California Department of Fish & Wildlife
U.S. Fish and Wildlife Service: Endangered Species Act (Section-6) Grant-in-Aid Program

Final Performance Report

1.	**State:**	California
	FBMS/FAIMS Grant No.	F11AP01065
	Grant Name:	Managing Reproduction and Genetic Diversity in the Ben Lomond and Contra Costa Wallflowers
	Grant Year:	2011
2.	**Report Period:**	8/1/2011 – 7/31/2014
	Final Report Due:	10/31/2014
	Grant Period:	8/1/2011 – 7/31/2014

3. Location of work: This project had field, greenhouse and lab components. The field portion was conducted at the Antioch Dunes National Wildlife Refuge (Contra Costa wallflower) and in the Santa Cruz Mountains (Ben Lomond wallflower). Greenhouse and lab components took place in the Department of Biology at Santa Clara University. Microsatellites were run at the Cornell University Core Lab.

4 Objectives and Expected Results:

The goal of this project was to identify reproductive requirements and distribution of genetic diversity and to continue monitoring of two endangered wallflower species, *Erysimum teretifolium* (Ben Lomond wallflower) and *E. capitatum* var. *angustatum* (Contra Costa wallflower).

- **Objective 1:** Reproductive Biology - Identify the cause of reproductive failure by characterizing the mating system (self-incompatible or not), the primary pollinators and the degree to which these plants rely on pollinators.
  Expected Results: Determine whether or not these species are self-incompatible and the degree of pollinator-mediated outcrossing. Primary pollinators will be identified for both taxa throughout their flowering season in natural populations.

- **Objective 2:** Genetic Diversity - Locate reservoirs of genetic diversity among populations of *E. teretifolium*, determine the genetic consequences of the 1978 bottleneck on genetic diversity of *E. capitatum* var. *angustatum* and assess its evolutionary distinctiveness compared to other varieties of *E. capitatum*.
  Expected Results: The distribution of genetic diversity will likely reflect the naturally fragmented populations of these species. The disjunct nature of sandhill habitats among more mesic forested habitats has likely lead to partitioning of genetic diversity between populations of *E. teretifolium*. Identifying the location of reservoirs of genetic diversity is essential in determining source material for reintroduction efforts. Furthermore, the researchers predicted that genetic diversity in *E. capitatum* var. *angustatum* has been influenced by the 1978 population bottleneck when the population was reduced to 28 individuals.

- **Objective 3:** Monitoring - Conduct annual surveys of all known and accessible occurrences of *E. teretifolium*. Many occurrences of *E. teretifolium* are on private property and access is not assured.
  Expected Results: Conduct annual surveys and record pertinent demographic information for all *E. teretifolium* occurrences to which access is granted.

5. If the work in this grant was part of a larger undertaking with other components and funding, present a brief overview of the larger activity and the role of this project. N/A

6. Describe how the objectives were met.

- **Objective 1:** Reproductive Biology - Identify the cause of reproductive failure by characterizing the mating system (self-incompatible or not), the primary pollinators and the degree to which these plants rely on pollinators.
    - In March, 2012, 270 *E. teretifolium* plants were obtained from Dr. Ingrid Parker (UC Santa Cruz). They were already 1.5 years old and some had flowered the previous spring and had been used in some controlled crosses under Dr. Parker's supervision. Since Dr. Parker's project was no longer funded, the researchers agreed to move the plants to Santa Clara University (SCU) and continue with the controlled crosses using the blocked experimental design that Dr. Parker initiated. Only 16 plants flowered in 2012. In 2012, the following five controlled cross treatments were attempted on the flowering plants: emasculation control, self-pollination, outcross within patch, outcross between patch, and outcross between populations. Only 10 of the 16 plants received all five treatments due to the limited number of potential pollen donors. The remaining six plants received as many pollination treatments as possible. In total, the researchers harvested fruits from 45 pollinations (not including emasculation controls).
    - In 2013, the collection of cultivated *E. teretifolium* plants that were obtained from Dr. Ingrid Parker in the previous year flowered profusely in spring and summer. Pollinations were conducted on an additional 33 plants, bringing the total number of pollinations to 161 when combining 2012 and 2013. In 2013, the researchers focused on self vs. within population vs. between population crosses.
    - When 2012 and 2013 data are combined, the result was a 6.5 times reduction in seed set in self-pollinations compared to outcross pollinations (within population and between population outcross treatments were pooled). The number of crosses producing zero seeds was 4.4 times higher for self-pollinations compared to outcross pollinations. These results are consistent with a self-incompatible mating system common in the mustard family and present in some diploid species of *Erysimum*.
    - The outcrossing treatments resulted in a 13 percent increase in seed set in the between population crosses versus the within population crosses. The difference is statistically significant based on the analysis conducted.
    - Self-incompatibility can also be diagnosed as the inability of pollen tubes to germinate and grow into stylar tissue following self-pollinations. Six self and outcross pollinations were made on four *E. teretifolium* plants in March 2014. The pistils were collected after 24 and 48 hours – sufficient time to detect pollen tube germination and growth in many other mustards. Three out of the six pollinations exhibited self-incompatibility. Two crosses exhibited pollen tube growth in both the self and the outcross pollination treatments. The sixth cross had no pollen tube growth in either the self or outcross pollinations. The crosses were repeated, but pistils were allowed to mature into fruits to determine if the self-pollinations produced any seeds. There was a strong correlation with the plants exhibiting self-incompatibility (no pollen tube growth under the microscope) and no or very few seeds produced. All outcross pollinations that exhibited pollen tube growth also produced normal amounts of seeds.
    - Outcrossing results were also examined in light of the geographic distance between the parents and the genetic distinctiveness of the parents. No correlation was found between seed production and geographic distance, nor between seed production and genetic divergence.
    - During peak flowering of *E. teretifolium* in spring 2012 and 2013, digital video cameras were used to document pollinators. In 2012, pollinator observations were made at three populations: Quail Hollow, SLVWD/Olympia, and Mt. Hermon. In 2013, pollinator observations were made at Quail Hollow and SLVWD/Olympia. Results indicate that this species does not appear to rely on any specialist pollinators and has a relatively high visitation rate. Approximately 57.8% of visits were by insects in the Order Hymenoptera (ants, bees, and wasps). Within Hymenoptera, the three most common function groups were "solitary bees," "large Bombus-like bees," and "large solitary bees." The other two common orders of visitors were Lepidoptera (butterflies and moths) (19.6%) and Coleoptera (beetles) (18.6%).
    - In May 2014, 92.9 "flower hours" of pollinator observations were recorded on *E. capitatum* var. *angustatum* at the Sardis Unit (Antioch Dunes National Wildlife Refuge). There were 79 visitors contacting reproductive parts of the flower, but 58 were from a type of pollen eating Coleopteran (beetle). After removing these visits because they are likely ineffective pollinators, Hymenoptera

was determined to be the most common visitor to *E. capitatum* var. *angustatum*, representing 76% of visits after excluding the pollen-eating Coleopterans.
    - Overall, it was determined that *E. teretifolium* is partially self-incompatible because of the dramatically reduced seed set following self-pollination coupled with the lack of self-pollen tube growth. This suggests that these plants require pollinators.
- **Objective 2:** Genetic Diversity - Locate reservoirs of genetic diversity among populations of the *E. teretifolium*, determine the genetic consequences of the 1978 bottleneck on genetic diversity of the *E. capitatum* var. *angustatum* and assess its evolutionary distinctiveness compared to other varieties of *E. capitatum*.
    - The researchers extracted DNA from a total of 662 individuals of *E. teretifolium* (n=485), *E. capitatum* var. *angustatum* (n=150), and *E. capitatum* var. *capitatum* (n=27). For *E. teretifiolum*, these DNAs represent wild collected leaf tissues representing eight populations (n=207) and greenhouse grown plants involved in the pollination experiment (n=278). Eight microsatellite loci were optimized, and preliminary genotyping revealed more than the two bands expected in a diploid individual. Analysis revealed that *E. teretifolium* and *E. capitatum* var. *angustatum* are hexaploids that can exhibit up to six alleles at a locus.
    - Due to hexaploidy in *E. teretifolium*, the researchers could not confidently determine genotypes, so the data was analyzed with the restriction model in Structure, treating each band as present or absent. A range of population clusters was tested using location priors and allowing for admixture. The number of population clusters that best fit the data was calculated.
    - Structure analysis identified *E. capitatum* ssp. *angustatum* and *E. teretifolium* as different species. Structure analysis for just the *E. teretifolium* samples indicates the most likely number of genetic groupings was two, followed closely by four.
    - AMOVA and F_{ST} were used to determine the distribution of genetic diversity within and among populations of *E. teretifolium*. The majority of genetic diversity in *E. teretifolium* can be found within populations (rather than between populations).
    - To determine if there was evidence of isolation by distance, the researchers compared the genetic distances between populations with their geographic distances. A genetic distance matrix of all individuals was created in PAUP v4.0. This genetic distance matrix was used to calculate the average genetic distance between all pairwise comparisons of populations. This population genetic distance matrix was compared to a geographic distance matrix. A Mantel nonparametric test was used to compare the geographic and genetic distance matrices. Results indicate no evidence of isolation by distance.
    - Twenty-three *E. capitatum* var. *angustatum* individuals from the Sardis Unit and four individuals from the Stamm Unit were genotyped following the same procedure used for *E. teretifolium*. Samples representing four populations of *E. capitatum* var. *capitatum* in the geographic vicinity of the *E. capitatum* var. *angustatum* populations were also included. For comparison, samples were included from *E. capitatum* var. *capitatum* populations in Oregon and Utah and from three other distinct species. Results of the genetic analysis indicate that *E. capitatum* var. *angustatum* is a genetically unique taxa, warranting taxonomic recognition as distinct from *E. capitatum* var. *capitatum.*

