plants can produce many racemes over the course of a flowering seasons, and both white- and pink-flowered plants developed small as well as large racemes. Comparisons here have focused on the largest inflorescences, in an attempt to compare the *maximum reproductive potential* of the two varieties as reflected by flower production.

The racemes of white-flowered plants were also more congested (Figure 10), with the raceme axis seldom visible between the flowers. Although not to the degree of *A. congdonii*, for example, pink-flowered ASAG racemes were clearly less congested than typical ASAG (Figure 11), with the raceme axis often visible between the lower flowers. We measured both the numbers of flowers per raceme (above), and the length of an inflorescence that actually included flowers (pink mean=4.7 cm ±0.7, n=39; white mean=5.1 cm ±1.3, n=120). With the average length of the flowering portion of white-flowered racemes only exceeding that of pink-flowered inflorescences by 0.4 cm, it's clear that the more congested inflorescence architecture of white-flowered plants, and not raceme length, accounts for the large difference in flower production.

Finally, although fruit production is governed as much or more by chance (as it requires pollination) than genetics, the number of fruit produced can still have taxonomic value. In the greenhouse, we discovered that white-flowered ASAG plants produced far more fruits than the pink-flowered plants growing with them. Not all of the white flowers produced pods in the greenhouse (8.7 ±4.3 fruits/raceme, n=120), but many did. In contrast, almost no pink-flowered plants produced fruit (0.3 ±0.9 fruits/raceme, n=39). Although the greenhouse was supposed to be free of insect pollinators, it's remotely possible some may have entered, potentially contributing to fruit set in white-flowered plants. However, white- and pink-flowered plants would have presumably stood equal chances of being pollinated if this had happened, and yet there was a substantial difference in fruit set between the floral types. So we're left with the conclusion that white flowers are more capable of spontaneous self-pollination than the larger pink flowers, possibly due to some aspect of floral architecture. It's admittedly not a particularly good character for use in field identifications, but still a significant biological difference between the varieties that further supports taxonomic separation.

Chromosome counts

Methods

ASAG root tips were examined from five greenhouse-grown, potted plants representing three populations (ASAGpop2, -4, and -5). From within each root preparation five cells, with intact cell walls, were surveyed for the number of chromosomes. [Note that we were unable to obtain chromosome counts for ASUM.]

The distal ends of freshly excised root tips from rapidly growing ASAG roots were saturated in PDB at 4°C for four to six hours. After rinsing in water, the pretreated root tips were soaked in Carnoy's solution for 30 minutes, and then hydrolyzed in HCl for 60 seconds. The roots tips were then transferred to a slide, and a small drop of acetocarmine was added to the tissue. Cover slips were "squashed" down, and cells were subsequently viewed at 1000x using a Leica DMLS compound microscope.

Results

All readable cells revealed a chromosome number of 2*n*=16. An example of the cells surveyed (from ASAGpop5) is illustrated by the photograph in Figure 14. While the anomalous ASAGpop5 with the pink flowers is clearly differentiated from the rest of ASAG by genetics and morphology, all populations of the species appear to share the same chromosome number.

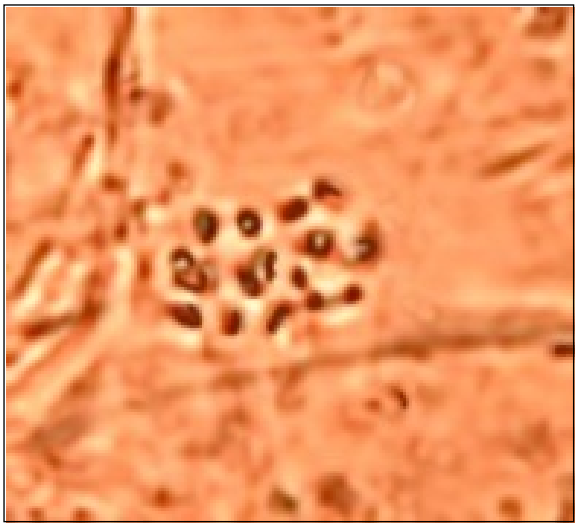

Figure 14. Mitotic cell division in root-tip of ASAG greenhouse plant, showing 2n=16.

