Morphological and Genetic Divergence of Morro Bay Kangaroo Rats

Agreement #P0485102 and #S0685103

Draft Final Report

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INTRODUCTION

This draft final report summarizes the methods, analyses, and results of work completed under Agreements # P0485102 and #S0685103. The primary objective of this work was to determine the evolutionary, genetic and morphological distinctness of the endangered Morro Bay Kangaroo Rat (MBKR, *Dipodomys heermanni morroensis*), relative to other neighboring subspecies of *Dipodomys heermanni.* Therefore, *D. h. morroensis* was compared to *D.h. arenae, D.h. jolonensis* and *D. h. swarthi* (Hall 1981). The most extensive comparison was made between *D.h. morroensis* and *D. h. arenae* since this subspecies is the putative closest relative of the MBKR (Grinnell 1922 and Boulware 1943).

The fundamental question addressed here is whether the MBKR is an Evolutionarily Significant Unit (ESU) under the definition provided by Moritz (1994). ESUs are defined as independent units of biological diversity, categorized as separate based on reciprocal monophyly in mtDNA or significant differences in nuclear allele frequencies. The independent nature of an ESU warrants separate conservation management (Moritz, 1994). However, Moritz (1994) notes that the ESU may be overly restrictive, and advocates a less restrictive unit of biological diversity termed the independent Management Unit (IMU). This term defines conservation units based on significant differences in allele frequencies between populations, even when the phylogenetic relationship or status is unknown (Moritz, 1994). This question is addressed here using mitochondrial DNA sequences and nuclear microsatellite allele frequencies.

The second fundamental question addressed here is whether museum records and data on soil type distribution can be used to locate extant populations of the four subspecies of *D. heermanni* within San Luis Obispo County. This question was addressed by determining the spatial distribution of sandy soils across San Luis Obispo using GIS, combining this information with data on previous museum collections, and then conducting site visits. The site visits were used to assess habitat quality, determine the presence of kangaroo rats sign, and to live trap. The entire recognized range of the MBKR occurs on sandy soil. In an effort to locate new MBKR populations, a GIS map of all sandy soil in close proximity to the MBKR's original range (as defined by Stewart and Roest 1960) was generated. New sites identified through this process were checked for potential habitat or obvious occupation by MBKR.

This report finds that there is statistically significant population genetic structure between subspecies of *D. heermanni* within San Luis Obispo County. Based on the analysis of microsatellite data we can confidently conclude that the MBKR is genetically differentiated from all other subspecies. Statistically significant differences in a) microsatellite allele frequencies, b) microsatellite individual assignment probabilities into populations, c) microsatellite estimates of migration rates, and d) mitochondrial DNA allele frequency differences and monophyly all indicate the MBKR is an ESU. Of all the populations sampled using microsatelliete DNA, the MBKR has lowest expected heterozygosity, mean number of alleles per locus, and nucleotide diversity. These results are consistent with the hypothesis of a historical (pre 1918) rather than a recent (post 1960) genetic bottleneck in the MBKR as proposed by Matocq and Villablanca (2001) in their mitochondrial DNA analysis.

This report finds that there is no evidence for an extant MBKR population within its recognized range. Importantly, it was discovered that some of the historical museum collection sites, as well as patches of sandy habitat, are East of Los Osos Creek and therefore *outside* of the currently recognized range. Further work in these locations is warranted. *D. heermanni arenae* is broadly distributed across the Pismo/Oceano/Nipomo/Guadalupe dune complex and only occurs in isolated patches in other portions of its SLO County range. *D. heermanni jolonensis* is present, but is not as broadly distributed as in the recent past. Both *D. h. arenae* and *D. h. jolonenis* populations are both being impacted by urban and agricultural development. *D. heermanni swarthi* is still abundant throughout large portions of its range.

The discussion addresses the potential advantages and disadvantages of translocating a related subspecies of the MBKR into MBKR habitat.

ANALYSIS AND RESULTS

Analyses and results are divided into five sections: Field Surveys, Scientific Collecting, Microsatellite Population Genetics, Mitochondrial Population Genetics, and Morphometrics.

FIELD SURVEYS

Habitat distribution and site evaluation

Distribution data were sought for *Dipodomys heermanni* in San Luis Obispo Co. in order to a) produce a compilation of all known and potential *D. heermanni* localities,b) determine the current status of previously existing populations, and c) identify possible locations for scientific collecting.

The locations of previously known populations of kangaroo rats were determined through a review of records from museum collections and of the literature. Museum records were compiled for vouchered specimens at the Museum of Vertebrate Zoology (M.V.Z., U.C. Berkeley), and the Aryan Roest Mammal Collection (A.R.M.C., Cal Poly San Luis Obispo). Field notes for the Berkeley specimens (available on line through the MVZ web site) were consulted in order to verify locations in the MVZ database. Field notes for the Cal Poly specimens were consulted in order to verify locations in the A.R.M.C. database. Field notes for these specimens are housed at the Santa Barbara Museum of Natural History, The literature on live trapping results is summarized in the Draft Revised M.B.K.R. Recovery Plan (USFWS 1999). Original references were consulted in order to verify locality data.

The locations of potentially new populations of kangaroo rats were determined through GIS mapping of the sandy soils within S.L.O. County. All areas with sandy soils were considered potential habitat for *D. heermanni*. Sandy soil in and adjacent to the range of the MBKR is shown in figure 1. Sandy soils within the south coastal San Luis Obispo County portion of *D. h. arenae*'s range are shown in Figure 2. The Edna site, shown in figure 2, represents the population of *D. h. arenae* geographically closest to *D. h. morroensis*. GIS themes were compiled in ArcView GIS (V 3.0) after being collected from http://lib.calpoly.edu:8080/gis/browse.jsp?browseType=theme. Vegetation themes were not

overlaid since fire or other disturbance can modify vegetation and transform areas with unsuitable vegetation into areas with suitable vegetation. The soil's potential to represent suitable habitat persists independently the vegetation that is currently present.

FIGURE 1. GIS map of sandy soils in and adjacent to the geographic range of the MBKR. The majority of the known geographic range of the MBKR occurs on Baywood Fine Sand. Note the patches of sandy soil east and south of the largest patch (adjacent to Morro Bay). A magnified, high resolution version of this map is available in the electronic media submitted with this report.

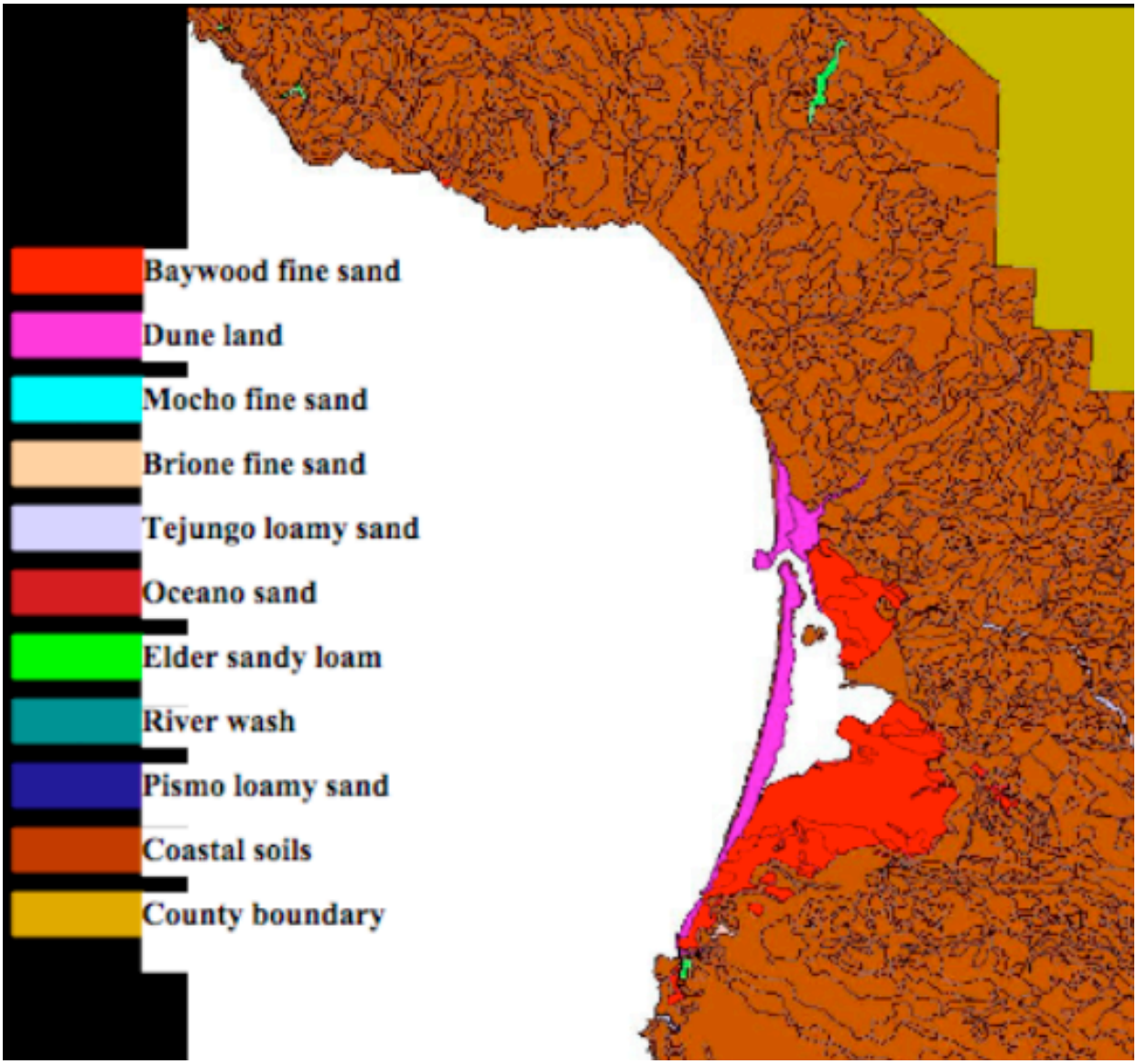
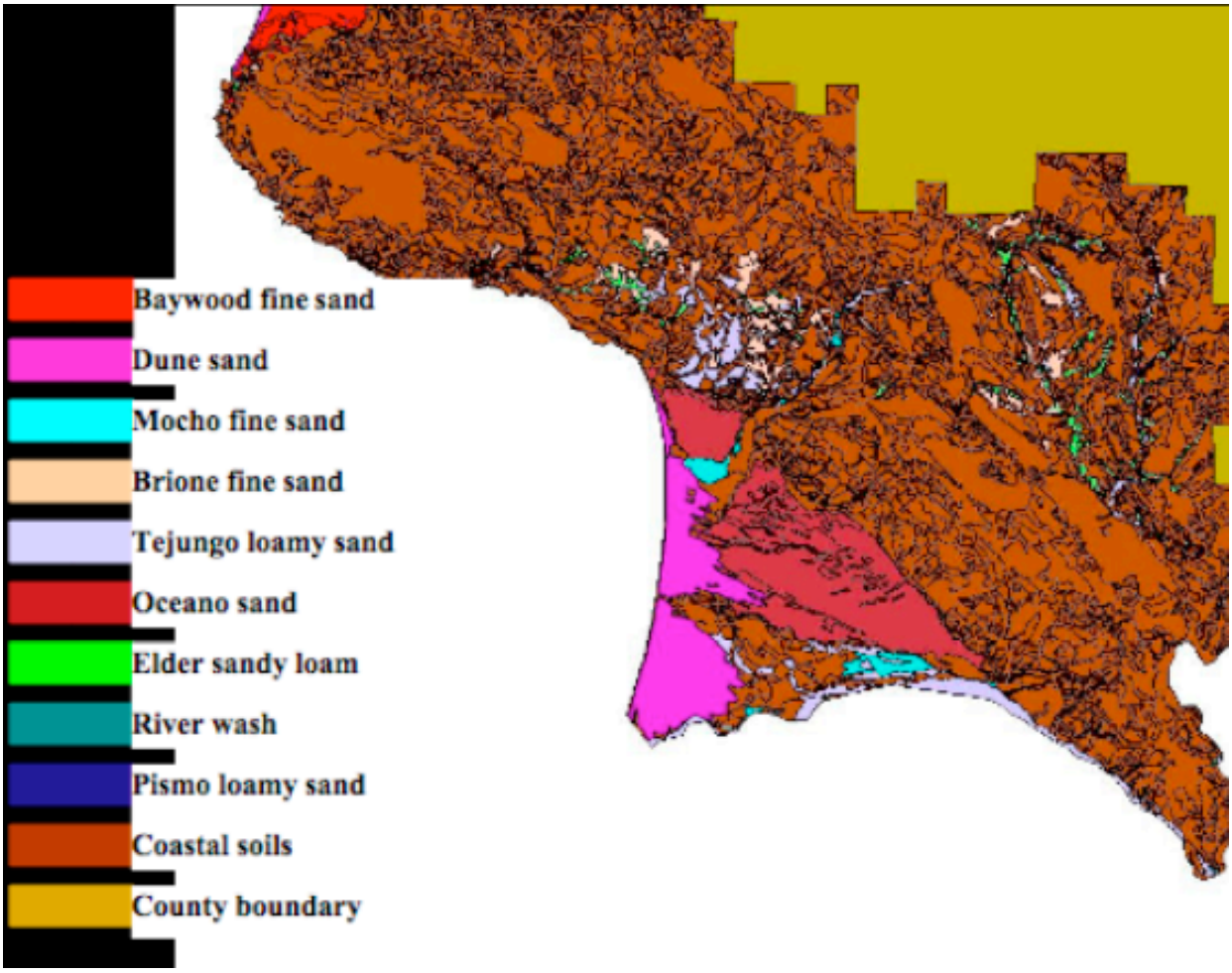


