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RESEARCH ARTICLE

Effects of urbanization on population genetic structure of western gray squirrels

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Abstract

Habitat loss and fragmentation due to urbanization are key contributors to the decline of biodiversity. The consequence of these factors is small, isolated populations that are more susceptible to deterministic and stochastic threats of extinction. There is an increasing trend in population reductions of the western gray squirrel (*Sciurus griseus*) in urban areas of Southern California, USA. Griffith Park (GP) contains one of the last urban populations of western gray squirrels (WGS) present in Los Angeles. We used hairtubes to collect hair of WGS at 3 sites within GP and at 5 sites outside of GP. Twelve microsatellite loci and a 550 bp segment of the mitochondrial control region were used to examine the genetic diversity within GP and among all sample sites, and to determine gene flow within GP. Results revealed subpopulations within GP have low levels of allelic richness at microsatellite loci ($A_R = 2.28–2.53$) and low mitochondrial haplotype diversity ($H_D = 0.000–0.271$). We found significant genetic differentiation ($F_{ST} = 0.109–0.156$, $p < 0.001$), high levels of relatedness within each GP subpopulation (0.399–0.633), and a lack of private alleles ($A_P = 0.09–0.27$) at microsatellite loci. Mode shifts in microsatellite allele frequencies and positive M-ratio tests provide evidence of bottlenecks within a GP subpopulation. The effective population size for GP ($Ne = 9.1$) highlights the effects of genetic drift on this isolated population. We suggest conservation efforts that could maintain these last extant populations of a native species in urban Los Angeles.

Keywords Bottleneck · Genetic drift · Microsatellite · Mitochondrial DNA · Relatedness · *Sciurus griseus*

Introduction

Loss of biodiversity continues to occur at an unprecedented and accelerated rate (Ceballos et al. 2015). Habitat fragmentation and destruction due to urbanization are the most prominent threats to biodiversity, and have resulted in the extinction of entire species or local populations (Forester and Machlis 1996; Vandergast et al. 2006; Allendorf et al. 2013; Galli et al. 2014). As habitats are fragmented into smaller discrete patches, populations residing therein are fractured into smaller populations that are susceptible to stochastic threats, such as changes in genetic, demographic, and environmental factors (Allendorf et al. 2013). Sufficient levels of genetic diversity are crucial to a population's long-term potential for survival (Bouzat 2010). Frankham (1995) emphasized that populations become more vulnerable to environmental stochasticity as the loss of genetic diversity increases. The loss of genetic diversity is caused by inbreeding, genetic drift, restricted gene flow, and small population size (Furlan et al. 2012). A negative feedback loop develops where reduced genetic variation leads to susceptibility to stochastic threats, causing a downward genetic spiral that lowers a population's fitness—a pattern referred to as the "extinction vortex" (Blomqvist et al. 2010).

California ranks as one of the four most ecologically degraded states in the country and contains eight of the country's most threatened ecosystems (Burge et al. 2016). Out of all the fifty states, California has the largest number of rare plants and animals (California Department of Fish and Game 2005). Southern California contains almost one-third of the state's native species, 38% of the state's invertebrate species, and is known as a biodiversity hotspot (Bunn

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et al. 2005; California Biodiversity Initiative 2018; California Department of Fish and Game 2005). Unfortunately, habitat loss and fragmentation caused by urbanization are among the foremost threats to biodiversity in southern California (Vandergast et al. 2006). The human population in Los Angeles County is over 10 million, with almost 4 million in the City of Los Angeles (United States Census Bureau 2016). As the human population increases in the southern California region, we can expect further habitat destruction (Ernest et al. 2014).

The extent to which habitat fragmentation and loss due to urbanization have impacted gene flow, genetic diversity, and the presence of wildlife in the southern California wildlife is documented across taxa. Large and wide-ranging species, such as carnivores, are notably affected by freeways, with isolation causing decreased genetic variation documented in southern California populations of bobcats (*Lynx rufus*), coyotes (*Canis latrans*), and mountain lions (*Puma concolor*) (Riley et al. 2006, 2014; Lee et al. 2012; Ernest et al. 2014; Benson et al. 2016). Studies on smaller, less mobile species also illustrate how urbanization in southern California impacts populations and reduces their genetic diversity. The endangered Stephen's kangaroo rat (*Dipodomys stephensi*) (McClenaghan and Truesdale 2002) was found to be negatively impacted by human development. Delaney et al. (2010) found three lizard species and one bird species which showed significant genetic differentiation also exhibited a decrease in gene flow among population patches due to urban fragmentation caused by highway development.

Within the urban sprawl of Los Angeles is Griffith Park (GP), the nation's largest municipal park, at 1703 ha (4210 acres) with habitats ranging from oak-walnut woodland to coastal sage scrub. Griffith Park is the most eastern part of the Santa Monica Mountains (SMMs), a range that extends from its western end at Point Mugu near Ventura to its eastern end at GP in Los Angeles. The expansion of Los Angeles can be traced back to gaining water rights of the Los Angeles River that emerges above ground on the northeastern end of the park (Eberts 1996). Since the late 1700′s there has been a history of human settlement and development of the land, starting with agriculture and culminating with high-density residential and commercial urbanization (Eberts 1996). To control flooding in the surrounding areas, the section of Los Angeles River bordering GP was lowered 8 feet, cemented, and channelized (Eberts 1996), and three major freeways now flank the park.

