CONSERVATION GENETICS OF THREE ENDANGERED VERNAL POOL PLANTS OF THE SANTA ROSA PLAIN, SONOMA COUNTY, CALIFORNIA

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ABSTRACT

We examined the population genetic structure of Sonoma sunshine (*Blennosperma bakeri*), Sebastopol meadowfoam (*Limnanthes vinculans*), and Burke's goldfields (*Lasthenia burkei*), three endangered annual plant species mostly restricted to vernal pools of the Santa Rosa Plain, Sonoma County. Conservation of existing populations and additional population establishment on protected mitigation lands are now under way. To inform these efforts, we developed nuclear DNA markers to characterize the distribution of genetic variation among populations in space and in time. Using 15 microsatellite markers we genetically surveyed 21 populations of *L. vinculans*, genotyping 577 individuals in 2006 and 182 individuals in 2008. 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 (FIT = 0.052), with most (82%) of the overall SRP population variation explained by within population genetic variation. We found notable temporal genetic structure between years in 6 tested populations (Fst ranging between 0.08 – 0.23). The Sonoma Valley population of *B. bakeri* was distinct from those on the Santa Rosa Plain (*PhiPT* = 0.19, P < 0.001) and notable genetic structure existed among populations within the Santa Rosa Plain (*PhiPT* = 0.11, P < 0.001). Range-wide, geographically separate populations of *L. burkei* were genetically distinct (F_{ST} = 0.22, P < 0.001). The variation was divided between the difference among Lake and Sonoma County populations (F_{ST} = 0.11), and within Sonoma county populations (F_{ST} = 0.11). We found evidence of temporal genetic structure at four sites sampled in both 2006 and 2008 (*PhiPT* range = 0.07 – 0.16). Our results inform conservation efforts as to the distinctiveness of populations for *ex situ* seed conservation, guide genetically appropriate seed inoculation of created pools, and will be used to infer possible explanations for genetic subdivisions to aid in management decisions. We recommend that each extant occurrence of these species be conserved and that seed movement be regulated and implemented according to the genetic information outlined here.

Key Words: *Blennosperma bakeri*, Burke's goldfields, conservation genetics, endangered plants, genetic structure, *Lasthenia burkei*, *Limnanthes vinculans*, Santa Rosa Plain**,** Sonoma sunshine, vernal pool.

INTRODUCTION

Many species of plants are closely associated with distinct, patchy microhabitats, such as vernal pools (Maliakal-Witt et al. 2005), that in recent decades have experienced profound human-caused habitat loss and degradation. Where microhabitats occur as discrete patches across the landscape, small-scale genetic divergence can occur through founding effects and genetic drift when gene flow is limited (Frankel & Soule 1980). The dispersal of pollen and seed are the primary mechanisms for gene flow in plants. When patches are lost within such a metapopulation fabric of patched, then gene flow among patches will decrease or cease altogether. If gene flow is decreased, and if microhabitat conditions differ among patches then genetic changes due to natural selection can result in further genetic divergence among plant populations (Soule & Mills 1998; Hufford & Mazer 2003).

One of the goals of conservation genetics is to maintain the micro-evolutionary, or adaptive

potential of the species by maintaining population genetic diversity within populations through time and its distribution among populations across space. Over time, a plant species can develop a differential distribution of genetic variation among populations across the landscape, termed genetic structure (Futuyama 1986; Slatkin 1987). We can determine the genetic structure of a plant species using selectively neutral genetic markers. Analysis of the genetic structure of populations helps us assess the status of the relative genetic diversity or adaptive potential among populations. Knowing whether a population is in decline and losing valuable genetic information, or whether it is genetically differentiated from other populations but thriving, the level of genetic structure among populations can inform our conservation decisions with respect to the possible translocation of individuals (i.e., seeds or plants) from one area to another to build up declining populations, the appropriate establishment of new populations, and design of possible seed collection scenarios for *ex situ* seed storage. Further, if genetic data are assessed relative to potential influences on genetic structure, then these results can be used to inform conservation efforts to maintain or simulate the ecological phenomena that have played a role in the micro-evolutionary development of the species.

Blennosperma bakeri Heiser, *Limnanthes vinculans* Ornduff, and *Lasthenia burkei* (Greene) Greene, are herbaceous annual plants limited to vernal pool and swale habitats mainly on the Santa Rosa Plain, Sonoma County, California. All are federally and State listed as endangered (Federal Register 1991). Extant natural populations of these species can persist in relatively small habitat fragments (e. g. within a single pool) on private property, or larger tracks of remnant vernal pool lands on preserves, or on recently established mitigation sites containing remnant natural and newly created vernal pools. We define population as an extant occurrence of a species and its seed bank at a distinct geographic location separated at minimum by 0.25 kilometers from the next nearest occurrence. Created pools contain populations that have been seeded by humans. All extant populations of both species are vulnerable to inappropriate site management and indirect impacts (e .g. change in hydrology on neighboring property).

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 (Holland 1978). Due to the loss of ~ 85% of seasonal wetlands on the Santa Rosa Plain (CH2MHILL 1990) most remaining natural occurrences are now more geographically isolated from each other than they were historically. A proportion of extant populations are now likely beyond the reach of inter- (or meta-) population pollination or seed dispersal mechanisms, thereby limiting gene flow. The goals of our research were (1) to determine the population genetic structure in order to inform conservation efforts as to the distinctiveness of populations for seed banking and *ex situ* conservation, and (2) 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 suites of neutral, nuclear DNA markers (Inter-Simple-Sequence Repeats (ISSRs) and Random Amplified Polymorphic DNAs (RAPDs) to characterize the genetic structure of these listed plants in space and in time.

METHODS

Study System

The Santa Rosa Plain extends west of Santa Rosa, north of Rohnert Park, south of Windsor and east of Sebastopol (Fig. 1). Historically, the Santa Rosa Plain was a contiguous and vast oak savannah, with vernal pools and swales spread throughout. These seasonal wetlands, which support a characteristic community of now largely endangered or threatened endemic plants and animals, become visible each spring as a succession of showy annual plants flower. 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). Vernal pools and swales today occur in the Santa Rosa Plain as remnant habitat in a matrix of agriculture, urbanization, and fragmented remains of valley oak (*Quercus lobata*) savannah, grassland, and persistent wetland vegetation.

Study Species

Burke's goldfields (*Lasthenia burkei*) is a spring-flowering herbaceous annual plant in the Asteraceae, which inhabits vernal pools and swales on the Santa Rosa Plain in Sonoma County and Lake County, California (Center for Plant Conservation 2007). As of 2008 there were 20 extant populations (CNDDB 2006; C. Sloop pers. obs., D. Wiemeyer & M. Lee pers. com). *Lasthenia burkei* is reported as strongly self-incompatible (Ornduff 1966) and is pollinated by insects (Ornduff 1966). Achene dispersal in *L. burkei* is little known, but the free phyllaries and the bristly pappus (Ornduff 1969) 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 within pools and between populations.

Sonoma sunshine (*Blennosperma bakeri*), also known as Baker's stickyseed, is a spring-flowering annual herb also in the Asteraceae. The achenes are sticky when wet (Ornduff 1964). The species is restricted to the Santa Rosa Plain and Sonoma Valley in Sonoma County. As of 2008 there were 24 populations believed to be extant (CNDDB, C. Sloop, pers. obs). We were only able to sample six of these extant populations in 2008. *B. bakeri* is reported as strongly self-incompatible and is insect pollinated (Ornduff 1964, R. Thorp & J. Leong, pers. com.). 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).