FIGURE 2. GIS map of sandy soils in south coastal San Luis Obispo County primarily within the range of *D. h. arenae*. The upper left of the figure contains the Southern most portion of Baywood Fine Sand within the MBKR's range. The curving lower edge of the figure corresponds with the Santa Barbara/San Luis Obsipo County line. The complex of Tejungo Loamy Sand and Brione Fine Sand (just above center in the figure) corresponds to the Edna site. This is the population of *D. heermanin areneae* that is geographically closest to the MBKR. A magnified, high resolution version of this map is available in the electronic media submitted with this report.



The GIS maps represent areas of possible kangaroo rat habitat within the county. Site assessments of some of these locations were conducted to determine if the sites are currently occupied by *Dipodomys*. Collection sites were selected from the suite of sites that were found to contain kangaroo rats. The site assessments were conducted by a) Visiting the site to determine if the site was still undeveloped and provided public access, b) Assessing the habitat to determine if the vegetation on site was suitable for kangaroo rats, c) Conducting a pedestrian survey for sign of *Dipodomys* (burrows, tail drags, pit caches, sand bathing sites, or scat), and d) Conducting live trapping. If an initial phase or step in the site assessment resulted in a negative result, then

further or subsequent steps in the assessment were not completed. No live trapping was attempted in areas that potentially contained MBKR habitat.

D. h. arenae distribution and site evaluations

Most of the range of this subspecies is within San Luis Obispo County. Some of the range also occurs in Santa Barbara Co. The recorded localities within S.L.O county are: 1) 3.5 mi S Oceano, Latitude and Longitude (Lat/Long) 35.047927, -120.599139, Collecting Date (Date) 1941; 2) Guadalupe, Oil Field N. of Santa Maria, Lat/Long 34.979550, -120.633200 Date 1995, these two localities (1 and 2) are synonymous; 3) Nipomo Mesa, 0.5 mi W Hwy. 1 and 0.5 mi SSE White Lake, Lat/Long 35.058832, -120.601075, Date 1990; 4) Phoenix Creek, 8.5 miles NE Arroyo Grande, Lat/Long 35.1862, -120.4541, date 1962; 5) 1 mile W. Edna, Lat/Long 35.202, -120.6297, date 1950; 6) Huasna River 12 miles E. of Arroyo Grande, Lat/Long 35.1391, -120.3604, date 1963.

Several of these historical collecting localities were evaluated for the presence of kangaroo rats. The Unocal Guadalupe oil field (1 and 2) has a thriving population of kangaroo rats based on sign and live trapping. The Nipomo Mesa site (3) is private property. No trapping was conducted there. But a site visit provided evidence for an extensive kangaroo rat population. The Phoenix Creek site (4) is private property and could not be accessed. The Edna site (5) had a very large population in 1950 (Roest unpublished manuscript and large reference collection). This location represents the site closest to Los Osos (and the MBKR) with a historical population of *D. h. arenae*. All of the property owners (N=5) that could have allowed access to the Edna site declined to allow live trapping or site evaluations. The current status of the Edna population is therefore unknown. The Huasna River site (6) was discovered to support a sparse population of kangaroo rats: eight kangaroo rats were captured in 225 trap nights at Huasna. It is possible that the Huasna population is sparse, but broadly distributed along the Huasna river bottom and flood plain.

Several sites that are not historical localities were identified through the GIS analysis and evaluated for potential habitat and the presence of kangaroo rats. These “new sites” include: 7) Price Canyon, Lat/Long 35.1442, -120.6302, which is open space for the City of Pismo Beach. The mouth of the Price Canyon (Easy of Hwy 101) has sandy soils and a mix of coastal scrub, oak woodlands and grasslands. One hundred and eighty trap nights produced no kangaroo rats. 8) Carpenter Canyon, Lat/Long 35.1522, -120.5765. This site is 3.1 miles by road South of east Corral de Piedras creek and is adjacent to low density housing. It is sandy soil with coastal scrub, oak woodland and exotic plantations. One hundred trap nights produced no kangaroo rats. 9) Southwestern Nipomo Mesa, Lat/Long 35.0179, -120.5647 is a site on the southern slope of the Nipomo Mesa. It is inland but contiguous with the Pismo/Oceano/Nipomo/Guadalupe dune complex (sampled by sites 1, 2, 3, 9, 10, 11, and 12). The site is undisturbed coastal scrub. One hundred and ten trap nights produced 15 kangaroo rats. 10) Guadalupe, Santa Maria River at Hwy 1, Lat/Long 34.9783: -120.5689, is a site 1.65 miles south of Southwest Nipomo Mesa site. It is contiguous with the Guadalupe dunes site, but only connected via the Santa Maria River bottom and flood plain. The habitat is disturbed, with kangaroo rat sign found only on the larger sand bars. One hundred and fifteen trap nights produced 11 kangaroo rats. This population could be ephemeral depending on flow and flooding rates in the Santa Maria River. 11) The

Oceano Dunes State Vehicular Recreation (ODSVRA) area was surveyed at three sites: Boy Scout Camp, Lat/Long 35.0410, -120.6131; Maidenform, Lat/Long 35.0353, -120.6213, and Pipeline Lat/Long 35.0545, -120.6229. Trapping was done with assistance from State Parks personnel. These 3 (ODSVRA) sites sample habitat that is contiguous across the State Vehicular Recreation Area, the Nipomo Mesa (3) and the new Southwestern Nipomo Mesa (9) sites. The habitat is disturbed and undisturbed coastal scrub. Four hundred and fifty trap nights resulted in the capture of 51 kangaroo rats. 12) Guadalupe Dunes National Wildlife Refuge, Lat/Long 35.0158, -120.6115. This site is contiguous with and 1 mile from the Maidenform site in the ODSVRA. The habitat is undisturbed Coastal scrub. One hundred and eighty trap nights resulted in the capture of 86 kangaroo rats. Trapping at the Guadalupe Dunes National Wildlife Refuge was done with a special use permit from the NWR.

Of the sites checked, only two, Huasna and the Pismo/Oceano/NipomoGuadalupe dunes complex contained *Dipodomys heermanni arenae*. Based on site visits, live trapping, and the distribution of sign, it is estimated that approximately 26.4 square miles (68 square km) or 16888.18 acres (6837.45 hectares) of the dune complex and Mesa is occupied by *D.h. arenae* (Figure 3). The average observed population density (based on mark recapture) across sites in this dune complex during 2005-2006 was estimated at 5.7 kangaroo rats per hectare. The Dune complex population *Dipodomys heermanni arenae* is thus estimated at roughly 39,200 individuals. This is in stark contrast to the low density (Huasna), patchy nature (possibly Edna and Santa Maria River), and unknown status (e.g. Edna, and Phoenix Creek) of other populations of *D. h. arenae*. Figure 2 shows that there are extensive patches of sandy soil inland of the Dune Complex. Specifically, from Price Canyon northward to Avila Valley, and Easy of Arroyo Grande, there are several patches and linear (creek and river) associations of sandy soils. These areas are in private property, but represent a potential focus for future research.

D. h. jolonensis distribution and site evaluations

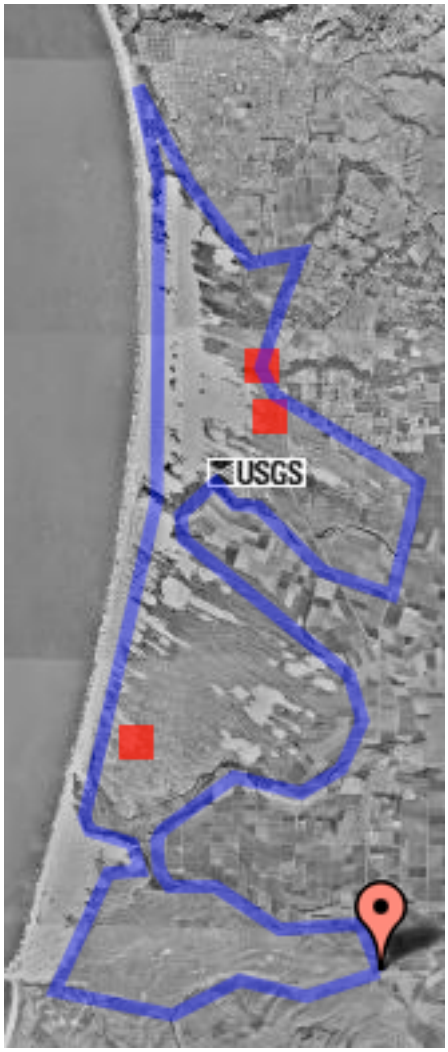
Most of the range of this subspecies is within Monterey County. The recorded localities in S.L.O county are: 1) Camatta Creek, 1450 ft, Lat/Long 35.446483, -120.288724, Date 1947; 2) 3.5 mi E and 0.5 mi N McChesney Mt., 1900 ft, Lat/Long 35.285637, -120.174110, Date 1947; 3) 2.3 mi E and 4.9 mi S Shandon, 370 ft, Lat/Long 35.581383, -120.327076, Date 1993; and 4) 2 miles S. San Miguel, Lat/Long 35.723588, -120.6973, data 1918.

The San Miguel area was chosen for site assessment given extensive public access to the Salinas River, and the potential for a large population of kangaroo rats. Sandy soil is abundant in this area, particularly along the Salinas River flood plain. The collecting site (4) is currently a residential development located between Hwy 101 and the Salinas River. Therefore, a seven-mile section of the Salinas River was surveyed from Wellsona Rd on the South, to Indian Valley Rd on the North. Both of these roads, as well as North River, and River Roads, were used for access. Suitable habitat was distributed almost along the entire edge of the Salinas River corridor. No sign of kangaroo rats was seen. Live trapping was conducted in the Salinas River flood plain at River Rd. (Lat/Long 35.755, -120.6874), and in the Salinas River flood plain at Wellsona Rd. (Lat/Long 35.698, -120.6872, and Lat/Long 35.6979, -120.6845). No Kangaroo rats were captured in 304 trap nights. Therefore, no evidence of Kangaroo rats was found over this 7 mile stretch of the Salinas River. In contrast, the flood plain of Big Sandy Creek, upstream

(east) of its confluence with the Salinas River (Lat/Long 35.7939, -120.7205, (with 200+ meter radius)), contained a thriving population of *D. h. jolonensis*. Twenty-five kangaroo rats were captured in 75 trap nights. This site is currently a protected area (Big Sandy Wildlife Area, C.D.F.G.) and Permission for Access and Small Mammal Trapping was obtained.