Griffith Park is home to a population of western gray squirrels (*Sciurus griseus*), a species native to the west coast of North America with a distribution extending from central Washington to Baja California (Escobar-Flores et al. 2011; Ingles 1947). In southern California, the WGS is currently distributed across the San Gabriel Mountains (SGM), parts of the San Bernardino Mountains, the Santa Ana Mountains, the San Jacinto Mountains, and in some areas of the SMMs, but becomes rare at low elevations (Cooper 2013). Before the advent of intense human development across the Los Angeles Basin in the twentieth century when oak woodland covered much of the northern portion of the basin, the WGS would have been much more widespread at lower elevations (Cooper and Mathewson 2009; Cooper 2013), as confirmed by the historical specimen record. As urbanization spread, WGS populations likely retreated to urban parks, wooded residential areas, and less-developed foothills (Cooper and Muchlinski 2015). Today, WGS are rare or completely absent from most areas below 1000′ elevation, and in much of the region, have been replaced by the non-native fox squirrel (FS) (Guthrie 2009; Muchlinski et al. 2009; King et al. 2010).

Conservation of the WGS is important because it is a keystone species that maintains native oak woodlands, is considered to be a niche specialist, and is a management indicator species for oak-conifer communities (Pacific Biodiversity Institute 2012; Cooper 2013; Cooper and Muchlinski 2015). The population of WGS in GP is isolated from the rest of a larger contiguous population in the SMM range and from other suitable habitat to the east due to major highways and geographical limitations, and this issue could be a factor in future survival (Cooper 2013). It appears to be further isolated within GP, with clusters of animals in a handful of moist, densely-wooded canyons, and few sightings elsewhere in the park (D.S. Cooper, pers. obs.).

We investigated the genetic effects of urbanization on the population of WGSs in GP by analyzing 12 polymorphic microsatellite loci and mitochondrial (mtDNA) control region haplotype variation. To assess the population's long-term viability, we calculated estimates of genetic diversity, Bayesian clustering, genetic divergence, and relatedness. The questions we addressed were: (i) Are the WGSs in GP genetically isolated from other populations? (ii) Is there gene flow among subpopulations in GP? and (iii) Is the WGS population in GP at risk for local extinction, and how might the population be augmented? Implications of our results may be valuable for management practices concerned with preserving an important low-elevation population of WGSs in southern California.

Methods

Field sample locations

Three sample sites within GP were chosen based on previous studies noting the presence of WGSs (Cooper 2013). Sites included Fern Dell (FD), Vermont Canyon (VC), and Boy's Camp (BC) (Fig. 1a). Observations at each site within GP

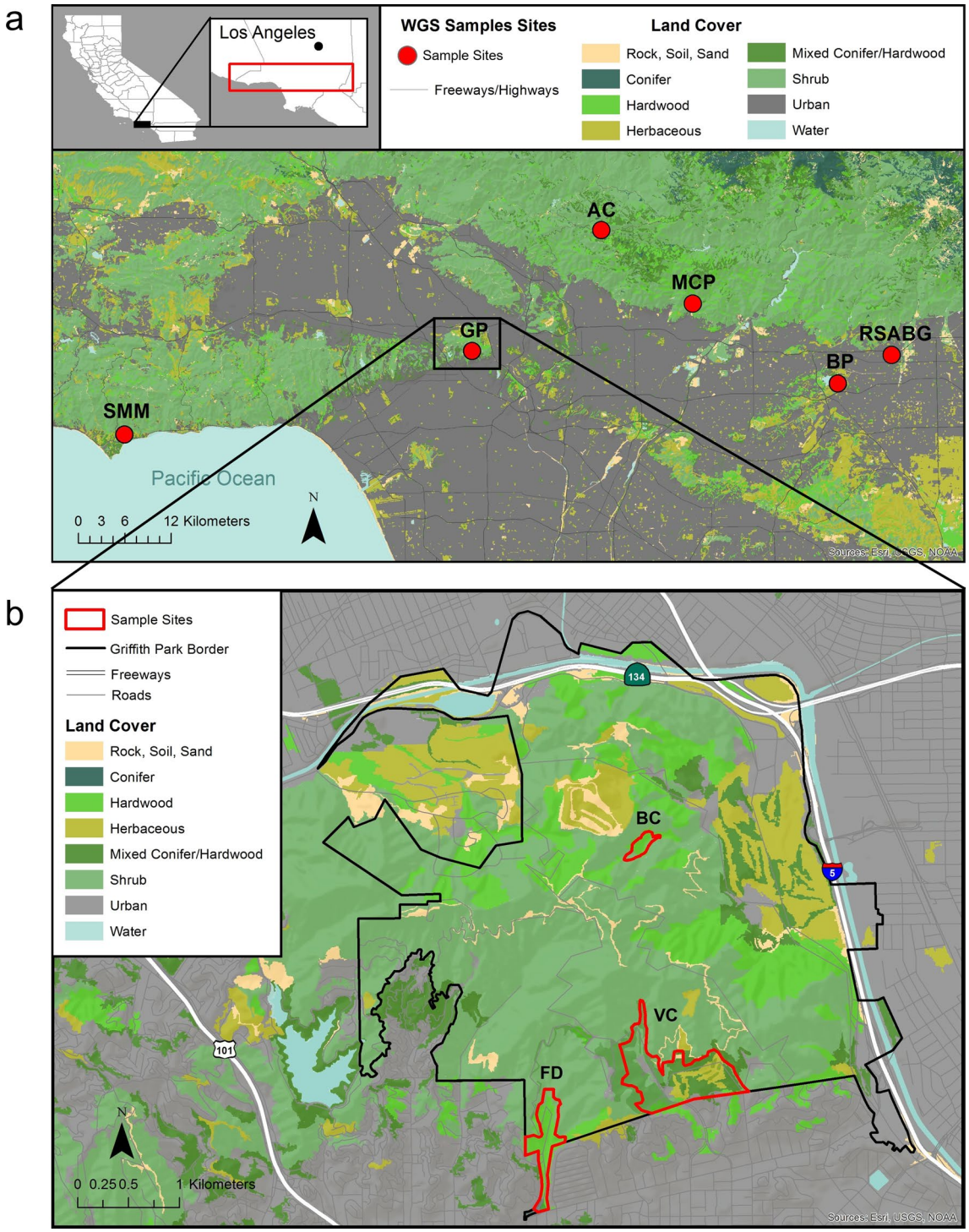

Fig. 1 **a** Field collection sample sites, excluding Kern County. *GP* Griffith Park, *SMM* Santa Monica Mountains, *AC* Angeles Crest, *MCP* Monrovia Canyon Park, *BP* Bonelli Park, and *RSABG* Rancho Santa Ana Botanic Gardens. **b** Griffith Park sample sites. *BC* Boy's Camp, *FD* Fern Dell, and *VC* Vermont Canyon

were done for a period of two weeks to assess areas for the presence of WGSs.