Insect floral visitors

Methods

We observed (or attempted to observe) insect floral visitors on ASAG and ASUM flowers for several afternoons at each study site (except ASAGpop5) in June and July of 2011 and 2012. Observations at ASAGpop5 were limited to brief observations on two days. Nothing relating to pollination was observed there due to the very small number of reproductive plants present (this issue has been discussed earlier in the report). Accordingly, the following results and discussion pertain only to the white-flowered populations (i.e., ASAGpop1-4).

Generally, pollinator observations were scheduled in half day increments, mostly in late morning and early afternoon. The coastal ridgeline habitats frequented by ASAG were commonly misty or overcast, especially in the mornings. Pollinators generally were most abundant on warm, windless, sunny days, and the chilly damp weather we experienced at some sites often decreased the abundance of potential pollinators at the time of observation. Also, the phenology of flowering for each study site was different (with Mendocino flowering earlier than Humboldt sites), reducing the ability to observe and collect data for pollinators from every location during each trip to the area.

Floral visitors and their actions were recorded, with representative specimens collected by net and transferred to a kill jar containing ammonium carbonate crystals (Figure 15). Ammonium carbonate produces carbon dioxide when exposed to moisture (such as the moisture produced by the respiration of a trapped insect), which smothers the insects quickly without damaging their physical characteristics. The specimens were then pinned, allowed to dry, and later identified with the help of the Oregon State Arthropod Collection staff, and entomologists at ODA.

Figure 15. Insect collections from ASAG study site in Humboldt County (July, 2011).

Sample visitation rates were determined by counting the number of each type of floral visitor during ten minute intervals. A total of 110 minutes of observations were taken at three field sites (ASAGpop1, -2, and -4), during which time every floral visitor was

recorded. Insect behavior was observed to determine the 'fidelity' of the insects visiting the plants. After the insect landed on a flower, the insect's behavior was recorded as follows:

1. The insect moved to another flower on the same plant (geitonogamy[4])
2. The insect moved to another flower on a neighboring ASAG plant (allogamy[5])
3. The insect moved to another flower on a different species, or flew out of sight

Results

The apparent primary pollinators of ASAG (and ASUM) included at least 3 species of *Osmia* (Figure 17)(solitary bees) and three species of *Bombus* (bumblebees), including *O. dolerosa, laeta, tristella,* and *B. sitkensis, vosnesenskii* (Figure 16)*,* and *mixtus.* These species are well-suited to pollinate legume flowers, and pollen loads carried by both *Osmia* and *Bombus* matched ASAG reference pollen (the identity of which we established using a scanning electron microscope at OSU). The bumblebees, in particular, can fly long distances, at the very least between neighboring ASAG patches within larger populations. Whether they could transfer pollen between distant populations is doubtful. *Osmia* bees are probably very local in their visitation. Other floral visitors were also observed, such as syrphid flies (family Syrphidae), wasps (order Hymenoptera), and hoverflies (*Bombillious major*), but these were considered to be incidental pollinators at best.

Principle pollinators of ASAG flowers. **Figure 16** (left*). Bombus vosnesenskii*. **Figure 17** (right). *Osmia* sp.

[4] Genetically self-pollination, but not autogamy (which is self-pollination without an insect vector)
[5] True out-crossing

Geitonogamy, or moving to another flower on the same plant, was the most common method of pollination observed (Figure 18). ASAG plants can be large (multiple stems up to 8 or more dm long) and floriferous (dozens or even hundreds of flowers open at once), so high levels of geitonogamy would seem unavoidable. As noted earlier (in the Morphometrics section), ASAG is self-compatible, so this visitation pattern is not necessarily detrimental. Between the two major pollinator types, i.e., *Osmia* and *Bombus*, about 73% of pollination events were geitonogamous.

The second most common method of pollination observed was allogamy, or moving to a flower on a different plant of the same species. This cross-pollination is genetically important, as it helps reduce inbreeding. Without crossing, inbreeding depression, already shown to be present in populations (see the Population Genetics section) may become even more prevalent. Between *Osmia* and *Bombus*, about 20% of pollination events involved floral visits from one plant to another.

Lastly, a non-faithful visit, i.e., moving to another plant species (or a movement where the insect disappeared before its destination was established by the recorder) was the least common among observation, making up about 7% of pollination events.