FIGURE 3. The portion of the Pismo/Oceano/Nipomo/Guadalupe dune complex estimated to be occupied by *D.h. arenae* (outlined in blue). This is the largest patch of habitat occupied by *D.h. arenae* within San Luis Obispo County. This represents the extant population of *D.h. arenae* that is geographically closest to the endangered MBKR of Los Osos. *D.h. arenae* from the Edna site would be geographically closer, but the status of this population could not be confirmed. A. Aerial photograph of the Pismo/Oceano/Nipomo/Guadalupe Dunes complex. B. Topographical map of the same area.

A.



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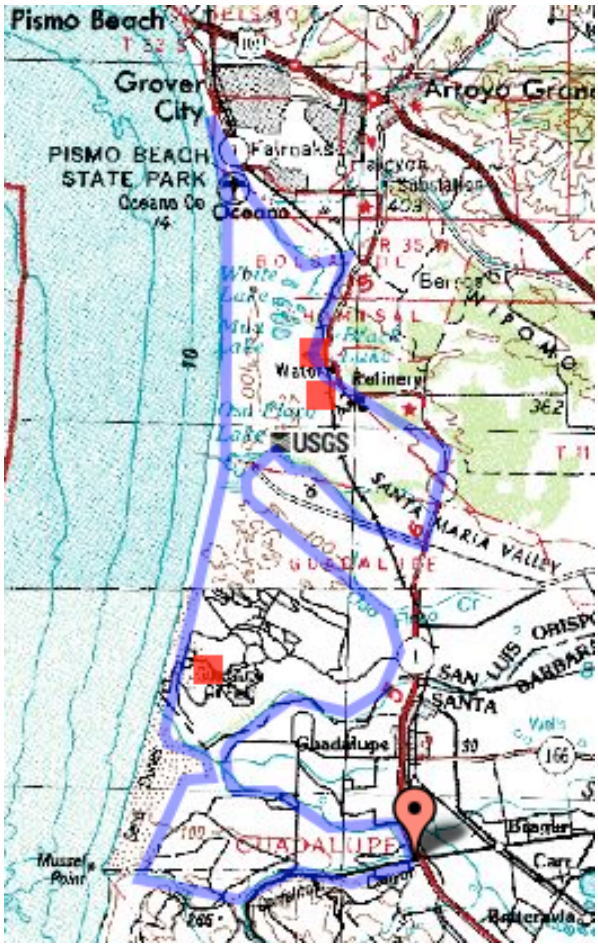


D. h. swarthi distribution and site evaluations

Most of the range of this subspecies is within San Luis Obispo County. The historical collection sites include: 1) 7 mi SE Simmler, Carrizo Plains, Lat/Long 35.279590, -119.899310, date 1911; 2) 9 mi W Simmler, Lat/Long 35.383299 -120.122876, date 1930; 3) La Panza Ranch, 11 mi W Simmler, 35.384359 -120.167494, date 1930; 4) Santiago Springs, 1.5 mi S and 8 mi E Simmler, Lat/Long 35.328304 -119.845250, date 1943; 5) San Diego Joe's, Lat/Long 35.328300 -119.845250 date 1931; 6) San Diego Creek, Temblor Range, Lat/Long 35.333410 -119.843370, date 2002. Site 6 is synonymous with sites 4 and 5; 7) 1.1 mi W and 0.5 mi N Temblor Peak, Lat/Long 35.070583 -119.509332, date 1991, 8) Beartrap Creek, 1.5 mi W San Juan Creek, Lat/Long 35.558351; -120.261440, date 1946; 9) 5 mi E and 4 mi S Shandon, Lat/Long 35.597750; -120.281730, date 1947; 10) 3.5 mi SE Cholame, Lat/Long 35.695428 -120.252334, date 1957; 11) Painted Rock, Lat/Long 35.146085 -119.860768, date 1954; 12) Beam Flat, Elkhorn Hills, Lat/Long 35.019117 -119.492483, date 1999; 13) 0.4 mi S Wells Ranch, Caliente Range, Lat/Long 35.044270 -119.894680, date 2001; 14) 13.3 mi NW (by road) New Cuyama, Lat/Long 35.044270 -119.894680, date 2001; 15) 1.75 miles E. Cuyama, Lat/Long 34.9338 -119.5848, date 1966; 16) 2 miles S. Crocker Grade, Carrizo Plains, Lat/Long 35.1547, -119.6899, date 1984; 17) Caliente Mtn, 5,000, Lat/Long 35.0354, -119.7601, date 1964; 18) Panaroma Hills, Elkhorn Plains, too vague for GPS coordinates, date 1986.

This subspecies is easily collected in San Luis Obispo Co., as evidenced by 6 sites from which it has been documented since the 1980s. This subspecies is extensively distributed over the Elkhorn Plains and Carrizo Plains in an area that is preserved under the Carrizo Plains National Monument. The San Diego Creek/San Diego Joe's/Santiago Springs (synonyms) site (at 35.3501, -119.8341) was evaluated in this study. There is a dense population of Kangaroo rats at that location: 23 individuals were trapped in 75 trap nights.

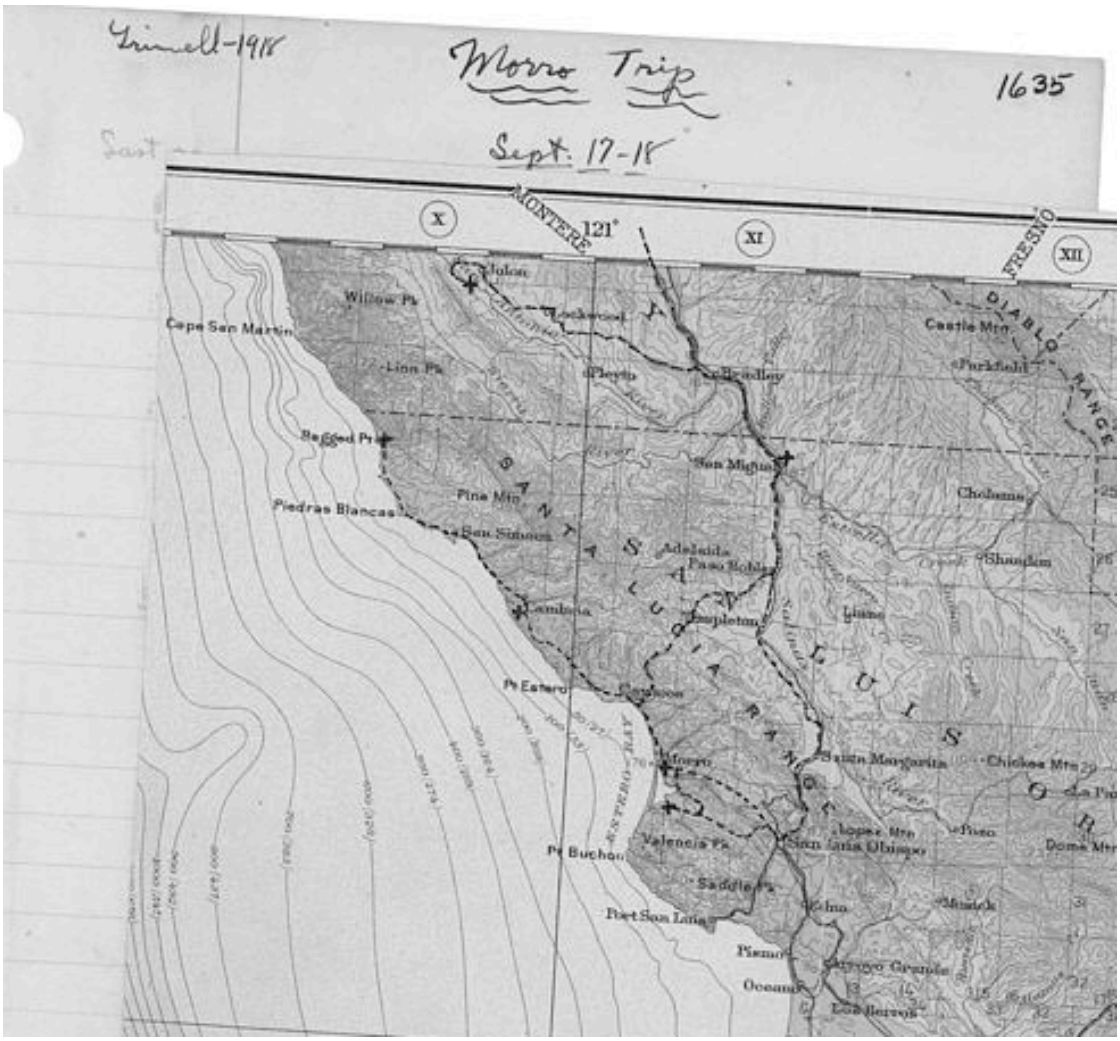
D. h. morroensis distribution and site evaluations

There are several historical collection sites for the Morro Bay Kangaroo rat. The locations described for that subspecies occur over a very small area compared to locations discussed for the preceding subspecies. For example, the San Diego Creek site (evaluated for *D.h. swarthi*) is approximately 1.5 miles long. This is on the same scale as the entire range of the M.B.K.R. Given this scale, and the current endangered status, an attempt was made to determine the exact location of all 16 historical M.B.K.R collection sites. Collection localities are grouped geographically in the discussion below.

1) 4.0 and 4.5 mi S Morro, date 1918. Both of these sites (including Lat/Long) are recorded with up to 1800 meters of uncertainly (*sensu* MVZ). As recorded, the sites are on a slope south of the recognized range of this subspecies (4 mi S Morro), and at the head of Hazard Canyon (4.5 mi S Morro), also outside of the recognized range. Either the MBKR once occurred in an area much larger than currently thought (e.g. including Islay Creek, Hazard Canyon, and the Los Osos Creek drainage), or there are errors or uncertainty in both the literature and databases. The locality uncertainty was resolved by consulting Grinnell's and Dixon's original field notes (1918). This collecting locality is not 4.5 miles *S*. of Morro (Bay) as reported in the literature and *labeled* in Dixon's field notes (1918, pg. 885-887), but is instead 4.0 miles *SSE* of Morro Rock

as *described* in Grinnell's field notes (1918 pg. 1648). A map presented by Grinnell shows the roads they traveled to the collection site (Figure 4). The distance to the collection site was 10 miles (sensu Grinnell) *by road* (via Turri Rd). That corresponds with 4 miles SSE of Morro Rock. This is presently near the intersection of Los Osos Valley Rd and South Bay Blvd. or between Lat/Long 35.3127: -120.8156 and Lat/Long 35.3036: -120.8216 (Figure 5). Thus, we can reject that MBKR occurred at the head of Hazard Canyon (error) in 1918. Likewise, we can reject the uncertainty reported as a 1.0 -1.2 mile variance (MVZ database).

FIGURE 4. A reproduction of Grinnell's field notebook (1918) showing the route to the Morro Bay Kangaroo Rat collecting locality. Notice the dotted line from Morro Bay to San Luis Obispo, and from San Luis Obispo to Los Osos. Then note the dotted line from Morro Bay to Los Osos (along Turry Rd). This Route is 4.0 miles SSE of Morro Rock and 10 miles by road from Morro Bay as described in the text of Grinnell's notes.