- **Objective 3:** Monitoring - Conduct annual surveys of all known and accessible occurrences of *E. teretifolium*. Many occurrences of *E. teretifolium* are on private property and access is not assured.
    - Nine populations of *E. teretifolium* were surveyed over the duration of the project. Eight of the populations were also included in the microsatellite study of genetic diversity. The ninth population, Quail Hollow County Park North, is a newly discovered, relatively small population that was not discovered until after the genetic sampling was complete. A new population was also discovered along a sandhill road cut on Highway 17 northbound between El Rancho Drive exit and Mt. Hermon Road exit. In some populations, population counts were divided into life history stages. The results of the population surveys are summarized in the following table:

Population	Date	Juveniles	1st Year Reproductive	2nd Year Reproductive	Total
Quail Hollow County Park – S	May 7, 2012	371	363	13	747
Quail Hollow County Park – N	April 15, 2013	22	34	5	61
	September 27, 2013	6	16	0	22
Geyer/Randal Morgan Preserve	June 3, 2012	n/a	n/a	n/a	~500
Olympia/San Lorenzo Valley Water District	May 17, 2012	486	157	3	646
Azalea Road (near S Ridge)	May 22, 2012	n/a	n/a	n/a	~450
Hwy 17N at El Rancho	May 15, 2012	21	12	2	35
Mount Hermon	May 31, 2013	19	119	5	138
Bonny Doon Ecological Reserve	April 15, 2013	10	1	0	11
	May 1, 2014	5	3	0	8
Sandhill Road & Glenwood Drive	July 18, 2014	9	5	0	14

- The researchers also surveyed for several historical collections, but they were not relocated. Personal communications between the researcher and Dr. Jodi McGraw indicate that the occurrences have not been relocated because of incorrect/vague original locality information, extirpation, or due to inaccessible private lands.

7. Discuss differences between work anticipated in grant proposal and grant agreement and that actually carried out with Federal Aid grant funds; include differences between expected and actual costs. N/A

8. List any publications or in-house reports resulting from this work.

Managing Reproduction and Genetic Diversity in the Ben Lomond and Contra Costa Wallflowers, Final Report dated July 31, 2014, submitted by Justen Whittall (attached).

9. Name, title, phone number, and e-mail address of person compiling this report

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Managing Reproduction and Genetic Diversity in the Ben Lomond and Contra Costa Wallflowers (Grant Agreement #P1182012)

Chalcedon checkerspot (*Euphydryas chalcedona*) pollinating the Ben Lomond Wallflower (*Erysimum teretifolium*) at Quail Hollow County Park in May, 2012. Photo: JW

Final Report
July 31, 2014

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I. Reproductive Biology of *Erysimum teretifolium*

The goal of assessing reproductive biology of *E. teretifolium* was to determine if *E. teretifolium* was self-compatible. Identifying self-incompatibility would be critical in determining how to manage or supplement existing populations and could guide the germplasm used in creating new populations of *E. teretifolium* to maximize long term population viability.

I.A. Captive Breeding Population

On March 27, 2012, I inherited ~270 *Erysimum teretfolium* plants from Dr. Ingrid Parker (UC Santa Cruz). They were from Geyer Quarry (aka Randall Morgan Preserve), Quail Hollow County Park, San Lorenzo Valley Water District (aka Olympia), and Bonny Doon Ecological Preserve. They were already 1.5 years old and some had flowered the previous spring and been used in some controlled crosses under Dr. Parker's supervision. Since her project was no longer funded, I agreed to move the plants to Santa Clara University (SCU) and continue with the controlled crosses using the blocked experimental design that she initiated. This was a great time savings since it takes the plants over a year to become reproductively mature when grown in standard greenhouse conditions.

Fig. 1. SCU's outdoor garden with newly arrived *E. teretifolium* from UCSC.

When the plants arrived at SCU, they were placed in an outdoor garden to encourage some late winter dormancy and hopefully synchronize flowering to facilitate the crosses (Fig. 1). When the first signs of buds appeared in late spring (5/29/2012), we moved all the plants to the SCU greenhouse where we could control the crosses without the influence of pollinators (Fig. 2).

I.B. Crossing Design

In 2012, only 16 of the plants flowered. Alongside research assistant, Miranda Melen (SJSU Master's student with Rachel O'Malley), we attempted to perform the following five treatments in 2012: emasculation control (anthers removed, no pollen added), self pollination (anthers removed, pollen from another flower on the same plant), outcross within patch (anthers removed, pollen from another plant collected within the same "patch" as determined by Ingrid Parker), outcross between patch (anthers removed, pollen from another plant collected from a different patch within the same population), and between populations (anthers removed, pollen applied from a flower off a plant from a distinct population). Only 10 of the 16 flowering plants received all five treatments due to the limited number of potential pollen donors. The remaining six plants received as many pollination treatments as possible. In total we harvested fruits from 45 pollinations (not including emasculation controls).

Although flowering in 2012 was significantly less than hoped for, it's not surprising given their lack of winter dormancy in the UCSC greenhouses. Luckily, we had very low mortality upon transferring to SCU (only 12/270 individuals died in the first year). Plants were transferred back to the SCU outdoor common garden near the end of September, 2012 to experience their first complete winter.

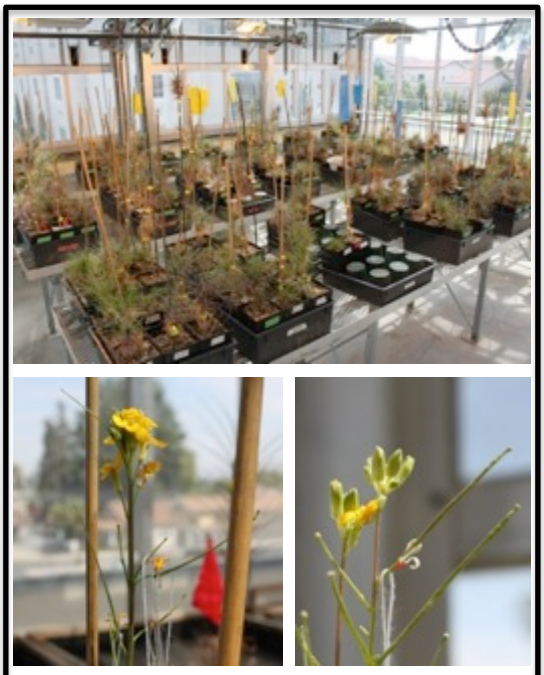

Fig. 2. SCU greenhouse housing the *E. teretifolium* crossing experiment & some successful pollinations. Flowers are marked with puff-paint and jewelry tags.

In 2013, the collection of cultivated Ben Lomond Wallflowers (*Erysimum teretifolium*) started by Ingrid Parker in the UCSC greenhouses flowered profusely in spring and summer after a complete winter outdoors. We conducted pollinations on an additional 33 plants bringing the total number of pollinations to 161 when combining 2012 and 2013. In 2013, we focused on self vs. within population vs. between population crosses (the difference of within patch and between patch was the least dramatic in 2012 so received lower priority in 2013). Emasculation controls on all of these plants consistently produced zero seeds indicating the greenhouse was pollinator free and our emasculation methods were also pollen-free. Although there is a small, third set of crossing data from the UCSC greenhouses conducted by Ingrid Parker and her assistants in 2011, these crosses were conducted under different environmental conditions (UCSC vs. SCU) using slightly different methodologies. Therefore, we did not include their results in the following analyses (although qualitatively, the results from the UCSC crosses are consistent with the results from the SCU results presented below).

I.C. Crossing Results

When 2012 and 2013 data from SCU are combined, the most remarkable result was a 6.5× reduction in seed set in self-pollinations (n = 43) compared to outcross pollinations (within population and between population outcross treatments pooled; n = 118; Kruskal-Wallis test, $p < 0.0001$; Fig. 3). The number of crosses producing zero seeds was 4.4× higher for self-pollinations (27/43 = 62.8%) compared to outcross pollinations (17/118 = 14.4%). These results are consistent with the preliminary work of Dr. Ingrid Parker where only 3 of 22 self-pollinations produced seeds (data not shown). The pattern suggests a self-incompatible mating system – common in the mustard family and present in some diploid species of *Erysimum*. Self-incompatibility in hexaploid *E. teretifolium* is somewhat unexpected since the self-incompatibility system frequently breaks-down following multiple duplications of the genes coding for the sporophytic incompatibility system (see Section **I.D. SRK Allele Sequencing** below).

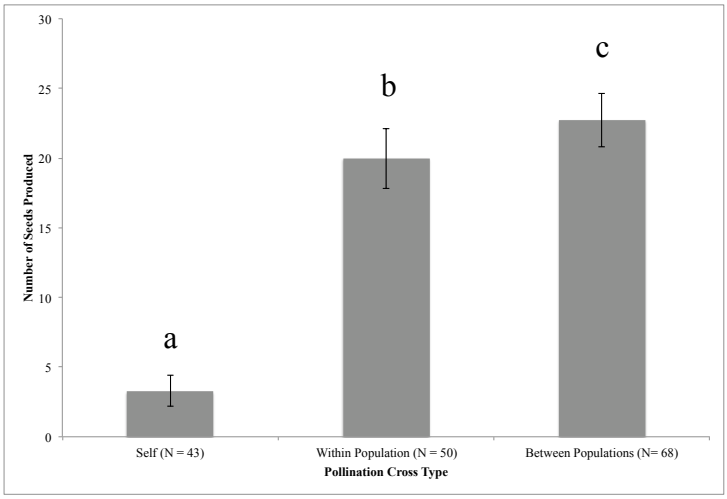

Fig. 3. Self pollinations produced 6.5x less seeds than the two outcross pollination treatments. Between population crosses produced 13% more seeds than within population crosses. Different letters indicate significant differences at $p < 0.05$.

