Genetic structure of three Endangered Plants of the Santa Rosa Plain: Burke's goldfields (*Lasthenia burkei*), Sonoma sunshine (*Blennosperma bakeri*), and Sebastopol meadowfoam (*Limnanthes vinculans*)

Debra R. Ayres
Section of Evolution and Ecology
University of California, Davis, CA 95616

Christina M. Sloop
Laguna de Santa Rosa Foundation
P.O. Box 7886, Santa Rosa, CA 95407

Prepared for:
Mary Ann Showers
(mshowers@dfg.ca.gov)
Lead Botanist
Habitat Conservation Division
California Department of Fish and Game
1416 Ninth Street, Room 1280
Sacramento, CA 95814

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Abstract:

Burke's goldfields (*Lasthenia burkei*), Sonoma sunshine (*Blennosperma bakeri*), and Sebastopol meadowfoam (*Limnanthes vinculans*) are herbaceous annual plants that inhabit vernal pool and swale habitats mainly on the Santa Rosa Plain of Sonoma County, California. All are Federally- and State-listed as endangered. The goal of our research was to determine the genetic variation between populations in order to inform conservation efforts as to the distinctiveness of populations for seed banking and *ex situ* conservation, and to infer possible explanations for genetic subdivisions to aid in management decisions. Using 15 microsatellite markers we genetically surveyed 21 populations of *L. vinculans*, genotyping 577 individuals. Our results indicate a substantial divergence and limited gene flow between Santa Rosa Plain (SRP) populations and an outlying Napa county population (mean F_{ST} = 0.180). Among SRP populations genetic variation is significantly lower (F_{IT} = 0.052), with most (82%) of the overall SRP population variation explained by within population genetic variation. *L. vinculans* shows relatively high genetic diversity at all sites (average H_{exp} = 0.65 ± 0.19, average H_{obs} = 0.53 ± 0.23, average number of alleles per locus = 7.419 ± 3.748), suggesting a large effective population size and an outcrossing breeding system for this species. We examined 307 *L. burkei* plants from 15 populations. We used 42 inter-simple-sequence-repeats (ISSR) and random amplified polymorphic DNA (RAPD) nuclear DNA markers to measure genetic characteristics. Species wide, we found that geographically separate populations of *L. burkei* were genetically distinct (F_{ST} = 0.22), using Analysis of Molecular Variance (AMOVA). Half of this variation (F_{ST} = 0.11) was due to the difference between Lake and Sonoma County populations; the other half was due to variation within county populations. (also F_{ST} = 0.11). We were able to examine only 64 individuals of *B. bakeri*, scattered among five populations, due to unforeseen extraction problems resulting in the poor quality of 80% of our DNA samples. Using 27 ISSR and RAPD markers, our preliminary assessment was that regional and local patterns of genetic variation exist in this species that merit further study in 2008 and are of conservation concern. Our troubleshooting of the extraction protocols has suggested areas where we can improve our methods so as to improve DNA quality in the 2008 survey.

INTRODUCTION

Population decline in most species is due to the degradation and loss of their habitats, often including diverse microhabitats (Foin *et al* 1998). Many species of plants are associated with distinct microhabitats (Maliakal-Witt *et al* 2005). Due to both adaptation and genetic drift, changes in habitat are often accompanied by genetic changes in plant populations (Hufford and Mazer, 2003, Soule and Mills 1998). Where microhabitats occur as discrete and small-scale areas, they create an opportunity for small-scale genetic divergence (Frankel and Soule 1980).

The differential distribution of genetic variation in plant populations (genetic structure) develops over time due to adaptation, genetic drift, gene flow and natural selection (Slatkin 1987, Futuyama 1986). Gene flow is determined primarily by the dispersal of pollen and seed. When gene flow is reduced, isolated populations diverge both due to natural selection and the chance effects of genetic drift. If gene flow is a common occurrence, the divergence of genetic structure is reduced. By analyzing differences in genetic structure between populations of plants, using selectively neutral markers, taking into account their breeding system and observations of potential causes of gene flow, we can determine the degree of genetic isolation between populations and the relative rate of microevolution between them. If genetic data are assessed relative to potential influences on genetic structure, results can be used to inform conservation efforts to maintain or simulate ecological phenomena that have played a role in the microevolutionary development of the species. Further, analysis of the genetic structure of populations can inform our conservation decisions with respect to possible translocation of individuals (i.e., seeds or plants) from one area to another, appropriate establishment of new populations, and design of possible seed collection scenarios for *ex situ* seed storage.

Lasthenia burkei, *Blennosperma bakeri*, and *Limnanthes vinculans* are herbaceous annual plants that inhabit vernal pool and swale habitats mainly on the Santa Rosa Plain. They are all Federally and State listed as endangered. Populations of the three listed plants are fragmented, with many populations persisting in small habitat fragments that are vulnerable to off-site impacts and inappropriate site management. Remaining populations are patchily distributed, either on privately owned sites, on recently established preserves, or on mitigation banks, where they occur in remnant natural pools or increasingly are seeded into newly created pools. Most remaining natural populations are now more geographically isolated from each other than they were historically, potentially beyond the reach of pollinators or seed dispersal mechanisms. The

goal of our research was to determine the genetic variation between populations in order to inform conservation efforts as to the distinctiveness of populations for seed banking and *ex situ* conservation, and to infer possible natural explanations for genetic subdivisions to aid in management decisions, such as the movement of seeds from remnant natural site to inoculate created mitigation pools.