2) 0.5 miles SW Los Osos School, Baywood Park, date 1964. The original Los Osos School site was at the intersection of Los Osos Valley Rd and Turri Rd. This is outside of the recognized range of the MBKR. Roest's field notes from 1961 place the Los Osos School North of Los Osos Valley Rd, and along the road now named Sombrero, or as he puts it "SW corner of Sec 9, R 11 E, T 30 S, on road near "gravel Pit" and just at beginning of rise in road to the east." Thus the collecting locality (Figure 5) would have been along Los Osos Valley Road, at Lat/Long 35.3065: -120.8043, or nearly exactly at the same location as site 1. 3) North of Los Osos Creek near Los Osos School, date 1961. This site is at Lat/Long 35.3067: -120.8033, is essentially the same as site 1 (Figure 5), and is represented by a road kill specimen. 4) Buckskin Drive, Lat/Long 35.3137, -120.8153, date 1983. This site is off of Buckskin Drive and represents the Buckskin site (Figure 5). Localities 1-4 are all clustered at or just beyond the Southeastern corner of the MBKR's range.

FIGURE 5. Historical collection sites for *D. h. morroensis* (numbers 1 – 4 described in text) at the Southeastern margin of the recognized range. Sites 1, 2, and 3 are East of Los Osos Creek and technically outside of the currently recognized range. The locations for sites 1, 2 and 3 are approximated to within 250 meters. The location of Site 1 has been erroneously reported in the literature. Site 4 is the Buckskin site. Figure 6 includes the area almost directly to the north of this figure.

5) 1.5 miles NE Los Osos Store, Baywood Park, date 1964. Roest's field notes indicate this store was at the intersection of Mountain View (now 17th St.) and San Luis Ave. Thus, the site

would have been on one of the small sandy hills that make up the extension, or at Lat/Long35.3226: -120.803. This site is East of Los Osos Creek and off of Turry Rd. as shown in Figure 6. 6) 1.5 mile E. Baywood Park, date 1961 is effectively the Santa Ysabel site at 35.3307: -120.8206 (Figure 6). 7) 2.25 miles E of Baywood Park, date 1958. This is the site of the "Turri Rd Extension," an area recognized as being on loamy and not sandy soil that is within the recognized range (Figure 6). This location is at Lat/Long 35.3251: -120.8036. 8) 1 mile SE Baywood Park 1968 is adjacent to the southern edge of the Junior High School site (Figure 6) at Lat/Long 35.3199: -120.8189.

FIGURE 6. Historical collection sites for D. h. morroensis (numbered 5, 6, 7 and 8 in the text) at the Northeastern margin of the MBKR's recognized range. Site 5, recorded by Stuart and Roest (1960), is the Northern most of two sandy hills East of Los Osos Creek that comprise part of the extension. Site 6 is Santa Ysabel. Site 7 is the loamy soil portion of the "extension." Site 8 is South of the Junior High site. Figure 5 includes the area almost directly South of this figure.

9) Baywood Park, date 1964, Lat/Long 35.3277: -120.8336. 10) East edge of Baywood Park, date 1980, Lat/Long 35.3263: -120.8255. 11) Los Osos, Bayview Heights, Lat/long 35.3039, -120.8386, date 1983. These three sites are represented in collections and in the live trapping literature. The two Baywood sites (9 and 10) are fully developed at this time. The Bayview Heights site is the location of the last known wild population.

12) 0.5 miles SW. Los Osos School, Baywood Park, date 1961. If we use the location of Los Osos School from Roest's notes, then this collecting site would have been near the Los Osos Oaks and sites 1, 2 and 3 at Lat/long 35.3021: -120.8117. There is an inconsistency in referring to this school as being in the town as Baywood Park. If the reference was to Baywood School, then the location would be Lat/Long 35.3249: -120.83, and would be more or less synonymous with site 9. This site is not discussed in Roest's notes and thus remains unconfirmed, though as it stands it does not appear to represent a novel site.

13) Morro Bay State Park, date 1961. This location name spans an area from North of the Turri Rd. Extension all the way to Black Hill just east of the Morro Bay city limts. This is a distance of 4.5 miles. In 1961 the Park boundary was closer to Black Hill, but the location cannot bee determined exactly. In 1918 Dixon (pg 883) comments that there is a sandy stretch south of Black Hill that contains burrows probably of *Perodipus* (*Dipodomys*) *morroensis* though he never trapped at the site. Thus the exact location of this site is unknown, but is clearly outside of the recognized range. 14) Morro Bay, date 1963. There are no field notes for this specimen and the exact locality is unknown. 15) Morro, date 1907 (Merriam 1907), represents the type locality for this subspecies. The field notes have not been consulted and this locality at present is to vague to locate.

16) Irish Hills Apartments, San Luis Obispo, 1980. This location is near the intersection of Los Osos Valley Rd and Madona Rd in San Luis Obispo. The specimen is identified as *D. heermanni*, with no subspecies designation. This specimen was discovered dead in a fallow field adjacent to a residential development. The site is at 35.2564: -120.6946, placing it 9.2 miles from the nearest known MBKR locality (1), and 4.25 miles from the nearest known *D. h. arenae* locality (Edna Valley).

For locality 1 (4 mi SSE Morro Rock), Grinnell states in his 1918 field notes that "I have never seen *Perodipus* (*Dipodomys*) sign thicker anywhere, especially in some once cleared land where the road crosses Los Osos Creek." Sites previously occupied by MBKR (as defined in the Draft revised Recovery Plan) were re-surveyed for this study. These sites included: Pecho South, Pecho North, Bayview, Los Osos Oaks, Buckskin, Junior High and Santa Ysabel. Visual surveys were conducted between April and June of 2006. Survey dates were chosen so that they were preceded by several days with minimal wind, such that tracks, tail drags and other sign would have a chance to accumulate. No sign of MBKR was detected during any of the site visits.

SCIENTIFIC COLLECTION

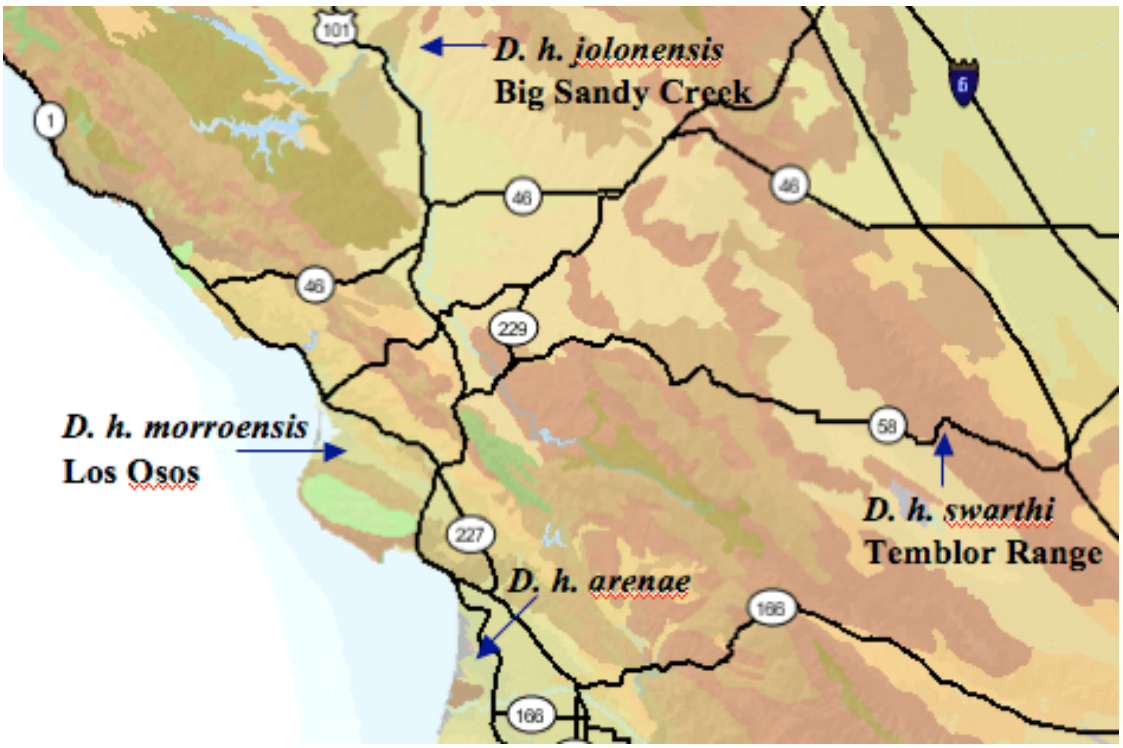
We determined whether nuclear DNA extracted from museum specimens from the A.R.M.C. would be of sufficient quality to be used for a microsatellite analysis. These specimens did *not* produce reliable or repeatable results (see below). Therefore, tissues from live trapped specimens or frozen tissue collections were required in order to complete the study. Specimens of *D.h. arenae, jolonensis and swarthi* were live trapped and collected in the field. Specific collecting sites for all subspecies/population are presented in Table 1 and shown in Figure 7. *D.h. arenae* from Guadalupe, UnoCal were collected by collaborators and donated to the Cal.

Poly. A.R.M.C. All of these specimens are vouchered and deposited in the Aryan Roest Mammal Collection at Cal Poly.

TABLE 1. Collecting localities for live-trapped kangaroo rats used in the microsatellite analysis. All specimens are vouchered with skin and skull and deposited in the Aryan Roest Mammal Collection at Cal Poly.

Subspecies	**Collecting Locality**	**Lat/Long**
D. h. arenae: N=8	Huasna River	35.1391, -120.3604
D. h. arenae: N=11	Southwestern Nipomo Mesa	35.0179, -120.5647
D. h. arenae: N=9	Guadalupe, Santa Maria River	34.9783, -120.5689
D. h. arenae: N=8	Guadalupe, Unocal Oil Field	34.9795, -120.6332
D. h. jolonensis: N=14	Big Sandy Creek	35.7939, -120.7205
D. h. swarthi N=10	San Diego Creek	35.3501, -119.8341

FIGURE 7. General collecting localities for kangaroo rats used in genetic analysis. For specific locations, including latitude and longitude see text and table 1.



Frozen tissues for MBKR were derived from individuals collected in the 1980s for the captive breeding program. These individuals were originally at Cal Poly (under Dr. Aryan Roest), and ultimately at the National Zoological Park (under Dr. Miles Roberts). Once all the individuals in that population were deceased, they were transferred to the Smithsonian Conservation Genetics Lab (under Dr. Robert Fleisher). A small piece of liver tissue was obtained from all of these MBKR individuals. The sampling locations for MBKR the are indicated in Table 2.

TABLE 2. Collecting sites for Morro Bay Kangaroo Rats used in the microsatellite analyses. Samples were provided through a tissue sharing agreement between the Lab of Molecular and Organismal Evolution at Cal Poly (under Dr. F. Villablanca) and the National Zoological Park and Smithsonian Conservation Genetics Lab.

Subspecies	**Collecting Locality**	**Lat/Long**
D. h. morroensis: N=1	Los Osos, Buckskin	35.3137, -120.8153
D. h. morroensis: N=10	Los Osos, Bayview	35.3039, -120.8386

MICROSATELLITE POPULATION GENETICS

The objective of the population genetic analysis was to determine the degree of genetic affinity between the MBKR and its geographic neighbors. Specifically, we quantify the amount of genetic diversity, genetic divergence, gene flow, and phylogenetic affinity between populations of *D.h. morroensis*, *arenae*, *jolonensis* and *swarthi*.

Microsatellite loci are ubiquitous in mammalian genomes. A microsatellite locus is defines as a 2, 3 or 4 nucleotide-long repetitive sequence. Alleles are defined by the number of these repetitive sequences that are found in a locus, or by the length of the microsatellite locus which contains the repeats. Since these loci are nuclear, individuals can be homozygous or heterozygous for microsatellite alleles. These loci show a high rate of mutation and are thus ideal for population genetic surveys between closely related taxa.