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 37 known occurrences (CNDDB 2006, C. Sloop pers. obs. (this study), S. Talley, pers. com.), and one known occurrence in Napa County. The species 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 collection site typically occurred in discrete associations within single pools or multiple adjacent vernal pools or swales. At each site, according to plant abundance and within site distribution, we collected between 12 and 60 plant samples (~ 35 per pool) for genetic analysis (Table 1). Thirteen populations were sampled for *L. burkei* in 2006, and six of the geographically most distant populations of *B. bakeri* were sampled in 2008 (Fig. 1, Table 1). Although each species is nearly endemic to the Santa Rosa Plain, there are *L. burkei* and *B. bakeri* populations located outside the Plain, which we sampled: two populations of *L. burkei* in Lake County, and one *B. bakeri* population in the Sonoma Valley. To determine temporal variation in genetic structure we re-sampled six sites for *Limnanthes vinculan*s, and five sites for *Lasthenia burkei* in 2008 (Table 1). We evaluated a total of 1400 plant samples.

Sampling in 2006 and 2008 began in March and ended in May. A plant sample consisted of two to three stems from a single plant, 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 County. 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

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.

Blennosperma bakeri and *L. 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 that degraded DNA even in the presence of the CTAB buffer. Longer incubation times resulted in DNA degradation.

Polymerase Chain Reaction (PCR) Conditions

Limnathes vinculans: 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. 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).

Blennosperma bakeri and *L. burkei*: We used both inter-simple-sequence-repeat primers (ISSR) and random amplified polymorphic DNA (RAPD) to amplify DNA fragments. $MgCl_2$ and annealing temperature were simultaneously optimized on an Eppendorf Mastercycler Gradient thermalcycler. For *L. burkei* 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}. For *B. bakeri* one ISSR primer – 812 (U British Columbia) – was used at an annealing temperature of 50 ^{o}C, and four RAPD primers – A7, B17, H5, (Operon) and 239 (U British Columbia) – were used at an annealing temperature of 38 ^{o}C.

PCR conditions, electrophoresis, and scoring: 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 uL 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 umol/L $MgCl_2$, 200 umol/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 presence or absence of polymorphic DNA fragments (bands).

Data Analysis

For *L. vinculans,* 577 individuals (21 populations, 2006) and 182 individuals (6 populations, 2008) were tested with the 15 most polymorphic loci (out of 25 loci) with < 5 % of missing

values. We used 33 bands for *L. burkei*, and 15 bands for *B. bakeri* after removing bands with replicate patterns or with > 5% missing values for all analyses. For *Limnanthes vinculan*s and *Lasthenia burkei* matrices of squared Euclidean genetic distances were calculated between all individuals per species. The matrices were then subjected to Analysis of Molecular Variance (AMOVA) to evaluate differences between-counties and within-counties in 2006. We used AMOVA and Principal Coordinates Analysis (PCA) to assess 2008 spatial population genetic structure in *B. bakeri,* and spatial and temporal population genetic structure in *Limnanthes vinculan*s and *Lasthenia burkei*, sampled both in 2006 and 2008, utilizing *F*st for *L. vinculans* co-dominant SSR marker data, and the *F*st analog *PhiPT* for non-codominant binary data for the other two species. We further assessed isolation by distance via a Mantel tests. All analyses were performed using Arlequin (AMOVA, Excoffier et al. 1992; http://lgb.unige.ch/arlequin/) and GenAlEx 6.1 software (Peakall and Smouse 2006).

Genetic Structure in Limnanthes vinculans:. 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*.

RESULTS

Blennosperma bakeri: Results indicated evident range-wide genetic structure (*PhiPT* = 0.19, $P < 0.001$) across six extant populations within Sonoma County (Table 2). Genetic structure solely among Santa Rosa Plain populations was about half as pronounced as range-wide estimates (*PhiPT* = 0.11, $P < 0.001$; Table 3). The outlying Sonoma Valley population, more than 20 km due south, was clearly distinct from Santa Rosa Plain populations (*PhiPT* ranged from 0.21 to 0.37; Table 4). The population at the northernmost extent of the range (Windsor) was least distinct among tested Santa Rosa Plain populations (*PhiPT* ranged between 0.05 and 0.09; Table 4), while Youth Park was most divergent (*PhiPT* ranged between 0.09 and 0.19; Table 4). Geographic distance predicted genetic distinction across Sonoma County supporting isolation by distance ($R^2 = 0.38$; Fig. 3, Wright 1943), however geographic distance did not explain distinctions among populations on the Santa Rosa Plain ($R^2 = 0.035$; Fig. 4).

Lasthenia burkei: There was substantial genetic structure among regional groups (across Sonoma and Lake counties) and among populations within groups. Over 78% of genetic variation was distributed within populations, resulting in a F_{ST} of 0.22 (Table 5). Half of this

variation was due to the clear difference between Lake and Sonoma County populations. The Manning Flat and Ployez (Lake County) populations showed distinct differences both from all of the Sonoma County (Santa Rosa Plain) populations (*F*st = 0.11, Table 5) and from each other (*F*st = 0.27).

Genetic difference comparisons among all Santa Rosa Plain populations in 2006 and 2008 clarified the patterns of variation (Table 5, Figs. 5 & 6). The westernmost (Dawson Ranch), and southernmost (Pellagrini/Wilkinson) *L. burkei* populations were genetically distinct (*PhiPT* = 0.10, P < 0.05; table 5, Fig. 1). The northernmost population at Windsor (Garcia) was genetically most distinct among Santa Rosa Plain populations in 2006 (*PhiPT* ranged from 0.088 to 0.27), but only three individuals could be sampled in 2006. The 2008 data for this population confirmed its distinctiveness with a samples size of 29 individuals (*PhiPT* ranged from 0.088 to 0.23; Table 5).

While the range-wide distinction between populations in Lake and Sonoma counties was likely due to isolation by distance of over 50 kilometers, geographic distance did not clearly explain population genetic structure across the Santa Rosa Plain (R^2 = 0.035, Fig. 7). The 2006 population analysis showed genetic sub-groupings within the Santa Rosa Plain: Windsor Garcia, SR Airport (runway), and Wood Road, were closely aligned, as were Wood Fulton, Piner Marlow, and Preakness Court (Figs. 5 & 6). Alton Lane and SR Airport Preserve, both sites that include native and constructed pools with seeded populations from other sites, were associated with this second group of populations (Figs. 5 & 6). The Maggi population, a natural pool, while geographically close to Wood Road, was genetically distinct from it (*PhiPT* = 0.13, Table 6), suggesting lack of gene flow between these geographically adjacent populations.

Comparisons of genetic variation between years (2006 and 2008) showed evidence of temporal genetic structure among all four tested Santa Rosa Plain populations. Comparisons between years at Wood Fulton and Piner Marlow were *PhiPT* = 0.07 for both (Table 5, Fig. 5), while genetic distinctions between years at Pellagrini/Wilkinson and Dawson Ranch were *PhiPT* = 0.16 for both (Table 5, Fig. 5).