To accomplish these goals we developed molecular genetic techniques to characterize and compare individual populations of the three listed plants. We adapted microsatellite markers recently developed for *Limnanthes alba* by Kishore et al. (2004) to examine fine scale and rangewide genetic structure of *L. vinculans*. We developed Inter-Simple-Sequence Repeats (ISSRs) and Random Amplified Polymorphic DNAs (RAPDs) markers to examine the amount and distribution of genetic variation throughout the range of *Lasthenia burkei* and *Blennosperma bakeri*.

METHODS

Study System

The Santa Rosa Plain extends west of Santa Rosa, north of Cotati, south of Windsor and east of Sebastopol (Figure 1). Historically, the Santa Rosa Plain was a contiguous and vast oak savannah, with vernal pools and swales spread throughout, becoming visible each spring once they filled with water, supporting a characteristic community of now largely endangered or threatened endemic plants and animals. These vernal pools and swales today occur as remnants in a matrix of agriculture, urbanization, and fragmented remains of valley oak (*Quercus lobata*) savannah, grassland, and persistent wetland vegetation.

The Santa Rosa Plain is the primary habitat of three endemic endangered vernal pool plants: *Lasthenia burkei*, *Blennosperma bakeri*, and *Limnanthes vinculans*, all State and Federal endangered plants. The future recovery of these three species is addressed in the Santa Rosa Plain Conservation Strategy (2005) that aims to create a long-term program to conserve their sensitive habitats and mitigate potential adverse effects due to future development on the Santa Rosa Plain. (http://www.fws.gov/sacramento/es/santa_rosa_conservation.html).

Housing development, agriculture, reclaimed water irrigation, and the cessation of livestock grazing on preserved sites have contributed to the severe decline and demise of a

majority of the area's pool fauna and flora within the last 50 years (CH2MHILL 1995). Altered hydrology, increased refuse, and foot traffic have contributed to site quality reduction in some areas. Remnant populations on private sites may constitute some of the few remnant high quality vernal pools on the Santa Rosa Plain, but these are currently not properly protected in the long-term, and quality may decline if management (e.g. horse or goat grazing) is changed over time by changing private owners. Intensive agricultural practices (including disking, vineyards, over-grazing, and orchards) have destroyed or damaged other vernal pool areas. In some cases pools spared in development projects in the Santa Rosa Plain area are declining in viability (D. Cook, pers. com). The floristic quality of the remaining pools has declined within the last 10-15 years in other, larger preserved areas due to the abrupt change of the historic site-specific grazing regimes to no grazing, which has greatly favored the establishment of exotic plant competitors (G. Cooley, pers. com.). Marty (2005) showed cattle grazing of exotic annual grasses in Central Valley vernal pools enhanced native plant diversity there. Further scientific study into the effectiveness of grazing regimes for Santa Rosa Plain vernal pool ecosystem viability is warranted for successful recovery of these listed plants.

Much of the original pool area in the Santa Rosa Plain has been destroyed either by intensive agriculture or by urban development (CH2MHILL 1995). The Santa Rosa Plain Conservation Strategy has identified areas for conservation. A reserve systems is being established by the Santa Rosa Plain Conservation Strategy Implementation Committee that includes historic (yet largely degraded) vernal pool preserves and sites where artificial pools have been created and where listed plants have been seeded within the past 10 years (S. Talley pers. com.). Mitigation and preservation banks currently include a mixture of natural and created pools/swales of variable habitat quality.

Study Species

Burke's goldfields (*Lasthenia burkei*)

Lasthenia burkei is a spring-flowering herbaceous annual plant in the Aster family (Asteraceae) that inhabits vernal pools and swales on the Santa Rosa Plain in Sonoma County, California and in Lake County, California (CPC, 2007). It is known from about 16 extant populations (C. Sloop pers. obs. (this study), D. Wiemeyer & M. Lee pers. com). *Lasthenia*

burkei is capable of surviving flooded conditions in the winter and spring, and often flowers with their roots and lower shoots still submerged (Ornduff, 1969a). The plant is listed as endangered under the California and federal Endangered Species Acts (Sec. 670.2, Title 14, California Code of Regulations; Federal Register 1991) due to threats to their habitat posed by development (Federal Register 1991). Several artificial habitats have been successfully created where *L. burkei* now grows (Federal Register 1991).

Lasthenia burkei is strongly self-incompatible and is therefore an obligate outcrosser (Ornduff, 1966). The anthers are well developed and the species is pollinated by insects such as flies, beetles, moths and solitary bees (Ornduff, 1966). Achene dispersal in *L. burkei* is little known, but the free phyllaries and the bristly pappus (Ornduff 1969a) suggest dispersal by attaching to the fur or feathers of passing animals (Ornduff 1966). It is also possible that the achenes float, which would allow for hydrological dispersal between populations.

Sonoma sunshine (*Blennosperma bakeri*)

Blennosperma bakeri also known as Baker's stickyseed, is a small (up to 12 inches in height), spring-flowering annual herb also in the Aster family (Asteraceae). When the achenes are moistened, surface papillae break open emitting filaments which swell with water to form a colloidal mass covering the achene making the seeds sticky when wet (Ornduff, 1964). The species is restricted to the Santa Rosa Plain and Sonoma Valley in Sonoma County. According to the California Natural Diversity Database, there are currently 22 populations believed to be extant. We were only able to access 10 extant populations in 2006 and are aware of only 5 other extant created sites. Several of the other populations have most likely been extirpated.