In comparing the two outcross treatments, there is a ~13% increase in seed set in the between population crosses (22.69 seeds/silique) versus the within population crosses (19.96 seeds/silique). This is a significant difference based on a non-parametric statistical analysis because of the non-normal distribution of seed counts (between population crosses n = 68; within population crosses n = 50; $p < 0.0001$; Fig. 3). We can dissect the differential seed set in the two outcross pollination treatments by examining characteristics of crosses producing the lowest and highest seed counts. There was only slightly fewer crosses producing siliques with zero seeds in the between population crosses compared to within population crosses (9/68 = 13.2% vs. 8/50 = 16.0%, respectively). When examining the top quartile of seed producing crosses among all outcross treatments (n=30 out of a total of 118), there was an excess of between population crosses (63%) compared to within population crosses (37%). Yet, due to unequal sample sizes of between and within crosses that were made, we would expect 57.6% of crosses in the top quartile to be from the between population crosses and 42.4% to be from the within population crosses. Regardless of the unequal sample sizes, we still find a 5% excess of between population crosses among the top seed producing crosses. Yet, the mean seed set in the top quartile is not significantly different when comparing the two outcrossing treatments (between populations = 42.2 seeds/silique; within population = 40.7 seeds/silique; t-test, $p>0.05$). Thus, it appears that weak differences in

both the lowest and highest seed producing crosses have contributed to the weakly significant difference in the two outcrossing treatments.

I.D. SRK Allele Sequencing

To confirm self-incompatibility, we initiated a collaboration with Dr. Jeremiah Busch at Washington State University (Pullman, WA). He has amplified the SRK locus and is using single-strand-conformation-polymorphisms to quantify allelic diversity at the self-incompatibility locus for a small set of parents in these crosses. Thus far, all samples produced single, strong bands following PCR amplification of the SRK locus (see Fig. 4A). Bands were then separated, excised and sequenced to compare the number, diversity and evolutionary history of the SRK locus in *E. teretifolium* (Fig. 4B and Fig. 4C). Unfortunately, no progress has been made in the past six months. It is unclear if, or when, we will have definitive results. Therefore, we have taken a more direct route to examining the likelihood of self-incompatibility by visualizing pollen tube growth on self versus outcross pollinations (see section **I.E. Pollen Tube Growth Experiment** below).

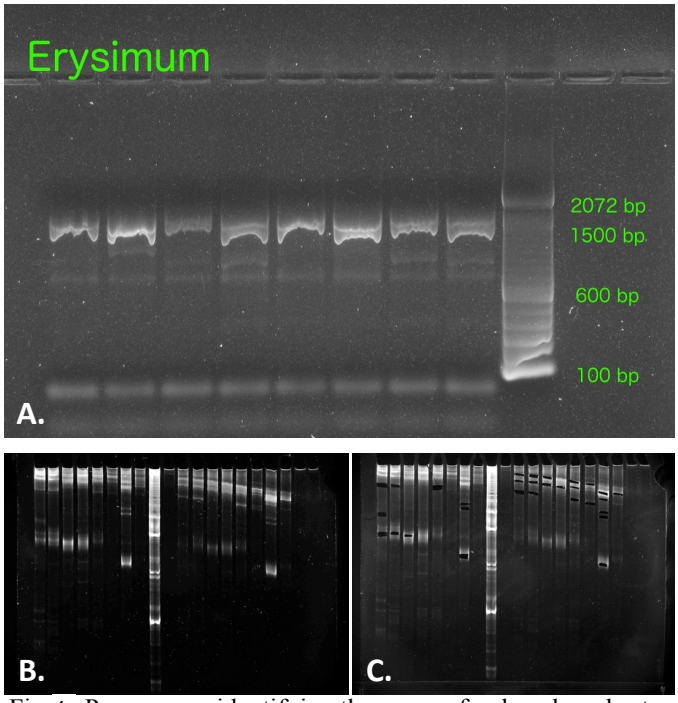

Fig.4. Progress on identifying the cause of reduced seed set in self-pollinated *Erysimum teretifolium*. (A) Agarose gel image of eight plants after amplification with mustard SRK primers shows single bands in the expected size range (1500-2000 bp based on ladder on right). (B) An SSCP gel was used to separate SRK PCR products into single strands of DNA. Bands of interest were excised from the SSCP gel as can be seen in (C). They will be sent to sequencing to determine if *E. teretifolium* is self-incompatible.

I.E. Pollen Tube Growth Experiment

Self incompatibility can also be diagnosed as the inability of pollen tubes to germinate and grow into stylar tissue following self pollinations. Therefore, we made six self and outcross pollinations on four plants in March 2014, then collected the pistils after 24 and 48 hours - sufficient time to detect pollen tube germination and growth in many other mustards. Pistils were fixed, then rinsed in NaOH and visualized using fluorescence microscopy at 40x magnification. Three out of six pollinations exhibited self-incompatibility (Fig. 5). Two crosses exhibited pollen tube growth in both the self and the outcross pollination treatments. The sixth cross had no pollen tube growth in either the self or outcross pollinations. The crosses were repeated, but pistils were allowed to mature into fruits to determine if the self-pollinations produced any seeds. There was a strong correlation with the plants exhibiting self-incompatibility (no pollen tube growth under the microscope) and no or very few seeds produced. All outcross pollinations that exhibited pollen tube growth, also produced normal amounts of seeds (10-30 seeds per silique).

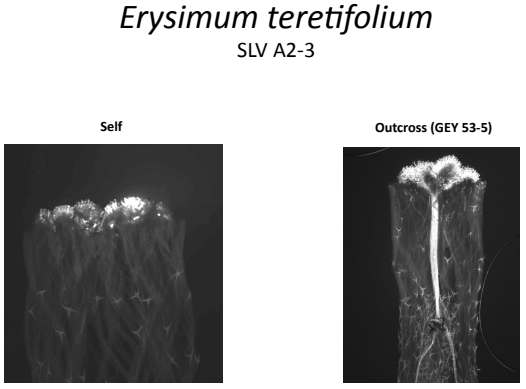

Fig. 5. Pollen tubes fail to germinate and grow after self-pollination (left) compared to outcross pollinations (right). Pollen and pollen tubes stain white with UV florescence microscopy (40x mag.).

I.F. Correlations with Geographic & Genetic Distance

Furthermore, we have examined these crossing results in light of the geographic distance between parents and the genetic distinctiveness of the parents. For the geographic distance analysis, we used the location of the original seed collection sites ground-truthed by Ingrid Parker, Miranda Melen and Justen Whittall in spring 2013. These GPS coordinates were used to determine straight-line geographic distances between parents used in all outcross treatments (n=118; Fig. 6). Geographic distances between parents of the outcross treated individuals ranged from 5m (within population crosses) to 8,237.7m (between population crosses involving the disjunct Bonny Doon population). The average geographic distance between parent plants was 579m and the median was 1,368m. We log transformed the geographic distances because of the leptokurtic nature of these

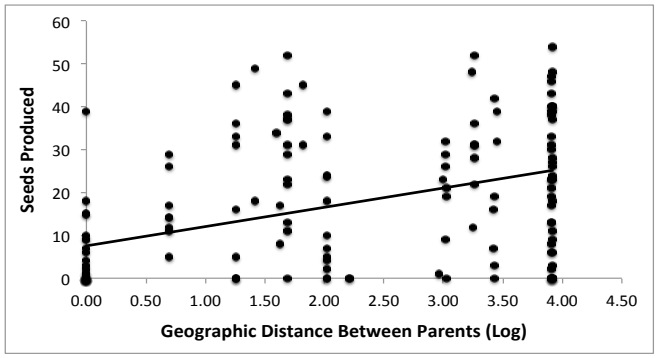

Fig. 6. No significant correlation between seed production and geographic distance among outcross treatments (Linear regression, n=118, $y = 4.4956x + 7.5556$, $R^2 = 0.19$, $p > 0.05$).

geographic distances. We conducted a range of regression analyses, none of which showed a significant correlation between seed set and log geographic distance. Linear regression explained less than 20% of the data ($r^2 = 0.19$; $p>0.05$; Fig. 6). The columns of datapoints in Fig. 6 represent repeated crosses between populations separated by the same geographic distance. Since seed production is discrete count data with several zeros, we also conducted a corrected version of the linear regression (quasi-Poisson) which accounts for the absorbing boundary of zero and an excess of crosses producing zero seeds. Even with this corrected version, there is no correlation between seed set and log geographic distance (quasi Poisson regression, n=118, t-value 1.189, p = 0.235).

To determine if there was a correlation between seed production and the genetic divergence of the parents for the *outcross treated individuals*, we used three heterospecific microsatellite primer pairs developed for the closely related *E. mediohispanicum*. Since *E. teretifolium* is a hexaploid, we could not determine heterozygosity at each locus. Instead, we relied simply on band presence or absence. In total, we identified 24 variable bands among the individuals used in this crossing experiment. If genetic divergence explains variation in seed set, we expect parents with different microsatellite bands to produce more seeds than parents with shared microsatellite bands. Since some bands were quite rare in some populations, we weighted our genetic divergence measure by the source population allele frequency based on tissue collected from wild individuals (see **II. Genetic Diversity** section below). Thus, if two parents shared a rare band, they were considered less genetically divergent than if two parents shared a high frequency band. There was very little correlation between seed count and genetic divergence, the latter only explaining 1.5% of the variation in seed count (linear regression n = 118, $r^2 = 0.0151$; $p > 0.05$, Fig. 7). In a more sophisticated statistical analysis accounting for the absorbing boundary of zero (quasi-Poisson) we found similarly non-significant correlations between seed count and genetic divergence (quasi-Poisson regression, n = 118, t=0.572, p = 0.569). It appears that genetic divergence at these microsatellite loci using our metric do not predict the number of seeds that will be produced among outcross pollinations.

