Determining the role of the seed bank in maintaining populations of a California grassland annual through prolonged periods of drought

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Executive Summary

Clarkia springvillensis, an endemic annual of California, exists in a region prone to periodic cycles of drought. Drought conditions seem to negatively affect species population size. When populations are small or are periodically reduced in size they become susceptible to the effects of genetic drift. As a consequence, populations or the species as a whole may lose genetic diversity. Low diversity is equated with a lack of adaptability and so is a concern to conservationists seeking to maintain species, such as *C. springvillensis*, for the long term.

In this study we evaluated the genetic diversity of *Clarkia springvillensis* and the importance of the seed bank. Seed banks are often important for maintaining populations and diversity. Tissue samples were taken from adults, seedlings grown from field collected seed and seeds from soil samples. All samples were assayed using starch gel electrophoresis. The genotype data collected was used to determine the genetic variability present and its distribution within and among populations. The total gene diversity present in the species was found to be similar to total gene diversity in other species with similar ecological traits. The genetic differentiation between populations of *C. springvillensis* was much lower than the differentiation that exists between populations of similar species indicating a striking genetic similarity between populations.

Populations of this species have been known to crash to zero for a year or more and then rebound to previous numbers. The evidence gathered in this study supporting the existence of a seed bank helps explain this phenomenon. Also, the variability found in the seed bank indicates that it is playing a role in maintaining genetic diversity within the species. When assessments of any site are made, in regards to this species, it will be important to realize that the absence of above ground individuals is not indicative of the absence of the species. Seeds could be present in the soil and will reestablish the above ground crop when local conditions improve or change. A more thorough examination of the seed bank would also be beneficial in order to determine the long term nature of the seed bank; how many years will seeds persist in the soil, and does the genetic variability change over time with varying conditions.

Overall, the larger sites have greater diversity than smaller sites but the difference between any of the sites is not great. The lack of diversity between populations is likely a historical consequence of the species response to climate change rather than an indication of species wide gene flow. The populations present today may have been separated for only a short period and therefore little differentiation has occurred. It will be important for management of this species to continue census activities to monitor the size and location of populations over time and also to assess the genetic component of populations periodically.

Introduction and Project Objectives

Characterizing and developing strategies to maintain the levels and distribution patterns of genetic variation in populations of rare species has become a major focus of conservation biology (Simberloff, 1988; Dole and Sun, 1992; Eguiarte, et. al., 1992; Soltis et. al., 1992). This interest in genetic variation centers around the idea that a species exhibiting low levels of variability will be less able to adapt to changes in the environment. The lack of adaptive ability would then, in turn, increase the species' likelihood of extinction (Beardmore 1983; Huenneke 1991; Van Treuren, Bijlsma et al. 1991).

Drought and other environmental stresses can negatively impact a rare plant species' genetic variability. By causing reductions in population size, stochastic events, such as drought, can render populations more vulnerable to inbreeding and genetic drift (Barrett and Kohn 1991). However, plant species that have evolved in an arid region subject to repeated cycles of drought may have evolved adaptations to buffer them against the adverse affects of bad years. A seed bank is one possible mechanism by which plants adapted to xeric environments might maintain population viability and genetic variation . The removal of individuals from risk in a given year by remaining in the seed stage could maintain a population's size and reduce the risk of extinction (Kalisz and Mcpeek 1993). These quiescent seeds would reduce the fluctuation in the number of individual genotypes, maintaining genetic variability , despite the fluctuation in the number of visible adult plants (Baskin and Baskin 1978).

Another possible means by which populations could maintain genetic variation is the phenomenon of heterozygous advantage. Small populations subject to periods of extreme heat or drought may be more likely to exhibit an advantage of heterozygous genotypes than populations existing in benign conditions (Lesica 1992). The retention of variability through this type of genotypic advantage would help counteract the effects of drift over time.

In this study we examine the impact of drought induced fluctuations in population size and the role the seed bank plays in maintaining the viability and genetic variability of populations of the California grassland annual *Clarkia springvillensis*. The specific questions to be addressed are: 1) What are the existing levels of genetic variation within and among existing populations? 2) Is there a shift in genotype frequencies as populations progress through the life cycle? and 3) What is the nature of the seed bank and how does it affect variability in populations of *C. springvillensis*.

Materials and Methods

The system

Clarkia springvillensis Vasek is a rare California grassland annual in the family Onagraceae. It is listed by the state of California as endangered and is a Category I federal candidate currently being assessed by the U.S. Fish and Wildlife Service for listing under the Endangered Species Act. The present known distribution of *C. springvillensis* is limited to eleven main sites along the Tule River drainage in the western Sierra Nevada foothills, all within the county of Tulare (Figure 1, Appendix 1). It should be noted that three of the sites listed in Appendix 1 are previously unreported ones located during our 1993 field season in Tulare County. We chose three of the eleven sites as primary sources of material for this study. These sites are, from Appendix 1, #'s 1, 2, and 8. They are referred to in this work as Springville Ecological Reserve (SCER), Gauging Station (GS), and Bear Creek (BC 2.7) respectively. SCER and BC 2.7 were chosen because of their large size relative to other populations. This was a concern as we wanted to have as little impact on these populations as possible. While GS is fairly small in size we thought it important to sample as it is in an area geographically distinct from the other sites (see Appendix 1A).

Populations of *C. springvillensis* have been known to fluctuate from as many as several thousands of plants to zero from year to year (Martin, 1991; CA Natural Diversity Data Base reports). These fluctuations do appear to be tied to winter and spring rainfall. Rainfall was high during the 1993 season and all populations that we were familiar with from the previous season exhibited higher numbers of individuals than the year before.

Collections and Analyses

We had in our possession a small number of *C. springvillensis* seeds that had been collected in 1985 by Dr. Frank Vasek. These seeds, as all seeds collected for this study, were stored in small brown coin envelopes that were subsequently placed in brown paper sacks and kept at room termperature. A germination trial was conducted at the University of Missouri in an attempt to recover seedling tissue from these specimen for isozyme analysis. The protocol used was the same as for all our *Clarkia* germinations. Four inch pots were filled with vermiculite and the seeds placed on the surface of the vermiculite. The pots were kept in a growth chamber with a 12-hour photoperiod at a day/night temperature of 72/42°F. In addition, we sent a sample of the seeds to the Ransom Seed Laboratory, Carpinteria, CA. for germination and viability tests. We also sent to the Ransom Seed Lab samples of seeds collected in 1992 and 1993 for similar tests.