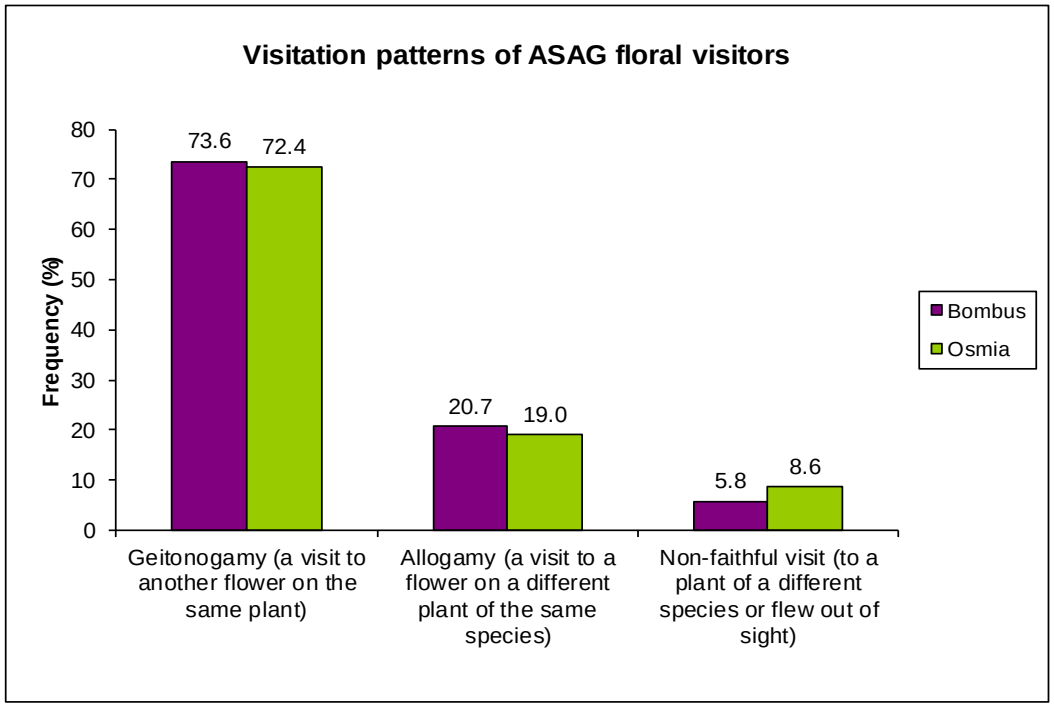

Figure 18. Post-visit behavior of ASAG floral visitors. See text for discussion.

Of the two major pollinator groups, *Osmia* species made about 7 visits per ten minute observation period, whereas *Bombus* made about 2.5 visits per ten minute observation period (Figure 19). However, these numbers may be deceiving. During the early-season observations, *Osmia* visits were far more prevalent than *Bombus*, while during the late-season observations the reverse was true. These two opposing observations did not balance each other out, because there were far fewer total

pollinators present in the late-season observations, thus creating a dataset that suggests *Osmia* were of much greater pollination importance than *Bombus*. More observation would be needed to confirm which pollinator is more important. Suffice to say, both are probably valuable to seed set in the species.

