a thorough sampling along Route 67 and limited the ability of the authors to assess whether the road itself acts as a barrier to gene flow.

The goal of this study was to primarily assess east-west connectivity across Route 67 and secondarily, north-south connectivity across Scripps Poway Parkway and Poway Road, two highly trafficked roads to the west of Route 67 (Fig. 1). We collected Mule Deer scat piles from both sides of these road segments timed to roughly coincide with the spring rutting and fall mating seasons, when Mule Deer tend to move greater distances (Anderson and Wallmo 1984). Using previously developed microsatellite loci, we investigated movement distances among individuals genetically identified and resampled throughout the study period. We also assessed the distances between siblings and parent offspring pairs identified through pedigree reconstruction to capture past movement or dispersal patterns, and examined the impacts of roads on population genetic structure throughout the study area.

Methods

Sampling and laboratory methods.—We collected scat piles in the spring (March-June) of 2015 and in fall (October) 2015, within a 100 km^2 region along Route 67 between Lakeside and Poway (Fig. 1), San Diego County, California, at sites where Mule Deer presence was previously confirmed by land managers, field researchers, members of the local community-based tracking team (San Diego Tracking Team; www.sdtt.org), or past successful collection efforts (Andrew Bohonak and Anna Mitelberg, unpubl. report). Upon arriving at the site, we searched for fresh deer sign (tracks and browse), which we tracked until we encountered fresh scat piles. Fresh scat appear shiny and smooth, versus older scat piles that appear dry and cracked (Mitelberg 2010). Previous studies suggest that fresher scat piles have higher amplification and genotyping success rates (Piggott 2005; Panasci et al. 2011). We air dried pellets of Mule Deer for two to four days at room temperature, and we collected the

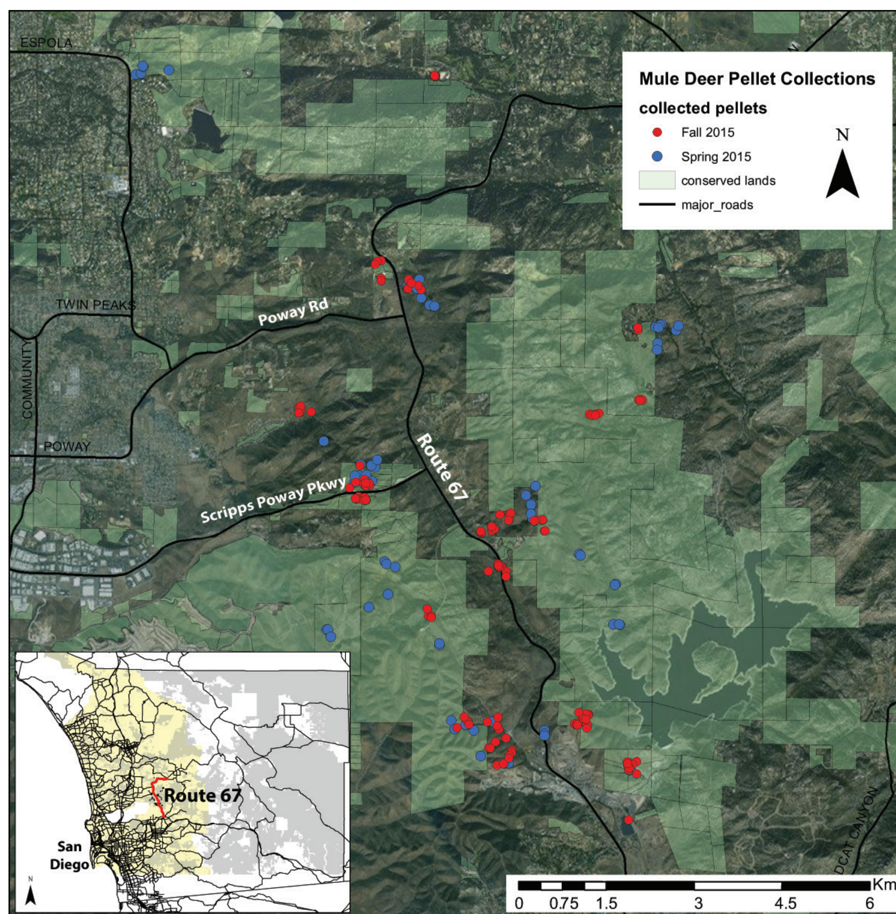

Figure 1. Location of study area showing major roadways and 238 scat piles collected of Mule Deer (*Odocoileus hemionus fuliginatus*). We assessed connectivity across California State Route 67, Scripps Poway Parkway and Poway Road, southern California. The insert shows the section of Route 67 examined in this study within San Diego County. The MSPA (Multi-Species Plan Area) is highlighted in yellow, and conserved lands across San Diego County are shown in gray. Road density and urban development increase to the west of the county, while open space and conserved lands increase to the east. (World Imagery Basemap source: ESRI, Digital Globe, GeoEye, Earthstar Geographics, CNES/Airbus DS, USDA, USGS, AEX, Getmapping, Aerogrid, IGN, IGP, swisstopo and the GIS User Community).