Starch gel electrophoresis was used to determine the variation present at several isozyme encoding loci. In order to compare the variation present in the seed bank, seedling and adult stages we collected tissue both in the field and from glasshouse raised individuals.

For the adult analyses, tissue was collected, during the second full week of April 1993, from individuals in the field at three locations; GS, SCER and BC 2.7. In each of these locations we ran transects through random points in the population and then sampled individuals at random points along the transects. We sampled fifty-nine, ninety-one and ninety individuals from GS, SCER and BC 2.7 respectively. These tissue samples (~ 2 leaves per plant) were packed on ice in the field and then express mailed back to the University of Missouri for immediate extraction and subsequent isozyme analysis.

In order to assess the role of the seed bank in maintaining the numbers and diversity of this species through time, soil cores were taken from the same three populations as above. We collected two sets of samples, each sample set being taken from random points along transects run through the populations. We collected the first sample set in April 1993, after germination of *C. springvillensis* had occurred. Any *C. springvillensis* seeds found in these samples could then be assumed to be members of a dormant seed bank. We collected the second sample set in July 1993, after the adult individuals in those populations had dispersed their seed. This second sample was taken so that we could make a comparison between total seeds in the soil versus quiescent seeds in the soil. A total of 110 samples were collected among the three populations and two sampling times. After collection, the soil samples were sieved to remove both large and very fine particles.

Clarkia seeds are fairly small, ~ 1mm x .5 mm., mimicking in size and color many of the soil particles remaining in the samples. Several strategies were tested for separating the *Clarkia* seeds from the sample matrix. The method chosen was one of general germination. Soil samples were placed in petri dishes lined with filter paper, watered, and, with covers in place, put in a growth chamber with a 12-hour photoperiod at a day/night temperature of 72/42°F. We monitored the samples daily, watering them and checking for *Clarkia* germination. When *Clarkia* seedlings emerged they were transplanted to cell packs filled with ProMix and transferred to the glasshouse where conditions closely approximated those in the growth chamber. Two to three weeks after transplanting, the seedlings were harvested and their tissues extracted for isozyme analysis.

Because stems with fruits will persist for at least one year in the field, we collected fruits from year-old stems to determine if they contained an above ground seed bank.

Tissue used to assess variability present in the seedling stage was collected in the following manner. Seeds were collected in July 1993 from several populations. Several seeds from each maternal plant were germinated and one individual from each resulting family was

selected randomly for tissue collection and isozyme analysis. Three populations were assayed in this manner; GS, SCER and Scicon South (SS). SS is located near SCER and BC 2.7.

All tissue samples in this study were extracted by grinding with a small mortar and pestle and mixing the resulting material with an extraction buffer from Gottlieb (1981). The extracts were absorbed onto filter paper wicks and stored at -80°C until each sample set was electrophoresed in turn. The procedures used for electrophoresis are summarized in Table 1. The following enzyme systems were assayed: Acid Phosphatase (ACP, one locus), Triose-phosphate isomerase (TPI, four loci), Aspartate-(alpha)-keto-glutarate transaminase (AAT, one locus), Glutamate dehydrogenase (GDH, one locus), Malate dehydrogenase (MDH, two loci), Shikimate dehydrogenase (SKDH, one locus), and Phosphoglucomutase (PGM, 3 loci).

Table 1
Summary of electrophoresis procedures

Gel Buffer System	Current (mA)	Volts (max)	Run Time (h)	Enzymes assayed
LiOH-boric acid[a] pH 8.3	75	13/cm	4.5	AAT, GDH, TPI
Histidine-Citrate[b] pH 7.0	35	--	7.5	MDH, PGM,SKDH
System 8-[c] pH 8.0	75	13/cm	4.5	ACP

a-buffer system from Scandalios 1969
b-buffer system from Gottlieb 1981
c-buffer system from Soltis 1983

Gene Diversity Statistics

We used the program Genestat 2 (Whitkus 1985) to calculate Nei's genetic diversity statistics by the methods of Nei and Chesser (1983). We calculated estimates of Ht, Hs, Dst, and Gst within and among the populations at the adult and seedling stage. H was calculated for seed bank seedlings and used for comparison of seed bank diversity with that of the adult population. Genetic distance (D) and genetic identity (I) (Nei 1972; Nei 1978) were also determined using Genestat 2. Values were calculated for distance and identity between all populations sampled.

F-Statistics

F-statistics as described by Nei (1987) were calculated using the program f.stat_v 3.2.3 by Holtsford (). This provides values indicative of the differentiation between groups and sub-groups and also provides coefficients of inbreeding.

Results

Germination Assays

In the germination trial using seeds collected in 1985, three of the fifty seeds being tested germinated. Results returned to us from the Ransom Seed Lab showed 0% viability in the 1985 seeds. The tests of 1992 and 1993 seeds showed high viability, 90% and 96% respectively, although germination rates varied greatly. Results from the Ransom Seed Lab tests are summarized in Table 2.

Table 2: Results of Germination and Viability Tests

	% Germination	% Dormant	Total % Viable*	# in Sample
1985 Seeds	0	0	0	50
1992 Seeds	84	6	90	50
1993 Seeds	24	72	96	50

* Viablility determined by tetrazolium staining
Tests conducted by Ransom Seed Laboratory

Year-Old Stems

Eighty-six year-old stems were collected along transect one in BC 2.7. There were a total of 219 fruits still attached to these stems. All the fruits were examined and contained a total of 129 seeds. Along transect two in BC 2.7 we collected thirty-nine stems bearing a total of 174 fruits. These fruits contained a total of 100 seeds. Twenty-five stems were collected from GS. They bore 127 fruits with 32 seeds. The fruits referred to had, by the time of collecting, dehisced and released most if not all of the seeds originally present. Any seeds found were in the basal most portion of the fruit.

Soil Seed Assays

Of the 110 soil samples collected, only 27 produced *Clarkia* seedlings in the germination trials. Sixteen of these were samples taken in April, 1993 (Pre-seed set) and eleven were samples taken in July, 1993 (Post-seed set). *Clarkia* did emerge from samples taken in all three populations, BC 2.7, GS and SCER. Not all the *Clarkia* that germinated were *C. springvillensis*, however. After being given time to develop, several of the transplanted seedlings proved to be *C. dudleyana*. *C. dudleyana* commonly occurs in sympatry with *C. springvillensis*. The total

number of *C. springvillensis* seedlings harvested from the April samples was 28. The total number harvested from the July samples was 38 (Table 3).