General microsatellite methodology

Several steps are required to produce and then validate the data prior to analysis. These steps are briefly discussed below as they are part of the procedure employed in the study. Details of the lab protocols can be found in Kornbluth (2007) or Villablanca (in prep.).

1) Literature survey. It can take 12-16 months to isolate a half dozen microsatellite loci from the genome of a novel species. Therefore, if microsatellite loci are known from a close relative to the species of interest, then it is most efficient to explore these loci first. The utility of heterospecific loci (those developed for another species) will depend on the time since divergence and rate of molecular evolution in the target species and in the species for which microsatellite loci were developed.

   A literature survey identified nine microsatellite PCR primer pairs from *D. spectabilis* and *D. ingens* that were developed by Davis et al (2000). *D. ingens* is in the same species group as *D. heermanni* (Grinell 1922, Carrasco 2000, Alexander and Riddle 2005), so it was hypothesized that the Davis et al. PCR primers would work on *D. heermanni*. Furthermore, a microsatellite approach was deemed potentially suitable for *D. heermanni* given the fine scale geographic resolution discovered in *D. ingens* (Loew et al. 2005), and in *D. spectabilis* (Winters and Waser 2003, Waser et al 2006) using the same set of microsatellite loci.

2) Trial amplifications of microsatellite loci in the target species using PCR. The primer pair for each locus was tested in *D. heermanni* to ascertain whether D. *ingens* and D. *heermanni* are closely related enough that primer sequences would be conserved across these three species. Trial amplifications were conducted on DNA extracted from frozen liver tissue (*D.h. arenae*, Guadalupe, UnoCal), and from museum skins (M.B.K.R. from the A.R.M.C.). Eight of the 9 primer pairs developed by Davis et al. (2000) cross amplified in *D. heermanni* (Table 3). When efforts were made to get the last locus to amplify (Di12F), the result was nonspecific amplification – multiple amplicons were produced making it impossible to identify the homolog fragment. Homology of the microsatellite loci is inferred by the size of the DNA amplicon.

3) Museum skins proved to be problematic. Several extractions had to be pooled to yield DNA concentrations high enough for PCR amplification. Unfortunately, multiple extractions multiply the chance for contamination. Also, even with multiple extractions, it was not possible to consistently amplify all loci from every individual, or to amplify the same alleles across replicates from any given individual. We attribute the problem to null alleles: alleles that do not amplify due to mismatch between template and primer DNA, or because of degraded template DNA. All data presented here are derived from PCR amplifications from DNA extracted from frozen liver or ear tissue.

4) Optimization of PCR reactions across all loci. PCR amplification conditions were optimized so that PCR product could be produced, and compared, across multiple taxa. The PCR conditions were optimized for all 8 loci that initially amplified in *D. heermanni*. The final PCR conditions used for all loci are presented in Table 3.

TABLE 3. Heterspecific microsatellite loci developed in *D. ingens and D. spectabilis* (Davis et al. 2000) that cross-amplified in *D. heermanni*. The repeat sequence (motif), optimal primer annealing temperature, amplicon size range (including 18bp of M13 sequence), and reasons for exclusion from analysis are provided. * indicates X – linked.

Locus	Repeat Motif	Annealing temp °C	Size Range (bp)	Included in Survey
Ds3	GT	62	190-212	Yes
Ds1	(GT)(AG)	66	194-218	Yes
Ds19*	GT	54	112-146	Yes
Ds28	GT	~47-54	~183-210	No – inconsistent results
Ds30	GT	62	237-336	Yes
Ds46	GT	~54-60	~147-188	No - inconsistent results
Di5	CAC	59	193-217	Yes
Di12E	(CA)(CAC)	66	203-252	Yes
Di12F	NA	NA	NA	No – did not amplify

5) Generation of multiple replicates of data for one individual. Amplicons were generated for all 8 loci in one single individual. These PCR fragments were used to optimize our use of the CEQ (capillary elecrophoresis for quantifying fragment size), to train, and to establish quality controls. The data generated through this exercise were used to determine which loci gave reproducible results. Only if a locus gave repeatable result across multiple amplifications from a single individual, was it considered informative for comparisons across individuals. Two loci (Ds28 and Ds46) were eliminated because they yielded inconsistent results (Table 3).

6) Survey population for genetic diversity. This step is necessary in order to determine is loci are variable within the target species. Common practice is to select a test population and screen it for variability in all loci that gave consistent amplification results. The series of frozen tissues for *D. heermanni arenae* from the Guadalupe, Unocal Oil Field site (N=8) was screened for genetic variability across all 6 loci that consistently amplified (Table 3). All the loci were found to contain allelic variation.

7) Generate Pilot data and test assumptions. A pilot data set was generated for all 6 loci (table 3) and all 7 populations (tables 1 and 2). The pilot data set can be more inclusive than the final data set. The *nature* of the genetic variation in the pilot data set has to be evaluated to determine whether these data fit assumptions that are commonly required by analytical/inference methods.

   The data were analyzed using a Bayesian method (MICROCHECKER) that estimates the probability of null alleles (alleles that failed to amplify) occurring in the data, and if this fraction is small, corrects the observed allele frequency (Van Oosterhaut et al 2004), to yields a corrected allele frequency for each population. This is the same approach used by Wilson and Rannala (2003). Null alleles were detected at a low enough frequency that actual allele frequencies within populations could be estimated from the observed frequencies.

   All loci in the pilot data set were then tested for deviations from Hardy-Weinberg equilibrium (HWE). To determine whether HWE was violated, the observed and expected allele frequency for each of the six loci over four subspecies (*D. h. arenae*, *D. h. morroensis*, *D. h. swarthi*, and *D. h. jolonensis*) and four geographic populations of *D. h. arenae* (Huasna, Nipomo, Guadalupe UnoCal, and Guadalupe Santa Maria River) were calculated using ARLEQUIN 3.01 (Excoffier et al., 2005). Potential departures from HWE were evaluated by comparing the observed and expected heterozygosity for every population at every locus. Significant departure was assessed using a Markov Chain estimation (1,000,000 iterations), and including a burnin of 100,000 steps (Guo and Thomson 1992). A global and sequential Bonferonni correction (Rice, 1989) was performed. If HWE was rejected in the same locus across every populations, then that locus would be dropped from further analyses. None of the loci showed a significant departure from HWE across all populations under either a global or sequential Bonferroni correction. Within populations, HW equilibrium was not rejected for any locus, in *D.h. arenae* Huasna, *D.h. arenae* Unocal, *D.h. arenae* Santa Maria River, *D.h. morroensis, D.h. jolonensis*, or *D.h swarthi*. Hardy-Weinberg equilibrium was rejected

for *Ds*30 (p=0.043) in *D.h. arenae* Nipomo, but this result is not significant under a Bonferroni correction. Therefore, the assumption of Hardy-Weinberg equilibrium does not appear to be rejected by these data.

All loci in the pilot data set were then tested for deviations from linkage disequilibrium (LD). ARLEQUIN 3.01 (Excoffier et al. 2005) was also used to test for linkage disequilibrium in genotypic data with unknown gametic phase. To perform this equilibrium test, an Expectation-Maximization (EM) algorithm was used. This algorithm looks at each population separately and utilizes maximum likelihood to resolve genotypic data into haplotypic data for two loci at a time (Excoffier and Slatkin 1995). The likelihood is then used in a likelihood ratio test to determine whether there is significant linkage disequilibrium between any two loci within each population (Excoffier and Slatkin, 1995). In *D. h. arenae* Huasna two pairs of loci showed significant linkage at the 0.05 level (Ds1 and Ds3, p = 0.006, and Ds1 and Ds30 p = 0.016). *D. h. arenae* Unocal showed significant linkage at two pairs of loci (Ds5 and Ds19 p=0.049) and Ds3 andDi12E p=0.049). *D. h. arenae* Santa Maria showed significant linkage in one pair of loci (Ds1 and Ds30 p=0.018). *D.h. morroensis* showed significant linkage in one pair of loci (Ds19 and Ds30 p=0.015). *D.h. jolonensis* showed significant linkage at one pair of loci (Ds30 and Di12E p=0.035). Yet, 1) none of these results were significant with a global or sequential Bonferroni correction for comparisons within each population, 2) no single locus or pair of loci consistently came up as significantly across all populations, and 3) *D.h. swarthi* had no locus pairs with any evidence for linkage. Therefore, the assumption of linkage disequilibrium does not appear to be violated by these data.

Analytical results of microsatellite DNA survey

1) Within locus diversity. The cumulative number of alleles in each locus is presented in Table 4. All loci were polymorphic with anywhere from 2 to 35 alleles per locus. The Di5 locus was monomorphic in some populations (*D. h. arenae* Huasna, *D. h. arenae* Nipomo, *D. h. arenae*, Unocal, and *D. h. morroensis*), but variable in others.

TABLE 4. Within population allele counts (number of alleles detected) for all loci found to be in HWE and LD. The cumulative number of alleles per locus is also indicated.

	Number of alleles per locus					
Population	Ds3	Ds1	Di5	Ds30	Ds19	Di12E
D.h.arenae Huasna	4	10	1	9	4	5
D.h.arenae Southwest Nipomo	7	7	1	10	6	6
D.h. arenae Guadalupe UnoCal	8	8	2	7	4	8
D.h.arenae Guadalupe SM River	5	7	1	7	5	4
D.h.morroensis	3	7	1	6	2	1
D.h.jolonensis	11	9	2	16	4	4
D.h.swarthi	4	8	2	6	5	6
Cumulative number of unique alleles	14	22	2	35	14	11

2) Within population diversity. Patterns of genetic diversity across the populations of *D. heermanni* are not uniform. One population, *D.h. morroensis*, was monomorphic at two loci. Monomorphic means there is only one allele at a locus. Three populations, *D.h. arenae* Huasna, *D.h. arenae* Nipomo, and *D.h. arenae* Santa Maria, were monomorphic at a single locus. While three samples, *D. h. arenae* Unocal, *D.h. jololensis* and *D.h. swarthi* showed some genetic diversity (polymorphism) at every locus evaluated.

The Morro Bay kangaroo rat has the lowest mean number of alleles per locus (3.333), which is significantly ($p = 0.02$) lower than the mean for the other 6 populations. Likewise, the expected heterozygosity (0.436) for *D.h. morroensis* is significantly lower ($p = 0.01$) than the average for the other 6 populations. The Morro Bay Kangaroo rat has the lowest nucleotide diversity (theta under the stepwise mutation model) of all the populations sampled. Though the MBKR's nucleotide diversity value (theta = 1.533) was not significantly lower than the mean (1.773 ± 0.164 (1 s.d.)) for the other 6 populations ($p = 0.11$).

This result, a significantly lower mean number of alleles, a significantly lower mean heterozygosity, and a non-significant difference in nucleotide diversity is the typical pattern seen in bottlenecked populations (Tajima 1983). This fits the theoretical expectation (Nei et al. 1975): rare alleles are lost first, gradually heterozygosity declines, and ultimately nucleotide diversity declines to reflect the new equilibrium population size. Unfortunately, if the nucleotide diversity is not significantly lower, then the MBKR will statistically have the same demographic history as that of the rest of the populations (Slatkin 1991). This means that it is possible to document that the MBKR has been through a bottleneck, may still be losing diversity to reflect the post bottleneck population size, but it may be impossible to date the bottleneck (Hey and Neilsen 2004)

3) Differentiation between subspecies. An Analysis of Molecular Variance (AMOVA) was conducted using ARLEQUIN 3.01 (Excoffier et al., 2005). This analysis compares predefined groups, where the groups were the four subspecies of *D. heermanni* sampled. Each subspecies was represented by a single population, except for *D. h. arenae* which was represented by 4 populations.