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, but are also the result of using a highly polymorphic suite of markers for this assessment. 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). 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. However, five of the geographically most isolated sites on the fringes of the distribution are more genetically distinct: Crinella (*F*st = 0.13) to the north, Laguna Vista (*F*st = 0.11) to the West, Desmond (*F*st = 0.09) and Theiller (*F*st = 0.06) to the south, and Horn (*F*st = 0.07) to the southeast. In addition, several populations sharing a common history as

preserves or mitigation sites are remarkably genetically similar (SWSR Preservation Bank, Wright Preserve, Todd Carinalli, Todd Road Preserve, *F*st < 0.03). Finally, the Haroutounian population (in the southeast) is genetically similar to the Henderson Preserve (*F*st = 0.03) 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. We examined the groupings of our true cluster estimation for *K* = 4 (Evanno et al 2005) of our 21 recognized populations:

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.

Comparisons of genetic variation between years (2006 and 2008) also showed evidence of temporal genetic structure among all six tested Santa Rosa Plain populations in this species. Comparisons between years showed high genetic distinctions at Haroutounian (Fst = 0.23), as well as at Air Center and Todd Rd Preserve (Fst = 0.14 for both). Genetic distinctions between years were notable yet slightly lower at Theiller and Henderson (Fst = 0.10 for both), and Grech (Fst = 0.08 Fig. 8).

DISCUSSION

All over the State of California, vernal pool ecosystems are in decline, mainly due to encroachment from urban development, threats posed by non-native plant competition, and other factors that degrade or destroy vernal pool habitat (Holland 1978). In Sonoma County, the 81,000 acre Santa Rosa Plain historically contained a vast network of vernal pools and swales. Human activity during the past 50-100 years has destroyed 85% of this network and has degraded the pools 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. To counter species decline in this vulnerable habitat, movement of seed inoculum of both species occurred between populations across the Santa Rosa Plain during the last 15 -20 years (C. Patterson &. S. Talley, pers. com).

The patterns of genetic structure we observed were the result of ancient 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. Unfortunately, we cannot know with any certainty which factors have played the dominant role in the current dispersion of genetic variation across the landscape.

The dispersal of seed and pollen are the main mechanisms for natural gene flow in plants. 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 long distance dispersal. 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. A small number of areas of the Santa Rosa Plain are prone to flooding in particularly rainy winters, which may also carry seeds long distances.

For *L. burkei* Ornduff (1966) lists 'moths, flies, beetles and solitary bees' as the main pollinators and for *B. bakeri*, he lists '*Andrena*, and *Nomada* solitary bees, bee flies, drone flies and small beetles' (Ornduff 1964). Solitary bees can have a close co-evolutionary relationship with the flowers they pollinate (Thorp 1990), but to what degree *L. burkei or B. bakeri* depend only on solitary bees for pollination is unclear. In general, insect pollinators are highly local in their activities resulting in the development of local genetic neighborhoods (Brunnet and Holmquist, 2007). However, pollinators may play a decisive role in the long-term recovery of these endangered plants irrespective of their role in genetic structure as a lack of pollinators may be a cause for population decline in highly outcrossing species.

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, and gene flow can be both beneficial and detrimental; it increases genetic variation and reduces inbreeding depression, however, gene glow from a genetically depauperate source can reduce genetic variation in a recipient 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, as is competition with other plants in vernal pools.

In the increasingly fragmented, degraded vernal pool system of the Santa Rosa Plain, we might predict contemporary loss of genetic variation in declining or small populations due to inbreeding, and the development of substantial genetic structure due to drift and reduced opportunities for natural gene flow. Countering these losses and developments would be human-aided dispersal and artificial mixing of seed from different populations. What we found was strong genetic structure across counties in *Limnanthes vinculans* and *Lasthenia burkei* and between the Santa Rosa Plain and Sonoma Valley in *B. bakeri*, likely due to natural isolation by distance over ~20 km. We found significant yet more moderate genetic structure across the Santa Rosa Plain for both *B. bakeri* and *L. burkei* that was not directly explained by isolation by distance.

The lesser degree of genetic distinctions and the absence of isolation by distance among Santa Rosa Plain populations is in concordance with human-aided gene flow, but can be

explained in several ways. First, remnants of historic gene flow stored in the seed bank influenced our measurement. Each growing season a proportion of historic seed, deposited within one to two decades ago, germinates among seeds deposited within the last five years. If population decline in recent years had caused any given population to become genetically more similar, the seed bank buffered this trend and masked the measurement of more pronounced genetic structure through its added variability from times when gene flow was more pronounced in past metapopulations. Seeds of both endangered species survive and germinate after storage for at least 0-13 years (Rancho Santa Ana Botanic Gardens, pers. com). Future studies of seed bank dynamics will shed more light on these important dynamics.

Second, the dispersal of pollen and seed are the main mechanisms for natural gene flow, and we need to take into consideration what is known about the pollen or seed dispersal of the species addressed here. Decreased or lack of pollination due to the absence of prime pollinators can decrease seed set in any given year and so decrease the overall seed bank and its genetic variability. Also, if pollinators do not travel far distances pollen primarily comes from within population sources, again influencing the level of variability of the seed bank. Solitary bees usually have a close co-evolutionary relationship with the flowers they pollinate (Thorp 1990), but to what degree *L. burkei* or *B. bakeri* depend only on solitary bees for pollination is unclear. For *L. burkei* Ornduff (1966) lists 'moths, flies, beetles and solitary bees' as the main pollinators, and suggests animal or water-aided achene dispersal. 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). A lack of pollinators may be a cause for population and seed bank decline and decreasing variability in these outcrossing species, and pollinators may play a decisive role in the long-term recovery of these endangered plants.

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 kilometers between vernal pools to find water, and so carry seeds from one population to another. If water dispersal of buoyant seed is the means of dispersal, sites that are hydrologically linked may share seeds each season. A small number of areas of the Santa Rosa Plain are prone to flooding in particularly rainy winters, which may also carry seeds long distances.

Less pronounced genetic structure among the Santa Rosa Plain populations is also due to movement of seed inoculum of both species within the last 15 -20 years between populations across the Santa Rosa Plain (C. Patterson &. S. Talley, pers. com). Such artificial gene flow once in place is then magnified over time, followed by natural gene flow and represented in the seed bank. For example, 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 had 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.

We found evidence for temporal genetic structure at for populations, suggesting that there is notable genetic variation stored in seed banks of these *L. burkei* populations. Genetic diversity of the seed bank indicates that temporal metapopulation dynamics are at work across the different climatic conditions present each year, triggering only a portion of genetically more similar individuals in a pool to germinate and grow and set seed in a given season. This variable environmental selective force assures that the seed bank remains diverse over time to assure that the population can persist through the varied environmental conditions of this harsh vernal pool ecosystem with its fluctuating hydroperiod, temperature, and solar radiation during the growing and pollination seasons.

While genetic structure was less pronounced across Santa Rosa Plain populations, there existed a significant amount of distinction between populations to warrant conservation of each population. The long-term assessment of population abundance and seed bank size, the presence of pollinators to ensure continued seed input into the seed bank, and the existence of threats that could cause population decline are important factors to consider in the management and ultimate recovery of these species. Long-term demographic monitoring is needed to determine whether populations are experiencing bottlenecks that would tend to reduce variation.

A long-term monitoring program of a subset of extant populations of both species was begun in 2007, and is now in its third year (http://www.citizen-science.org). Before this, the last extensive range-wide surveys were done in the 1980s (Waaland et al 1990; CNDDB 2006) and much has changed in this system since that time. Current surveys include estimates of target plant abundance and cover within pools, the composition of the within pool and adjacent upland flora, presence and type of pollinators, potential threats, and site management regime.