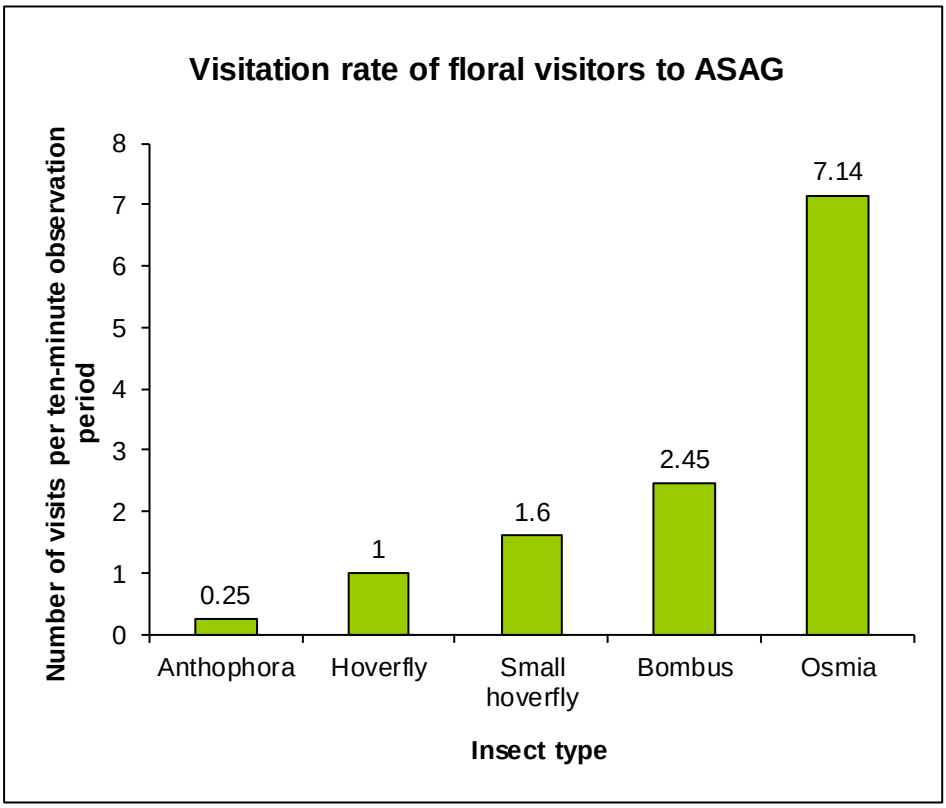

Figure 19. Floral visitation by principle pollinators of ASAG. See text for discussion.

Life history observations

Plant longevity and survival

Our observations support the previous reports describing the seral life history of ASAG populations. Plants are known to germinate after disturbance, form sometimes extensive populations (depending on the site), and then vanish within a few years as forest succession recreates an intact canopy and understory at the site. We were able to observe populations in different demographic stages in 2011 and 2012, although none were recently emerged. From 2011 to 2012, we estimate populations had declined by a third to a half in terms of reproductive individuals. Based on when these sites were first reported, it appears that individual plants probably rarely live more than five or six years.

Survival of populations, conversely, is most likely very long-term. The first known population of the species, described by Barneby (1957), was extirpated (above ground, at least) in 1957, not to appear again for 30 years (Berg and Bittman 1988). Based on this

well-known report, and subsequent observations of the species elsewhere, ASAG appears to spend the vast majority of its existence in the forest seed bank, where it is evidently capable of surviving for decades until sprouting in response to fire or logging. How many "hidden" populations of the species exist is anyone's guess.

Flower and seed production

Immediate and successful reproduction is especially key to the success of plant species that only appear intermittently in response to disturbance. Populations take a risk just by germinating, as this depletes the seed bank without any guarantee of replenishment. Many disturbance followers hedge their bets by attempting to produce large plants and high numbers of seed, even if this strategy may be more apt to attract seed predators or other herbivores. ASAG appears to be one of them.

In the greenhouse, we noted that white-flowered ASAG outproduced pink-flowered plants in terms of flowers per inflorescence ($mean_{white}$ = 31.8 ±6.8, n=120 *versus* $mean_{pink}$ = 17.1 ±2.9, n=39). Although white-flowered plants produced almost twice as many flowers in a raceme as the pink-flowered plants (Figures 10 and 11), both floral types were very fecund and are clearly geared for substantial seed output under optimal conditions. Pink-flowered plants did not produce much fruit in the greenhouse, but the white-flowered plants produced an average of 8.7 (±4.3) fruits/raceme (n=120) under cultivation. Remember, however, that this was based on measurements of the three largest inflorescences on plants, so we felt it may not be representative of the reproductive output in nature.

To get an idea of reproduction in the field, we randomly selected white-flowered plants at the four study sites (plant size had no bearing, and the only condition was they had to be reproductive), and measured the number of inflorescences per plant, the number of flowers per inflorescence, and then the number of fruits produced by an inflorescence. Since we needed to sample at different times over the summer, the same plants were not necessarily available for all three sets of measurements. We also lumped the data for all 4 sites. Our overall sample size for each trait was 62.