Table 3:Seed Bank Germinations

Sample taken	April (Pre-seed set)	July (Post-seed set)
Total:	28	38
Mean:	1.75 plants/sample	3.45 plants/sample
Range of seeds/sample	0-5	0-10
Std. Dev.	.930	2.67
N	16	11

$p<.05$

Isozyme Assay

We examined 13 loci encoding 7 different enzyme systems. Of those 7 enzymes, GDH, with one loci, was monomorphic in all individuals from all the populations sampled. AAT was monomorphic in the adults and seedlings sampled but showed a small amount of variation in seed bank individuals. Three of the four TPI loci were monomorphic in all individuals. One locus for MDH, specified as MDH-slow, showed polymorphism in adults and seedlings but was monomorphic in all seed bank individuals. Only loci that were polymorphic for any given group (adults, seedlings, seed bank) were used in calculating diversity statistics. A summary of allele frequencies is given in Appendix 2.

Gene Diversity

Gene diversity values for all groups assayed are summarized in Table 4. Overall, seedlings had higher total gene diversity (Ht) than did the adults. Seed bank individuals had higher diversity than both seedlings or adults. The Gst values, which measure differentiation between populations, ranged from 0 to .0346. These values are quite low compared to other species with similar ecological traits. H (gene diversity) values for individual populations varied between adults, seedlings and the seed bank. However, at each life stage, plants from GS consistently showed the lowest gene diversity and SCER exhibited the highest. Direct comparison of gene diversity values (H) between groups is shown in Table 5. Mean number of alleles per polymorphic loci varied slightly between life stages but not significantly.

Table 4: Gene Diversity in *C. springvillensis*

	H	Ht	Hs	Dst	Gst
Adults		.2507	.2420	.0087	.0346
GS	.213				
SCER	.243				
BC 2.7	.271				
Seedlings		.3121	.3015	.0106	.0341
GS	.195				
SCER	.282				
SS	.264				
Seed Bank (Pre)		.3554	.3562	.0000	.0000
GS	.3012				
SCER	.4539				
BC 2.7	.3368				

H= mean gene diversity for individual population
Ht= total gene diversity in the species (at the given life stage)
Hs=avg. gene diversity within populations
Dst=avg. gene diversity between populations
Gst=gene differentiation between populations

Table 5: Comparison of gene diversity between life stages

Population	N	k_p	H
GS (seed bank)	7	2.1	.301
GS (seedling)	15	2.4	.195
GS (adult)	59	2.3	.213
SCER (seed bank)	9	2.3	.454
SCER(seedling)	11	2.4	.282
SCER(adult)	91	2.3	.243
BC (seed bank)	12	2.5	.337
BC(adult)	90	2.3	.271
SS(seedling)	20	2.3	.264

N: sample size k_p:# of alleles/polymorphic loci H:mean gene diversity

Genetic Identity and Distance

Genetic distances and genetic identities were calculated between all the populations for adults and for the seed bank. The distance between adult populations was very short, but it was in accordance with biogeographic vicariance (Table 6). Bear Creek and SCER, which are only separated by 500m, were most closely related. Gauging Station, which is 8km from SCER, had a greater genetic distance. Calculations for the seed bank showed the same trend.

F-statistics

Weir and Cockerham's F-statistic values closely matched corresponding gene diversity values. For the adults, Fst (degree of differentiation of pops) was .044. Fis and Fit were .211 and .246 respectively. The average inbreeding coefficients for adults, by population, were GS-.254, SCER-.099 and BC-.206. For seedlings Fst, Fis and Fit were .033, .320 and .342 respectively. Inbreeding coefficients were, GS-.447, SCER-.421 and SS-.130. The seed bank samples (pre-seed set) had the following values: Fst-.0082, Fis-.2709, and Fit-.2769. Inbreeding coefficients were GS-.171, SCER-.432 and BC-.273. These results are summarized in Table 7.

Table 6: Genetic Identities (upper triangle) and Genetic Distances (lower triangle) *C. springvillensis* adults

	GS	SCER	BC
GS		.980	.974
SCER	.020		.995
BC	.026	.005	

Table 7: F-statistics and inbreeding coefficients

	f(s.e.)	Fst	Fis	Fit
Adults		.044*	.211*	.246
GS	.254 (.023)			
SCER	.099 (.039)			
BC	.206 (.037)			
Seedlings		.033	.320*	.342
GS	.447 (.186)			
SCER	.421 (.158)			
SS	.130 (.034)			
Seed Bank (pre)		.0082*	.2709	.2769
GS	.171 (.075)			
SCER	.432 (.168)			
BC	.273 (.117)			

P<.05, within columns, there are significant shifts in these values

Discussion

One of the goals of this study was to determine the change in genetic variation in *C. springvillensis* over time. Specifically, we wanted to determine the level of variation present before and after the latest cycle of drought in California. The seeds we proposed to use for assaying variation present in 1985, prior to the most recent drought, proved to be inviable. However, the tests done to establish viability in these and the 1992 and 1993 seeds yielded valuable information. Eight years is apparently beyond the maximum for maintaining viability in seeds stored in the conventional manner described in the methods section of this report. This will be an important consideration if the decision is made to maintain an *ex situ* seed bank for this species. A more stringent protocol for the storing of these seeds, such as cold storage, may be necessary to maintain long term viability. A maximum for seed viability in the soil, however, cannot be taken from these results.

Results from the 1992 and 1993 seeds also yielded important information regarding dormancy in *C. springvillensis* . The initial germination and viability tests were conducted in September 1993, only two months after the 1993 seeds had been collected in the field. The low percentage germination but high viability found in the 1993 seeds indicates that this species possesses some form of innate dormancy. That is, the seeds require a period of "after-ripening' before they will respond to normal germination conditions ((Silvertown 1987). Seeds from the same 1993 accession did respond favorably, with a germination rate over 90%, when we exposed them to germination conditions identical to those of the September trial, in December 1993, five months after their collection. The results from the 1992 seeds, showing 6% dormancy, supports the idea that this species is capable of producing at least a small dormant seed pool.