To summarize, it seems likely that the patterns of genetic structure we observed 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 declining in recent decades all remnant populations are to be considered high on the conservation priority list. Even if there are only slight trends of population differentiation as in some *Lasthenia burkei* and *Limnanthes vinculans* populations, there is sufficient evidence that all of the microhabitats should be conserved to maintain the highest possible level of genetic diversity of these species. Current abundance data show relatively large populations in 2008 (Fig. 1), but in all cases increasing environmental pressures and the change in or absence of appropriate management regimes may cause the decline and ultimate loss of populations.

To avoid additional loss of genetic variation by the artificial movement of seed inoculum among or within the vicinity of genetically distinct populations, the translocation of seed to inoculate newly created vernal pools on mitigation banks should become highly regulated. It should only occur from source sites that have been genetically tested, and that are found to be in relatively close genetic alignment. Seed from a geographically more distant site is likely

more distinct, yet 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 an initial 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 these 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, better, more detailed knowledge, based on their population biology and the dynamics of vernal pool ecosystems, 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 (e.g. yearly seed set, viability, germination)
    - Pollination ecology (e.g. 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 (e.g. size, input, output, genetic variation, germination cues)
    - Seed dispersal mechanisms (e.g. 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 versus 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 genetic alignment and proximity to the creation site.

- The results from this study should serve as initial guidance for seed movement activities

- More sites should be genetically tested and 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 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 with invasive grassland species and inappropriate 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.

ACKNOWLEDGEMENTS

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Table 1: Site name, population size estimate, and number of plants sampled from each site per year per species. *Population size estimates were conducted differently between years; in 2008 a detailed standardized protocol was followed. **Population contains created pools with seed inoculum from other sites.

Species	Site Name	Population size 2006*	Sampled 2006	Population size 2008*	Sampled 2008
Blennosperma bakeri					
B. bakeri	Sonoma Valley Regional Park	> 5,000	-	700,000	32
B. bakeri	Youth Community Park	> 2,000	-	11,408	28
B. bakeri	Windsor	> 2,000	-	288,932	28
B. bakeri	Wood/Fulton	*no data*	-	7,667	28
B. bakeri	Haroutounian	> 2,000	-	1,198,821	20
B. bakeri	Maggi	> 2,000	-	111,590	28
B. bakeri	**Total**		**-**		**164**
Lasthenia burkei					
L. burkei	Wood Road Mitigation Site	-	35	-	-
L. burkei	Alton Lane**	-	35	-	-
L. burkei	Preakness Court	> 1000	35	168,369	-
L. burkei	Santa Rosa Airport Runway	> 10,000	34	-	-
L. burkei	Santa Rosa Airport Preserve**	*no data*	35	-	-
L. burkei	Maggi	> 1000	35	8,039	35
L. burkei	Piner & Marlow	> 10,000	60	139,598	35
L. burkei	Pellagrini/Wilkinson	> 20,000	47	1,102,505	35
L. burkei	Windsor - Garcia	> 10,000	35	809,339	-

Species	Site Name	Population size 2006*	Sampled 2006	Population size 2008*	Sampled 2008
L. burkei	Wood/Fulton	> 5,000	35	68,798	35
L. burkei	Dawson Ranch	> 2,000	30	276,048	30
L. burkei	Manning Flat – Lake Co.	> 10,000	35	-	-
L. burkei	Ployez Winery – Lake Co.	> 5,000	35	-	-
L. burkei	**Total**		**486**		**170**
Limnanthes vinculans					
L. vinculans	Hale	> 5000	30	-	-
L. vinculans	SWSR**	> 1000	30	-	-
L. vinculans	Napa Valley	>5000	30	-	-
L. vinculans	Desmond**	> 1000	30	-	-
L. vinculans	Horn mitigation site	> 2000	30	-	-
L. vinculans	Crinella	~200	15	-	-
L. vinculans	Alton Lane **	-	30	-	
L. vinculans	Theiller CDFG property	> 1000	28	4,820	35
L. vinculans	Todd-Carinalli**	-	30	-	-
L. vinculans	FEMA/ Broadmoor Acres	> 5000	28	60,000	-
L. vinculans	Grech property	< 300	30	4,969	35
L. vinculans	Laguna Vista	19	19	-	-
L. vinculans	Todd rd preserve	~1000	30	222,237	30
L. vinculans	Magee Mitigation Site	-	28	-	-

Species	Site Name	Population size 2006*	Sampled 2006	Population size 2008*	Sampled 2008
L. vinculans	Haroutounian (Open Space District)	~100	15	133	12
L. vinculans	Wright Preservation Bank	-	28	-	-
L. vinculans	Air Center (National Guard)	~ 1000	30	5,375	35
L. vinculans	Shiloh Mitigation Site	-	30	-	-
L. vinculans	Henderson Preserve	> 500	28	4,683	35
L. vinculans	Balletto	~500	30	16,078	-
L. vinculans	Alpha farm/Poncia	~200	28	4,380	-
L. vinculans	**Total**		**577**		**182**

Table 2: AMOVA results for six populations of *Blennosperma bakeri* within Sonoma County, five located within the Santa Rosa Plain, one in Sonoma Valley.

Pop	**Wood_Fulton**	**Maggi**	**Windsor**	**Youth_Park**	**Haroutounian**	**Sonoma_Valley**
n	28	28	28	28	20	32
SSWP	83.357	82.071	76.714	72.143	68.350	70.875

Summary AMOVA Table

Source	**df**	**SS**	**MS**	**Est. Var.**	**%**
Among Pops	5	104.245	20.849	0.660	19%
Within Pops	158	453.511	2.870	2.870	81%
Total	163	557.756		3.530	100%

Stat	**Value**	**P(rand >= data)**
PhiPT	0.187	0.001

Probability, P(rand>=data), for PhiPT is based on permutation across the full data set.

PhiPT = AP / (WP + AP) = AP / TOT

Key: AP = Est. Var. Among Pops, WP = Est. Var. Within Pops

Table 3: AMOVA results for five populations of *Blennosperma bakeri* within the Santa Rosa Plain, Sonoma Co. (excluding outlying Sonoma Valley).

Pop	**Wood Fulton**	**Maggi**	**Windsor**	**Youth Park**	**Haroutounian**
n	28	28	28	28	20
SSWP	83.357	82.071	76.714	72.143	68.350

Summary AMOVA Table

Source	**df**	**SS**	**MS**	**Est. Var.**	**%**
Among Pops	4	50.372	12.593	0.364	11%
Within Pops	127	382.636	3.013	3.013	89%
Total	131	433.008		3.377	100%

Stat	**Value**	**P(rand >= data)**
PhiPT	0.108	0.001

Probability, P(rand>=data), for PhiPT is based on permutation across the full data set.

PhiPT = AP / (WP + AP) = AP / TOT

Key: AP = Est. Var. Among Pops, WP = Est. Var. Within Pops

Table 4: Pairwise Population Matrix of *PhiPT* (*F*st analog for dominant markers) values for each population of *Blennosperma bakeri.* All values above the diagonal indicate the level of statistical significance.