F_{ST} is the population inbreeding coefficient traditionally used to quantify population genetic structure. It is based exclusively on evaluating allele frequency differences between populations. Molecular data provide an advantage over traditional frequency data in that models of molecular evolution can be specified for genetic markers. Application of a model of evolution allows additional information to be extracted from an analysis. Microsatellites are thought to evolve by the stepwise mutation model, therefore the R_{ST} statistic is more appropriate for AMOVA analysis of microsatellite data than is F_{ST} (Slatkin 1995). This statistic compares the frequency of alleles (like Fst), as well as the number of step separating alleles, where each step is defined as a single increase or decrease in the number of repeats in an allele. An AMOVA analysis was used to partition genetic variance via R_{ST}, and to determine whether there is significant genetic subdivision between the subspecies of *D. heermanni* sampled. The significance of R_{ST}, was evaluated with 1,000 permutations. The AMOVA provides protection from type I

error in subsequent comparisons between pairs of subspecies. Significance for F_{ST}, (frequency based analysis) was likewise evaluated with 1000 permutations. Fisher's exact test was done with a 4,000,000 step MCMC approach including a 100,000 step burnin.

The AMOVA analysis of four subspecies, *D. h. arenae, morroensis, jolonensis* and *swarthi*, revealed a significant R_{ST} value indicating significant population genetic subdivision. 1) 13.88% ($p < 0.000$) of the total genetic variance was attributed to between subspecies. 2) 84.68% of the total genetic variance was attributed to within populations ($p = 0.000$). The variance partitioned among the four populations of *D. h. arenae*, 1.44% ($p = 0.445 \pm 0.015$), was not significant. In terms of the R_{ST} values, MBKR was most different from *D. h. jolonensis* ($R_{ST} = 0.250$, $p = 0.001$), then *D. h. swarthi* ($R_{ST} = 0.110$, $p = 0.021$), and then not significantly different from *D. h. arenae* ($R_{ST} = 0.082$, $p = 0.0645$). A comparison of *D. h. morroensis* to the four populations of *D. h. arenae* shows that *D. h. morroensis* is significantly different from two populations (*D. h. arenae* Huasna, $R_{ST} = 0.146$, $p = 0.033$, and *D. h. arenae* Unocal, $R_{ST} = 0.093$, $p = 0.044$), but not significantly different from two others (*D. h. arenae* Nipomo, $R_{ST} = 0.034$, $p = 0.108$, and *D. h. arenae* Santa Maria, $R_{ST} = 0.056$, $p= 0.076$).

We can conclude from the R_{ST} analysis that the MBKR is significantly different from the *D. h. jolonensis* and *D. h. swarthi* subspecies, and significantly different from some (*D. h. arenae* Huasna, *D. h. arenae* Unocal) but not all (*D. h. arenae* Nipomo, *D. h. arenae* Santa Maria) populations of *D. h. arenae*. It is considered likely that the MBKR would be found to be significantly different from all populations of *D. h. arenae* if a larger number of loci or individuals were included in this survey. Yet, even at this time, we can conclude that the MBKR is most similar to the *D. h. arenae* Nipomo population.

An F_{ST} analysis and Fisher's exact test of non-differentiation were conducted. These tests perform all pairwise comparisons (compare all populations to every other population). Both of these tests are based entirely on frequency differences. Neither test considers the degree of differentiation between alleles. Neither test was significant overall. Some of the pairwise population comparisons were significant, but not following the Bonferonni correction.

4) Differentiation among populations. The R_{ST} analysis indicates that there is significant population genetic subdivision between the subspecies. Yet this analysis was conducted presupposing that the number of genetic units is known, and equal to the number of geographic samples ($n = 7$). If any of the populations are not significantly different from one another, then this supposition is not correct. Meaning that allele frequencies within populations, which arethe bases for an F_{ST} and R_{ST} analysis, were not calculated correctly. For example, the *D.h. arenae* populations may not all be differentiated from one another and thus allele frequencies should be calculated after collapsing them into less than 4 groups. Therefore, an analysis was performed to estimate the number of genetic groups in the sample of 7 geographic populations

The number of genetic groups within the set of sampled geographic populations was estimated using a Bayesian analysis (Bayesian Analysis of Population Structure BAPS V 4.1.4 Corander 2007). The number of groups (K) and the allele frequencies (p_i) within groups are jointly estimated from the data (X). The prior probability of individual assignments to groups can be defined as uniform (equal across locations). Alternatively, the prior probability of individual assignments to groups can be based on the geographic location from which a sample was obtained thus generating a non-uniform prior. Both approaches (uniform and non-uniform priors) were used here.

First, an analysis was conducted with uniform priors (Corander et al. 2003, 2006) were the maximum number of populations (K max) was set as a range of values from 1 to 7. Seven runs were conducted one for each of the 7 values of K (from K=1 to K=7). Each run made four estimates of P(X|K). The results for all estimations (7 runs and 4 replicates each) were merged and the K value of the 10 most visited partitions (and their Log maximum likelihood (logML) values) are retained. K values that differed by a Bayes Factor (exponential of the absolute difference in logML) of < 2.5 were considered to not have significantly different probabilities (Raftery 1995). Three replicate of this analysis were performed in order to assure convergence and stationarity.

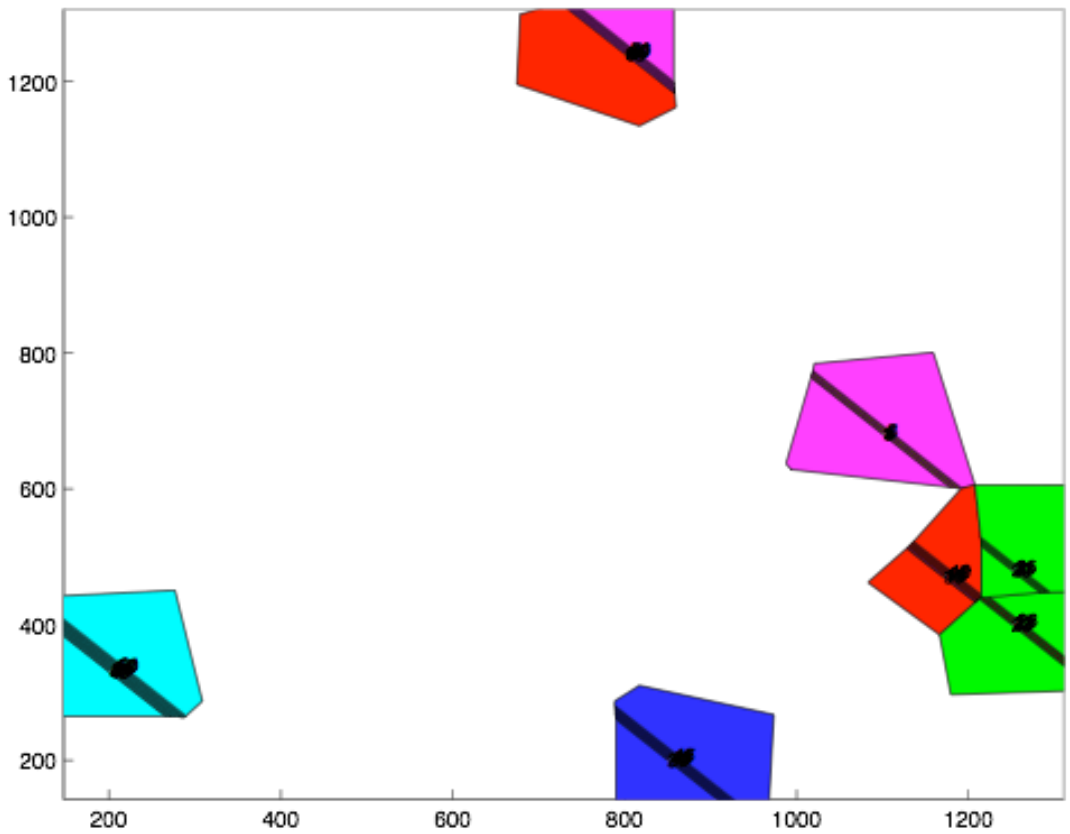
Second, the same analysis was conducted except that non-uniform priors conditioned on the geographic origin (location) of each individual (Corander et al. *accepted*) were used. The geographic coordinates of each location were generated by superimposing a grid on a map of sampling localities. This analysis allows for increased power in detecting population structure since some information about possible groupings is included. It also allows for a visual representation of the structure (Voronoi tessellation).

When uniform priors were used and K max was allowed to range from 1 to 7, K max was consistently estimated at K = 7 (logML = - 1748.1, p = 0.97876; logML = - 1741.8, p = 0.9998; logML = - 1746.5, p = 0.9977 for the 3 replicates). When non- uniform priors were used, and K max was allowed to range from 1 to 7, K max was consistently estimated at K = 6 (logML = -1841.6, p = 0.99863) with the exact same result across all three replicates. K equal to 6 had a significantly better log likelihood than a K equal to 7 (difference in logML of > 2.5). Thus, the sample of 7 geographic populations most likely represents 6 genetic groupings (Figure 10). The difference between the uniform prior analysis (K = 7) and the non-uniform (conditional) analysis (K = 6) is primarily a function of individuals that have a high admixture probability (see below), or have divergent genotypes. The differences consistently include three individuals from *D.h.a* (Nipomo), one from *D.h.j*., and one *D.h.s*.

FIGURE 10. The Voronoi tessellation represents a grid of geographic distances with the 6 genetic populations distributed over space. Taxonomic names are indicated above or below the geographic cluster. Shared color indicates membership in the same genetic group. The tessellation shows that individual *D. h. morroensis* (blue), and *D. h. jolonensis* (teal) are genetically and geographically distinct entities relative to all other populations sampled. *D. h. swarthi* (red and pink) shares alleles with *D. h. arenae* from Huasna (pink), and *D. h. arenae* from Nipomo (red). While *D. h. arenae* from

Guadalupe (Unocal and S. M. River) are very similar to one another and distinct from the other samples. It should be noted that there are 6 genetic populations inferred, but that these do not match cleanly with the geographic or taxonomic sampling.

D. h. swarthi



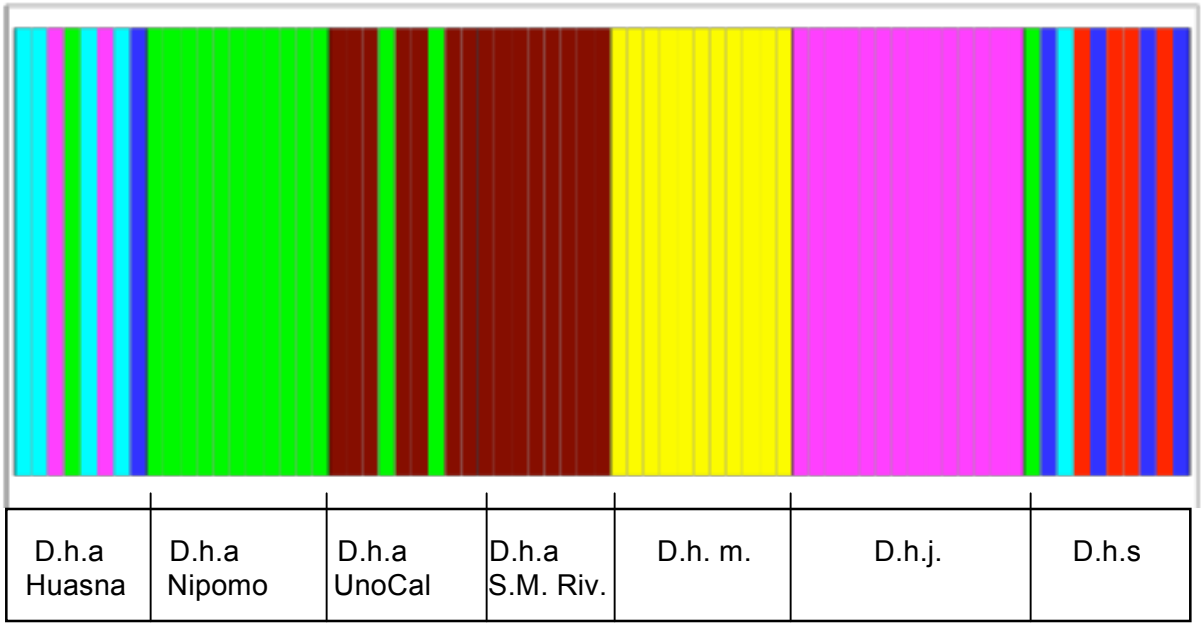
D.h. jolonensis *D. h. morroensis* *D. h. arenae*

From the Bayesian Analysis of Population Structure and the Voronoi tessellation we can conclude that the MBKR is one distinct genetic group, as is *D.h. jolonensis*. In contrast, we can conclude that the taxonomic and geographic distinctions between *D.h. swarthi* and populations of *D.h. arenae* are likely not valid. In particular it appears that there is a lack of full genetic distinction between *D.h. swarthi* and *D.h. arenae*.