C. springvillensis does exhibit several characteristics associated with species possessing seed banks. It is an annual, early successional species that produces small compact seeds with a hard seed coat. While some of these seeds were shown to remain above ground in year-old fruits, the importance they have for the population is difficult to determine. Of significance are the results from the soil census. Although we obtained *Clarkia* seedlings from only a small subset of the soil samples taken the results show that viable seeds do reside in the soil at all of the sites sampled. Our sampling procedure may have attributed to the small number of samples that produced*Clarkia* seedlings. All the soil cores were taken from randomly chosen points at each site. In many cases, these points were not located in or near patches of existing plants. Many of these microsites may simply have not been exposed to the seed rain of *Clarkia springvillensis* at the site.

The gene diversity values for adults, seedlings and seed bank individuals is consistent with values reported in other studies for species with similar ecological traits (Table 8)(Loveless

and Hamrick 1984). The comparison values presented in Table 8 are for species similar to *C. springvillensis* with the exception that the species in these other studies were not necessarily rare nor subject to dramatic shifts in their numbers. Therefore, our results are encouraging from a conservation perspective in that *C. springvillensis* still possesses a fair amount of diversity, despite its small population sizes and fluctuations in numbers. The seed bank, in all cases, exhibited higher diversity (Ht) than seedlings or adults. Perhaps this is a result of multi-generation contributions to the seed bank or a result of mutation occurring in the seed bank over time. Levin (1990), has suggested that a buildup of mutations in the seed bank could increase genetic variability in populations. However, the diversity decreases as the plants progress through their life histories. It is possible that selection is playing a role in mediating the amount of variability seen in the adults of populations. These results argue against the phenomenon of heterozygous advantage occurring in these populations as the trend would be exactly the opposite if this were the case. The F_{it} values which are comparable to H_t values showed greater diversity in the seedlings followed by the seed bank. These discrepancies could be due to the small sample sizes involved in both the seedling and seed bank pools. The inbreeding coefficients (Table 7) in the majority of cases did track the diversity levels found in each group. GS had the lowest diversity and the highest inbreeding coefficients. Interestingly, this was the smallest population surveyed. SCER, the largest population, had the highest diversity and lowest coefficient of inbreeding. There are some deviations from these trends, but again these may be attributable to small sample size.

Table 8: Comparison values of gene diversity

	Total Diversity	Diversity w/in pops.	Differentiation betwee
	Ht	Hs	Gst
C. springvillensis (adults)	.251	.242	.035
Outcrossers	.251	.214	.118
Hermaphrodite	.284	.161	.389
Sexual	.261	.170	.300
Annual	.264	.136	.430
Endemic	.272	.202	.227

Comparison values from Loveless and Hamrick, 1984

Although the diversity levels found in *C. springvillensis* are comparable to species with similar traits, the differentiation between populations was lower than expected. Both the Gst and Fst values, which measure gene differentiation between populations, were at or near .04 for adults. This means that only 4% of the variation in the adults of the species is due to between population differences. The differentiation values decrease to 0 in the seed bank individuals. There are at least three possible explanations for the lack of differentiation between these seemingly distinct populations. One is that there is significant gene flow between populations. This would minimize the possibility of differentiation occurring. Secondly, the species may have a more contiguous range than what is apparent form the adults in the field. In effect, a seed bank that is more widespread than above ground individuals in any given year could have a mediating effect. A third consideration is that the isolation of these populations is a fairly recent occurrence. If this were the case then the populations show little differentiation only because there has not been time enough for drift or selection to have a significant effect.

The possibility of gene flow between populations would be difficult to dispute if only the between population data were considered. However, we have found that if patches within the populations are examined (McCue-Harvey, et.al., work in progress) gene flow is not occurring between patches within the same population and so is not likely to be occurring between populations.

The second and third possibilities are somewhat linked. If the species were only recently isolated then soil seeds may exist in the areas between existing populations. However, our findings from soil samples do not strongly support this. We do have some evidence though, to support the hypothesis that the present day populations of the species have only recently been isolated. This idea has its basis in climate change that has occurred in the region. During the period from AD 1500 to 1920, there was a southward shift in circulation patterns in North America (Sanchez and Kutzbach 1974). This resulted in a significant regional decrease in precipitation with a slight increase in temperature. During this time period a xeric adapted species such as *C. springvillensis* may have been favored and spread beyond its present narrow range. Only when the climate began to return to present day norms would it recede to its small enclaves. So it is possible that *C. springvillensis* has been distributed in its present fashion for less than a century.

Since we can develop rough estimates for the effective population size and the time of divergence, it is possible to estimate F_{st} (Nei 1987:217) for the populations studied. F_{st} measures the differentiation between populations and is related to the time of divergence and the effective population size as follows: $F_{st}=1-e^{-t/2Ne}$. Using effective population sizes of 200, 300, and 1000 for the patches of Gauging Station, Bear Creek, and SCER respectively and an

estimated time of divergence of 80 years the Fst were calculated. The estimated Fst of populations within the species is 0.0512, while the actual value is 0.0421. These appear to be in close agreement, therefore they lend support to the model's validity.

This model is consistent with low levels of differentiation between recently isolated groups and does not contradict the assertion of restricted gene flow. In addition, it is possible that gene flow between generations (i.e. seed bank effects) is constraining differentiation between groups by maintaining gene frequencies in the population over time and countering the effects of drift.

In summary, despite the recurring cycles of drought that occur in the region occupied by *C. springvillensis* , the species does not at this time exhibit the detrimental effects which might be expected when small populations are further reduced in size by stochastic events. The levels of diversity present in *C. springvillensis* are consistent with levels found within ecologically similar species. There is little differentiation between populations indicating that drift has not had a significant impact. This can in part be attributed to the presence of a seed bank which is buffering the populations from the effects of bad years. In addition, consideration should be given to the possibility that climatic change in the region has only recently affected the isolation of the present populations and that could account for the lack of differentiation seen.