Wild plants produced a mean of 26.4 (±32.5) inflorescences per plant, 23.7 (±12.0) flowers per inflorescence, and 16.7 (±8.4) fruits per inflorescence. Compared to the greenhouse-grown plants, natural populations produced 25% fewer flowers per raceme, but almost twice as many fruits per inflorescence. We didn't count the number of racemes produced by greenhouse plants, so have no comparison there.

The increase in fecundity for field plants makes sense if we assume that even though ASAG is genetically self-compatible, and can self-pollinate to a degree (based on the greenhouse work), optimal pollination is facilitated through visiting insects. White-flowered plants grown in cultivation (without insect pollinators) only produced pods on 27% of their flowers, while those we sampled in the field produced fruit a (comparatively) whopping 70% of the time. What this tells us is that even an isolated ASAG plant can probably manage to produce seed, if pollinators don't notice it, and that large patches of plants (generally more attractive to insects) can presumably ratchet up the reproductive effort significantly over self-pollination.

Finally, we sampled pods of white-flowered plants and counted the number of seeds they produced, and calculated a mean of 4.7 seeds (±2.4, n=900) per fruit. So in terms of what we might expect to be returned to the seed bank after an ASAG population has run its course, a hypothetical population of 1,000 reproducing plants in existence for 5 years could potentially generate 10,360,680 seeds (calculated from our means: 26.4 racemes per plant x 16.7 pods per raceme x 4.7 seeds per fruit x 1,000 plants x 5 years). Even if the majority of these are eventually lost to predation or disease over the years, it's not hard to see how ASAG seed banks can persist for long intervals between disturbances.

Pre-dispersal seed predation and herbivory observations

Risk factors that can impact seed bank regeneration in ASAG after mass germination events include predation of flowers and fruit by insects, and loss of foliage and flowers to ungulate herbivory.

To investigate these threats, pods from three field sites (ASAGpop1, -2, and -4) were selected in a non-biased way and returned to the lab for later examination. Using a random number generator, pods were then randomly selected for dissection from those collected (n=262 pods sampled for all three sites). Any viable seeds present and not destroyed were returned to the field site. Pre-dispersal seed predation evidence was then recorded in one of four categories:

1. Frass (insect debris) found in pod
2. Larvae found in pod (often inside a seed full of frass)
3. Chewed ends of pod (with ovules destroyed)
4. Oviposition site

The percent of pods damaged by predation was determined as the percentage of pods exhibiting one of the four evidence types (above) out of the total number of pods sampled.

To estimate the damage to ASAG populations from browsing by deer or elk, we used a three-level intensity rating for herbivory (n=80 plants randomly selected from those same 3 study sites). Stems were rated as having *no damage*, *having partial damage*, and having *complete damage*. No damage meant no evidence of herbivory was present, partial damage meant that some herbivory was evident (but some inflorescences were still present), and complete damage meant that the plant was browsed until there were no inflorescences left. The mean of these three intensity levels was compared across the three populations.

Results

Pre-dispersal seed predation was recorded from flowers at all three study sites. Frass was the most common seed predation evidence, found in 14.6% of the sampled pods, followed by live or dead larvae, found in 11.1% (Figure 20). Two types of larvae were found alive in the pods. One was a moth caterpillar (Lepidoptera), and the other a weevil larvae (Curculionidae). Other members of the weevil family were found both live, on inflorescences, and dead, in pod collection bags. Weevils are known to be seed predators, and are commonly found feeding on or mating in seed bearing plants.

After one week of inadvertent incubation in the lab, dozens of tiny wasps hatched inside of the pod collection bags that still contained some unsampled fruits. These were identified as members of the Scelionidae, a group of wasps known to be insect egg parasitoids, which are commonly used as biocontrol agents for agricultural crops (Alim and Lim 2009). The wasps are not harming the plants, but are probably focusing on populations of seed predators, such as the weevils.