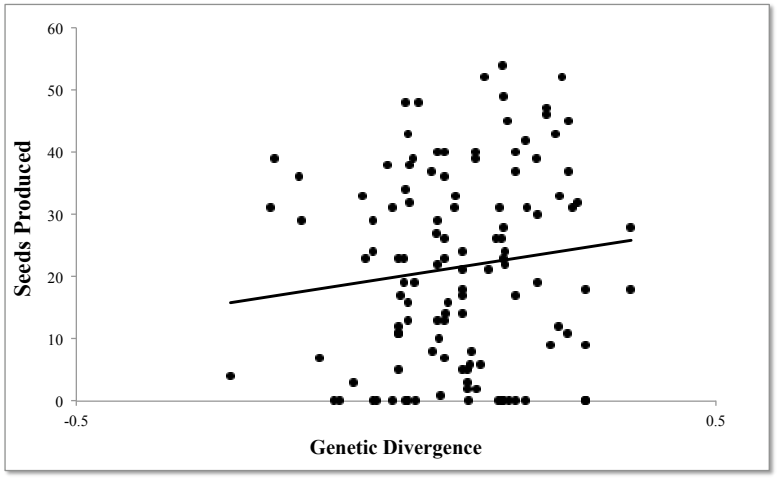

Fig. 7. No significant correlation between seed production and genetic divergence among outcross treatments (Linear regression, n=118, y = 16.005x + 19.965, $R^2 = 0.015$, $F_{1,116} = 1.778$, p = 0.185).

I.G. Reproductive Biology Conclusions

Overall, we conclude that *E. teretifolium* is partially self-incompatible because of the dramatically reduced seed set following self-pollination coupled with the lack of self-pollen tube growth. This suggests that these plants require pollinators (consistent with the high floral visitation rates – see **II. Pollinator Observations** below). Thirteen percent more seed is produced in crosses involving parents from different populations when compared to within population crosses. There is neither a correlation between seed production and geographic distance, nor between seed production and genetic divergence.

II. Pollinator Observations

The objective of observing pollinators of *E. teretifolium* was to determine pollinator diversity, frequency and efficiency. This complements the reproductive biology results and can help inform conservation and management decisions if specialized pollinators are identified or if visitation rates are high and pollinators are essential.

II.A. Study Sites & Methods

During peak flowering of *E. teretifolium* in Spring 2012 and 2013, we documented pollinators using digital video cameras. In 2012, we made pollinator observations at three populations: Quail Hollow (5/7/12), SLVWD/Olympia (5/18/12), and Mt. Hermon (5/25/12). In 2013, we recorded pollinator observations at Quail Hollow (3/27/13 & 3/29/13) and SLVWD/Olympia (3/29/13 & 5/31/13). In total, we collected approximately 54.5 hours of video footage. Since each camcorder was focused on between one and 17 flowers (median = 7 flowers/camcorder), we collected a total of 1108.8 "flower hours" of pollinator observations.

Each camcorder was placed approximately 50 cm from one or two flowering inflorescences at an angle of between 0 degrees (horizontal) to 30 degrees below horizontal and recorded floral visitors from sunrise to sunset (Fig. 8). Sunrise temperatures in May were ~42F making it unlikely that pollinators would be on the wing before the start of our recordings. Furthermore, no nocturnal pollinators were observed during repeated visits to these populations after sunset (Whittall, personal observation), yet no formal pollinator observations were conducted past sunset.

Fig. 8. Camcorder set-up & student assistants Miranda Melen & Gavin Powell(B).

Videos were downloaded to an iMAC and converted to Quicktime format using iMovie. Quicktime clips were watched at 1-4x speed. We recorded all insect visits (and one arachnid) that made contact with flowers' reproductive parts. We extracted the duration of visit and captured 1-3 still images of the pollinator for later identification. Pollinators were identified with the assistance of entomologist colleagues. For analysis, we grouped pollinators into functional guilds within each taxonomic Order.

II.B. Pollinator Identifications & Visitation Results

We documented 506 floral visitors that contacted reproductive parts in 2012 and 2013 combined (0.46 visits/flower/hr). After identifying pollinators to Order (see Fig. 9 for exemplars), we estimate approximately 57.8% of visits are by Hymenoptera. Within the Hymenoptera, the three most common functional groups were "solitary bees" (42.2%), "large Bombus-like bees" (9.5%) and "large solitary bees" (5.0%; Fig. 10). The other two common Orders of visitors were Lepidoptera (19.6%) and Coleoptera (18.6%). Only 0.6% of visitors remain unidentified, yet they are preserved in digital clips and still images (available from JBW by request).

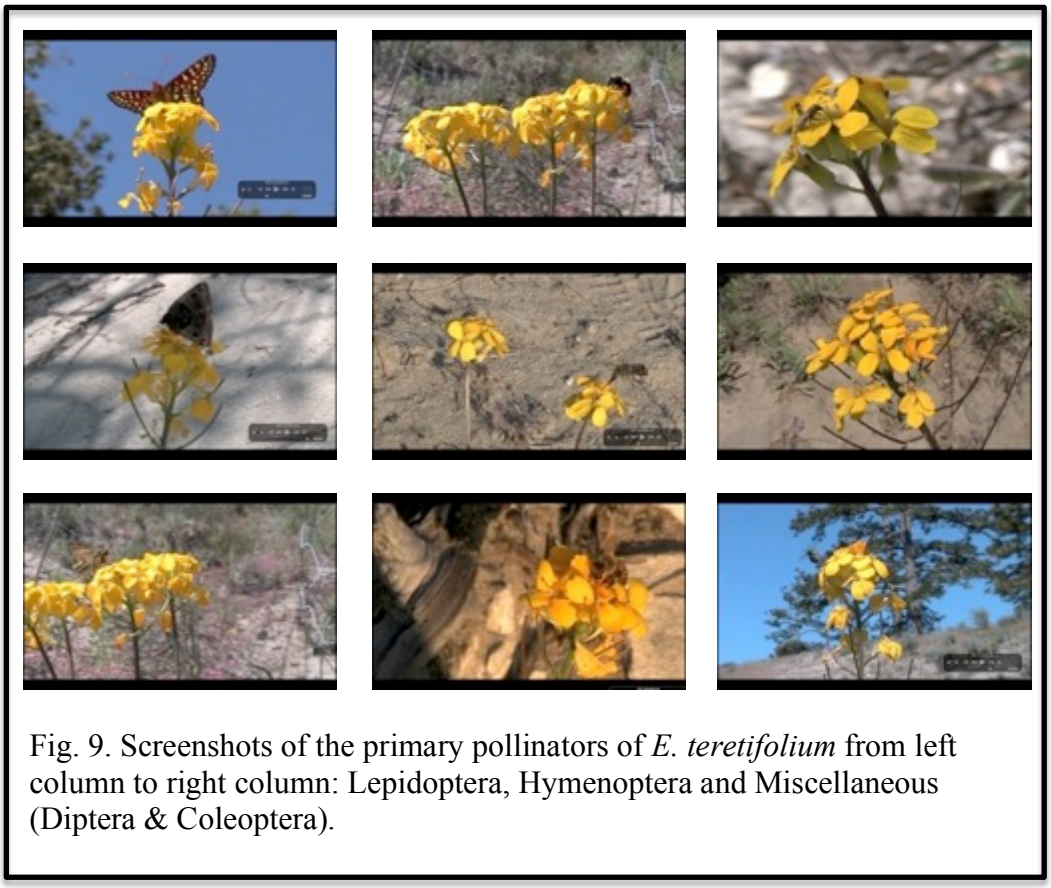

Fig. 9. Screenshots of the primary pollinators of *E. teretifolium* from left column to right column: Lepidoptera, Hymenoptera and Miscellaneous (Diptera & Coleoptera).

The effectiveness of each pollinator type at removing and depositing pollen can be approximated by the amount of time spent on each flower (longer visit durations correlate with increased pollen removal & deposition). Visitation durations ranged from one second to 31 minutes and 43 seconds (Coleoptera, Mordellidae, tumbling flower beetle). The median visit duration was nine seconds (average = 57 seconds). The longest average flower visitor was by Hemiptera (aphids and leaf hoppers = 3 minutes, 3 seconds). Coleoptera and Hymenoptera both had similar average visit durations (1 minute, 6 seconds and 1 min, 5 seconds, respectively).

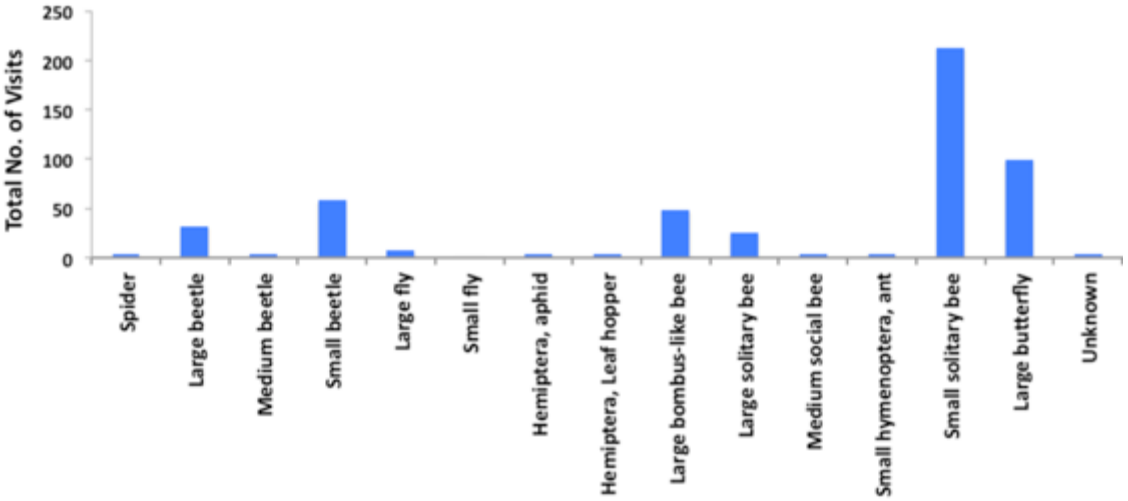

Fig. 10. Number of visits to *E. teretifolium* in 2012 and 2013 divided into functional pollinator guilds.