Recommendations

The successful management of *Clarkia springvillensis* will require the incorporation of knowledge of the species ecological and evolutionary history as well as the monitoring of present day demography and genetic structure. During our 1993 survey of Tulare County we discovered three previously unreported populations of *C. springvillensis*. Perhaps, this is a result of a more diligent search than had been previously conducted or perhaps it is simply the result of conducting our survey in the first wet season occurring in seven years. Given that previously known populations have crashed to zero for several years and then rebounded it is not unlikely that the new sites we reported exhibited no visible above ground individuals in prior years. Their subsequent reestablishment is likely due to a below ground seed bank. Because this phenomenon does occur, and we have shown that viable seeds do reside in the soil, multi-year surveys of *C. springvillensis* should be conducted before concluding that the species has gone extinct in any particular area. A more thorough examination of the seed bank would also be beneficial in order to determine the long term nature of the seed bank; how many years will seeds persist in the soil, and does the genetic variability change over time with varying conditions.

The genetic diversity of the species at the present time is apparently good. Our study also indicates that most of the variation present in the species is present in any given population. If maintaining variation in *C. springvillensis* is a management goal then this might be done by

preserving a few of the larger populations. However, it should be kept in mind that two of the smaller populations exist along a separate drainage from the others. While they may not be highly differentiated at this point, they may in the future become locally adapted and exhibit a higher degree of differentiation. A periodic genetic assessment of populations along both drainages will aid in making management decisions as well as providing information of interest to ecologists and evolutionists.

In addition to the recommendations relating to the genetic welfare of this species we would like to add a few comments based on personal observations. In the past, the typical recommendations made for conservation of this species has been to post signs indicating the presence of a sensitive species and to fence the population. We agree that these are certainly worthwhile endeavors. However, at several sites (GS and the flume site (Camp Wishon map)) where fences have been erected the plants are no longer growing within the boundaries of the fence. This leads us to conclude that this species can and will migrate in relatively short periods of time. If fencing is to be used as a primary means of protecting the plants at these sites then regular monitoring and adjustment of the fences will be necessary. Also, at several sites (GS, SCER, the flume site), *C. springvillensis* occurs sympatrically with *C. unguiculata*, a more common species of *Clarkia* and the putative progenitor of *C. springvillensis*. This is a previously unreported phenomenon with potentially important implications for the management of *C. springvillensis*. It has been shown in glasshouse experiments (Vasek, 1964) that it is possible for the two species to hybridize. Hybrids are generally sterile, however the potential for genetic swamping of one species by the other exists (Ellstrand, 1992). This may be an important area of research for conservation management.

Finally, this species has probably expanded and receded within its range in response to regional climate. We have proposed that drier conditions may actually be conducive to the spreading of the species. On the other hand, seemingly more benign conditions may provide an advantage to species adapted for wetter climes. Those species could help affect a decrease in the numbers of *C. springvillensis*.. In this case the winking out of *C. springvillensis*. populations or the eventual extinction of the species would be a natural evolutionary endpoint. Certainly though, human related activities such as development, road maintenance or cattle grazing, that negatively impact the species should be controlled and monitored when plant conservation is the goal.