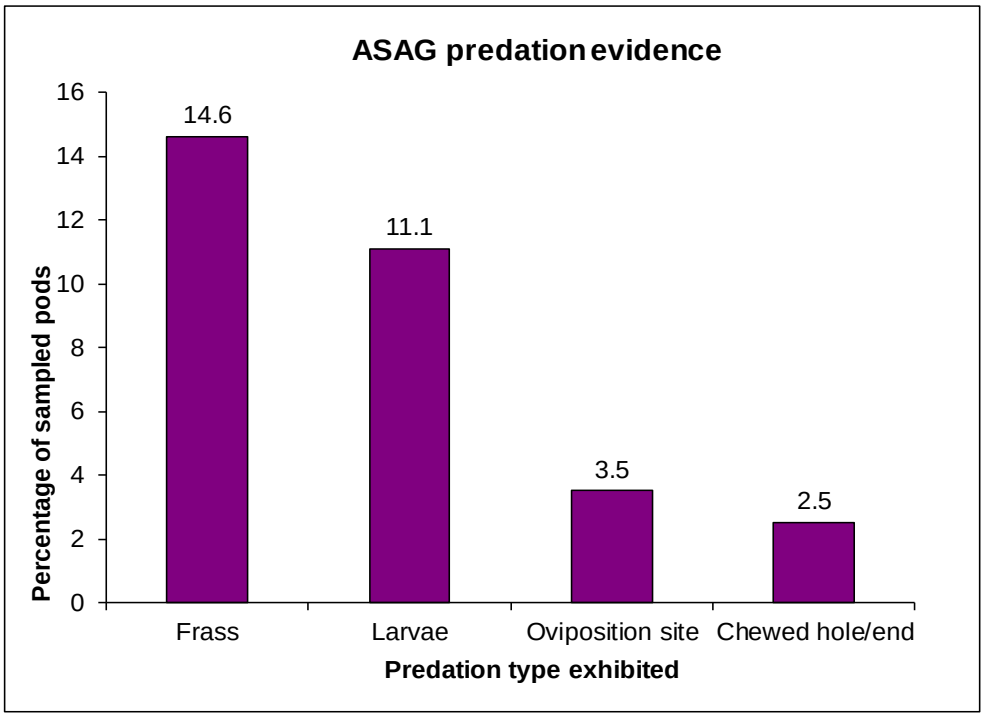

Figure 20. Pre-dispersal damage to ASAG fruits by fruit predators, by damage class. See text for discussion.

Herbivory was observed in all three ASAG populations sampled (Figure 21). Browsing intensity varied by site and year over the course of the project, and this snapshot from 2011 shows that populations can be significantly impacted by mammalian herbivores. Efforts to exclude deer or elk for the short periods that populations are above ground and reproducing may be one of the most important conservations measures to ensure adequate reproduction and seed bank protection.

Seed germination and viability

In addition to collecting data on seed production, we wanted to know if the seeds ASAG produces are generally viable, especially in light of the potential for inbreeding depression reported here earlier (which can depress the germinability of seeds in some cases).

To investigate seed viability and germination requirements, seeds were collected from open-pollinated plants from study site ASAGpop3. All seeds used in this evaluation