Three locations with known populations of WGS outside of GP selected as sampling sites for comparison included Rancho Santa Ana Botanic Garden (RSABG), Bonelli Park (BP), and the SMMs. Sample sites in the SMMs consisted of a riparian area in Malibu near Ramirez Canyon and a mobile home development and tourist area, called Paradise Cove.

Total sample sites were GP, BP, RSABG, SMM, MCP, AC, and KRN (Fig. 1b; KRN not shown).

Field collection

Once locations within sample sites were determined to have a consistent presence of WGSs, hair collection tubes, called hairtubes, were deployed. Hairtubes are a non-invasive method for collecting hair samples to obtain DNA (Taberlet et al. 1997; Garcia-Alaniz et al. 2010; Henry and Russello 2011) and are a type of hair snare designed for small mammals, such as squirrels and shrews (Pocock and Jennings 2006; Finnegan et al. 2007; Fimbel and Freed 2008; Mortelliti and Boitani 2008; Schwingel and Norment 2010; Pacific Biodiversity Institute 2012). Hairtubes were constructed per design schematics used by the Pacific Biodiversity Institute (Pacific Biodiversity Institute 2012) which consisted of baited 40.6 cm (12 in) plastic pipes with tape mounts on the upper inside. Bait included walnuts or a peanut butter and oatmeal mixture. The tubes were either anchored on the ground at the base of trees or were placed on tree branches and were checked every 2–4 days for the presence of hair.

Hairtube deployment took place from August 2014 to May 2016. A total of 117 samples [114 hair and 3 tissue (road kill)] were collected from 9 locations (6 outside of GP and 3 within) (Table 1). Of the 117 samples, 87 were obtained from GP. Hair from WGSs was successfully obtained using hairtubes from these sites.

Hair sample identification and DNA extraction

WGS hair was distinguished from non-target species by colored banding patterns on the hair shaft. Hair samples identified as WGS were stored in wax paper and zip-loc bags until extraction of DNA. If a sample was identified as being mixed with non-target species, that sample was discarded. It was assumed that all WGS hair on the tape was from a single individual until a later time when microsatellite results could reveal multiple individuals in a hair sample. While Henry et al. (2011) and Kendall (2012) used hair orientation in the same direction and grouping on mounting tape as an assumption of individuals being sampled, and then discarded samples where hair was grouped in different orientations, personal observations and video showed that an individual WGS will repeatedly go into the tube to get bait, leaving overlapping patterns of hair.

Hair was removed from tape mounts by tweezers and care was taken to observe that follicles were intact before placing hair into 2.5 ml microcentrifuge tubes containing elution (AE) buffer. Preliminary tests showed a minimum of 10 hairs were needed to ensure sufficient DNA quantities for downstream procedures. DNA from hair samples was extracted using DNeasy Blood and Tissue Kit protocols (QIAGEN, Valencia CA) with modifications. Instead of one elution step at 200 ul, samples were eluted twice at 75 ul. DNA was then quantified using a high sensitivity kit in a Qubit Fluorometer (Life Technologies, Carlsbad, CA). All sample processing and DNA extractions were done in a room separate from other PCR activities to reduce the possibility of contamination. DNA extractions were stored at − 20 °C.

Mitochondrial DNA polymerase chain reaction

Universal mtDNA primers for vertebrates (Kocher et al. 1989), L15926 (5′-TCAAAGCTTACACCAGTCTTGTAA ACC-3′) and H16340 (5′-CCTGAAGTAGGAACCAGA TG-3′), were used to amplify a 550 base pair fragment of the control region (D-loop) of mtDNA. A 25 ul reaction was done using 12.5 ul 2.0 × Apex Taq Master Mix (Genesee Scientific, San Diego CA), 1 ul of each primer at 10 mM

Table 1 Western gray squirrel (*Sciurus griseus*) samples used in this study

Sample sites	Abbreviation	Latitude	Longitude	Number of hair samples	Number of samples for mitochondrial DNA	Number of samples for microsatellites DNA
Vermont Canyon	VC	34° 7′11.82"N	118°17′29.49"W	40	26	25
Boy's Camp	BC	34° 8′28.30"N	118°17′46.80"W	24	16	16
Fern Dell	FD	34° 6′44.08"N	118°18′25.48"W	23	14	18
Bonelli Park	BP	34° 5′13.98"N	117°47′49.81"W	17	5	5
Rancho Santa Ana Botanic Garden	RSABG	34° 6′38.14"N	117°42′574.37"W	5	2	5
Santa Monica Mountains	SMM	34° 1′22.72"N	118°47′12.63"W	5	5	–
Monrovia Canyon Park	MCP	34°10′29.84"N	117°59′13.69"W	2	2	–
Angeles Crest[a]	AC	34°15′58.41"N	118° 8′38.08"W	1	1	–
Kern County[a]	KRN			1	1	–
Total				117	71	69

[a]Opportunistic collections from roadkill or a wildlife rehabilitator

concentration, 3 ul DNA template, and 7.5 ul MiliQ water. The profile for PCR reactions was as follows: initial denaturation at 96 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 47 °C for 1 min, and 72 °C for 1 min, with a final extension of 72 °C for 8 min. An Applied Biosystems 799 thermocycler was used for mtDNA PCR reactions. PCR products were visualized on a 1% agarose gel. Samples were sent to Laragen Inc. (Culver City, CA USA) for post-PCR clean up and sequencing on an ABI 3730XL sequencer (Applied Biosystems, Foster City, CA). Sequences were edited and aligned using Geneious version 8 (Kearse et al. 2012). Sequences were subject to BLAST searches against the NCBI database to verify they were WGS and compared to DNA extractions from WGS tissue.