Wood Fulton	Maggi	Windsor	Youth Park	Haroutounian	Sonoma Valley	
0.000	0.027	0.004	0.001	0.001	0.001	**Wood Fulton**
0.033	**0.000**	0.003	0.001	0.001	0.001	**Maggi**
0.049	0.055	**0.000**	0.001	0.002	0.001	**Windsor**
0.193	0.191	0.088	**0.000**	0.001	0.001	**Youth Park**
0.128	0.134	0.067	0.121	**0.000**	0.001	**Haroutounian**
0.358	0.369	0.304	0.205	0.242	**0.000**	**Sonoma Valley**

Table 5: AMOVA results for 14 populations in two counties, Lake vs. Sonoma, of *Lasthenia burkei* in 2006.

Source of Variation	df	Sum of Squares	Percentage of variation
Among counties	1	66.736	11.2
Among populations within counties	12	194.222	10.67
Within populations	293	1216.361	78.13
Fixation indices			
FSC		0.12016	
FST		0.21869	
FCT		0.11199	

Table 6: Pairwise Population Matrix of *PhiPT* values for each Santa Rosa Plain population of *Lasthenia burkei* in 2006 and 2008. All values above the diagonal indicate the level of statistical significance. Underlined values indicate the level of temporal genetic structure within populations across years.

	Alton ln 06	**Dawson 06**	**Maggi 06**	**Preakness 06**	**Pellagrini Wilk 06**	**Piner Marlow 06**	**SR airport runway 06**	**SR airport preserve 06**	**Wood Fulton 06**	**Wood road 06**	**Piner Marlow 08**	**Dawson 08**	**Pellagrini Wilk 08**	**Wood Fulton 08**	**Windsor Garcia 08**
Alton ln 06	**0.000**	0.001	0.003	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Dawson 06	0.147	**0.000**	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Maggi 06	0.072	0.183	**0.000**	0.001	0.001	0.001	0.001	0.003	0.001	0.001	0.001	0.001	0.001	0.056	0.001
Preakness 06	0.051	0.115	0.094	**0.000**	0.004	0.001	0.002	0.001	0.094	0.001	0.001	0.001	0.001	0.002	0.001
Pellagrini Wilk 06	0.082	0.103	0.110	0.043	**0.000**	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Piner Marlow 06	0.076	0.108	0.084	0.055	0.102	**0.000**	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
SR airport runway 06	0.166	0.132	0.157	0.139	0.194	0.125	**0.000**	0.001	0.001	0.005	0.001	0.002	0.001	0.001	0.001
SR airport preserve 06	0.047	0.161	0.060	0.056	0.117	0.056	0.150	**0.000**	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Wood Fulton06	0.098	0.166	0.064	0.015	0.059	0.068	0.192	0.073	**0.000**	0.001	0.001	0.001	0.001	0.001	0.001
Wood road 06	0.163	0.096	0.127	0.084	0.134	0.096	0.077	0.149	0.102	**0.000**	0.001	0.001	0.001	0.001	0.001
Piner Marlow 08	0.115	0.157	0.132	0.102	0.166	**0.071**	0.103	0.112	0.121	0.084	**0.000**	0.001	0.001	0.001	0.001

	Alton ln 06	Dawson 06	Maggi 06	Preakness 06	Pellagrini Wilk 06	Piner Marlow 06	SR airport runway 06	SR airport preserve 06	Wood Fulton 06	Wood road 06	Piner Marlow 08	Dawson 08	Pellagrini Wilk 08	Wood Fulton 08	Windsor Garcia 08
Dawson 08	0.103	**0.156**	0.145	0.143	0.159	0.103	0.128	0.120	0.167	0.157	0.079	**0.000**	0.001	0.001	0.001
Pellagrini Wilk 08	0.109	0.176	0.099	0.115	**0.155**	0.092	0.125	0.110	0.147	0.136	0.038	0.079	**0.000**	0.001	0.001
Wood Fulton08	0.055	0.132	0.026	0.047	0.088	0.064	0.125	0.084	**0.069**	0.096	0.083	0.102	0.055	**0.000**	0.001
Windsor Garcia 08	0.127	0.226	0.150	0.154	0.174	0.128	0.142	0.140	0.181	0.160	0.133	0.124	0.131	0.088	**0.000**

Table 7: AMOVA results for 21 populations in two counties Napa vs. Sonoma of *Limnanthes vinculans* ($P < 0.01$).

Source of Variation	**df**	**Sum of Squares**	**Percentage of variation**
Among groups	1	65.542	13.54
Among populations within groups	19	221.677	4.48
Within populations	1133	3320.145	81.98
Fixation indices			
FSC			0.05177
FST			0.18018
FCT			0.13542

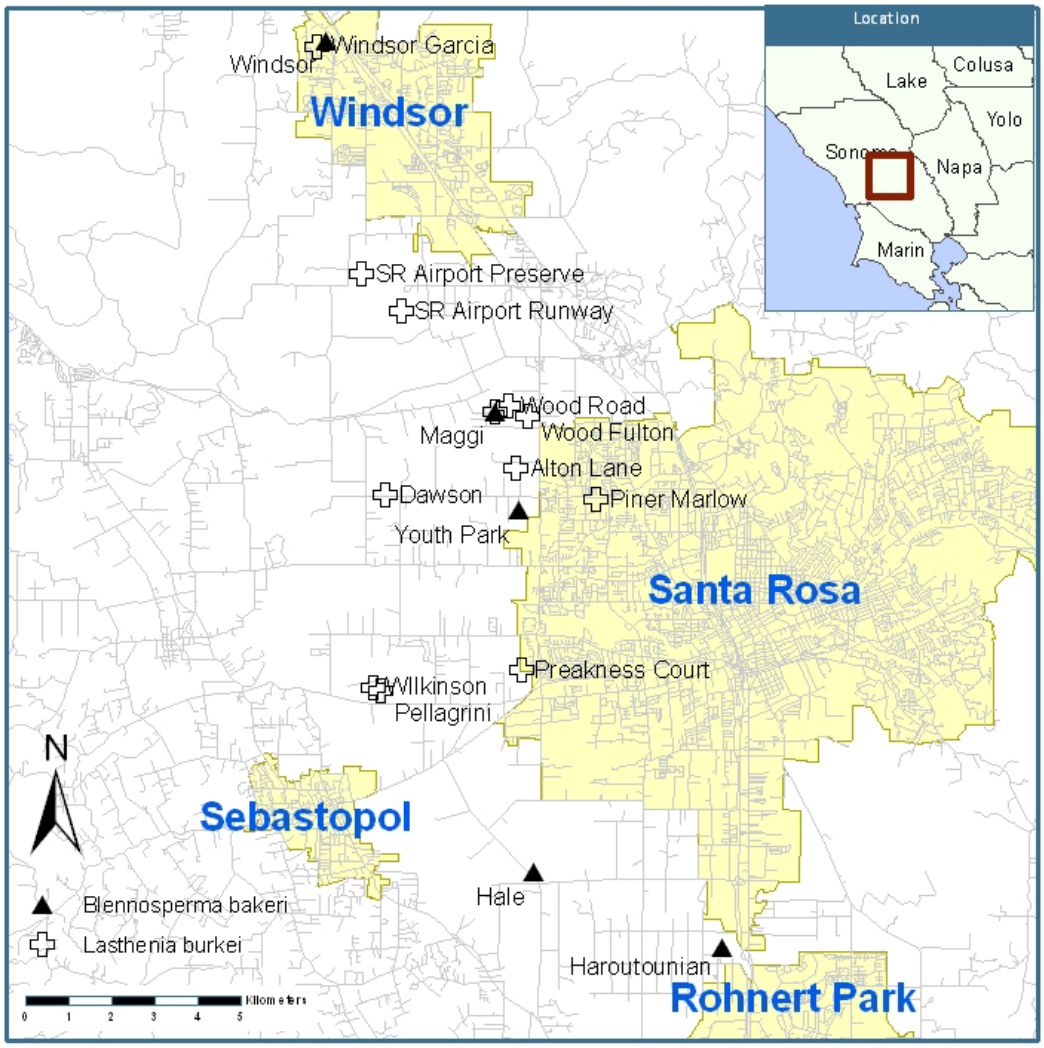

Fig. 1: Map of *Blennosperma bakeri* and *Lasthenia burkei* study populations on the Santa Rosa Plain, Sonoma County (122°46'26.249"W, 38°27'49.249"N). The Sonoma Valley (*B. bakeri*) and two Lake County (*L. burkei*) populations are not shown.