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Appendix 1

Locations of *Clarkia springvillensis* populations

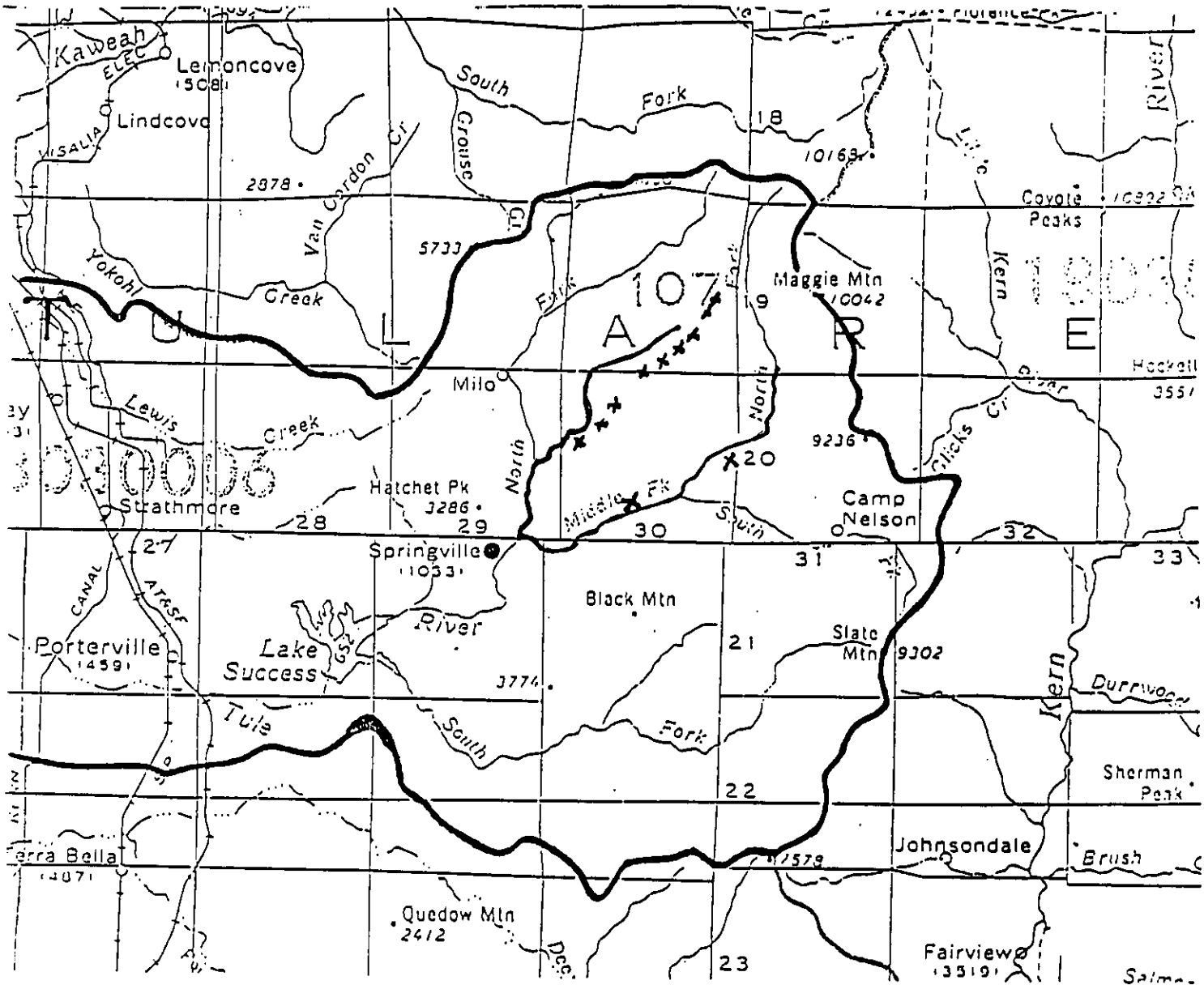


Figure 1: Upper Tule River Drainage

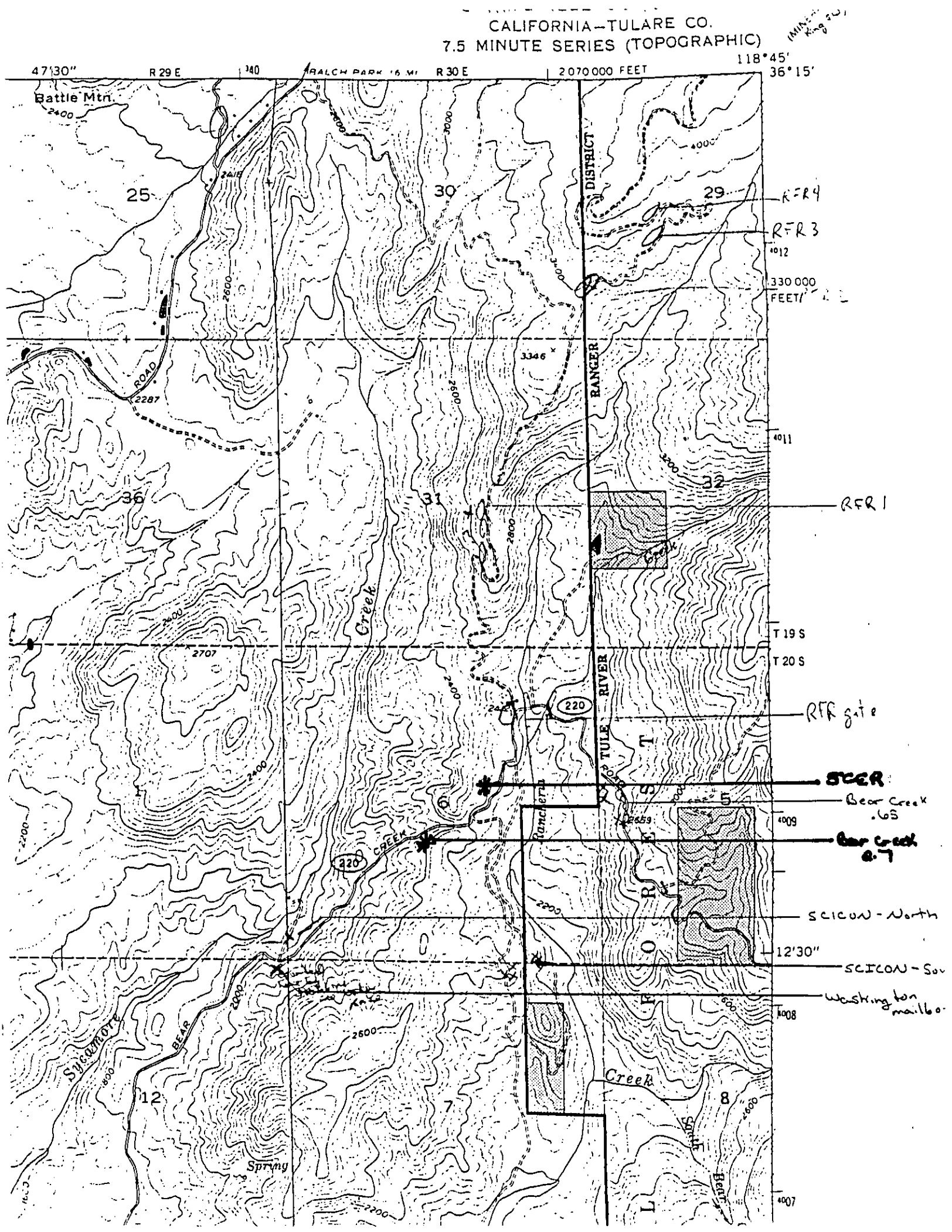
x=approximate locations of *C. springvillensis* populations

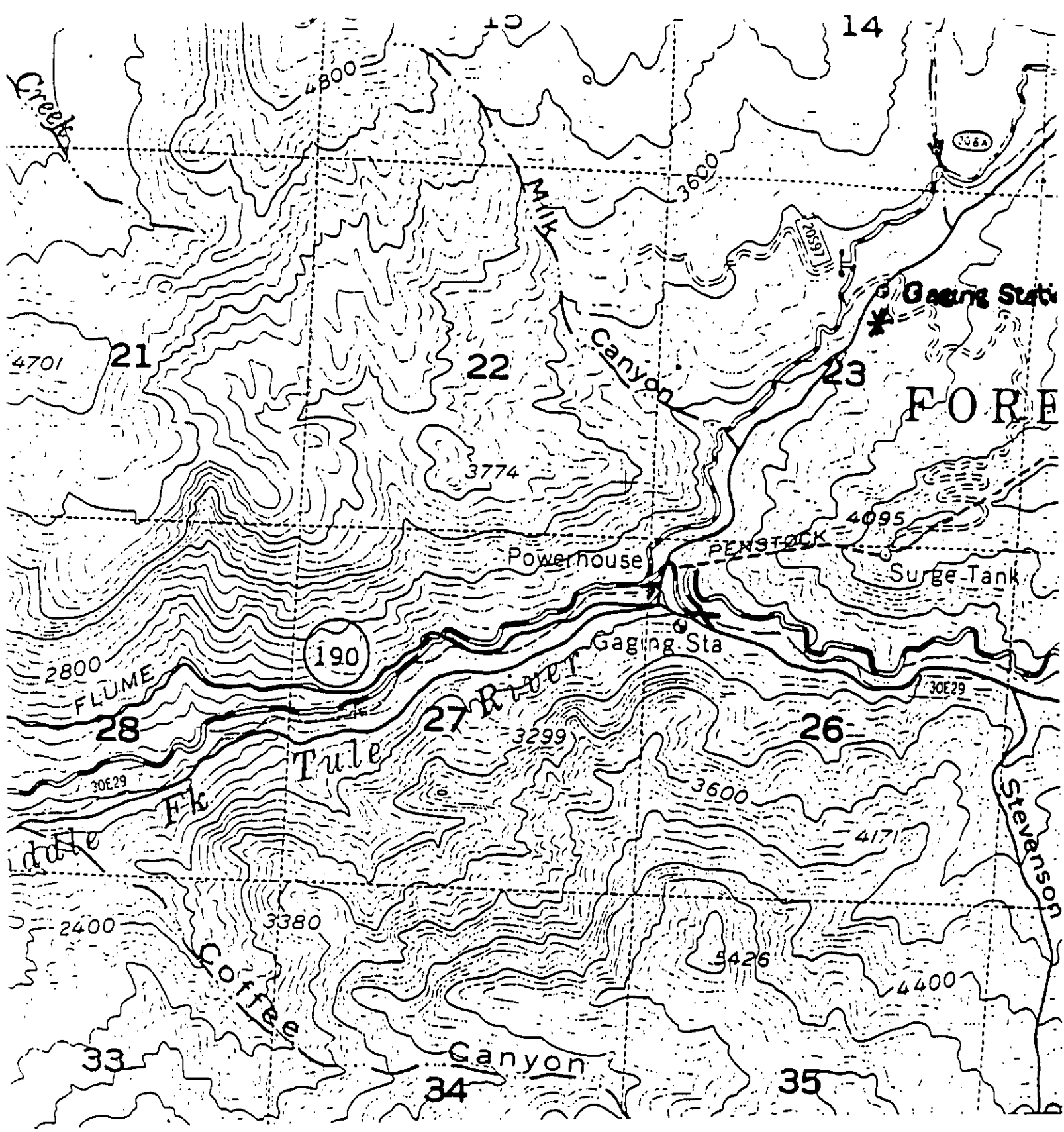
Appendix 1: Extant Populations of Clarkia springvillensis
Tulare County, California