5) Admixture between populations. Microsatellites sample unlinked loci. As a result, an individual's genotype may be an admixture of alleles from multiple locations – alleles within loci are not inherited as a single linkage group. This admixture documents migration and subsequent interbreeding. This analysis partitions each individual's genotype across all previously defined groups (N =6), while also showing the geographic location from which the individual was sampled. This analysis is helpful in illustrating

the connectivity between populations, and the basis for the patterns we see in the tessellation diagram (Figure 11), but it is not a formal estimate of gene flow.

FIGURE 11. Admixture diagram. Each genetic grouping (N = 6 from the BAPS analysis) is assigned a separate color. Each geographic population is identified at the bottom of the figure. Geographic populations are demarked by the vertical lines separating the taxon names. Each individual is represented as one column. Admixture is represented as colored columns that don't match the color of the rest of the taxon sample. Note that only *D.h. morroensis* (yellow) shows zero evidence of admixture: all samples of *D.h.m* are yellow, and yellow does not occur in any other geographical samples. In contrast, *D.h arenae* Huasna and *D. h. swarthi* show abundant evidence for admixture.



Genetic exchange is evident in the genetic assignment of individuals overlaid on the geographic designation of populations (Figure 11). *D. h. morroensis* is the only sample that does not show evidence of admixture with locations or taxa. At the other extreme, *D. h. swarthi* and *D. h . arenae* Huasna appear to be geographically and genetically open populations.

6) Gene flow estimate between populations. Gene flow was estimated as the migration rate between populations using BAYES-ASS+ (Wilson and Rannala 2003). This is a Bayesian approach that uses MCMC to estimate the posterior probability of the migration rates between populations. This method simultaneously solves for the probability that each individual is a non-migrant (class 0), migrant (class 1), or the product of migration and interbreeding (class 2), *and* identifies the population that is most likely to have produced the observed genotype. This approach is used to estimate migration between populations over the very short term, as in migration and gene flow (migration plus interbreeding) over the last 1-5 generations. Migration is estimated as m_{lq}, the fraction of individuals in population *q* that are migrants from population *l*. As such the value of m is influence both

by the number of migrants and by the size of the population that is accepting these immigrants (i.e.: the relative proportion of migrants to the total population).

This method does not assume Hardy-Weinberg equilibrium (Wilson and Rannala 2003). In addition, it is superior to other methods (such as Fisher's exact test) because the observed allele frequencies are used as priors in a Bayesian estimation of the allele frequencies. The estimated allele frequency is then used rather than the observed allele frequency. Thus, sampling error (and its association with sample size) is explicitly considered. Null alleles are known to exist in this data set (identified with the method of Oosterhout et al. 2004). The allele frequencies for loci with null alleles have thus been estimated (Oosterhout et al. 2004) using the Bayesian approach proposed by Wilson and Rannalla (2003).

An initial BAYES-ASS+ analysis was done using 3,000,000 steps of the MCMC sampler, with a 30,000 step burn-in to avoid autocorrelation, and sampling at every 1500 steps in the Markov Chain. Each time the chain is sampled, the observed value is selected if it has a higher probability than the last value. A new value is also accepted randomly (even if it has a lower probability) at set intervals in the chain. Delta is set by the user and defines the maximum amount a parameter can change each iteration. If this maximum is set too low, then the chain will not accept as many changes (sampling will be constrained to occur in a limited portion of the distribution). Bayesian methods perform best when between 40% and 60% of the steps in the chain result in a change (Wilson and Rannala 2003). Delta values of 0.30 were found to satisfy this requirement for the *Dipodomys* data set (47%-56% of all changes were accepted depending on the parameter being estimated). This analysis was repeated 10 times and evaluated for convergence and stationarity.

Results from the 10 replicates showed some variation. Individuals were assigned to one population in one run and another population in another run. Likewise, gene flow estimates differed to some degree between replicated. The 3,000,000 step chain was not sufficient for convergence and stationarity. Therefore, the results presented here are from a single analysis in which delta was set at 0.30, sampling frequency along the chain was at 2,000 step intervals with a 100,000,000 step chain of which 10,000,000 was burnin. This analysis was replicated three times to confirm convergence and stationarity. The single long run is a good summary of the variation seen in the 10 shorter replicates, and seems to simultaneously provide results that were seen as alternative solutions in the shorter runs. For example, subsets of *D.h. swarthi* are assigned alternatively to one population or another across each of the short chains. In the long chain *D.h. swarthi* individuals are simultaneously assigned to all of these populations.

In an assignment test, an individual can be assigned to multiple populations, the only constraint being that the cumulative probability of assignment for each individual cannot exceed 1.0. Table 6 shows results of the individual assignments for all cases that have >5% posterior probability. Most populations have at least some individuals that appear to be derived directly from migration (class 1), or from migration and interbreeding (class 2). Only individuals from two populations are exclusively assigned as non-migrants

(class 0) to the actual population from which they were sampled. Those belong to *D.h.morroensis*, and *D.h. arenae* Nipomo. Thus, there is < 5% chance that individuals from *D.h.morroensis*, and *D.h. arenae* Nipomo are migrants or the result of migration and interbreeding, and more than a 95% chance that these individuals are non migrant recruits from within the same population.

Table 6. Results from the BAYES-ASS+ assignment test. The number of times (preceding parenthesis) than an individual from the sampled population was assigned to a receiving population at a particular time class (within parenthesis). Time class 0= non-migrant, 1=migrant, 2=product of migration and interbreeding. For example 3(2) indicated three individuals from the population on the row were assigned to the second age category in the population of the column. Each individual can be assigned to multiple populations or to multiple age categories, so long as the cumulative assignment probability is = 1. With no gene flow, all values would be on the diagonal, and every population would only contain time class 0 assignments. Only assignments with a > 5% probability are shown. * Indicates that all individuals in the population only assigned as non-migrants to that same population (i.e.: no evidence for gene flow out of the population). Population names in italics are for subspecies of *D. heermanni*. Names that are not in italics represent population of *D. h. arenae*.

	Population to which an individual was assigned with more than 5% probability							
Population Sampled	Huasna	Nipomo	Unocal	SM River	Morroensis	Jolonensis	Swarthi	Sample size
Huasna	8(0)	1(1)1(2)		1(2)		7(1)1(2)	1(2)	8
Nipomo		11(0)*						11
Unocal		3(1) 1(2)	9(0)	9(1) 3(2)				9
SM River			8(1) 4(2)	8(0)				8
morroensis					11(0)*			11
jolonensis	14(1) 8(2)					14(0)		14
swarthi	7(1) 10(2)	6(1) 10(2)	1(2)	1(2)	3(2)	4(1) 8(2)	10(0)	10

The bayesian estimates of migration rates (m_{ql}) between populations of *D.heermanni* are shown in table 7. This table only shows significant values of m_{ql}. Significance means that the 95% confidence intervals for migration does not include zero, and the value is greater than what is expected by chance alone (Wilson and Rannala 2003). All values of non-migration (retention of individuals within a population) are also shown, whether significant or not. Estimates of m between pairs of populations are not necessarily symmetrical for two reasons. First, the amount of gene flow need not be equal in both directions. Second, each of the receiving populations may have a different effective population size, such that even if they were exchanging the same number of migrants, the *proportion* (*m*) of the population that is immigrant could be different.

TABLE 7. Mean and 95% confidence interval of the migration rate between populations of *D. heermanni* estimated from the posterior distribution of the MCMC analysis of

Bayes-Ass+. Values represent m_{ql} which is the proportion of individuals in population q that are migrants from population l. Diagonal values represent the fraction of non-migrant ("migrants" when q and l are the same population), and values above the diagonal represent the fraction of migrants in the receiving population. Note that the values should sum to 1 for the columns (receipt cannot exceed 100%), but will not sum to 1 since not all values are significant. Non-significant values are not shown.

Source population (l)	Mean and 95% confidence interval of migration rate (m_{ql}) between populations of D. heermanni						
	Receiving population (q)						
	Huasna	Nipomo	Unocal	S.M. River	Morroensis	Jolonensis	Swarthi
Huasna	0.863 (0.740-0.984)						0.114* (0.010-0.218
Nipomo		0.756* (0.650-0.863)					0.095* (0.024-0.164)
Unocal			0.697* (0.667-0.727)	0.131* (0.016-0.244)			
S.M. River			0.223* (0.275-0.171)	0.808 (0.675-0.941)			
Morroensis					0.974* (0.969-0.997)		
Jolonensis						0.976* (0.953-0.998)	
Swarthi	C.I. includes zero.	0.168* (0.054-0.280)					0.747* (0.667-0.827)

* Denotes a value that does not include zero, and is outside of the 95% confidence limit of the value that might be expected by chance alone in a sample of 7 populations (Nm = 0.833 (0.675 - 0.992) within populations and 0.0.0277 (-0.0000001 - 0.144) between populations.

The results of the Bayesian estimation of migration rates (Table 7) indicate that 1) *D. h. morroensis* and *D. h. jolonensis* are the only subspecies that do not show a significant genetic connection to other subspecies. The proportion of the population that is retained (non-migrant) is approximately 97%. 2) There is significant gene flow between *D. h. arenae* Santa Maria River and *D. h. arenae* Unocal. The proportion of the population attributed to retention (between 70%-80%) and gene flow (between 13% and 22%) sum to approximately 93%. This result is not surprising given the close proximity and habitat continuity between these populations. 3) There is significant gene flow between *D. h. swarthi* and *D. h. arenae* Huasna, and between *D. h. swarthi* and *D. h. arenae* Nipomo. Approximately 94% of the *swarthi* individuals can be attributed to retention (75%) and gene flow (20%). Thus, *D. h swarthi* appears to be genetically connected to inland populations of *D. h. arenae*. This connection may be facilitated by sandy washes along the Cuyama, Santa Maria and Huasna Rivers which are all part of the same drainage. There is some reciprocity in gene flow between *D.h. swarthi* and *D. h arenae*, though the full extent cannot be estimates since not all of the values are significant (Huasna and *D. h. swarthi* Table 7).

MITOCHONDRIAL DNA POPULATION GENETICS

In recent years the interpretation of mtDNA results has changed dramatically with regard to monophyly and the recognition of evolutionary significant units (ESUs). Simply put, replication of mtDNA results is now required in order to validate those results (Carstens and Knowles 2005). The perspective is that a single locus represents a single outcome of the stochastic evolutionary process (Carstens et al. 2007). Sampling a single locus, given the potential variation in outcome, provides a single estimate. The accuracy of an estimate is established when the same outcome is obtained through independent replication of the results. Since the mtDNA genome is a single linkage group, independent replication of mtDNA results can only be done using nuclear DNA analyses.

The theoretical need for replication is based on the fact that monophyly should occur faster in mtDNA than in nuclear DNA (Palumbi et al. 2001, Rosenberg 2003, Hudson and Turelli 2003). Because mtDNA reaches monophyly first, monophyly in mtDNA is no guarantee of monophyly in nuclear loci (Baker and Palumbi 1994, Tosi et al. 2003, Crochet et al. 2003). But, the opposite is true: nuclear monophyly takes longer and therefore should only occurs after mtDNA monophyly. So, showing monophyly in mtDNA does not provide for any conclusions regarding nuclear DNA. But, showing monophyly in nuclear DNA allows the conclusion that mtDNA is also monophyletic. Because of this recent shift in perspective, it was judged that the mtDNA analysis originally proposed would not be as robust as the microsatellite results (above) supplemented with the mtDNA results of Matocq and Villablanca (2001).