We can also estimate the number of within plant visits (geitonogamy) that would be likely to lead to self-pollination – a consequence of having several flowers on a plant open simultaneously. In 2012, we recorded 140 out of 231 visits where pollinators moved between flowers within the digital camcorder frame (61%). We did not note this for 2013. Coupled with the previous mating system results, this high rate of geitonogamous selfing (which is a minimum since we cannot be sure that pollinators entering the frame didn't come from the same plant outside of the frame) could be costly to *E. teretifolium* by covering the stigma with self-pollen that has a low probability of germinating, growing and eventually producing viable seeds.

II.C. Pollinator Observations Conclusions

Erysimum teretifolium is visited by a diversity of insect pollinators. As far as we can tell from multiple years, at multiple times during the flowering season, in multiple populations, this species does not rely on any specialist pollinators. It has a relatively high visitation rate (0.46 visits/fl/hr). If each flower lasts three days and pollinators are active for 50% of the time from 9:00am until 6:00pm (very conservative estimates), then each flower would be visited more than six times (0.46 visits/fl/hr x 4.5hrs/day x 3 days). In comparison, we collected 92.9 flower hours of pollinator observations on *E. capitatum* var. *angustatum* in May, 2014 at the Sardis Unit (Antioch Dunes National Wildlife Refuge). There were 79 visitors contacting reproductive parts of the flower, but 58 of these were from a type of pollen eating Coleopteran (Dermestidae). After removing these visits because they are likely ineffective pollinators, the visitation rate was 0.23 visits/fl/hr, or half of that for *E. teretifolium*. Yet, similar to *E. teretifolium*, Hypmenoptera was the most common visitor to *E. capitatum* var. *angustatum* representing 76% of visits after excluding the pollen-eating Coleopterans. Hymenopteran visitors were primarily bumblebees, honeybees, and solitary bees. In both species of *Erysimum*, the likelihood of geitonogamous selfing is very high with more than half of

visits arising from movements of the pollinator between flowers of the same plant. The consequence of geitonogamy in a largely self-incompatible mating system is decreased seed set.

III. Genome Sizing

We needed to compare the genome size of *E. teretifolium* with other *Erysimum* species with known genome sizes and chromosome counts to confirm its polyploid nature. It has 2n=36 chromosomes suggesting it may be a hexaploid. A polyploid of this nature would complicate genotype assignment during microsatellite genetic analysis. It would be impossible to differentiate homozygotes from heterozygotes, yet we could still score microsatellite alleles as present or absent.

We confirmed polyploidy in *E. teretifolium* by estimating the genome size using flow cytometry (in collaboration with Dr. Aru K. Arumuganathan, Benaroya Research Institute at Virginia Mason, Seattle WA). Leaf tissue of *E. teretifolium* from four populations consistently produced 2c = 2.92 pg (range 2.82 – 3.06) which is substantially larger than genome sizes estimated for Eurasian *Erysimum* species with smaller chromosome counts (*E. scoparium* 2n=28 & 2c = 1.08 pg; *E. bicolor* 2n=28 & 2c = 1.16 pg; *E. chieranthoides* 2n=16; 2c = 1.66 pg; http://data.kew.org/cvalues/).

IV. Microsatellite Genetic Diversity

The goal of assessing genetic diversity in *E. teretifolium* was to identify reservoirs of variation among isolated sandhill outcrops. We expected to find the partitioning of microsatellite diversity comparable to island species given the disjunct nature of the Zyante Sandhills (compounded by recent habitat destruction, extirpation and increased population fragmentation). This data is important in locating source populations for establishing new populations with the greatest chances of long-term persistence. Genetic barriers among populations would suggest substantial uniqueness of individual populations, whereas genetic homogeneity among populations could suggest that most source populations are representative of the species genetic variation.

IV.A. Microsatellite Sampling & DNA Extraction

We extracted DNA from a total of 662 individuals of *E. teretifolium* (n=485), *E. capitatum* var. *angustatum* (n=150) and *E. capitatum* var *capitatum* (n=27). For *E. teretifolium*, these DNAs represent wild collected leaf tissues (n=207) and greenhouse grown plants involved in the pollination experiment (n=278). The wild collected *E. teretifolium* samples are from eight populations representing the extent of the range of this species (Bonny Doon, Quail Hollow County Park, Azalea Road, Sandhill Rd/Glenwood Dr, Olympia, Mount Hermon, Geyer/Randall Morgan Preserve, and Hwy 17).

The DNAs were highly concentrated and very pure – two essential criteria for microsatellite analysis. The average concentration of DNA for all the extractions is 58.4ng/uL with only one sample below 5ng/uL (samples >5ng/uL have worked previously for microsatellite PCR reactions). We measure DNA purity using the ratio of absorbance at 260nm to 280nm expecting a ratio of 1.8 or above when there is no contamination. The average purity ratio for these DNA extractions is 1.85 with only three samples scoring below 1.5 (a minimum purity score for proceeding with microsatellite analysis).

IV.B. Microsatellite Amplification, Separation, Scoring & Analyses

We optimized eight microsatellite loci originally developed for *E. mediohispanicum* from the Sierra Nevada of Spain. Each locus now produces a single, strong PCR product in *E. teretifolium* and *E. capitatum* var. *angustatum*. Preliminary genotyping of 24 individuals per locus repeatedly revealed more than the two bands expected in a diploid individual like we saw in our *E. mediohispanicum* positive controls. We estimated as many as six bands in most of the loci for both North American taxa suggesting some history of polyploidy. Chromosome counts for the entire *Erysimum capitatum* alliance including *E. teretifolium* & *E. capitatum* var. *angustatum* is 2n=36 compared to *E. mediohispanicum* (2n=14) thereby confirming a complex history of polyploidy in the *E. capitatum* alliance since the arrival of the *E. capitatum* ancestors from Eurasia. The *E. mediohispanicum* samples consistently produce just one or two microsatellite alleles as expected from a diploid.

Thus, *E. teretifolium* and *E. capitatum* var. *angustatum* are hexaploids that can exhibit up to six alleles at a locus. Because of the polyploidy, we were not able to assign genotypes

(homozygotes vs. heterozygotes), yet we were still able to compare allelic diversity within and among populations – the primary objective of the microsatellite study. Several of the analytical methods proposed also work with "dominant" markers equivalent to data produced by amplified fragment length polymorphisms (AFLPs) or restriction fragment length polymorphims (RFLPs). Therefore, we proceeded with high-throughput microsatellite genotyping of the wild populations and supplemented with some individuals from the greenhouse crossing study in order to include the now largely extirpated Bonny Doon population.

As part of her Senior Honor's thesis, Julie Herman (SCU undergraduate research assistant) and I examined *E. teretifolium* and *E. capitatum* var. *angustatum* for four of the eight microsatellite loci to determine the distribution of genetic diversity within and among populations. We genotyped 233 *E. teretifolium* individuals which represent eight populations (11-43 individuals per population). We included 27 individuals of *E. capitatum* var. *angustatum* to confirm the analysis methods could distinguish this species as a distinct genetic cluster. PCR amplifications were carried out using three microsatellite primer pairs developed for the European *E. mediohispanicum* according to the methods described in that original paper (the fourth locus appears in one of these three primer pair PCR products as a distinctly sized fragment). Alleles were separated on an ABI3730 with a LIZ600 size standard, and lengths were determined using PeakScanner Software v1.0 (Life Technologies).

To confirm the reliability of our microsatellite fragments as genetic markers, we examined the inheritance of most scored fragments by genotyping five controlled crosses for all four loci. Crosses were chosen to maximize insights across all four loci. On average, 12.7 F1 offspring per locus were genotyped to determine whether markers were inherited in a predictable fashion (n = 1 - 4 offspring per cross). Of 24 fragments initially detected, 16 were examined in the F1 generation. We removed any fragments that appeared in offspring that were not present in the parents from later analysis.

Due to hexaploidy in *E. teretifolium*, we could not confidently determine genotypes, so we analyzed the data with the restriction model in Structure treating each band as present or absent. A range of population clusters (k = 1-10) were tested using location priors and allowing for admixture (ngen=10^6, 5 replicates per k-value, burnin=$5x10^5$, lambda=2.1237 determined empirically). The number of population clusters that best fit the data was calculated using the Δk method of Evanno *et al.* (2005) in Structure Harvester (Earl *et al.* 2011). Runs with identical parameters were conducted including samples from the closely related wallflower, *E. capitatum* var. *angustatum* (ERCAAN), to ensure the model could differentiate these taxa.

IV.C. *Erysimum teretifolium* Microsatellite Results – Bayesian Genetic Clustering

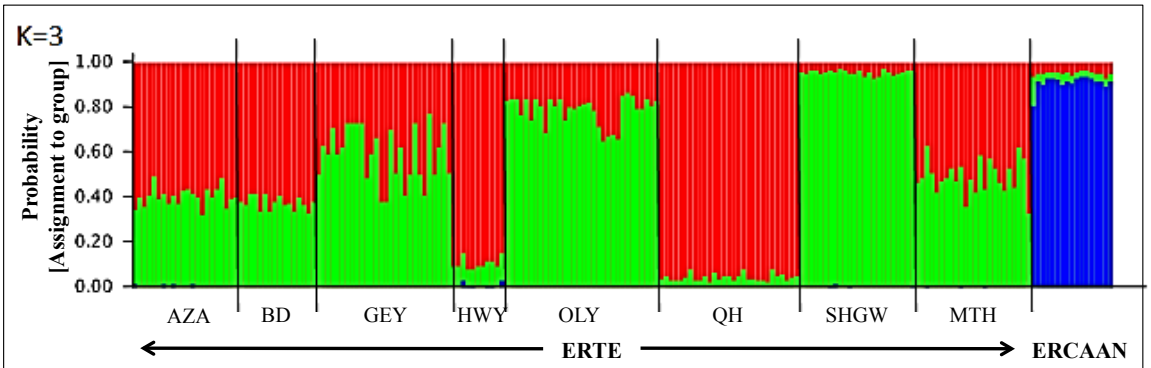

Fig. 11. Structure analysis of microsatellite data reflects the division between two *Erysimum* species and admixture within *E. teretifolium*. *E. capitatum* var. *angustatum* (blue, ERCAAN) and *E. teretifolium* (red and green, ERTE) are correctly separated as different species.