Blennosperma bakeri is strongly self-incompatible, as is its congener *B. nanum*, despite abundant self-pollination in both species. Pollinators of *B. bakeri* include bees of the genera *Andrena* and *Nomada*, bee-flies, drone flies, and small beetles (reported in Ornduff 1964). Hybrids between the two *Blennosperma* species were highly pollen sterile and produced no seed. Seed dispersal was inferred to be within a small radius of maternal plants based on the dispersion of naturally occurring flower-color variants of *B. nanum*, although the sticky seeds could provide for occasional wider dispersal by animals (Ornduff, 1964).

Sebastopol meadowfoam (*Limnanthes vinculans*)

Limnanthes vinculans is a small (up to 12-inch tall), multi-stemmed herb of the false meadowfoam family (Limnanthaceae). The species is endemic to the Santa Rosa Plain, Sonoma County, with one known occurrence in Napa County. It is found in seasonally wet meadows, swales and vernal pools. *Limnanthes alba, L. douglasii* var. *nivea* and *L. vinculans* all share the same floral morphology and this is most likely associated with their almost exclusive out-crossed breeding system and their sharing of the same bee pollinators in the family Andrenidae (Ornduff 1969b). *Limnanthes vinculans* nutlets are densely clothed with short, broad tubercles, which may aid in animal or water dispersal (Ornduff 1969b), but no specific seed dispersal mechanism is recognized at this time.

Sampling Design

Plants at each site typically occurred in discrete associations within either a single or within multiple adjacent vernal pools or swales. At each site, according to plant abundance and within site distribution, we collected between 15 and 60 plant samples (~ 35 per pool) for genetic analysis (Table 1). An attempt was made to sample most to all extant populations of the three listed plants. In only two cases site access was not obtained. Although each species is nearly endemic to the Santa Rosa Plain, *L. burkei* and *L. vinculans* had populations located outside the Plain, which we sampled. Ten sites were sampled for *B. bakeri*; 21 sites for *L. vinculans*, and 15 sites for *L. burkei* for a total of 1,585 plant samples. Sampling began March 20, 2006 and ended two months later on May 19, 2006.

A plant sample consisted of two to three stems, which were placed into individually labeled zip-lock bags. Plants were collected haphazardly from throughout each site by walking linear or circular transects throughout the target plant's area of distribution and collecting a plant sample at equidistant intervals to cover the complete distribution area (this was variable in each case due to changing distribution densities from site to site). Pool circumference, transects, and sample points were noted using GPS (geographic positioning systems). Due to the numbers of samples, and the fragile nature of the tender stems, plant samples were initially processed as quickly as possible in Sonoma. This initial processing consisted of weighing out 200 mg of plant material, wrapping the material in a labeled foil envelope, and freezing. Extra plant material was

kept in the collection ziplock bag and also frozen. Frozen samples were transported to the UC Davis lab about every 2 weeks and placed into a -80^{o} C freezer until DNA extraction.

DNA Extraction Protocols and Polymerase Chain Reaction (PCR) Conditions

We planned on using Qiagen DNeasy Plant MiniKit for all our DNA extractions according to the instructions that came with the kit. The Qiagen protocol had to be modified to handle previously frozen material. It is important to prevent plant material from thawing unless it is immersed in a buffer that neutralizes plant secondary chemicals. During thawing, cells and vacuoles within the cells are ruptured by ice crystals, releasing phytochemicals which can potentially degrade DNA. To prevent thawing, we kept the foil envelope of plant material in the deep freeze, or on ice, until we were ready to go forward with the extraction.

The first step in the Qiagen extraction technique, after weighing out the plant material, is to pulverize it in liquid nitrogen. To do this, we quickly scraped the frozen material off the foil and into a mortar. Approximately 1 ml of liquid nitrogen was poured over the sample, which was then ground into powder while hard frozen using a pestle. The first Qiagen buffer was added to the mortar before the sample thawed, and the extraction proceeded according to Qiagen's instructions.

Test extractions were made on all three species shortly after their collection and resulted in DNA that was measurable using a spectrophotometer. However, this extraction protocol reliably produced adequate amounts of high quality DNA for *L. vinculans* only. Protocols for *L. burkei* and *B. bakeri* are described following methods for *L. vinculans*.

Limnanthes. vinculans: We genetically surveyed 21 private and public *L. vinculans* sites, 20 on the Santa Rosa Plain and one outlying population in Napa County. We adapted a suite of highly variable SSR (simple sequence repeat or microsatellite) markers characterized for *L. alba* by Kishore *et al* (2004). We initially tested 25 of the most polymorphic markers for use with *L. vinculans* using PCR (polymerase chain reaction), and ultimately genotyped 577 individuals with 15 polymorphic marker loci (Table 2). PCR reactions were performed according to the methods in Kishore et al (2004), and using fluorescently labeled forward primers. PCR products were then sized using an ABI 3730 96-capillary DNA analyzer and ABI GeneMapper 3.0 software (Applied Biosystems, Cupertino, CA).