Mitochondrial DNA analysis

There were 73 unique individuals identified by microsatellite analysis, but due to only having one sample per site for AC and KRN, samples from these sites were excluded from haplotype diversity and genetic differentiation estimates. To estimate the level of genetic differentiation and divergence among sites with adequate sample sizes, the program DnaSP 5.10.01 (Librado and Rozas 2009) was used to estimate the number of haplotypes, haplotype diversity (H_D), and number of segregating sites (S). PopART (Leigh and Bryant 2015) was used to generate a median joining network for all sequenced haplotypes.

Microsatellite polymerase chain reaction

Samples that were verified as WGS though mitochondrial analysis were subject to microsatellite DNA analysis. We originally tested 19 microsatellite loci on a subset of WGS samples. However, only thirteen of these microsatellite primers (Supplemental Table S1), designed for WGSs by K. Warheit (personal communcation 2007), were used to assess genetic variation in WGSs. A multiplex pre-amplification PCR method was used to increase the success of amplification (Piggott et al. 2004; Sharma et al. 2013). Five multiplexed PCR reactions were conducted based on primer annealing temperatures and then the PCR products were used as template DNA for the second amplification. Pre-amplification PCR was done in 25 ul reactions using 12.5 ul QIAGEN Multiplex Master Mix (QIAGEN Multiplex PCR kit, Valencia CA), 5 ul DNA, 0.075 ul of each unlabeled primer at 10 mM concentration, 0.2 ul 10 × BSA, and 7.5 ul MiliQ water. The conditions for PCR were as follows: initial denaturation at 95 °C for 15 min, followed by 40 cycles of 95 °C for 30 s, annealing temperature for 30 s, and 72 °C for 1 min, with a final extension of 72 °C for 7 min.

A second amplification was performed for each locus in 15 ul reactions, with 1.2 ul undiluted template from pre-amplification, 7.5 ul 2.0 × Apex Taq Master Mix (Genesee Scientific, San Diego CA), 0.45 ul of a labeled forward primer at 10 × concentration, 0.45 ul of an unlabeled reverse primer at 10 × concentration, 0.2 ul 10 × BSA, and 4.3 ul of MiliQ water. An unlabeled oligonucleotide, M13(− 21), was attached to the forward primer as described by Schuelke (2000) and was complementary to the universal fluorescent-labeled probe (FAM, PET, VIC, and NED) which is incorporated into PCR products in later cycles. PCR conditions were as follows: initial denaturation at 95 °C for 3 min, followed by 40 cycles of 94 °C for 30 s, annealing temperature for 30 s, and 72 °C for 1 min, with a final extension of 72 °C for 7 min. An applied Biosystems 799 thermocycler was used for microsatellite PCR reactions. PCR products were checked on a 2% agarose gel. Fragment Analysis was done at Laragen Inc. (Culver City, CA) on an ABI 3730XL sequencer (Applied Biosystems, Foster City, CA), using LIZ 500 size standard.

Microsatellite analysis

Alleles were scored in Geneious version 8 (Kearse et al. 2012) and samples that had more than two peaks at a locus were removed from subsequent analyses. Using a microsatellite add-in in Excel, identical samples were identified and removed from the data set. Samples that had a greater than 80% match to other samples, due to missing one or two genotypes and samples that were missing more than five genotypes, were removed. The removal of samples with missing genotypes was to prevent biases in estimates for allele and genotype frequencies.

We screened for errors in scoring due to stuttering, large allele drop-out, and null alleles with Microchecker (Oosterhout et al. 2004). A locus that had null alleles across all three subpopulations was removed from the data set. The software program GENEPOP 4.1(Raymond and Rousset 1995; Rousset 2008) was used to test for Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium within populations, using default parameters. GENETIX (Belkhir et al. 2004) was used to calculate F_{ST} values across all populations for all 12 loci (Raymond and Rousset, 1995; Rousset 2008).

GENETIX (Belkhir et al. 2004) was used to estimate observed and expected heterozygosity (H_O and H_E) at each locus and for all loci within each sampling location. The numbers of alleles (A) were calculated and F_{IS} was estimated per locus. We estimated allelic richness and private alleles for the populations of WGSs in GP, the SMMs, and BP with HP_RARE (Kalinowski 2005) which incorporates rarefaction, a method that takes sample size into account. The program ML-Relate (Kalinowski et al. 2006) was used to estimate the relationships between all pairs of individuals sampled in GP. The most likely relationship for four possible scenarios, parent/offspring (PO), full sib (FS), half

sib (HS) and unrelated (U), was used to determine the proportion of these relationship classes within and among GP subpopulations.

Population-level comparisons were done to estimate the effective population size (Ne) in GP. The program LDNE Version 1.31 (Waples and Do 2008) was used to estimate the effective population size Ne of GP, both as a whole and as separate subpopulations. Using genetic linkage disequilibrium data, an unbiased estimate of breeding individuals is computed with parametric confidence intervals (Waples and Do 2008).

To examine whether the population of WGS in GP have experienced a bottleneck, we used BOTTLENECK v1.2 (Piry et al. 1999), which tested for allele frequency distribution to determine whether it was L-shaped or had mode-shifts, and for heterozygote excess using the one-tailed Wilcoxon sign rank test. Three mutation models, Infinite Allele Mode (IAM), Two Phase Model (TPM), and Stepwise Mutation Model computed the distribution of the heterozygosity expected from the observed number of alleles, given the sample size under the assumption of mutation-drift equilibrium (Piry et al. 1999). The TPM was set with 90% of mutations being Stepwise Mutation Model and 10% being multistep, with a variance of 12 as recommended by Garza and Williamson (2001). We also calculated the M-ratio using software M_P_VAL (Garza and Williamson 2001) where M is the ratio of the mean number of alleles (k) to the range in allele size (r). The significance of M is determined by comparing it at a critical value, M_C, generated by the program CRITICAL_M (Garza and Williamson 2001) which calculates the distribution of M values from theoretical populations assumed to be in mutation–drift equilibrium. If the M-ratio is lower than M_C, there is evidence of a bottleneck.