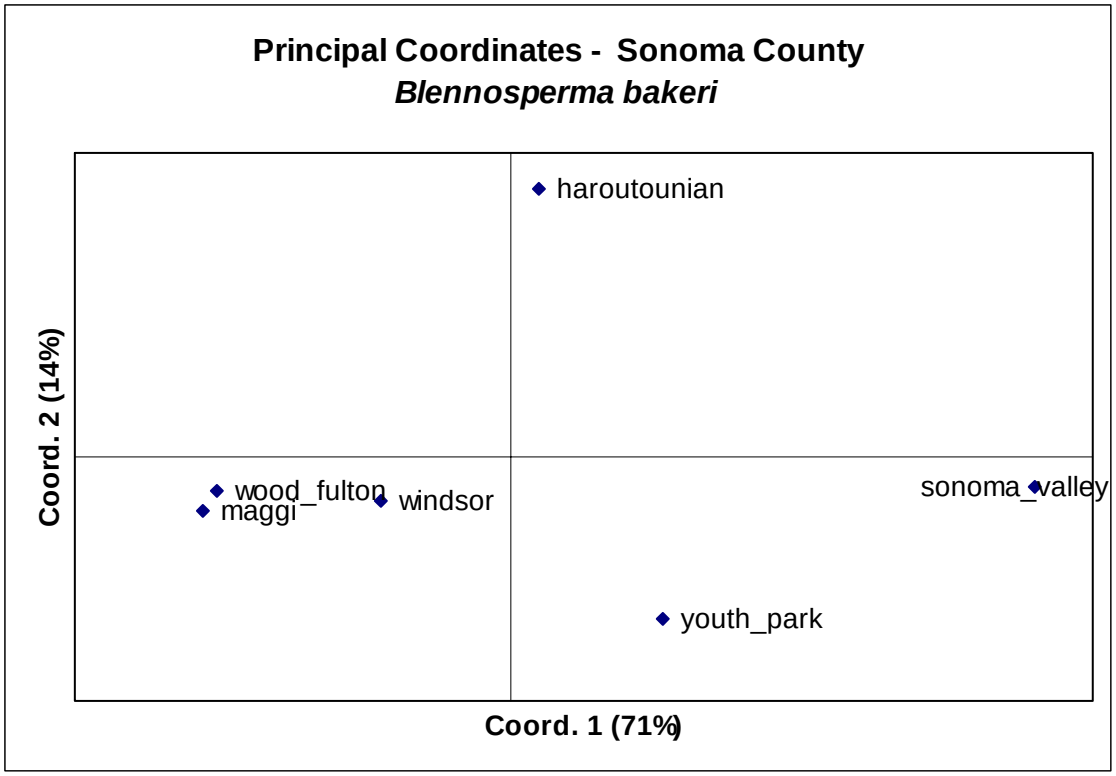

Fig. 2: Principal Coordinate Analysis of six *Blennosperma bakeri* extant populations in 2008 based on a population genetic distance matrix with standardization. Axes 1 and 2 explain 81 % and 14 % of the variation, respectively.

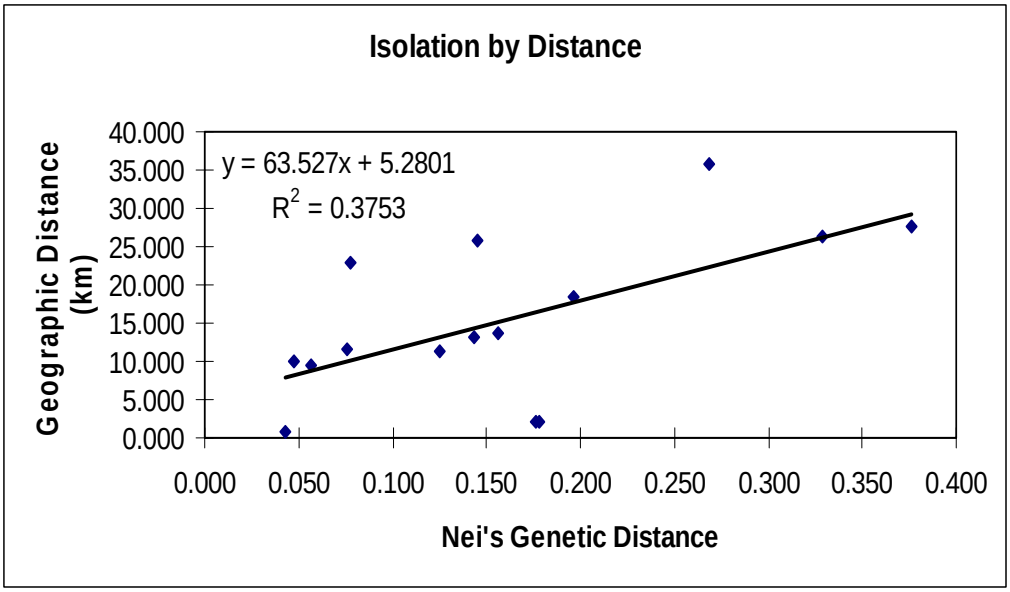

Fig. 3: Isolation by Distance Analysis of five populations of *Blennosperma bakeri*, range-wide including Sonoma Valley.

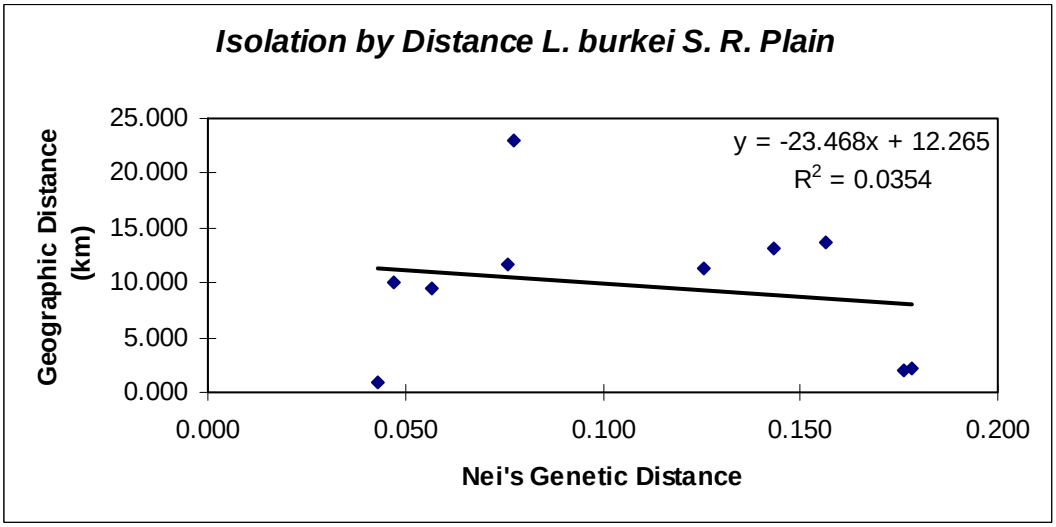

Fig. 4: Isolation by Distance Analysis of five populations of *Blennosperma bakeri* within the Santa Rosa Plain.