Population ID#	Reported By	Date First Reported	Location
1+	Stebbins, J.	1984*	Along Bear Creek Rd., across from SCICON entrance, 0.35 miles W of Sequioia NF boundary--(Springville Clarkia Ecol. Reserve).
2+	Stebbins, J.	1985 *	1.45 miles along PG&E Rd to penstock and gauging station, E of the Wishon Fork of Tule River. *7.5' Camp Wishon (also Camp Nelson NW) T20S R30E SW1/4 NE 1/4 Sect. 23*
3	Stebbins, J.	1985*	Along Hwy 190, 0.8-0.9 miles W of Wishon Rd., between Springville and Camp Nelson. Directly upslope and under PG&E transmission line and adjacent to flume for SCE hydro project. *7 1/2' Camp Wishon T20S R30E SE 1/4 NW 1/4 Sect. 27*
4	Stebbins, J.	1985*	Along Bear Creek Rd., 2 miles NE of Balch Park Rd.(County Rd M-220), 1.45 miles S. of Rancheria Fire Rd., opposite Washington family mailbox. *15' Springville T20S R29E NE1/4 NE1/4 Sect. 12*
5	Stebbins, J.	1988*	Entrance to Rancheria Fire Rd., 0.1 miles W of jct. of Bear Creek and Rancheria Creek. Patch continues 0.1 miles S along Bear Creek Rd. *7.5' Springville T20S R30E SE 1/4 NE 1/4 Sect. 7*
6	Stebbins, J.	1990*	Several sites in and around SCICON Conservation and Education Camp in Bear Creek Canyon. First large population .5 miles from SCICON entrance. *7 1/2' Springville T20S R30E Center and SE 1/4 , NE and SE 1/4 Sect. 7*
7	Stebbins, J.	1990*	Dillon Ranch Rd. (M256) 0.1-0.2 miles W of Balch Park Rd. On both sides of road. *7 1/2' Springville T19S R29E NE 1/4 NE 1/4 Sect. 25*
8+	Holtsford, T.	1992*	Bear Creek Rd., 2.6 miles NE of Balch Park Rd., 0.75 miles S of Rancheria Fire Rd. *15' Springville T20S R30E NE1/4 SW1/4 Sect. 6*
9	Holtsford, T. McCue, K.	1993	Several patches along Rancheria Fire Rd. Sited 1.05, 1.9, 2.2, and 2.7 miles N of USFS gate at entrance to road. *7.5' Springville T19S R30E NE 1/4 SW 1/4 Sect. 29.*
10	Holtsford, T. McCue, K.	1993	Along Balch Park Rd., E of jct with Rancheria Fire Rd. Patches 4, 4.1, 4.15, 4.7 miles from jct..*7.5' Kaweah SE T19S R30E NW 1/4 NW 1/4 Sect. 20.*
11	Holtsford, T. McCue, K.	1993	Along Balch Park Road (County Road M-220), .65 miles N of Rancheria Fire Road. *15' Springville T20S R30E SW 1/4 NW 1/4 Sect. 5*

* Confirmed extant May 1993 by Holtsford and McCue +Sampled for this study

Appendix 1A
Topographic Map Locations of *C. springvillensis* populations


CALIFORNIA—TULARE CO.
7.5 MINUTE SERIES (TOPOGRAPHIC)
47'30"
R 29 E
R 30 E
2070000 FEET
118°45'
36°15'
Battle Mtn.
25
30
29
DISTRICT
RANGER
RFR4
RFR3
4012
330 000 FEET
3346
ROAD
2287
36
31
32
RFR1
Creek
4011
T 19 S
T 20 S
2707
TULE RIVER
220
RFR gate
SCER
Bear Creek .6S
Bear Creek .7
5
4009
Ranchería
CREEK
SCICON - North
12'30"
SCICON - South
Washington mailbox
4008
Sycamore
BEAR
Creek
12
7
8
Spring
South Beart
4007



Camp Wishon 7 1/2' Quad Tulare County

Appendix 2
Allele Frequencies determined from Isozyme Data for *Clarkia springvillensis*

Allele frequencies for adults sampled in field

ALLELE FREQUENCIES FOR LOCUS:MDHF

GROUP	ALLELE 1	2
GS	.938	.061
SCER	.966	.034
BC	.989	.011

ALLELE FREQUENCIES FOR LOCUS: MDHS

GROUP	ALLELE 1	2
GS	.983	.017
SCER	1.000	.000
BC	1.000	.000

ALLELE FREQUENCIES FOR LOCUS: SKDH

GROUP	ALLELE 1	2	3	4	5	6	7	8
GS	.120	.139	.565	.167	.000	.000	.009	.000
SCER	.006	.125	.644	.162	.050	.006	.000	.006
BC	.013	.133	.456	.291	.108	.000	.000	.000