Three parameters were needed for TPM (θ, p_g, Δ_g): (i) $\theta = 4 N_e \mu$, where N_e = effective population size and μ = mutation rate, (ii) p_g = the probability of changes greater than one step, and (iii) Δ_g = the size of non-one-step changes. Parameters for mutation rates and effective population sizes were varied in order to have an adequate range in theta values. Two mutation rates were used, $\mu = 5.0 \times 10^{-4}$ mutations/generation/locus (Garza and Williamson 2001), which is a common estimate of microsatellite mutation, and $\mu = 5.0 \times 10^{-3}$ mutations/generation/locus (Busch et al. 2007), which is an estimate that reflects the potential faster rate of molecular evolution in rodent species. We tested $N_e = 5$, 50, 100, and 500, giving us a wide range of θ values (0.001–10.0). The probability of changes greater than one step was $p_g = 0.10$ and the mean size of mutations larger than a single step was $\Delta_g = 3.5$ as suggested by Garza and Williamson (2001).

To estimate the level of genetic partitioning among populations, STRUCTURE v.2.3 (Pritchard et al. 2000) was used to test for Bayesian clustering within the population in GP and clustering among all sample sites. We estimated the number of groups (K) in GP and among all sample sites, and assessed the relationships between the inferred subpopulations and any geographical features that could affect gene flow (Smith 2011). For estimates of K using GP's three subpopulations, 25 replicates were done for 3, 2, and 1 groups (K). The Markov chain Monte Carlo (MCMC) ran for 500,000 iterations with a burn-in period of 50,000. For estimates of K including all five sample sites, 40 replicates were done for groups (K) 5–2. The program STRUCTURE HARVESTER (Earl and von Holdt 2012) was used to calculate the best-fit K. We then used the fullsearch algorithm in CLUMPP (Jakobsen and Rosenberg 2007) to find the optimal ancestry proportions across independent STRUCTURE runs. Results were visualized with DISTRUCT (Rosenberg 2004).

Results

Mitochondrial DNA analysis

We sequenced a total of 6 haplotypes from samples of WGSs across nine sites (GenBank accession MN923254–MN923259). Low haplotype diversity was found in GP and SMM relative to other sites (Table 2). A single haplotype (A) was found across many southern California sites and was the dominant haplotype in GP. Haplotype B was also found, at lower frequency, in GP. BP, MCP, and RSABG had the highest levels of haplotype diversity (Table 2). The median joining haplotype network (Fig. 2)

Table 2 mtDNA haplotypes for western gray squirrels (*Sciurus griseus*) in the study sites*

Population	N	S	H	H_D
BC	16	0	1	0.000
FD	14	0	1	0.000
VC	26	1	2	0.271
BP	5	10	2	0.600
MCP	2	9	2	1.000
RSABG	3	11	2	0.667
SMM	5	0	1	0.000
Total	71	12	5	0.348

*Angeles Crest and Kern County were not included in the analysis due to only having one sample per site

Data include the number of individuals (N), segregating sites (S), number of haplotypes (H), and haplotype diversity (H_D)

Study sites include *BC* Boy's Camp, *FD* Fern Dell, *VC* Vermont Canyon, *BP* Bonelli Park, *SMM* Santa Monica Mountains, *MCP* Monrovia Canyon Park, *AC* Angeles Crest,

showed that the most common haplotype (A) was found in all locations except SMMs and KRN.

Microsatellite analysis

Hardy Weinberg equilibrium and linkage disequilibrium

After omitting duplicate samples and removing samples with missing genotypes, 73 unique individuals were obtained from six locations. Due to low sample size in RSABG (3), MCP (2), AC (1), and KRN (1), only 66 individuals were used in microsatellite analyses (56 GP, 5 BP, and 5 SMM). Only 12 microsatellite loci were included in analyses due to one locus (Sgr-C222) having a null allele. Locus Sgr-C222 showed significant evidence of deviation from the HWE across all populations. It was also determined to have a null allele in all populations, so it was removed from the data set. Microchecker (Oosterhout et al. 2004) results indicated that locus Sgr-D237 also had a null allele, however only in VC. Since that locus was in HWE in all populations, it was not omitted from the data set. Calculations in GENEPOP 4.1 (Raymond and Rousset 1995; Rousset 2008) for linkage disequilibrium (LD) with the Holm-Bonferroni sequential correction (Holm 1979) revealed significant LD in only 15 out of 330 pairwise locus comparisons across all populations (Supplemental Table S2). This allowed us to utilize 12 loci as independent measures of variation and structure in our study.