We removed four of the 24 originally scored microsatellite fragments because they appeared in F1 progeny, but were not present in the parents. For the 233 *E. teretifolium* individuals from eight populations plus a small sampling of *E. capitatum* var. *angustatum* as controls, Structure analysis identified three genetic clusters (K=3; Fig. 11). One cluster splits *E. captiatum* var. *angustatum* from *E. teretifolium* samples (blue vs. red/green) indicating the method works to distinguish genetically unique samples.

Structure analysis for just the *E. teretifolium* samples indicates the most likely number of genetic groupings was k = 2, followed closely by k = 4 with a higher standard deviation (Fig. 12). The ΔK for k = 2 and k = 4. The k = 2 analysis revealed 75.11% of individuals were admixed, with individuals from QH and SHGW having the lowest percent of individuals admixed (9.52% and 16.67%, respectively; Fig. 12). The k = 4 analysis revealed 100% admixture for all individuals in all populations, with an average maximum assignment probability of 0.657 ± 0.010 (Fig. 12). The k = 8 analysis, reflecting the number of populations sampled, had the fourth lowest ΔK and exhibited 100% admixture for all individuals in all populations, with an average maximum assignment probability of 0.452 ± 0.010. Populations representing distinct genetic clusters were often at the margins of the species distribution.

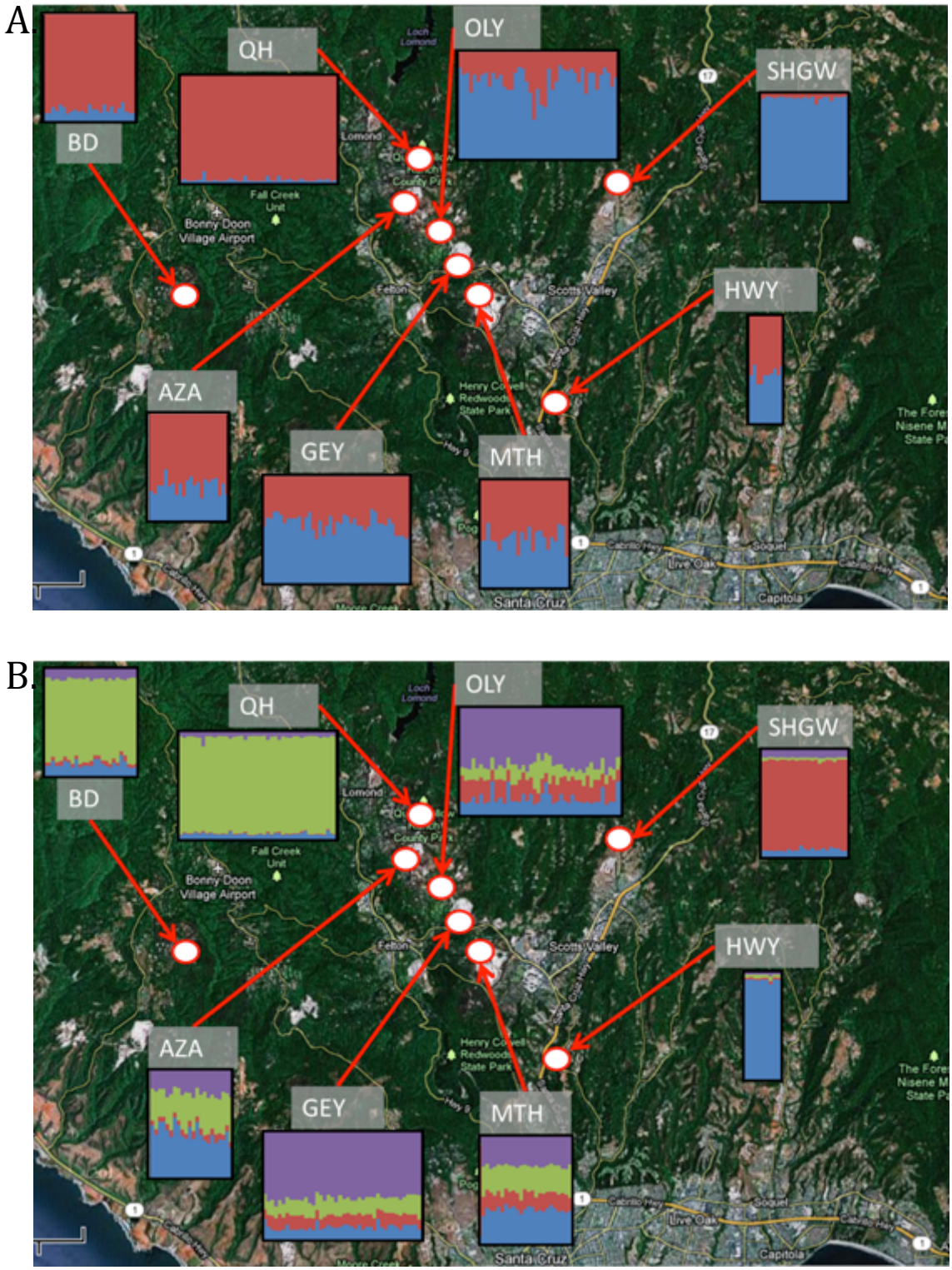

Fig. 12. Only *E. teretifolium* data suggests there are either two (A) or four (B) genetic clusters. The height of the bars indicates the probability of assignment to a cluster. Different colors indicate different genetic clusters. Each bar is a separate individual sampled. Peripheral populations (HWY, SHGW, and BD/QH) appear most genetically distinct in the k=4 analysis (B).

Figure 12 shows the geographic distribution of the four genetic clusters identified by Structure. Although all populations have some admixture, the most genetically distinct groupings are on the margins of the species range (HWY, QH/BD, SHGW). The central populations (MTH, GEY, AZA, OLY) are more evenly admixed. This pattern could arise from an ancestral polymorphic population at the center of the species range with subsets of genetic diversity in descendant populations that emerge around the periphery.

IV.D. *Erysimum teretifolium* Microsatellite Results – AMOVA, Fst & Isolation by Distance

To determine the distribution of genetic diversity within and among populations of *E. teretifolium*, we used AMOVA and F_{ST}. The AMOVA was carried out with genetic clusters predicted by the k=4 grouping in Structure. The majority of molecular variation exists within populations (83.79%; Fig. 13). Only 4.95% of genetic variation exists among populations, whereas 11.26% of genetic variation is explained by the four Structure-identified groupings.

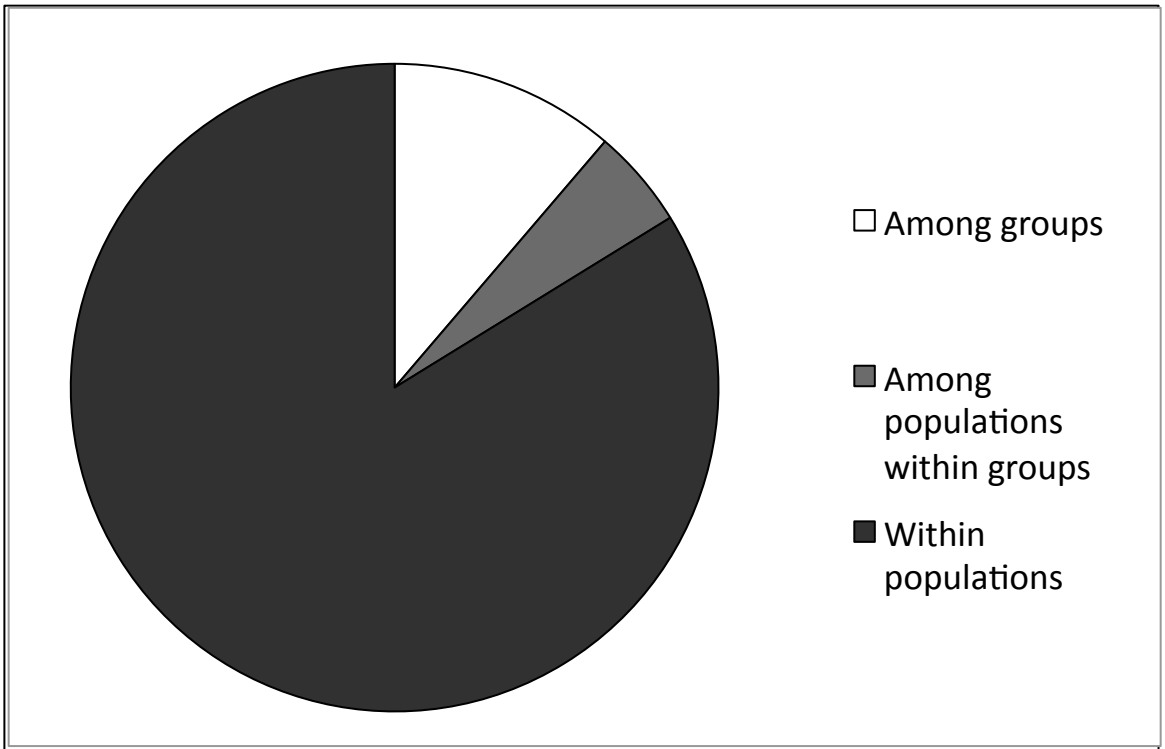

Fig. 13. The distribution of genetic diversity within populations, among populations and among groups (the four genetic clusters identified by Structure). The majority of genetic variation is found within populations.

For genetic subdivision, pairwise F_{ST} averaged 0.153 (0.008 – 0.431) with 26 out of the 28 comparisons being statistically significant ($p < 0.05$). F_{ST} within the central cluster of populations averaged 0.055 (0.008 – 0.106). In comparison, F_{ST} between peripheral populations and the central cluster of populations averaged 0.200 (0.034 – 0.431).