epithelial cells within two weeks of collecting pellets in the field (we stored dried pellets at 8° C for up to two weeks before performing this step). To collect epithelial cells from the surface of scat pellets, we dropped 3–5 scat pellets at a time into a small plastic bag and covered the pellets with approximately 2 mL of phosphate buffered saline solution (1X PBS). We proceeded to gently rub the surface of the pellets through the plastic bag to dislodge cells off the pellet, being careful not to break up the pellet. As some PBS was absorbed during this process, we added additional PBS as needed to keep the liquid volume in the bag at about 1 mL. We removed and disposed of the used pellets using tweezers and added more unprocessed pellets from the same scat pile to the bag, repeating the process until we washed about 12–20 pellets from a single scat pile in the same bag. We centrifuged this volume of PBS (about 1 mL), containing intestinal cells from 12–20 deer scat pellets for 2 min at 13,000 RPM to further concentrate the intestinal cells at the bottom of the tube. We transferred all but the bottom 250 µL of this solution to a 1.5 mL tube and stored at -20° C for future extraction attempts if necessary. We stored the remaining 250 µL at -20° C and extracted it within the following month using the DNA IQ kit (#DC6700; Promega, Madison, Wisconsin) according to the standard protocol (with the modifications that cell lysis was performed at 95° C and the lysed cell solution was filtered through a spin basket filled about half way with cheesecloth to filter out any sediment). To help detect contamination problems should they arise, each batch of washes included a negative control with only PBS and each batch of extractions included a negative control with water.

We genotyped at 15 previously developed microsatellite loci (Valero 2004; Pease et al. 2009; Mitelberg 2010) and a sex specific marker (Gilson et al. 1998) in a single multiplex PCR using the Qiagen Multiplex PCR Plus kit (#206152; Qiagen, Hilden, Germany). Each 5 µL reaction contained 1.5 µL DNA, 2.5 µL Master Mix, 0.375 µL of primer mix containing all 16 primer sets in optimized proportions (see Appendix 1; #450056; ThermoFisher, Carlsbad, California), and 0.625 µL of water. Cycling conditions were as follows: 95° C for 5 min; followed by 37 cycles of 95° C for 30 s, 56° C for 3 min, 72° C for 30 s; and a final 68° C extension for 30 min. Each set of PCR reactions included two positive controls (one male extraction and one female extraction) and two negative controls. Eton Biosciences (San Diego, California) performed fragment analysis on an ABI 3730xl following submission of a 1.5 µL aliquot of the PCR product, combined with 10 µl formamide (#4311320,ThermoFisher, Carlsbad, California) and 0.5 µL of GeneScan 500 LIZ size standard (#4322682; ThermoFisher, Carlsbad, California). We compiled the raw data (chromatographs) into genotypes using GeneMapper v. 4.0 (Applied Biosystems, Foster City, California).

To identify and eliminate genotyping errors, we initially genotyped all scat piles twice at all loci; we analyzed these initial genotypes with RELIOTYPE (Miller et al. 2002), a software program that implements a maximum likelihood algorithm to assesses the reliability of the multilocus genotype based on allele frequencies and recommends a replication strategy for those samples failing to pass the 99.49% reliability criteria. At this point, we discarded all samples requiring more than six PCR replicates. We genotyped all remaining samples again (according to RELIOTYPE recommendations) and ran the data through RELIOTYPE a second time, following which we discarded all samples failing to yield a reliable genotype. We used GIMLET 1.3 (Valière 2002) to reconstruct consensus genotypes for all scat piles with reliable DNA fingerprints.

Identifying capture and recapture events.—We grouped the consensus genotypes for all scat piles with reliable DNA fingerprints by genotype, with missing alleles considered as any other alleles. Within this set of scat piles, we identified unique individual genotypes using GIMLET's group by genotype algorithm. We further sorted the remaining scat piles into two categories: either they were resampling events resulting from repeated sampling of the same scat pile of an individual at the same site and date, or they were true recaptures of the individual at a different site and/or date. We considered all scat piles collected within 1–2 d of each other and that matched the same unique genotype as resampling events; in these cases, we reduced all resampling events of an individual mule deer to one capture event with the first scat pile processed in the laboratory serving as a representative of that capture event. To verify whether resampling distances differed from recapture distance, we also examined the time span (in days) between all resampling and recapture events. These fell within three clustered time periods: either on the same day or the next day (within 1–2 d), within 16–26 d (within 1 mo), or within 130–200 d (across seasons). We examined box-plots of distances between samples for each of these three groups.

We implemented CERVUS v.3.0.7 (Kalinowski et al. 2007) on the subset of unique genotypes to calculate the average probability that two unrelated individuals (PID) and the more conservative average probability that two siblings (PSIB) in the present data set could have identical genotypes. We eliminated Locus B (which was found to have a high likelihood of null alleles) from all remaining analyses sensitive to methodological artifacts such as null alleles. We conducted all further analyses using the remaining 14 microsatellite markers. We used CERVUS to calculate all microsatellite summary statistics (Table 3).