Lasthenia burkei: DNA was extracted using the modified CTAB extraction described in Ayres and Ryan (1997) with an added modification – we did not incubate the samples due to the presence of aerobic chemicals in *L. burkei* that apparently can degrade DNA even in the presence of the CTAB buffer. Longer incubation times resulted in DNA degradation. Both inter-simple-sequence-repeat primers (ISSR) and random amplification of polymorphic DNA (RAPD) were used to amplify *L. burkei* DNA fragments. $MgCl_2$ and annealing temperature were simultaneously optimized on a Eppendorf Mastercycler Gradient thermalcycler; four ISSR primers – 823, 825, 827 and 845 (University of British Columbia) – were used at an annealing temperature of 55 ^{o}. Two RAPD primers – A11 and F13 (Operon Technologies) – were used at an annealing temperature of 42 ^{o}. We used a final set of 42 bands for our genetic analyses.

PCR conditions were: 94^{o} for 90 sec followed by 40 cycles of 94^{o} for 15 sec, the optimized annealing temperature for 30 sec, and 72 ^{o} for 2 min. Reaction volumes of 15 mL contained 10% by volume $MgCl_2$-free 10X reaction buffer A (Promega, Madison, Wisconsin), 0.6 units Taq polymerase (Promega, Madison, Wisconsin), 360 pico units primer (University of British Columbia), 3 mmol/L $MgCl_2$, 200 mmol/L each dATP, dCTP, dGTP, and dTTP (Promega, Madison, Wisconsin), and 30 ng genomic DNA. Most reactions were repeated twice to confirm consistency. Following electrophoresis on 1.5% agarose gels, DNA was stained with ethidium bromide and visualized under UV light. Gels were hand-scored for polymorphic DNA fragments.

Blennosperma bakeri: As PCR reactions using the test extractions were successful, we extracted all *B. bakeri* samples using the Qiagen method. Both inter-simple-sequence-repeat ISSR primers and random amplification of polymorphic DNA (RAPD) were used to amplify *B. bakeri* DNA fragments. $MgCl_2$ and annealing temperature were simultaneously optimized on a Eppendorf Mastercycler Gradient thermalcycler; one ISSR primers – 812 (U British Columbia) – was used at an annealing temperature of 50 ^{o}. Four RAPD primers – A7, B17, H5, (Operon) and 239 (U British Columbia) – were used at an annealing temperature of 38 ^{o}. PCR reaction conditions, electrophoresis, gel imaging and scoring were as above for *L. burkei*.

Troubleshooting extraction protocols. Once we began to run large-scale genotyping gels it became clear that the DNA quality was poor in over 80% of our samples. We experimented with various extraction methods (e.g. proteinase K method, CTAB) using frozen surplus plant material and got an adequate quantity and quality of DNA using the same protocol we used for

the *L. burkei* samples, i.e. CTAB without an incubation period. In addition, we think part of the problem may be that some thawing of plant material may have occurred at some point in the protocol – during transport of frozen material to UC Davis from Sonoma or during the transfer of plant material from the foil envelope to the mortar – that resulted in the release of plant chemicals which degraded the DNA. To avoid this in the future we would proceed with the extraction using fresh material only, and employ the no-incubation CTAB method that worked best in our troubleshooting experiments. We were not able to redo the study using the new protocol as, unfortunately, in many cases the surplus material was not enough for a full second extraction.

Data Analysis

Analysis of Molecular Variance (AMOVA). A matrix of squared Euclidean genetic distances was calculated between all individuals. The matrix was then partitioned into sub-matrices using Arlequin software ((Excoffier et al. 1992; http://lgb.unige.ch/arlequin/). For *L. burkei* a total of 27 loci (out of 42 loci) were used in the analysis after removing loci with replicate patterns or with > 5% missing values. For *L. vinculans,* 577 individuals were tested with the 15 most polymorphic loci (out of 25 loci) with < 5 % of missing values. The sums of squares in the matrix and submatrices yield sums of squares for the various hypothetical divisions in the population. The sums of squares were placed into an analysis of variance framework allowing for hypothesis tests of between-group and within-group differences at several hierarchical levels corresponding to Wright's F-statistics. The analysis was divided into ecologically meaningful groups (e.g. two groups for *L. burkei*, one for the Lake County populations and one for the Sonoma County populations; and two groups for *L. vinculans*, one for the Napa County population, one for the Santa Rosa Plain populations), and analyses were run to determine genetic divergence between the groups and in populations within each county. Pairwise population comparisons (F_{ST}) were calculated with this program and portrayed using the SAHN clustering program of NTSYS.

AMOVA was not performed on *B. bakeri* due to lack of within population replication. Instead, genetic distance among *individuals* was calculated using the Euclidean distance metric implemented with NTSTSpc (version 2.10 Exeter Software, Setauket, NY). Hierarchical patterns of genetic distance were portrayed using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster analysis.

Genetic Structure. A model-based Bayesian clustering method was also applied to all *L. vinculans* haplotypes using STRUCTURE software (Pritchard and Wen 2004, Falush *et al* 2003, Pritchard *et al* 2000). In this analysis individuals are probabilistically assigned to either a single cluster (the population of origin), or more than one cluster (if there is admixture). The program assumes the neutral unlinked markers to be in Hardy-Weinberg equilibrium and linkage equilibrium and that recent migration would likely produce departures from Hardy-Weinberg equilibrium and linkage equilibrium. STRUCTURE identifies the *K* unknown populations (genetic clusters) of origin of individuals and concurrently allocates all individuals to populations, giving their 90% confidence intervals. STRUCTURE was run using the 'admixture model' and correlated allele frequencies, with a burn-in period of 10,000, followed by 100,000 iterations. To detect the true number of clusters (*K*) we followed the graphical methods and algorithms outlined in Evanno *et al* (2005). Under the assumption that the sampled plants belong to an unknown number of *K* genetically distinct clusters, we used priors from 2 to 22 to estimate the average posterior probability values for *K* (log likelihood; ln L) for 20 runs each. This method established $K = 4$ as the true value of *K*.