There was no significant correlation between population census size and allelic richness per sampled individual (Fig. 14; Spearman rank correlation, $r_s = -0.47619$, df = 6, p = 0.233.).

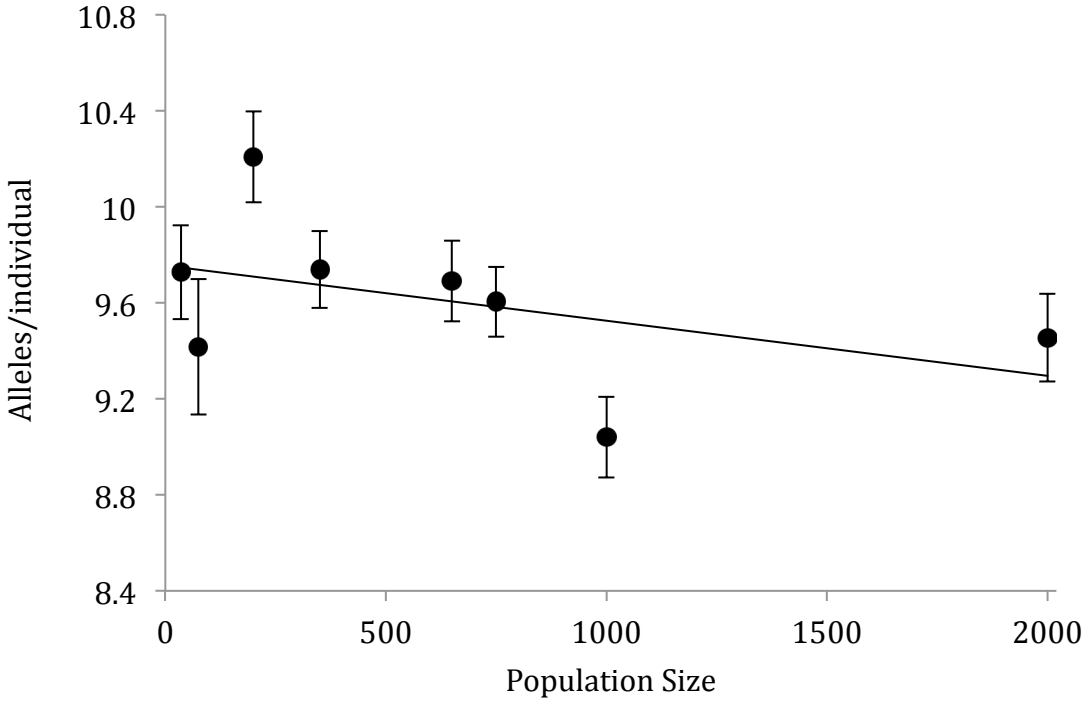

Fig. 14. Effects of population size on marker variants per individual by population. Error bars represent standard error of the mean. Equation of regression is $y = -0.0002x + 9.7553$ (Spearman rank correlation; r_s=-0.45238, $p > 0.05$)

Isolation by distance for all population comparisons was not significant (Fig. 15; Mantel test, t = 1.142, $p > 0.05$). Isolation by distance without the geographically distinct Bonny Doon population was significant ($r^2 = 0.561$; Mantel test, t = 2.232, p = 0.005). After excluding the Bonny Doon comparisons, $F_{ST} / (1 - F_{ST})$ increased by 0.07 per kilometer.

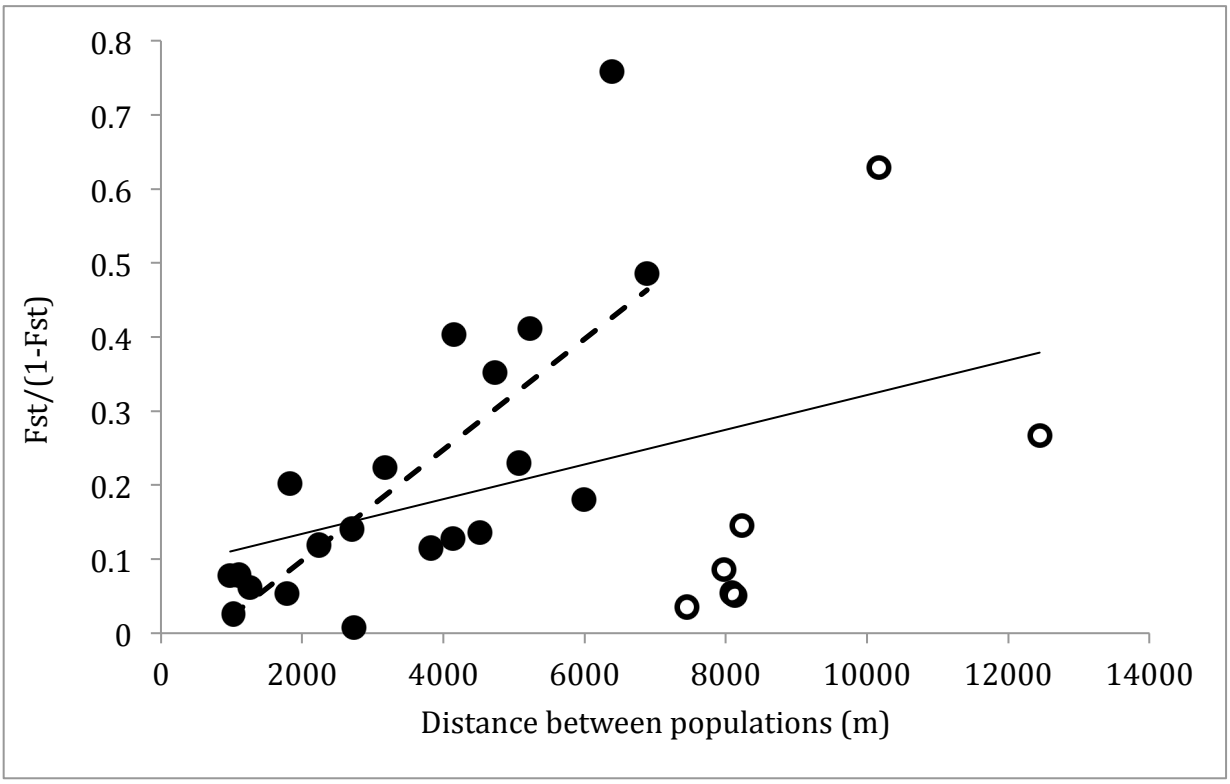

Fig. 15. Red line depicts non-significant linear regression of all samples (Mantel test); equation of regression is y = 2E-05x + 0.0873. Purple line depicts linear regression where comparisons with Bonny Doon are excluded (Mantel test; p=0.005); equation of regression is y = 2E-05x + 0.0873

IV.E. *Erysimum capitatum* var. *angustatum* Microsatellite Results

Twenty-three *E. capitatum* var. *angustatum* individuals from the Sardis Unit (ERCAAN-Sardis) and four individuals from the Stamm Unit (ERCAAN-Stamm) were genotyped for the same four microsatellite loci described above for *E. teretifolium*. We also included samples representing four populations of *E. capitatum* var. *capitatum* in the geographic vicinity of the *E. capitatum* var. *angustatum* populations (ERCA-CA: Mt. Diablo, Mt. Hamilton, Vacaville/Fairfield, and Clear Lake). We expect one or more of these to be most similar to ERCAAN. For comparison, we also included *E. capitatum* var. *capitatum* from the Columbia Gorge (OR) and Snowbird (UT) as ERCA-Non-CA. Three other distinct species were also included: *E. ammophilum* (ERAM – Sunset Beach), *E. menziesii* (ERME-Marina and Asilomar), *E. teretifolium* (see above).

Structure analysis followed the same parameters as described above for *E. teretifolium*. Structure Harvester clearly indicates that there are three distinct genetic clusters with admixture (Fig. 16). The two ERCAAN populations are very similar in their assignment probabilities. Furthermore, they are genetically distinct, but most similar to, the more admixed samples of local *E. capitatum* var. *capitatum*. The difference between ERCA (CA) and ERCAAN is comparable to the species differences when comparing ERAM, ERME and ERTE. This strongly suggests ERCAAN is a distinct genetic unit and deserves taxonomic recognition.

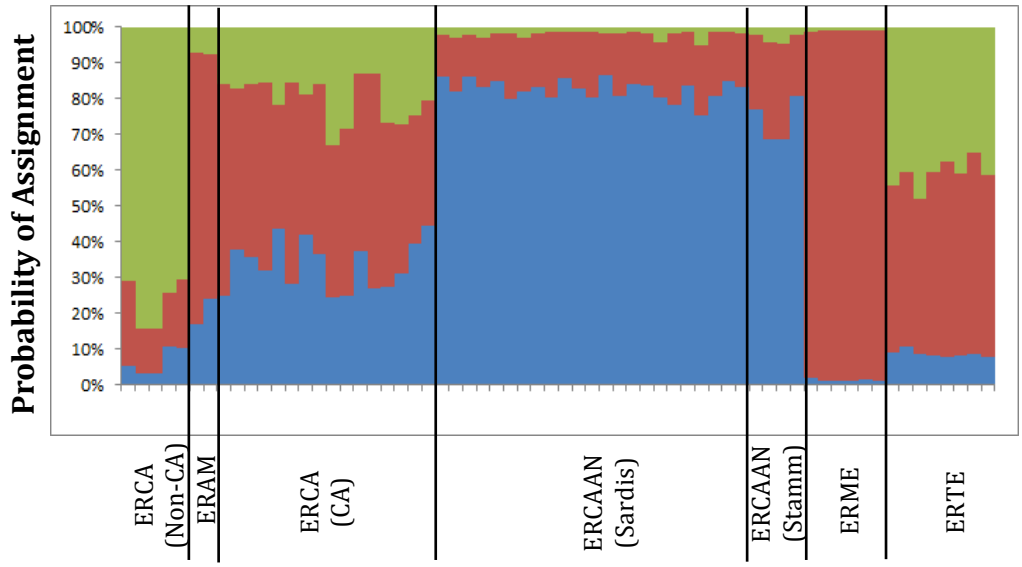

Fig. 16. Twenty-seven *Erysimum capitatum* var. *angustatum* (ERCAAN) samples from the Sardis Unit and Stamm Unit were genotyped for four microsatellite loci. They were compared with *E. capitatum* samples from adjacent populations in California (ERCA-CA) and samples collected outside of California (ERCA-Non-CA). Three other closely related species from California are also included in this Bayesian Genetic Clustering analysis: *E. ammophilum* (ERAM), *E. menziesii* (ERME) and *E. teretifolium* (ERTE) for comparison.