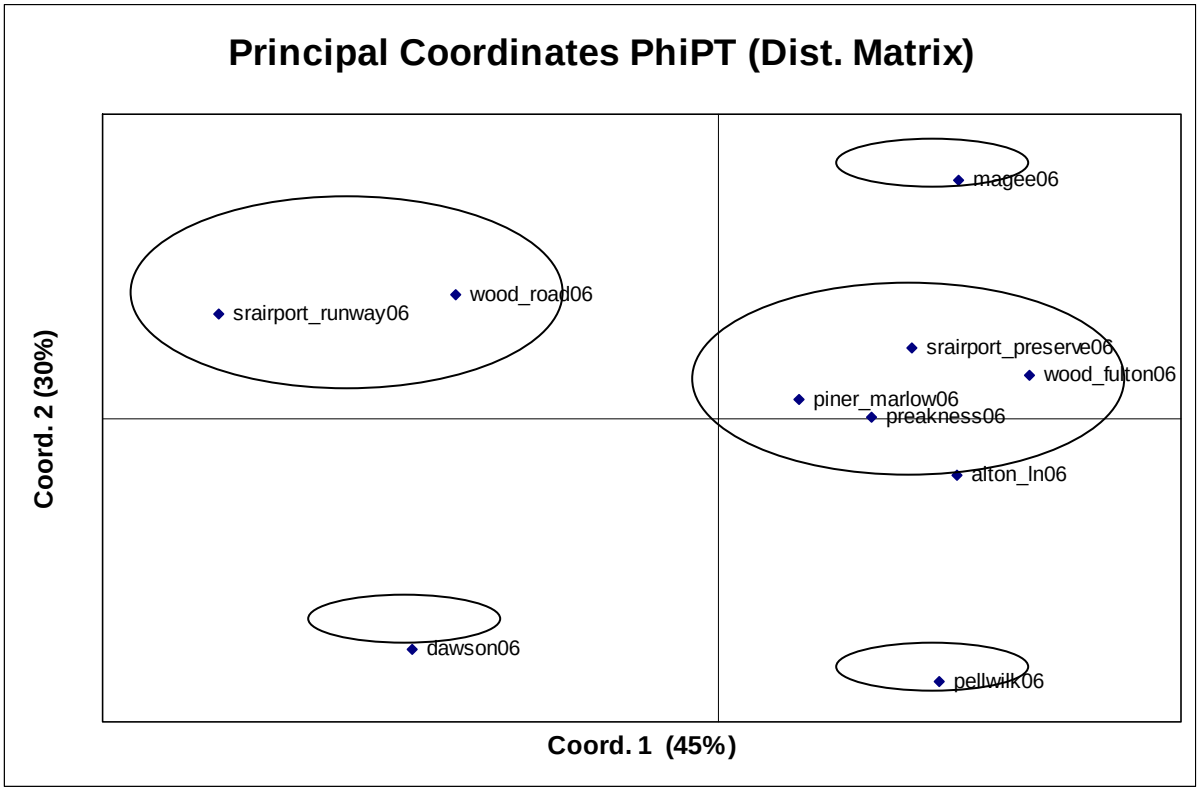

Fig. 5: Principal Coordinate Analysis of 2006 Santa Rosa Plain *Lasthenia burkei* populations, based on a population genetic distance matrix with standardization. Axes 1 and 2 explain 45 % and 30 % of the variation, respectively.

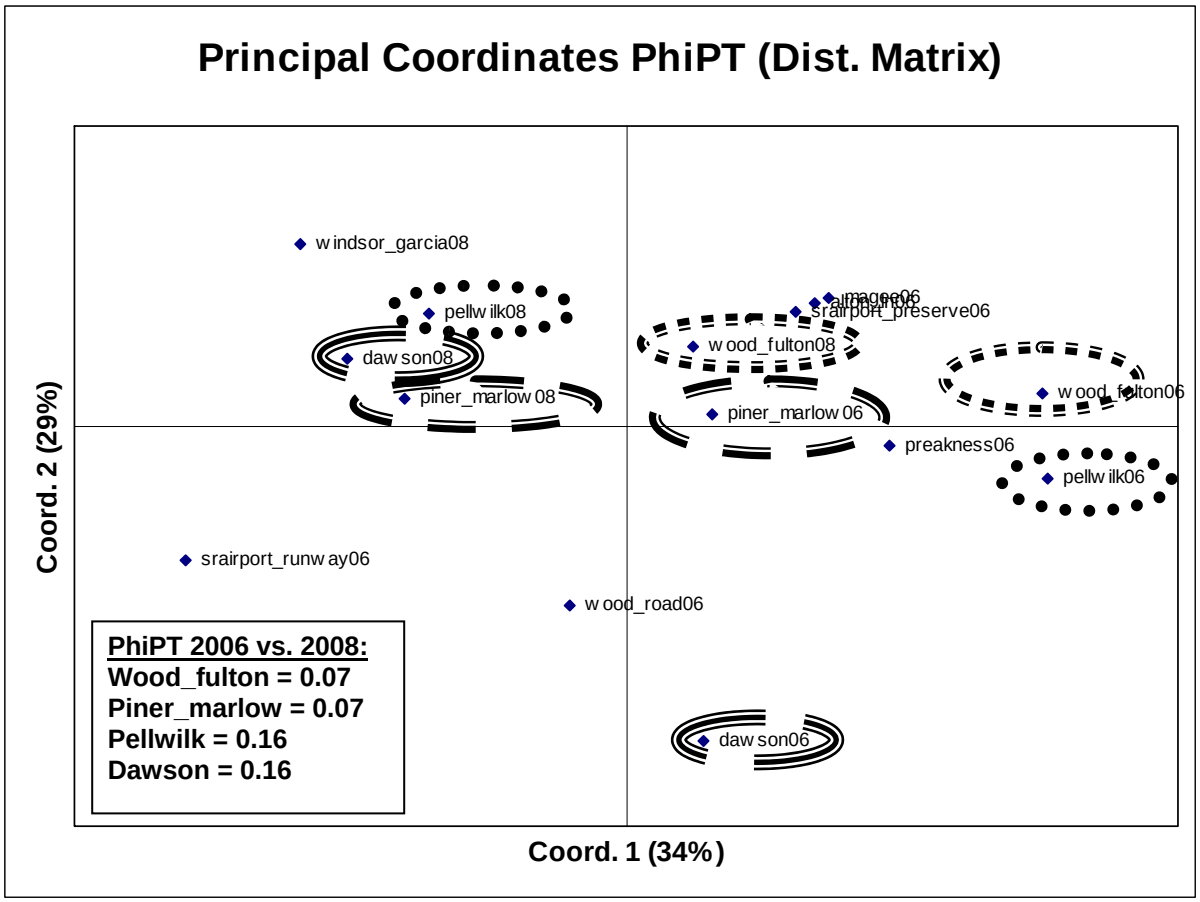

Fig. 6: Principal Coordinate Analysis of all Santa Rosa Plain *Lasthenia burkei* populations surveyed in 2006 and 2008 based on a population genetic distance matrix with standardization. Axes 1 and 2 explain 34 % and 29 % of the variation, respectively. Circled populations indicate temporal genetic structure analysis of four Santa Rosa Plain *Lasthenia burkei* populations: *PhiPT* values indicate the temporal genetic difference between individuals from the same populations sampled in two different years.