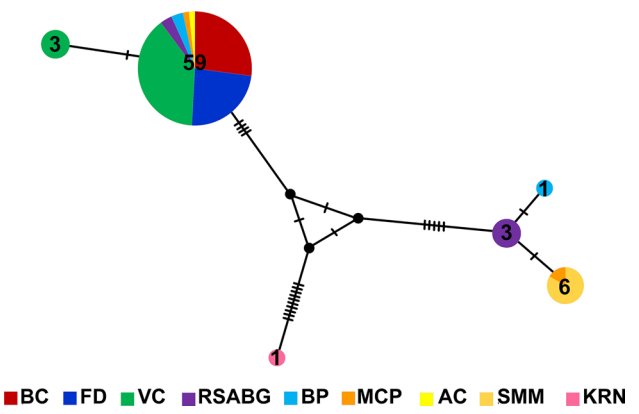

Fig. 2 A mtDNA haplotype network for western gray squirrels (*Sciurus griseus*) in nine sample sites. The different haplotypes are labeled A–F. Colors correspond to locations where haplotypes were found. The size of circles is proportional to the numbers of individuals (shown in circles) which have that haplotype. *BC* Boy's Camp, *FD* Fern Dell, *VC* Vermont Canyon, *BP* Bonelli Park, *SMM* Santa Monica Mountains, *MCP* Monrovia Canyon Park, *AC* Angeles Crest, and *KRN* Kern County

Heterozygosity, allelic richness, and private alleles

Table 3 shows a summary of population and locus data. Estimates of H_O did not reveal low heterozygosity. Mean H_O was higher than mean H_E for all populations except BP. A significant excess of heterozygotes was found in BC ($p < 0.05$). The inbreeding coefficient, as measured by F_{IS}, showed negative F_{IS} values in BC, FD, and SMM. The only populations that didn't have an excess of heterozygotes were VC and BP, which had mean F_{IS} values of 0.003 and 0.108, respectively.

The average number of alleles for all populations ranged from 2.70 to 3.33, with BC and FD subpopulations having the lowest and highest values, respectively. Subpopulations within GP had the lowest average allelic richness (A_R) estimates (2.280–2.300) compared to SMM (3.140) and BP (2.710). Allelic richness was estimated using a rarefaction method, which takes differences in sample size into consideration when comparing populations. This same method was used to compare the average number of private alleles in each population (A_P). Both BC and FD had the lowest relative average (0.090 and 0.270, respectively). Overall, animals within GP had the lowest estimates of A_R, and A_P, with BC having the lowest H_E, A_R, and A_P, but the highest H_O.

Relatedness

Maximum likelihood estimates of relationship classes among WGS in GP (Table 4) were elevated within GP subpopulations. Results for subpopulations showed that BC had the highest proportion of full sibling relationships (22.5%), parent–offspring relationships (17.5%), and the second highest half-sibling relationships (23.3%). For animals in BC, the total proportion of relatedness to all its relationships were 63.3%, indicating high levels of relatedness. The WGS in FD had the lowest relatedness percentage and the lowest parent–offspring relationships (9.15%). The second highest levels of relatedness were found in VC, with the lowest parent–offspring percentages (9.8%). Overall pairwise relationship percentages between subpopulations estimated that animals in BC and FD were 20.8% related- the highest likelihood estimate, while animals in BC and VC were 93.75% unrelated, showing the least amount of relatedness among all subpopulations. Overall, animals in BC and FD were the most related among subpopulations.

Effective population size

The program LDNE (Waples and Do 2008) calculated the effective population size (N_e) using the lowest allele frequency with a critical value of 0.05 (Table 5). The linkage disequilibrium method estimated that the WGSs in GP, as a single population based on 56 individuals, had N_e of 9.1, with a range of 6.1–12.8 based on a 95% CI. When

Table 3 Locus and population description for western gray squirrels (*Sciurus griseus*) from different study sites. Information for each population includes numbers of individuals at each locus (N), the number of different alleles per locus (A), expected and observed heterozygosity (H_E and H_O), F_{IS}, allelic richness (A_R) with its standard deviation (A_{SD}), and private alleles (A_P)

Populations						
Locus		BC	FD	VC	BP	SMM
B216	(N)	16	18	25	5	5
	A	3	4	4	4	4
	H_E	0.619	0.616	0.394	0.700	0.640
	H_O	0.563	0.778	0.400	0.800	1.000
	F_{IS}	0.123	−0.236	0.004	−0.032	−0.481
D118	(N)	15	17	25	5	5
	A	1	2	1	3	3
	H_E	0.000	0.360	0.000	0.620	0.340
	H_O	0.000	0.353	0.000	0.800	0.400
	F_{IS}	–	0.050	–	−0.185	−0.067
D7	(N)	16	17	25	5	5
	A	3	4	5	3	2
	H_E	0.529	0.652	0.374	0.460	0.480
	H_O	0.813	0.588	0.360	0.200	0.000
	F_{IS}	−0.512	0.128	0.059	0.636	1.000
D211	(N)	15	16	24	5	5
	A	3	3	2	2	2
	H_E	0.180	0.272	0.492	0.480	0.500
	H_O	0.200	0.313	0.292	0.800	0.600
	F_{IS}	−0.077	−0.119	0.425	−0.600	−0.091
B208	(N)	16	17	24	5	5
	A	3	4	3	4	4
	H_E	0.600	0.701	0.666	0.700	0.660
	H_O	0.938	0.588	0.917	0.800	0.800
	F_{IS}	−0.541	0.190	−0.358	−0.032	−0.103
D2	(N)	16	18	25	4	5
	A	3	4	3	4	2
	H_E	0.354	0.631	0.466	0.656	0.480
	H_O	0.438	0.611	0.560	0.750	0.800
	F_{IS}	−0.207	0.060	−0.181	0.000	−0.600
D237	(N)	16	18	25	5	5
	A	2	2	2	4	3
	H_E	0.430	0.475	0.435	0.660	0.460
	H_O	0.375	0.667	0.080	0.600	0.600
	F_{IS}	0.159	−0.378	0.823	0.200	−0.200
B205	(N)	15	16	24	4	4
	A	4	4	5	3	5
	H_E	0.691	0.539	0.613	0.531	0.781
	H_O	0.933	0.438	0.750	0.250	1.000
	F_{IS}	−0.320	0.219	−0.203	0.625	−0.143
D10	(N)	16	18	25	5	5
	A	3	3	3	2	2
	H_E	0.555	0.495	0.550	0.180	0.420
	H_O	0.500	0.444	0.800	0.200	0.600
	F_{IS}	0.130	0.131	−0.439	0.000	−0.333
D216	(N)	15	16	25	4	4
	A	3	3	3	4	2
	H_E	0.504	0.471	0.250	0.563	0.219
	H_O	0.800	0.625	0.280	0.500	0.250