Genetic diversity. Due to the codominant nature of SSR markers genetic diversity of *L. vinculans* populations could be determined using Arlequin 3.1 software. We calculated observed and expected heterozygosities, and the number of alleles per locus for each survey site. As co-dominant marker data were not available for *L. burkei*, we compared population genetic diversity by reporting the average number of pairwise differences in markers states (present or absent) for each population for the 27 loci evaluated by Arlequin. Populations with higher average pairwise differences have more genetically variable individuals than populations with lower average pairwise differences.

RESULTS

Lasthenia burkei

The use of AMOVA quantified variation between regional (county) groups and populations of *L. burkei*. As shown in Table 3, an average of 78.13% of genetic variation was within populations, resulting in an F_{ST} of 21.67%, indicating substantial genetic structure among populations. Half of this variation, 11%, was due to the difference between Lake and Sonoma County populations; the other 11% was due to variation within county populations. We calculated the number of migrants per generation from F_{ST} according to the formula: $F_{ST} = 1/(4Nm + 1)$ (Wright, 1943) and found migration in excess of 1 individual per generation both between and within counties, on average. In general, Nm estimates greater than 0.5 indicate that migration is adequate to prevent genetic divergence of populations due to drift (Slatkin, 1987).

Genetic difference comparisons among all populations clarified the patterns of variation (Figure 1, Table 3). The Manning Flat and Ployez (Lake County) populations showed distinct differences both from all of the Sonoma County populations and from each other. In Sonoma County, the westernmost (Dawson Ranch), and southernmost (Pellagrini) *L. burkei* populations were genetically distinct; the northernmost population at Windsor (Garcia) also was unusual but only three individuals could be sampled. A population cluster apparently extends from the northwest to the southeast (Windsor Garcia – SR Airport (runway) - Wood Road) meeting another group of populations extending southward (Wood Fulton – Piner Marlow – Preakness Court) from the center of the Sonoma county population distribution. Alton Lane and SRA Preserve, both constructed populations, were associated with this second group of populations. The Maggi population, a natural pool, while geographically close to Wood Road, was genetically distinct from it ($F_{ST} = 0.13$, Table 5), suggesting lack of gene flow between these adjacent populations.

The most genetically variable population, as assessed by the population having the greatest average number of differences between individuals, was the artificial population at the Santa Rosa Airport Preserve. This may indicate the inoculation of seeds from more than one source at this restoration site, or may reflect the addition of seed from a different population to an extant native population at the site. In Sonoma County, populations generally averaged over 8.5 differences while the two Lake County populations averaged less than 7.5 differences.

Limnanthes vinculans

Limnanthes vinculans showed relatively high genetic diversity at all sites (average H exp = 0.65 ± 0.19, average H obs = 0.53 ± 0.23, average number of alleles per locus = 7.419 ± 3.748). At only four sites (Shilo Ludwig, Magee Ludwig, Crinella, Alpha Poncia) was H obs significantly lower than H exp (alpha = 0.05). These results show high heterozygosity and imply high natural or artificial gene flow via out-crossing, and a large effective population size. AMOVA showed high population genetic variation between Santa Rosa Plain populations and the outlying Napa population, explaining 14% of the overall variation (F_{ST} = 0.180; Table 4). Among Santa Rosa Plain populations genetic variation is significantly lower (F_{IT} = 0.052), with most (82%) of the overall Santa Rosa Plain population variation explained by within population genetic variation. There are no clear trends or geographic groupings among Santa Rosa Plain populations (Figure 2). However, five of the geographically most isolated sites on the fringes of the distribution are more genetically distinct: Crinella (F_{ST} = 0.13, Figure 3) to the north, Laguna Vista (F_{ST} = 0.11, Figure 3) to the West, Desmond (F_{ST} = 0.09, Figure 3) and Theiller (F_{ST} = 0.06, Figure 3) to the south, and Horn (F_{ST} = 0.07, Figure 3) to the southeast. In addition, several populations sharing a common history as preserves or mitigation sites are remarkably genetically similarity (SWSR Preservation Bank, Wright Preserve, Todd Carinalli, Todd Road Preserve, F_{ST} < 0.03, Figure 3). Finally, the Haroutounian population (in the southeast) is genetically similar to the Henderson Preserve (F_{ST} = 0.03, Table 6) population (in the northwest) despite their geographical isolation from each other, suggesting long-distance gene flow or artificial movement of seeds. Some of these similarity groupings are further confirmed in the Bayesian ordination (Figure 4). We examined the groupings of our true cluster estimation for K = 4 (Evanno et al 2005) of our 21 recognized populations (Figure 4):

- Cluster 1 (green): Air Center, Alton Lane, Balletto, Crinella, SWSR Preservation Bank, Shilo Ludwig, Todd Carinalli, Todd Road Preserve;
- Cluster 2 (blue): Alpha Poncia, Desmond, Grech property, Hale, Theiller, Yuba Dr/FEMA;
- Cluster 3 (red): Haroutounian, Horn, Henderson Preserve, Laguna Vista, Magee Ludwig, Wright Preserve;

- Cluster 4 (yellow): Napa

Cluster 1 again groups SWSR Preservation Bank, Todd Carinalli and Todd Road Preserve. Cluster 2 includes all of the southern and southwestern populations (Theiller, Desmond, Grech Property). Cluster 3 again aligns Haroutounian with Henderson Preserve. Cluster 4 shows the outlying Napa population as distinct.