Pedigree reconstruction.—We used the maximum likelihood pedigree reconstruction software COLONY v.2.0.5.9 (Jones and Wang 2010) to identify potential full siblings and parents. To assess stationarity in the pedigree solution, we performed two independent

COLONY runs with the following parameters: female and male polygamy, with inbreeding, long run, full-likelihood analysis method, medium likelihood precision, no sibship scaling or sibship prior; all other parameters were set to default. We used allelic frequencies and error rates calculated over the larger San Diego Southern Mule Deer population (Andrew Bohonak and Anna Mitelberg, unpubl. report). To avoid exclusion of parent-offspring pairs based on a single allele, we assigned to all loci the minimal recommended false alleles rate of 0.0001. We set the expected probability of detecting a father or mother to 0.05 and 0.15, respectively. We measured the Euclidean distance between resampling events, recaptures, first order relatives, and all unique captures in ArcMap 10.2.2 (Esri, Redlands, California). We assessed whether distances between recapture events and first order relatives differed from that among all pairs of non-related unique captures using t-tests of differences between means, calculated in DataDesk 6 (Ithaca, New York, New York; Velman 1997).

Population structure.—The use of population genetic analyses aimed at detecting population structure provides an indirect method for inferring gene flow, i.e., movement and successful reproduction, or obstacles to it. Given the complexity of detecting population structure at the limited scale of this study for a large, mobile mammal such as the mule deer, we applied multiple analyses and used a consensus approach to determine population structure (Pearse and Crandall 2004). We employed two types of analyses, exact tests for population differentiation and individual clustering analyses, to indirectly assess whether Route 67 and/or either of the two highly trafficked roads in our sampling area function as barriers to mule deer gene flow. Because these kinds of analyses can result in biased conclusions in data sets containing related individuals, we randomly removed one of each pair of full siblings identified during pedigree reconstruction from the input files.

First, we used exact contingency tests to assess whether allele frequencies were significantly different among geographic groupings (Raymond and Rousset 1995). We performed Fisher's exact tests in GENEPOP (Rousset 2008) using 10,000 dememorisations, 100 batches, and 5,000 iterations per batch. In this type of analysis, groups are determined *a priori*, and the alternative hypothesis that these groups are genetically divergent is tested. We tested two scenarios: an East-West scenario, in which gene flow is limited by Route 67, resulting in two groups, East of Route 67 and West of Route 67; and a Roads scenario, in which gene flow is limited by Route 67, Poway Road, and Scripps Poway Parkway, resulting in four groups (North of Poway Road, South of Poway Road, South of Scripps Poway Parkway). Second, we performed individual-based clustering analyses in STRUCTURE v.2.2.4 (Pritchard et al. 2000; Falush et al. 2003), using an admixture model with correlated frequencies. We estimated the probability of K = 1–7 clusters using 1,000,000 Markov chain Monte Carlo (MCMC) iterations following a 500,000 iteration burn-in, with 10 replicate runs per K to verify consistency across chains. Individual-based clustering analyses search for the optimum number of gene pools based solely on individual genotype, without or with minimal value assigned to *a priori* population structure hypotheses. We combined replicate runs using CLUMPAK (Kopelman et al. 2015), which we also used to assist us in finding the preferred K using both, the method of Evanno et al. (2005) and Pritchard et al. (2000).

Results

Sampling and genotyping.—We collected 238 scat piles, 87 in the spring and 151 in the fall (Fig. 1). All 238 scat piles were extracted and genotyped. Although sample sizes were roughly equal on the east and west sides of Route 67, we were able to find and collect more pellets in the southern portion of the study area. About 53% (126) of the collected scat piles yielded reliable genotypes. Individual genotypic data and collection coordinates can be downloaded from the USGS (http://dx.doi.org/10.5066/F7KW5D32).

Capture and recapture events.—For the 15 loci in this study, the probability of PID of 2.5 × 10-10 and PSIB of 6.9 × 10-5 were both very low and well within reasonable limits of 0.01 to 0.0001 recommended for genotypes in natural populations (Waits et al. 2001). Excluding locus B, the number of alleles per locus ranged from 2 to 10, with an average of 4.21 alleles per locus (Table 1). Observed heterozygosity per locus ranged from 0.319 to 0.851, with an average of 0.563 (Table 1).

We identified 71 unique individuals (45 females and 26 males). We sampled 28 individuals more than once (some multiple times) for a total of 55 resampling or recapture events. Of these, we considered nine to be true recapture events (sampled at least 15 d apart). We considered the rest to be resampling events (sampled within one day of each other). With the exception of a few outliers, distances between samples acquired within one day tended to be closer together than those in the within month and among seasons groupings, and 95% confidence intervals around median distances did not overlap (Fig. 2). Of the 71 individuals, we found 38 on the east side of Route 67. Of the remaining 33 individuals that we found on the west side, 19 were south of Scripps Poway Parkway, 11 between Scripps-Poway Parkway and Poway Road, and three north of Poway Road (Fig. 3).

Six individuals (three males and three females) that we detected in the spring were recaptured during the fall collection season. The remaining three recapture events occurred within the same season. The average distance between recapture events was 816 m (ranging from 190 m to 1,564 m, Table 2). The average distance between recapture events for females and males was