Table 3 (continued)

Populations						
Locus		BC	FD	VC	BP	SMM
	F_{IS}	−0.563	−0.299	−0.098	0.250	0.000
A101	(N)	16	16	18	5	5
	A	2	4	4	3	3
	H_E	0.500	0.561	0.529	0.580	0.560
	H_O	0.500	0.500	0.500	0.200	0.800
	F_{IS}	0.032	0.140	0.084	0.714	−0.333
A7	(N)	14	15	25	4	4
	A	3	3	3	3	2
	H_E	0.651	0.287	0.618	0.531	0.500
	H_O	0.857	0.333	0.680	0.750	0.500
	F_{IS}	−0.284	−0.129	−0.079	−0.286	0.143
Total	Mean A	2.75	3.333	3.167	3.25	2.833
	H_E	0.468	0.505	0.449	0.555	0.503
	H_O	0.576	0.52	0.468	0.554	0.613
	F_{IS}	−0.187	−0.02	0.003	0.108	−0.101
	A_R	2.28	2.53	2.3	3.14	2.71
	A_{SD}	0.622	0.506	0.534	0.743	0.95
	A_P	0.09	0.27	0.06	0.75	0.66

BC Boy's Camp, *FD* Fern Dell, *VC* Vermont Canyon, *BP* Bonelli Park, and *SMM* Santa Monica Mountains

Table 4 Relationship comparisons of western gray squirrels (*Sciurus griseus*) in Griffith Park, Los Angeles, CA

	Percent Relatedness				
Population	FS	HS	PO	U	R
BC	22.5	23.3	17.5	36.7	63.3
FD	5.88	24.84	9.15	60.13	39.87
VC	14.1	20.7	9.8	55.4	45.0
BC:FD	1.0	16.7	3.1	79.2	20.8
BC:VC	0.5	5.0	0.75	93.75	6.3
FD:VC	0.4	9.4	4.0	86.2	13.8

Percentage of pairwise comparisons that fall into a particular relationship class. Relationships are full sibling (FS), half sibling (HS), parent-offspring (PO), unrelated (U), and total relatedness (R)

BC Boy's Camp, *FD* Fern Dell, and *VC* Vermont Canyon

comparing the larger population to the subpopulations, BC's N_e was low, 1.4. This was in contrast to populations within FD and VC, whose N_e estimates were very similar to one another, 6.2 and 6.6, respectively.

Bottleneck

The Wilcoxon sign rank test for heterozygosity excess using the IAM (Infinite Alleles Model), TPM (Two-Phase Model), and SMM (Stepwise Mutation Model) in the program BOTTLENECK was significant with IAM for GP (all subpopulations combined), BC, and FD (Table 6). For the TPM and SMM, only BC had significant heterozygosity excess. A mode shift was only detected in BC. M-ratio results from MP_VAL indicated that bottlenecks occurred in BC and FD depending on the effective population size and mutation rate

Table 5 Estimates of effective population size for western gray squirrels (*Sciurus griseus*) in Griffith Park, Los Angeles, CA

	GP (n = 56)	BC (n = 16)	FD (n = 14)	VC (n = 26)
Harmonic mean sample size	54.1	15	15.7	22.5
Independent Comparisons	356	179	347	233
Estimated N_e	9.1	1.4	6.2	6.6
95% CI for N_e	6.1–12.8	1–2.1	2.8–12.8	2.9–12.6

Alleles at a frequency of 0.05 or more were only used

GP Griffith Park, *BC* Boy's Camp, *FD* Fern Dell, and *VC* Vermont Canyon

Table 6 Heterozygosity excess tests and mode shift estimates for bottlenecks in populations of western gray squirrels (*Sciurus griseus*) in Griffith Park. Computations used three mutation models, Infinite Allele Mode (IAM), Two Phase Model (TPM), and Stepwise Mutation Model (SMM)

Population	IAM	TPM	SMM	Mode
BC	0.001	0.003	0.04	Shifted
FD	0.021	0.101	0.545	Normal (L-shape)
VC	0.062	0.259	0.681	Normal (L-shape)

Mode was based on Wilcoxon Test for probabilities with the assumption that all loci fit the TPM. *BC* Boy's Camp, *FD* Fern Dell, and *VC* Vermont Canyon

Table 8 Pairwise estimates for differentiation among five study sites. F_{ST} below diagonal, $p < 0.05$ above diagonal, based on 1000 permutations

	BC	FD	VC
BC	–	[a]	[a]
FD	0.108	–	[a]
VC	0.156	0.116	–

[a] = FST significantly greater than zero after sequential Holm-Bonferroni correction; $p < 0.0001$

BC Boy's Camp, *FD* Fern Dell, and *VC* Vermont Canyon

assigned in the analysis (Table 7). When we used the slower mutation rate ($\mu = 0.0005$), M-ratio values were significantly lower than M_C values ($p < 0.05$) for BC using $N_e = 5$–500 and for FD using $N_e = 5$–100. The faster mutation rate ($\mu = 0.005$) yielded significant M-ratio values for BC using $N_e = 5$ and $N_e = 50$, while FD only had a significant M-ratio for $N_e = 5$. Our M-ratios support the heterozygosity excess test under all mutation models and mode shift estimates for bottleneck in BC. The significant M-ratios for FD, under certain conditions, contradict the lack of bottleneck shown in the Wilcoxon sign rank test and allele mode shift estimates. Holm-Bonferroni sequential correction was done in cases where multiple tests were performed.

Population structure

F_{ST} estimates, using frequency data in GENETIX (Belkhir et al. 2004), revealed significant levels of genetic differentiation among all three subpopulations of WGSs in GP (Table 8). Mean pairwise F_{ST} within GP showed subdivision among the subpopulations with values ranging from 0.10856 to 0.15640. Notably, BC and FD had higher F_{ST} with pairwise comparisons with VC even though VC is geographically in between BC and FD.