ALLELE FREQUENCIES FOR LOCUS: PGM1

GROUP	ALLELE 1	2
GS	.975	.025
SCER	.933	.056
BC	.892	.091

ALLELE FREQUENCIES FOR LOCUS: PGM2

GROUP	ALLELE 1	2
GS	.837	.163
SCER	.949	.006
BC	.955	.017

ALLELE FREQUENCIES FOR LOCUS: PGM3

GROUP	ALLELE 1	2	3
GS	.962	.000	.038
SCER	.763	.051	.179
BC	.702	.045	.253

ALLELE FREQUENCIES FOR LOCUS: ACP1

GROUP	ALLELE 1	2
GS	.842	.158
SCER	.605	.395
BC	.639	.361

Allele frequencies for seedlings grown in glasshouse from field seed

ALLELE FREQUENCIES FOR LOCUS: MDHF

GROUP	ALLELE 1	2	3
GS	.967	.000	.033
SCER	.900	.050	.050
SS	.889	.111	.000

ALLELE FREQUENCIES FOR LOCUS: MDHS

	ALLELE	
GROUP	1	2
GS	.967	.000
SCER	1.000	.000
SS	1.000	.000

ALLELE FREQUENCIES FOR LOCUS: SKDH

	ALLELE			
GROUP	1	2	3	4
GS	.167	.067	.433	.333
SCER	.000	.375	.500	.125
SS	.000	.167	.722	.111

ALLELE FREQUENCIES FOR LOCUS: PGM1

	ALLELE		
GROUP	1	2	3
GS	1.000	.000	.000
SCER	.857	.000	.143
SS	.929	.071	.000

ALLELE FREQUENCIES FOR LOCUS: PGM2

	ALLELE		
GROUP	1	2	3
GS	.833	.111	.056
SCER	1.000	.000	.000
SS	.875	.000	.125

ALLELE FREQUENCIES FOR LOCUS: PGM3

	ALLELE		
GROUP	1	2	3
GS	1.000	.000	.000
SCER	.722	.111	.167
SS	.656	.125	.219

ALLELE FREQUENCIES FOR LOCUS: ACP1

	ALLELE	
GROUP	1	2
GS	.833	.167
SCER	.500	.500
SS	.781	.219

ALLELE FREQUENCIES FOR LOCUS: TPI2

	ALLELE		
GROUP	1	2	3
GS	.500	.500	.000
SCER	.438	.313	.250
SS	.583	.333	.083

Allele frequencies for all soil seeds, grouped by population

ALLELE FREQUENCIES FOR LOCUS: MDHF

	ALLELE	
GROUP	1	2
BC	1.000	.000
GS	.975	.025
SCER	1.000	.000

ALLELE FREQUENCIES FOR LOCUS: PGM1

	ALLELE	
GROUP	1	2
BC	.842	.158
GS	.950	.050
SCER	.694	.306

ALLELE FREQUENCIES FOR LOCUS: PGM2

	ALLELE		
GROUP	1	2	3
BC	.947	.000	.053
GS	.800	.200	.000
SCER	.909	.091	.000

ALLELE FREQUENCIES FOR LOCUS: PGM3

	ALLELE		
GROUP	1	2	3
BC	.579	.053	.368
GS	.976	.000	.024
SCER	.795	.023	.182

ALLELE FREQUENCIES FOR LOCUS: SKDH

	ALLELE				
GROUP	1	2	3	4	5
BC	.139	.278	.444	.139	.000
GS	.025	.050	.700	.100	.125
SCER	.023	.250	.636	.091	.000

ALLELE FREQUENCIES FOR LOCUS: ACP1

	ALLELE	
GROUP	1	2
BC	.808	.192
GS	.900	.100
SCER	.722	.278

ALLELE FREQUENCIES FOR LOCUS: AATF

	ALLELE		
GROUP	1	2	3
BC	.955	.000	.045
GS	1.000	.000	.000
SCER	.861	.056	.083

ALLELE FREQUENCIES FOR LOCUS: TPI2

	ALLELE		
GROUP	1	2	3
BC	.550	.450	.000
GS	.500	.450	.050
SCER	.231	.731	.038

Allele frequencies for soil seeds, grouped by sample time (Pre and Post seed set)

ALLELE FREQUENCIES FOR LOCUS: MDHF

	ALLELE	
GROUP	1	2
PRE	.981	.019
POST	1.000	.000

ALLELE FREQUENCIES FOR LOCUS: PGM1

	ALLELE	
GROUP	1	2
PRE	.712	.288
POST	.938	.063

ALLELE FREQUENCIES FOR LOCUS: PGM2

	ALLELE 1	2	3
GROUP	.788	.173	.038
PRE	.944	.056	.000
POST			

ALLELE FREQUENCIES FOR LOCUS: PGM3

	ALLELE 1	2	3
GROUP	.741	.037	.222
PRE	.833	.014	.153
POST			

ALLELE FREQUENCIES FOR LOCUS: SKDH

	ALLELE 1	2	3	4	5
GROUP	.037	.148	.648	.130	.037
PRE	.074	.235	.544	.088	.059
POST					

ALLELE FREQUENCIES FOR LOCUS: ACP1

	ALLELE 1	2
GROUP	.714	.286
PRE	.871	.129
POST		

ALLELE FREQUENCIES FOR LOCUS: AATF

	ALLELE 1	2	3
GROUP	.950	.050	.000
PRE	.933	.000	.067
POST			

ALLELE FREQUENCIES FOR LOCUS: TPI2

	ALLELE 1	2	3
GROUP	.536	.464	.000
PRE	.325	.625	.050
POST			

Glossary

F-statistics

f: inbreeding coefficient

F_{st}: genetic variation among populations (s) relative to the variation in all populations

F_{is}: inbreeding coefficient of an individual relative to a population (s)

F_{it}: inbreeding coefficient of an individual relative to all populations (t)

Gene diversity statistics

H: mean gene diversity or average heterozygosity

H_t: total gene diversity (all populations being considered)

H_s: average within population gene diversity

G_{st}: coefficient of gene differentiation; describes the relative magnitude of gene differentiation between populations

Knowledgeable Persons

The following people have some knowledge of *Clarkia* in general and of *C. springvillensis* specifically. All were helpful to us during the course of this study.

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