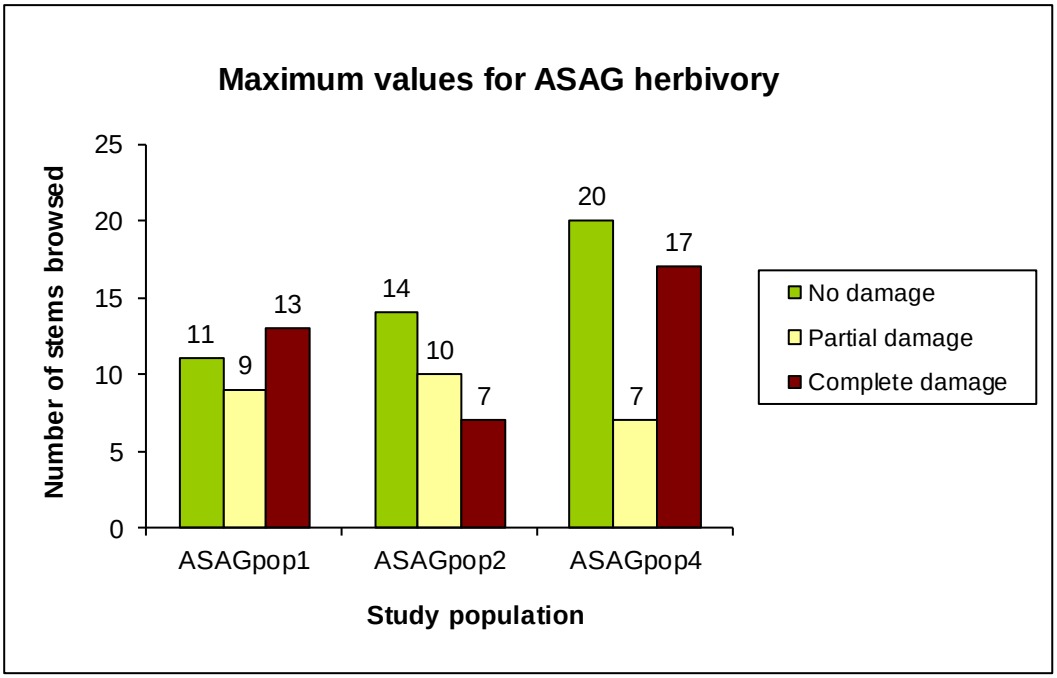

Figure 21. Browsing of ASAG by deer and elk at three study sites See text for discussion.

were first scarified by nicking the seed coats with a scalpel to facilitate imbibition (a common practice when conducting germination tests on hard-seeded legume species such as milk-vetches). Six field-collected maternal lines were represented from study site ASAGpop3 to enhance diversity in the sample, with one batch of seed (n=10) from each mother used in each of three treatments. Each line was kept in separate, well-watered petri dishes that were lined with filter paper.

One set of six petri dishes (equaling 12 dishes total, representing the six wild maternal lines) was exposed to each of three treatments: *ambient greenhouse conditions* (varying between roughly 14 and 30°C night and day, though not constant); *stratification in a 4°C coldroom*; and *constant coldroom stratification set to alternate* (at half-days) *between 15 and 25°C*. The seeds receiving stratification were chilled at 4°C for three weeks, and then placed in ambient conditions in the greenhouse.

Results

Germination data are shown in Figure 22. There was a statistically significant difference between treatment groups as determined by one-way ANOVA (F=12.99, p=.0005). An LSD (95%) post-hoc test confirmed that the germination percentage for seeds that were chilled at 4°C was significantly higher than for seeds exposed to the ambient or alternating temperatures. There was no statistically significant difference between the latter two treatments.

Of the three treatments, the 4°C stratification was most successful, with 98% germination. A few stratified seeds germinated while still in the cold room, but most germination took place (in a short burst) after being placed in the warmer greenhouse. Seeds kept at ambient greenhouse temperatures and in the alternating coldroom

temperatures began to germinate within the first week, achieving 43% and 38% germination, respectively. They stopped germinating after two to three weeks.

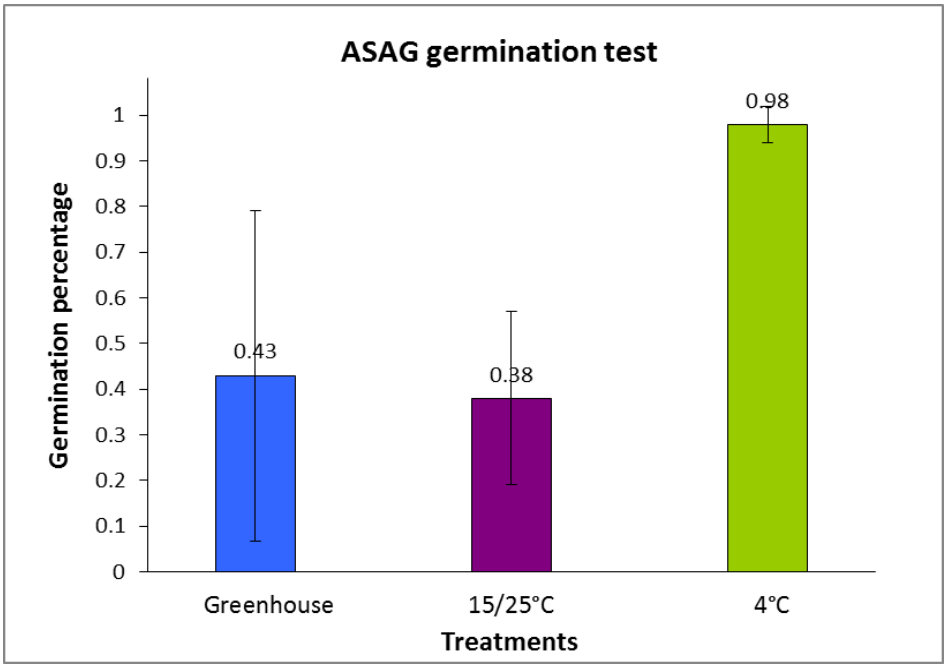

Figure 22. Germination results for ASAG seed exposed to three treatments. See text for details.