Blennosperma bakeri

Preliminary analysis of *B. bakeri* revealed the existence of spatial genetic structure in this species as well (Figure 5). At the local scale, members of the Windsor population, located to the extreme north of the species range, tended to occur in the same cluster. There was also a trend for larger spatial scale affiliations. For example, the Windsor population was included within a larger grouping comprised of plants from the north and central areas of the species range. However, the strength of these relationships can't be estimated due to lack of replication within populations.

DISCUSSION

All over the State of California, vernal pool ecosystems are in decline, mainly due to encroachment from urban development, the threats posed by non-native plant competition and other factors that degrade or destroy vernal pool habitat. In Sonoma County, the 81,000 acre Santa Rosa Plain (SRP) historically contained a vast network of unique vernal pools and swales. Human activity in the recent 50-100 years has destroyed 85% of the SRP vernal pools and has degraded the ones that remain due to a lack of appropriate management (CH2MHILL 1990). Remaining habitats now support five federally listed endangered species and many sensitive plant species. Development pressures on the Santa Rosa Plain are high, and land values are also high. Generally, there is a lack of contemporary range-wide surveys and most demographic records for the SRP endangered plant species are out of date (Waaland et al 1990, CNDDB 2006).

In theory, the population genetics of vernal pool plants may be summarized as follows (adapted from Elam, 1998):

1. Small, fragmented populations will diverge genetically due to genetic drift.

2. Inbreeding will reduce genetic variation and increase genetic structure.
3. Reducing gene flow can increase inbreeding, reduce genetic diversity, and increase structure. Gene flow can be both beneficial and detrimental. Gene flow increases genetic variation and reduces inbreeding depression, however, gene glow from a genetically depauperate source can reduce genetic variation in a target population, prevent local adaptation, or break up co-adapted gene complexes.
4. Natural selection can counter the effects of gene flow; soils are strong agents of selection in short lived plants. Competitive environment is another strong habitat factor in vernal pools.

In the increasingly fragmented, degraded vernal pool system of the SRP, we might predict loss of genetic variation in small populations due to inbreeding, and substantial genetic structure due to drift where populations are small, and due to reduced opportunities for natural gene flow. What we found was high genetic diversity and moderate to low genetic structure in the SRP for *L. burkei* and *L. vinculans*.

Based on a single measurement of genetic variation, we can't know whether genetic variation is stable, increasing or decreasing. Population size fluctuations, breeding system, and seed bank contributions can all affect genetic variation. Long-term demographic monitoring is needed to determine whether populations are experiencing bottlenecks that would tend to reduce variation. The last extensive surveys were done in the 1980s (Waaland et al 1990) and much has changed in this system since that time. The breeding system of all three species is either obligately or highly outcrossing, which would minimize reductions of genetic variation due to inbreeding. Seeds of all three endangered species survive and germinate after storage for 0-13 years (Rancho Santa Ana Botanic Gardens, pers. com). It is not clear which environmental cues cause some seeds to germinate earlier than others *in situ*. We can assume that some seeds have likely stayed in the soil for at least the last decade, and that plants from such 'historic seeds' represented a proportion of the plants we sampled, increasing the effective population size in addition to maintaining genetic variation via "temporal" gene flow. Further studies of seed bank dynamics will shed light on these important dynamics of the reproductive ecology of all three vernal pools species addressed in this report.

The dispersal of pollen and seed are the main mechanisms for natural gene flow. In order to evaluate the results of our study we need to take into consideration what is known about the seed or pollen dispersal of the three species addressed here.

Ornduff (1969b) describes nutlets of *L. vinculans* as 'densely clothed with short, broad tubercles,' that may aid in flotation or animal dispersal, and he points to solitary bees (Andrenidae) as the main pollinators. For *L. burkei* he lists 'moths, flies, beetles and solitary bees' as the main pollinators, and suggests animal or water-aided achene dispersal (Ornduff 1966). For *B. bakeri*, he refers to the stickiness of seeds when wet, 'aiding in dispersal via animals, and to '*Andrena*, and *Nomada* solitary bees, bee flies, drone flies and small beetles' as the main pollinators (Ornduff 1964). It is unknown whether seed dispersal in these vernal pool species is due to animals or to dispersal via floating in water. Either system may lend itself to relatively great distances traveled. An animal (e.g. waterfowl) may travel several miles between vernal pools to find water, and so carry seeds from one population to another. If water dispersal of buoyant seed is instead the means of dispersal, sites that are hydrologically linked may share seeds each season. Some areas of the Santa Rosa Plain are prone to flooding in particularly rainy winters, which may also carry seeds long distances.

Solitary bees usually have a close co-evolutionary relationship with the flowers they pollinate (Thorp 1990), but to what degree *L. vinculans, L. burkei or B. bakeri* depend only on solitary bees for pollination is unclear. Moths, flies, beetles, and other bee species (e.g. honey bees) also may play an important role in pollen dispersal, but the specific determination of the pollination ecology for all three species addressed here has not been determined, and is a highly important topic of future study. Estimating effective pollen dispersal distances with this potential array of pollinators is a daunting task. Further, a lack of pollinators may be a cause for population decline in these three highly outcrossing species. Therefore, pollinators may play a decisive role in the long-term recovery of these endangered plants irrespective of their role in genetic structure.