IV.F. Microsatellite Genetic Diversity Conclusions

In general, the island-like nature of the Zayante sandhills does not appear to effect the genetic distribution in *E. teretifolium* in the same way as oceanic islands affect genetic diversity. Instead, the vast majority of genetic diversity is contained within populations, with very little distributed among populations and genetic clusters. This is consistent with an obligate outcrossing mating system (i.e. largely self-incompatible) described in section **I.G. Reproductive Biology**. The small amount of remaining genetic diversity appears to identify peripheral populations as genetically unique.

We applied the same microsatellite loci to compare *E. capitatum* var. *angustatum* to geographically adjacent populations of *E. capitatum* var. *capitatum* and other closely

related species. The Bayesian genetic clustering strongly supports *E. capitatum* var. *angustatum* as genetically unique, yet there is no clear evidence of genetic subdivision within this species across the two existing populations. Thus, *E. capitatum* var. *angustatum* warrants taxonomic recognition as distinct from *E. capitatum* var. *capitatum*.

V. Population Surveys

We surveyed as many populations that we could access in 2012 and 2013. In some populations, we divided our counts into life history stages – juvenile, 1st year reproductive and 2nd year reproductive. Individuals were counted as 2nd year reproductive if there was an infructescence from the past year. Since these often break off, this is an underestimate of the number of 2nd year reproductive individuals, but since the number was non-trivial, we included it as a conservative estimate of the frequency of repeat flowering. Upon returning to the some populations in 2013, we recorded the number of reproductive individuals for comparison to 2012. In addition, we searched for historical collections and potential populations based on habitat similarities to known populations.

V.A. Populations Counted By Life History Stage

Population	Date	Juveniles	1st Year Repro.	2nd Year Repro.	Total
Quail Hollow Co. Park - S	May, 7, 2012	371	363	13	747
Quail Hollow Co. Park – N	April 15, 2013	22	34	5	61
Quail Hollow Co. Park – N	Sept. 27, 2013	6	16	0	22
Geyer/Randall Morgan Pres.	June 3, 2012	na	na	na	~500
Olympia / SLVWD	May 17, 2012	486	157	3	646
Azalea Rd. (near S Ridge)	May 22, 2012	na	na	na	~450
Hwy 17N @ El Rancho	May 15, 2012	21	12	2	35
Mount Hermon	May 31, 2013	19	119	5	138
Bonny Doon Ecological Pres.	April 15, 2013	10	1	0	11
Bonny Doon Ecological Pres.	March 1, 2014	5	3	0	8
Sandhill Rd. & Glenwood Dr.	May 13, 2012	17	23	2	42
Sandhill Rd. & Glenwood Dr.	July 18, 2014	9	5	0	14

V.B. Populations Where Only Reproductive Individuals Were Counted

Population	Date	Reproductive Individuals
Quail Hollow Co. Park - south	March, 27, 2013	93
Geyer/Randall Morgan Preserve	March 29, 2013	60
Hwy 17 northbound @ El Rancho	March 29, 2013	1

V.C. Historical Populations Surveyed Where No Plants Were Found

Jameson Creek Rd. from Hwy236-Empire Grade	May 22, 2012
Little Basin Rd. from Hwy 236 to Boulder Cr. Country Club	May 22, 2012
Forest Park, 2 mi west of Boulder Creek	May 22, 2012
Marion Rd. near Zayante School	April 15, 2013
Vista Robles Rd. near Ben Lomond	April 15, 2013

V.D. Population Surveys Conclusions

We surveyed nine populations across 2012 and 2013. Eight of these were also included in the microsatellite study of genetic diversity. The ninth population, "Quail Hollow Co. Park – N" is a newly discovered, relatively small population that was not discovered until after the genetic sampling was complete. We also discovered a new population along a sandhill road cut on Hwy 17 northbound between El Rancho Dr. exit and Mt. Hermon Rd. exit.

A very large population across Quail Hollow Rd. from Quail Hollow County Park at the quarry (known as "South Ridge", "West Ridge", and "North Ridge") is privately owned and the wallflowers there are monitored by Dr. Jodi McGraw. I could not ascertain permission to survey this population, but the Azalea Rd. population is immediately adjacent and genetically representative. Jodi McGraw monitors it annually and reports on it to the mining company every two years. Her latest report from 2012 is available upon request.

We searched for several historical collections, but they were not relocated. Personal communications with Dr. Jodi McGraw indicates that these occurrences have not been relocated because of incorrect/vague original locality information, extirpation or due to inaccessible private lands.

Erysimum capitatum var. *angustatum* is surveyed annually by Susan Euing at Antioch Dunes National Wildlife Refuge. We accompanied her in 2013 and 2014 during her census with numerous volunteers. She indicates the population is stable at both the Sardis Unit and Stamm Units. Survey results are available from her by request.

VI. Recommended Strategies to Conserve and Recover Endangered Wallflowers

In the sub-sections below, I highlight the conservation implications shared by both endangered wallflowers studied herein (*E. teretifolium* and *E. capitatum* var. *angustatum*). Following that, I make conservation and recovery suggestion that are unique to each species.

VI.A. Implications For Both Endangered Wallflowers

We have studied the mating system, pollinators, and genetic diversity in *E. teretifolium* and, to a lesser extent, in *E. capitatum* var. *angustatum*. The self-incompatible mating system documented in *E. teretifolium* indicates this species relies on pollinators for reproduction. Because of its close relationship to *E. capitatum* var. *angustatum*, both species most likely share self-incompatibility inherited from a common ancestor. Future reproductive success of these species rests on consistent availability of a diversity of pollinators. Fortunately, pollinator observations of both species revealed visitors from several insect Orders and numerous functional pollinator guilds. Therefore, ensuring a diverse pollinator assemblage will be critical for long-term population persistence of both species.

VI.B. Implications Unique to *E. teretifolium*

Population surveys revealed several small populations of *E. teretifolium*, especially on the perimeter of the species range. Small populations of self-incompatible species can spiral into extirpation if the diversity of self-incompatibility alleles drops below a critical threshold and the plants fail to reproduce. This may have been a contributing factor to the reproductive failure of the *E. teretifolium* population at Bonny Doon Ecological Preserve over the past 3-4 years. Interestingly, the primarily peripheral populations that are often small are also the most likely to harbor genetically unique individuals based on our microsatellite genetic diversity study (although most of the genetic diversity is harbored within populations).

The diminishing population at Bonny Doon Ecological Preserve would be an excellent target for future supplementation/reintroduction efforts. The land is preserved with restricted access to the public, it is being managed with endangered species needs in mind (Terris Kasteen, pers. comm.) and has a history of supporting several very large populations of *E. teretifolium*. Momentum is growing for such an effort (a stakeholders meeting is planned for August, 2014). Our genetic diversity analyses can be used to guide seed collection. Although the majority of genetic diversity can be found in any of the populations, in order to maximize sampling of genetic diversity, some germplasm should be included from the genetically distinct peripheral populations (e.g., HWY and SHGW).We have already optimized germination, growth, and transplantation conditions (in captivity) during the current study. Furthermore, our crossing experiments have generated a large number of seeds with known parentage that could be used in such an effort without impacting most natural populations.

Lastly, future conservation efforts should be established to (1) monitor, and supplement as necessary, the small peripheral populations to avoid extirpation and (2) to continue to acquire populations on private land. In regards to the latter, the Santa Cruz County Land

Trust should be applauded for preserving the Geyer Quarry population (now the Randall Morgan Preserve) and a recent property on the west side of Bean Creek Rd. in Scotts Valley that reportedly has a population of *E. teretifolium* (Jodi McGraw, pers. comm.). I am visiting this newly acquired property on July 31, 2014.

VI.C. Implications Unique to *E. capitatum* var. *angustum*

Erysimum capitatum var. *angustum* has always been restricted to a single population at the Antioch Dunes National Wildlife Refuge. It is divided into two halves by a private. industrial property. Although this species is very rare, reserve managers, agency employees, and volunteers have been actively conserving this species at several levels. Susan Euing has been conducting annual wallflower counts there for several years (data available from her upon request). They have recently converted a large area of unoccupied habitat into pristine dune habitat in collaboration with the Army Corps. of Engineers when they dredged a channel in the San Francisco Bay. Lastly, they have been successfully cultivating and transplanting young *E. teretifolium* seedlings across the population. These plants grow vigorously and flower profusely in the wild (Whittall, personal observations in 2013 & 2014). Overall, the population is being actively and responsibly managed and is relatively stable or growing.

The largest threat to *E. capitatum* var. *angustatum* is taxonomic. Authors of the recent taxonomic treatment in The Jepson Manual 2 synonymized this endangered species with the very widespread, *E. capitatum* var. *capitatum*. Given the genetic, geographic and edaphic uniqueness of the wallflowers at Antioch Dunes National Wildlife Refuge, I strongly disagree with this treatment. We will soon publish the microsatellite genetic diversity study described herein that clearly indicates that *E. capitatum* var. *angustatum* is genetically distinct from geographically adjacent and out-of-state populations of *E. capitatum* var. *capitatum*. At that time, I will share our findings with The Jepson Manual 2 editors in hopes that they will reconsider the decision to synoymize this taxon with a widespread relative. In the meantime, I urge practitioners to continue to acknowledge *E. capitatum* var. *angustatum* as it was treated in the first Jepson Manual.

Erysimum teretifolium at Quail Hollow County Park in 2012