As previously recommended by Bencie (1997), cold stratification and scarification are both beneficial for germination. While cold stratification is not strictly required, exposure to colder temperatures appears to enhance and intensify the germination response in the species. A correlation between higher germinability and cold winter temperatures is especially common in species from areas with dry summer climates (deserts, Mediterranean, etc.). However, being disturbance followers (and growing in sunny openings), ASAG populations do appear in relatively drier and hotter microsites within the redwood forest, so perhaps this is not so surprising. The 98% germination achieved under the cold-stratified treatment is impressive, and suggests that many (if not the great majority) of ASAG seeds that are produced in nature enter the seed bank as live and viable seeds. It would be interesting to expand this study and include additional populations, and (especially) seeds produced by autogamy in the greenhouse, in light of the potential for inbreeding depression in highly selfed populations.

Long-term populations trends

An initial goal for the project was to try and establish long-term monitoring plots that could be used to track population trends and condition over time. As we learned more about the species and its habitat, it became apparent that this was both impractical and unnecessary. As discussed earlier, ASAG is a species that responds to disturbances that create forest openings. Plants germinate and grow rapidly, reproduce for one or

several years, and then populations fade as the habitat shifts back to a closed canopy forest through succession.

The goal of long-term monitoring of rare plants is generally to try and establish, with the aid of a technique such as population viability analysis (PVA), whether populations are declining, increasing, or holding steady, and then, if possible, to link these trends to local environmental conditions. In the case of threatened or endangered species, we might try to establish a connection between population status (and projected demographic status) and a particular land use action (or inaction), such as grazing, logging, fire suppression, etc. (see Lesica 1995, for an example, and Menges 2000).

With ASAG, the life history of the species largely dictates the direction populations will trend. They will appear, flourish, and then die out and return to the seed bank, unless disturbance at the site continues, in which case population decline may be postponed. But the end result is inevitable. Monitoring this progression via a series of plots may document the phenomenon at a given site, but it's unlikely to provide us with information that we essentially didn't already know.

Probably the simplest alternative to a plot study, for maintaining a demographic record for the species, is to simply keep track of populations and their sizes as they occur, by conducting a yearly census from the time a population first appears until it has pretty much disappeared. By adapting the approach described here, an annual estimate could be made concerning how much seed is being produced, relating this to any obvious threats (such as herbivory), and then determine whether or not supplemental disturbance (through grading, fire, or whatever seems appropriate and feasible) or protection of the site (e.g., via enclosures to eliminate browsing) might enhance short-term seed production.

The most serious threats to the species are (1) exclusion of periodic site disturbance, which is needed to stimulate growth and reproduction, and (2) anything that interferes with seed production when populations are in their active, reproductive phases. For the most part, ASAG can probably co-exist in harmony with current land management practices in the montane redwood forest the species inhabits. Active forest management and harvest activities that open up sites are clearly associated with appearances of the species (evidently mimicking infrequent natural disturbances such as wind storms and fire), but these are largely over with by the time ASAG is reproducing and setting seed.

The goal of land managers should be to focus on post-harvest protection of sites through fencing where necessary (to reduce seed loss to browsers), and continued short-term maintenance of roads and landings, to extend the flowering and reproductive period for populations. Areas known to have harbored populations, and which have presumably reverted to seed bank status, should excluded for major construction projects, or anything that would physically alter the site to such an extent that ASAG could not grow there in the future. This holds especially true for the novel variety of ASAG (ASAGpop5), known from the single location near Gualala.

Overall, the investment in both time and money to effectively manage ASAG should be minimal. The forest practices currently in place at Mendocino and Humboldt Redwood Companies, and the California Department of Forestry, seem sufficient for the long-term conversation of the species.

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