On the Santa Rosa Plain the question remains whether the relatively high level of gene flow our data showed is historic or contemporary, that is, whether it occurred due to consistently high natural movement of pollen and seed over the centuries, or through seed movement by human activities during the past few decades, or both. The historic and genetic accounts are in accord: substantial numbers of seeds have been moved between sites to inoculate newly created

wetlands and to supplement degraded populations (S. Talley & C. Patterson, pers. comm.) in both *L. vinculans and L. burkei*. A large part of this movement has taken place in the central area of *L. vinculans* distribution between highly genetically similar populations: Todd Road Preserve, Todd Carinalli, SWSR Preservation Bank (S. Talley, pers. com). Similarly, in *L. burkei*, the reason for the genetic similarity between the Alton Lane and the Santa Rosa Airport Preserve populations, despite their geographic separation, may be that both populations were, at least in part, artificially constructed, and have received similar seed inoculum. The lack of direct genetic similarity to a natural population, and the high diversity of the Airport Preserve population could indicate that the mixing of seed inoculum from different sources has occurred. Doing so would result in populations that do not closely resemble any single existing population but instead resemble each other and contain a diversity of genotypes.

The other scenario is that the detected gene flow levels at least in part reflect micro-evolutionary natural gene flow. The four fringe populations at the edges of the *L. vinculans* distribution may reflect this as they are more isolated than all other populations. At one site, Laguna Vista, there has been speculation of a recent 'founding' (planting) event. While the specific population history dynamics at the Laguna Vista site are unclear and highly debated, our data showed this population to be among those most distinct from the majority of Santa Rosa Plain populations. Whether this distinction is due to its naturally reduced population size in recent years coupled with lack of gene flow, or is due to the fact that it was planted at the site from only a few propagules within the last few years is unclear and cannot be specifically ascertained from this study. However, the other remaining 'fringe' populations (Horn, Crinella, Desmond, Theiller) are likely not recent founding events and have not been the target of human seed movement, to our knowledge. These populations may have somewhat restricted gene flow being situated too far from most other populations for effective pollen or seed exchange making them more distinct.

Further, AMOVA results suggested that the Napa population represents a potential evolutionarily significant unit and should therefore be targeted as a conservation priority (Fraser and Bernatchez 2001). Further studies of this Napa population regarding the possibility for a potential hybridization with *L. douglasii* spp. *nivea* or perhaps even *L. bakeri* (Ornduff 1969) is further recommended, as leaf morphology in some plants of this population showed similarities between these species (G. Cooley pers. com).

AMOVA determined a significant amount of geographically meaningful genetic variation and structure in *L. burkei* populations throughout its range. As expected, gene flow is most limited between the geographically distant populations in Sonoma County and Lake County, and there is further evidence of reduced gene flow between the two Lake county populations. In addition, trends of population structure exist on the Santa Rosa Plain.

To summarize, it seems likely that the patterns of genetic variation and structure we have found are the result of historical gene flow by natural dispersal of seeds (and pollen) across a pristine landscape, "temporal" gene flow from reserves in the soil seed bank, circumscribed gene flow due to recent habitat fragmentation, and increased gene flow due to human restoration activities.

Due to the fact that Santa Rosa Plain vernal pools have been continuously declining in recent decades all remnant populations are to be considered high on the conservation priority list. Even if there seems to be effective gene flow on the Plain at this point in time, as in most surveyed *L. vinculans* populations, or only slight trends of population differentiation as in some *L. vinculans* and *L. burkei* populations, there is sufficient evidence that all of the microhabitats should be conserved to maintain the highest possible level of genetic diversity of these three species.

The movement of seed to inoculate newly created vernal pools on mitigation banks should become highly regulated and should only occur from source sites that have been genetically tested and are in relatively close proximity to the creation site. If seed is brought in from a site that is geographically more distant the likelihood of genetic distinction increases, however in some cases geographic distance does not reflect the potentially existing structure (e.g. Maggi vs. Wood Road). The results from this study should serve as further guidance to guide seed movement activities and a working database should be developed to effectively direct this important restoration process. In order to attain a better understanding of gene flow among populations of all three species focused studies on their reproductive and pollination/pollinator ecology, seed dispersal mechanisms, seed bank dynamics, and grazing dynamics should be undertaken as quickly as possible.

Recommendations

The data we presented here are an important part of the needed information to guide these endangered species towards recovery. While threats to the habitat of these species will persist within both protected and unprotected sites, a better, more detailed knowledgebase on their ecology and vernal pool ecosystem dynamics will allow a clearer picture of how to restore and manage the remnant populations over the long term.

We therefore urge the following steps:

- Further research into the specific reproductive ecology of each species investigating:
  - Breeding success (yearly seed set, viability, germination…)
  - Pollination ecology (dependency on specific pollinators, importance of large contiguous flower displays to attract sufficient pollinator numbers, phenology…)
  - Pollinator ecology (of those highly important in successful pollination)
  - Seed bank dynamics (size, input, output, genetic variation, germination cues…)
  - Seed dispersal mechanisms (relative importance of pollen versus seed dispersal…)

  This will allow a better evaluation of the long-term viability of populations and their potential for extinction vs. recovery.

- The movement of seed inoculum in vernal pool creation on mitigation banks should become highly regulated and should only occur from source sites that have been genetically tested and are in close proximity to the creation site.
  - The results from this study should serve as further guidance for seed movement activities
  - A working database should be developed to effectively direct seed inoculation of new and restored sites.