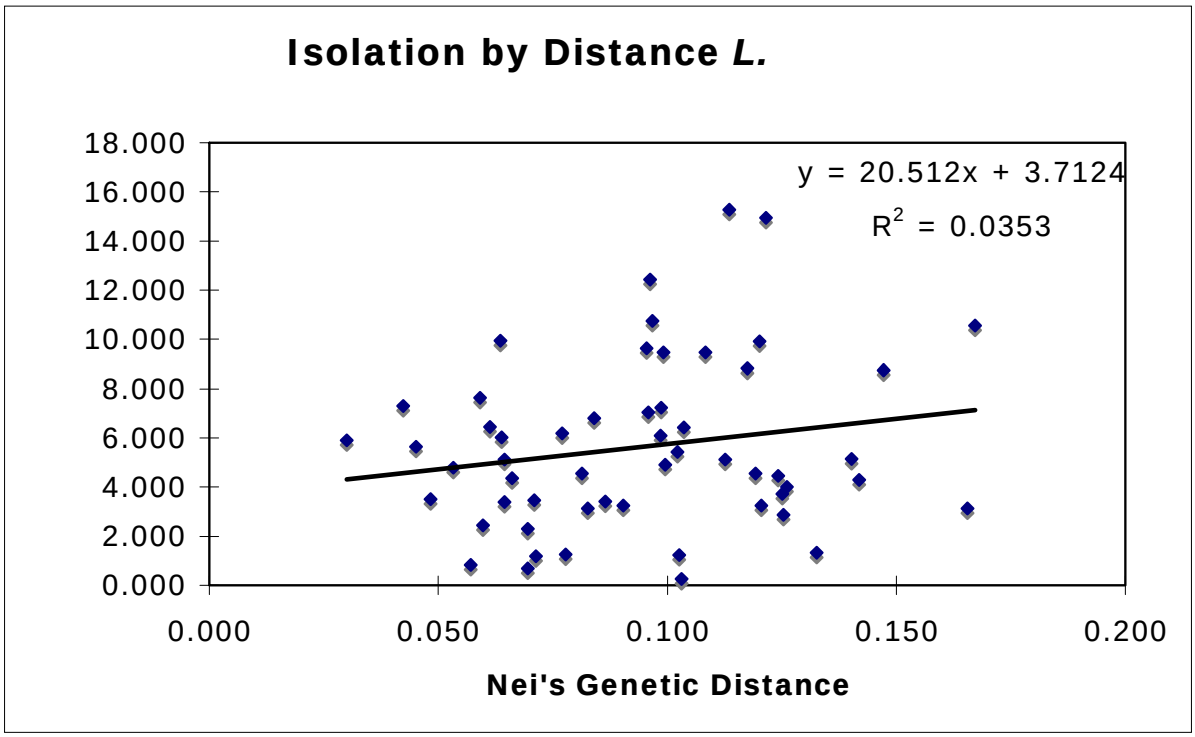

Fig. 7: Isolation by Distance Analysis of Santa Rosa Plain populations of *Lasthenia burkei*.

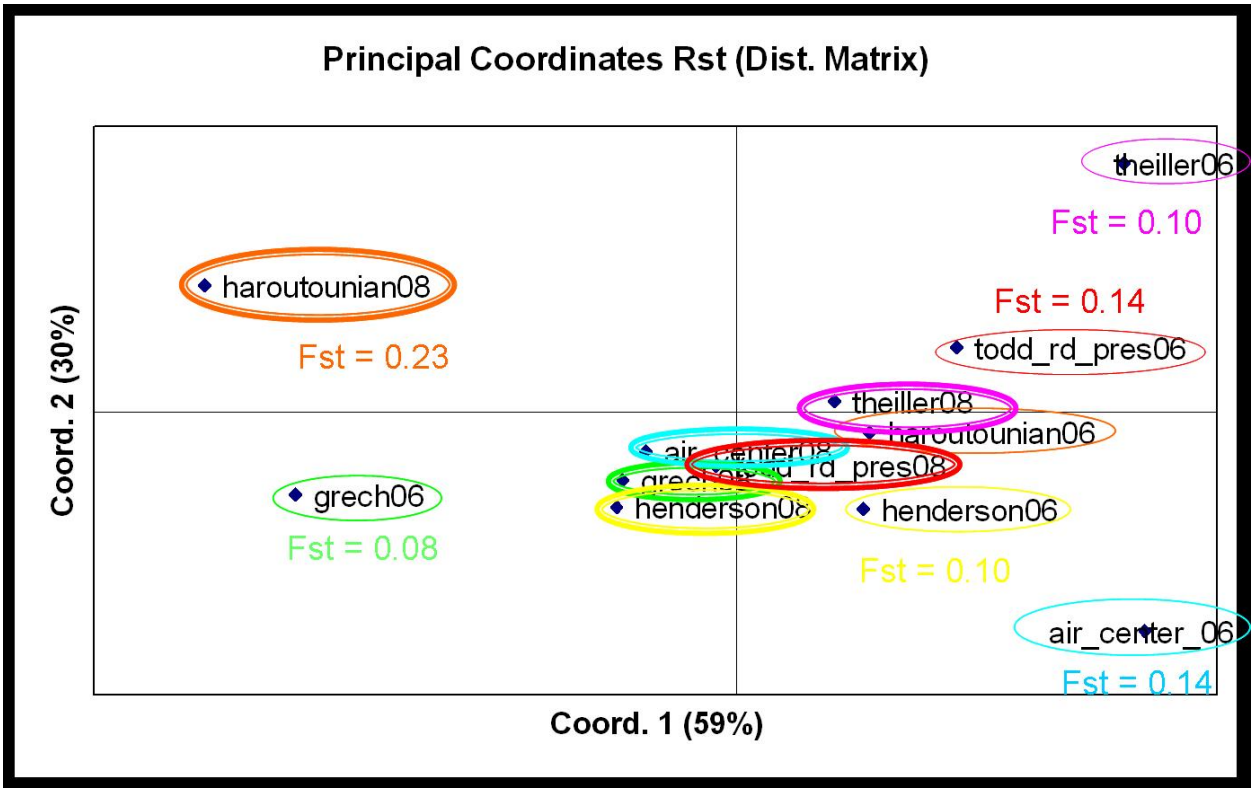

Fig. 8: Principal Coordinate Analysis of all Santa Rosa Plain *Limnanthes vinculans* populations surveyed in 2006 and 2008 based on a population genetic distance matrix with standardization. Axes 1 and 2 explain 59 % and 30 % of the variation, respectively. Circled populations indicate temporal genetic structure analysis of four Santa Rosa Plain *Limnanthes vinculans* populations: *Fst* values indicate the temporal genetic difference between individuals from the same populations sampled in two different years.