In regards to monophyly, Matocq and Villablanca (2001) provide a very useful result. These authors sequenced 443 nucleotides of mtDNA (including portions of the control region and cytochrome b) from 24 *D. heermanni*. A sample of 16 *D.h. morroensis* produced 2 haplotypes defined by 1 segregating site. A sample of 8 *D.h. arenae* yielded 6 haplotypes defined by 19 segregating sites. Importantly, none of the haplotypes were shared between subspecies. Given these two sample sizes, the lack of shared haplotypes, and the number of differences between the sequences, the probability of monophyly is >95% (Takahata 1990, Takahata and Slatkin 1990). Therefore, the MBKR qualify as an ESU based on mtDNA monophyly.

Nuclear monophyly can be defined as fixed differences between all alleles cross all loci. In other words, no alleles can be shared between populations that are reciprocally monophyletic. We know from the current microsatellite work (above) that this is not the case in *morroensis*: most alleles are shared and only some are private. Therefore, even if mtDNA is monophyletic in *D. heermanni*, we know that nuclear DNA is not. Thus, the MBKR is defined as an ESU based on significant nuclear allele frequency differences, and mtDNA monophyly.

Matocq and Villablanca (2001) found that the MBKR had less mtDNA diversity than *D. h. arenae.* A sample of 8 *D.h. morroensis* museum skins (1910s) showed the same low level of genetic diversity as 8 modern (1980s) Specimens. Matocq and Villablanca (2001) argue the MBKR show a historically low level of genetic diversity in mtDNA. This low level of diversity dates from the 1910s and thus predates the current population decline of the MBKR (post 1960s) and suggests that the bottleneck was historical. If the hypothesis regarding a historical bottleneck is valid, then one would predict that nuclear DNA would show low diversity within MBKR relative to other populations of *D. heermanni*. The microsatellite data demonstrate that the MBKR has the lowest expected heterozygosity, nucleotide diversity, and number of alleles

per locus of all the populations of *D. heermanni* sampled herein. This result is consistent with the hypothesis of a historical rather than a recent population genetic bottleneck.

MORPHOMETRICS

A morphometric analysis of *D. heermanni* subspecies from S.L.O. County was previously conducted by R. Risser (1976). Thus, the analysis will not be replicated here. Risser found that *D.h. morrensis* is marginally, but significantly different from *D. h. arenae* in regards to cranial characters. He used the same cranial characters that were used by Grinnell (1922) and Boulware (1943). Cranial characters are much better for distinguishing between the subspecies than are external morphological characters. The principle external characters (identified by Grinnell 1922) include darker overall color, especially in the auricular band at the base of the external nares, the greatly reduced lateral tail stripe (reduction in white), and the generally absent hip stripe. Yet, these external morphological characteristics are also found in some of the *D.h. arenae* currently in the A.R.M. Collection at Cal Poly. It is not clear whether these shared morphological characters are of taxonomic importance or simply reflect ecological convergence in coastal habitats.

DISCUSSION

Validity of the Dipodomys heermanni morroensis taxon

All three forms of evidence considered here, morphology, mitochondrial DNA and nuclear microsatellite DNA, indicate that the Morro Bay kangaroo rat is a distinct subspecies. Morphological differences are small but significant (Risser 1976). Unfortunately, in spite of the fact that there are morphological differences, these differences are not diagnostic. Morphological overlaps between *D. h. morroensis* and *D. h. arenae* do occur. It is not currently clear whether the overlap reflects common ancestry or independent adaptation to similar coastal environments. Mitochondrial DNA is shown to have a high probability of reciprocal monophyly in *D. h. morroensis* and *D. h. arenae*. Monophyly would be sufficient to establish *D. h. morroensis* as an Evolutionarily Significant Unit (ESU). Microsatellite DNA analyses also establish *D. h. morroensis* as an (ESU). Specifically, a) The R_{ST} analysis indicates that the MBKR is significantly different in allele frequencies (weighted by SMM) from *D. h. jolonensis*, *D. h. swarthi*, and some populations of *D. h. arenae.* b) The Bayesian assignment test, conditioned on collecting localities, assigns all samples from Los Osos to *D. h. morroensis*. c) The Bayesian admixture analysis of multilocus data reveals that *D. h. morroensis* is the only genetic population in the sample that does not contain genotypes from other populations. d) The Bayesian migration estimate excludes the possibility that there is gene flow between *D. h. morroensis* and any of the other populations in the sample. The lack of significant difference between *D. h. morroensis* and some populations of *D. h. arenae* in the R_{ST} analysis is attributed to sample size (i.e.: insufficient power to discriminate). In contrast, the Bayesian analyses reject genetic cohesion between *D. h. morroensis* and other samples of *D. heermanni* with statistical significance.

The MBKR is genetically differentiated from all other subspecies of *D. heermanni* in the sample. Thus it is difficult to decide which subspecies or population is most closely related to the

MBKR. The R_{ST} analysis suggests that the MBKR is most similar to *D. h. arenae* from Nipomo and then to *D. h. arenae* from the Santa Maria River. The Bayesian assignment supports some shared ancestry between the MBKR and *D. h. swarthi*. The traditional (R_{ST}) and contemporary (Bayesian) perspectives are even more difficult to reconcile given the evidence for reciprocity in gene flow between *D. h. arenae* and *D. h. swarthi* (table 7). Deciding which of these populations is genetically most similar to the MBKR seems a bit arbitrary at this point. Especially given that there is only weak evidence for *any* genetic connection between the MBKR and other populations. From an ecological perspective, it could be argued that given the greater ecological similarity between Los Osos and Nipomo, versus the Carrizo Plains or Cuyama Valley, members of the *D.h. arenae* Nipomo population are most likely to be adapted to habitats similar to those occupied by the MBKR.

Demographic history of the MBKR

Matocq and Villablanca (2001) found that the MBKR had less mtDNA diversity than *D. h. arenae.* The low level of diversity in the MBKR was observed in samples from the 1910s and thus predates the current population decline of the MBKR (post 1960s). This pattern suggests that the MBKR experienced a historical bottleneck. Across the microsatellite loci surveyed here, the MBKR was found to be significantly lower in the mean number of alleles per locus, and in expected heterozygosity, but not in nucleotide diversity when compared to the other 6 populations. This result indicates that the MBKR has experienced a historical bottleneck, but that genetic diversity is still declining and does not yet reflect the post bottleneck equilibrium population size. Thus, in spite of the fact that the bottleneck is historical, it was not in the sufficiently distant past to keep the MBKR from currently experiencing increasing levels of inbreeding. This evidence for increased inbreeding provides the only empirical support to date for the hypothesis (USFWS 1999) that the MBKR's decline could in part be due to genetic factors.

Population genetic structure of D. heermanni subspecies.

The Bayesian assignment test (conditioned on collecting locality) and the estimate of migration rates indicate that *D. h. morroensis* and *D. h. jolonensis* are not significant connected to other populations via gene flow. In contrast, there is evidence for significant gene flow between *D. h. swarthi* and *D. h. arenae* Huasna, and between *D. h. swarthi* and *D. h. arenae* Nipomo. This result is a bit surprising and was not expected *a priori*. This connection could be facilitated by sandy washes along the Cuyama, Santa Maria and Huasna Rivers since they are all part of the same drainage. If so, these corridors for genetic cohesion in *D. heermanni* have been previously unrecognized. Indirect evidence to support river that river washes are a possible conduit for gene flow includes the fact that samples of *D. heermanni* can be found in dry riparian corridors. *D.h. jolonensis*, *D. h. arenae* Santa Maria, and *D. h arenae* Huasna were all collected in dry riparian habitats. *D. h. goldmanni* has previously been collected by the author in a dry riparian tributary of the Salina River. The hypothesis that dry river washes serve as corridors for genetic cohesion between populations of *D. heermanni* deserves direct testing. It has always been informally presumed that the MBKR dispersed to Los Osos from the Nipomo Dunes Complex via a coastal dune route. It seems plausible that they colonized Los Osos via an overland riparian route instead.

Current extant populations and potentially occupied habitat patches

A review of the literature suggests that a portion of the MBKRs original range lies outside of its currently recognized range. Patches of sandy soil East of Los Osos creek are know to have been inhabited by kangaroo rats. Agricultural lands North and East of Los Osos creek have a history of producing kangaroo rats now found in museum collections (figure 5). There are two sandy hills Southeast of the "extension" that contained kangaroo rats in the late 1950s (figure 6, Stewart and Roest 1960). Importantly, these areas are likely to experience anthropogenic disturbance at a level consistent with high density kangaroo rat populations. Efforts should be directed to ascertain whether MBKR do or could occur on sites with sandy soil outside of the recognized historic range, especially those areas shown in figure 12.

Figure 12. Areas of MBKR habitat within, adjacent to, and outside of the currently recognized range, which are suggested for additional field survey effort. These areas (outlined in white) have one or more of the following characteristics: a history of MBKR occupation, sandy soil, or agricultural use that might be consistent with MBKR occupation.

There is still uncertainly about the original distribution of the MBKR. Some sites, such as Morro Bay State Park, have not been re-checked since the presence of MBKRs was originally documented. Sandy soils do occur adjacent to the city of Morro Bay, and Morro Bay State Park. These areas, and those identified in figure 12, need to be evaluated before any definitive statement can be made regarding whether the MBKR is extinct or extant. Trapping and survey efforts over the last seven years have failed to produce any wild kangaroo rats of definitive sign of their existence.

SPECIFIC MANAGEMENT RECOMMENDATIONS REGARDING A TRANSLOCATION OF KANGAROO RATS

Genetic data argue against a translocation if the MBKR is extant

Morro Bay kangaroo rats are genetically distinct from all other neighboring populations of kangaroo rats. As such, there is no straight-forward genetic equality or exchangeability that can be invoked to justify the translocation of kangaroo rats from outside of the historic range into the range of the MBKR. If the MBKR is extant, and a translocation of kangaroo rats into it's range results in interbreeding it is impossible to predict whether the outcome will be a "genetic rescue" or a breakdown of adapted gene complexes. At this time it seems advisable to place efforts into determining if the MBKR is or is not extant, rather than into evaluating the relative probability of a rescue versus a breakdown.

Ecological merits of translocation

If the MBKR is extinct, then the genetics become irrelevant: there would be no MBKR population to rescue or reduce in fitness. Under this scenario, the justification for a translocation is purely ecological.

Kangaroo rats at large have been shown to effect vegetation structure, plant cover, species composition, and species richness across multiple spatial and temporal scales (Davidson and Lightfoot 2006) with a trickle down to the vertebrate community structure. Goldingay et al. (1997) clearly articulate "We argue that they (kangaroo rats) deserve special emphasis because they have been shown to play key roles in ecosystem structure and composition… It is clear that preservation of kangaroo rats and their plant communities will not be achieved through simply setting aside blocks of habitat as reserves. Conservation areas will require active management in order to maintain existing biodiversity, but further studies are required to ascertain how best to manage these communities." Indeed, basic ecosystem research is essential even to establish the keystone role of a species. For example it is known that other species of rodents may be able to compensate and provide the keystone role in absence of kangaroo rats (Ernest and Brown 2001). Thus, the conservation of a kangaroo rat in Morro Bay is not only a taxonomic issue, but an ecological issue as well. It is possible that a translocation of kangaroo rats would re-introduce important ecological mechanisms back into the Los Osos coastal dune scrub ecosystem. If there is no species effecting a compensation for the loss of the MBKR's ecological role, then, without a translocation it would be impossible to mitigate the community wide ripple effects from the extinction of this seed predator.

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