- Further scientific study into the effectiveness of various site appropriate grazing regimes for the Santa Rosa Plain vernal pool ecosystem viability is warranted for successful recovery of these listed plants. This will help to avoid loss of populations due to competition or other negative effects of invasive grassland species and inappropriate or lacking management techniques.

- Include the remaining unique vernal pool systems of the Santa Rosa Plain as an extension of the Central Valley vernal pool classification efforts (Barbour et al 2005). A better understanding of the natural vernal pool vegetation associations will allow us to restore and recreate the appropriate natural communities, rather than just targeted habitats for a few specific species. Specific community members may play important roles in facilitating other members of the community to thrive.

We feel that these urgent steps will be crucial to realize the ultimate long-term recovery of these three endangered species.

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Table 1. Sites, site ID, and number of plants sampled from each site for three species.

Species	Site	Site Name	Site ID	# sampled
BLBA	1	Alton Lane	**AL**	35
BLBA	2	Youth Community Park	**YP**	35
BLBA	3	Sonoma Valley Regional Park	**SO**	35
BLBA	4	Windsor	**WI**	35
BLBA	5	Horn mitigation site	**HO**	35
BLBA	6	Hale	**HA B**	35
BLBA	7	Wood Road Mitigation Site	**WR**	35
BLBA	8	Todd-Carinalli	**TC-B**	35
BLBA	9	Haroutuonian (Open Space District)	**HAR**	35
BLBA	10	Maggi	**MA**	35
			Total BLBA	**350**
LIVI	1	Hale	**HA**	35
LIVI	2	SWSR	**PB**	38
LIVI	3	Napa Valley	**N**	40
LIVI	4	Desmond	**DE**	40
LIVI	5	Horn mitigation site	**HO-L**	35
LIVI	6	Crinella	**CR**	20
LIVI	7	Alton Lane	**AL-L**	35
LIVI	8	Theiller CDFG property	**TH**	35
LIVI	9	Todd-Carinalli	**TC**	35
LIVI	10	FEMA/ Broadmoor Acres	**YD**	45
LIVI	11	Grech property	**GP**	35
LIVI	12	Laguna Vista	**LV**	19
LIVI	13	Todd rd preserve	**TR**	50
LIVI	14	Magee Mitigation Site	**ML**	35
LIVI	15	Haroutounian (Open Space District)	**HAR-L**	15
LIVI	16	Wright Preservation Bank	**WP**	60
LIVI	17	Air Center (National Guard)	**AC**	35
LIVI	18	Shiloh Mitigation Site	**SH**	35
LIVI	19	Henderson Preserve	**HP**	34
LIVI	20	Balletto	**BA**	35
LIVI	21	Alpha farm/Poncia	**AFP**	35
			Total LIVI	**746**

Species	Site	Site Name	Site ID	# sampled
LABU	1	Wood Road Mitigation Site	**WR-L**	35
LABU	2	Alton Lane	**AL-LA**	35
LABU	3	Preakness Court (east of Wright PB)	**PC**	35
LABU	4	Santa Rosa Airport Runway	**SRA**	34
LABU	5	Santa Rosa Airport Wildflower preserve	**SRAP**	35
LABU	6	Maggi	**MA-L**	35
LABU	7	Lake County	**PL**	35
LABU	8	Lake County	**MF**	35
LABU	9	Piner & Marlow	**PM**	60
LABU	10	Pellagrini	**PE 1**	35
LABU	11	Pellagrini	**PE**	35
LABU	12	Windsor - Garcia	**WG**	3
LABU	13	Wilkinson	**WI**	12
LABU	14	Wood/Fulton	**WF**	35
LABU	15	Dawson Ranch	**DA**	30
			Total LABU	**489**
			Grand Total	**1585**

Table 2. SSR markers for *L. vinculans* adapted from Kishore et al (2004) showing marker ID, GenBank accession number and the total number of alleles per locus for *L. vinculans*.

SSR Marker	GenBank Accession Number	N alleles
LS88	BV007079	22
LS98	BV007088	22
LS106	BV007096	31
LS166	BV007132	40
LS318	BV007214	43
LS321	BV007217	30
LS354	BV007245	17
LS356	BV007247	33
LS378	BV007265	23
LS402	BV007285	25
LS457	BV007316	23
LS466	BV007320	14
LS483	BV007326	9
LS583	BV007382	18
LS614	BV007399	20

Table 3. AMOVA results for 14 populations in two counties, Lake vs. Sonoma, of *L. burkei*. (Nm for F_{ST} species-wide = 0.94)

Source of Variation	d.f.	Sum of Squares	Percentage of variation	Nm
Among groups	1	66.736	11.2	1.98
Among populations within groups	12	194.222	10.67	2.09
Within populations	293	1216.361	78.13	
Fixation indices				
FSC	0.12016			
FST	0.21869			
FCT	0.11199			

Table 4. AMOVA results for 21 populations in two counties Napa vs. Sonoma of *L.* v*inculans* (Nm for F_{ST} species-wide = 1.13)

Source of Variation	d.f.	Sum of Squares	Percentage of variation	Nm
Among groups	1	65.542	13.54	1.60
Among populations within groups	19	221.677	4.48	5.53
Within populations	1133	3320.145	81.98	
Fixation indices				
FSC	0.05177			
FST	0.18018			
FCT	0.13542			