Bayesian clustering from STRUCTURE indicated $K = 2$ populations based on all five populations and $K = 2$ based on only subpopulations within GP (Fig. 3). STRUCTURE HARVESTER (Evanno et al. 2005) detected the number of groups (K) that best fit the data and produced two plots (Supplemental Figure S1). Both plots show that the greatest change in probabilities was between $K = 2$ and $K = 3$, indicating $K = 2$ was the best-fit K.

Table 7 M-ratio analyses for populations of western gray squirrels (*Sciurus griseus*) in Griffith Park, Los Angeles, CA

		$\mu = 0.005$			$\mu = 0.0005$		
Population	N_e	M_C	M	p	M_C	M	p
BC	5	0.852	0.759	**0.002**	0.866	0.759	**0.001**
	50	0.779	0.759	0.026	0.852	0.759	**0.001**
	100	0.740	0.759	0.091	0.841	0.759	**0.002**
	500	0.642	0.759	0.692	0.782	0.759	**0.025**
FD	5	0.852	0.796	**0.007**	0.861	0.796	**0.003**
	50	0.779	0.796	0.070	0.852	0.796	**0.007**
	100	0.735	0.796	0.210	0.841	0.796	**0.011**
	500	0.631	0.796	0.855	0.778	0.796	0.075
VC	5	0.853	0.880	0.112	0.866	0.880	0.081
	50	0.788	0.880	0.459	0.854	0.880	0.116
	100	0.748	0.880	0.715	0.841	0.880	0.148
	500	0.677	0.880	0.992	0.787	0.880	0.458

N_e = effective population size, M_C = critical M, and p = percent of time you expect a smaller ratio at equilibrium

M_C values were obtained through theoretical mutation-drift populations

BC Boy's Camp, *FD* Fern Dell, and *VC* Vermont Canyon

Bold values indicate significant at $p < 0.05$ after sequential Holm-Bonferroni correction

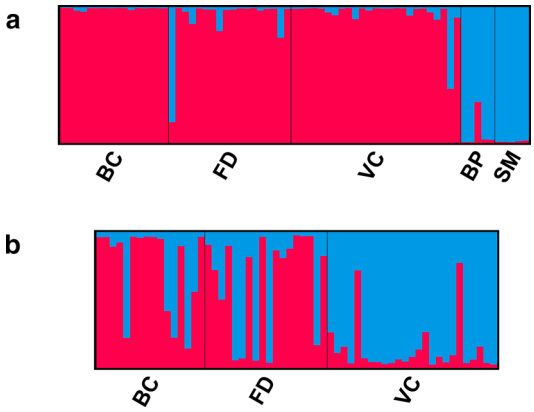

Fig. 3 **a** K = 2 Population clustering among western gray squirrels (*Sciurus griseus)* in five sample sites. *BC* Boy's Camp, *FD* Fern Dell, *VC* Vermont Canyon, *BP* Bonelli Park, and *SMM* Santa Monica Mountains. **b** K = 2 Population subdivision for WGSs within Griffith Park. *BC* Boy's Camp, *FD* Fern Dell, and *VC* Vermont Canyon

Discussion

Maintenance of biodiversity in urban settings is a major challenge with the contemporary increases in urbanization on a global scale. We investigated nuclear and mitochondrial genetic diversity in an isolated population of WGS from GP, a remnant open space in one of the largest urban sprawls (Los Angeles, CA USA). We found that the population of WGS in GP contains low levels of genetic variation, has low effective population sizes and a high proportion of related individuals. However, we did not find consistent signs of recent population bottlenecks across all sampled subpopulations. These results point towards a long history of isolation and genetic vulnerability for this remnant population of WGSs and suggest a high conservation priority for this population given the numerous forces that are contributing to regional declines of this species.

Mitochondrial lineages

Only two haplotypes were found in 56 individual WGSs sequenced from GP. This low level of variation is indicative of low female effective population. Across our limited regional sampling (southern California) a total of 5 unique haplotypes were found. The number of WGS haplotypes found in the Los Angeles area sample sites was slightly lower than the number of FS haplotypes found in the Los Angeles area in Claytor et al. (2015). Haplotype diversity (0.7959) and nucleotide diversity (0.0120) were greater in FS samples than that of WGS samples. This provides an interesting comparison as FS have been introduced in Los Angeles from multiple sources since the early twentieth century (Claytor et al. 2015) and have become a dominant component of the Los Angeles urban ecosystem. This difference in genetic diversity, however, is consistent with Furlan et al. (2012) and Cardoso et al. (2009) who observed that the loss of genetic diversity can be more prominent in endemic populations compared to recently introduced populations. Given the geographic limitations of the WGS sampled in this study, it is difficult to assess any geographic patterns in diversity or structure. The observed haplotype diversity is likely a remnant of historically larger populations and effective population sizes, while the low sequence diversity is likely due to more recent decrease in population and effective population sizes. As there has not been a comprehensive study of genetic variation across the range of the WGS our ability to make any inference on historical connections of GP or any other populations sampled in this study is limited. Future work on this species should sample from both large and small-scale geographic areas so that regional comparisons can be made.

Nuclear genetic variation

Observed heterozygosity and allelic richness suggest small effective population sizes of WGS in GP. We observed lower heterozygosity and allelic richness in samples from GP when compared to BP and SMM, the latter after correcting for differences in sample size. Within GP we found lower levels of microsatellite variation for the BC site, which could be due to a more recent bottleneck in this population (see below). Low levels of variation for heterozygosity and allelic richness are associated with small or recent reductions in effective population size (Frankham 2005; Hare et al. 2011). Attempts were made to sample greater numbers of WGS individuals from adjacent areas with more contiguous habitat (e.g. Santa Monica Mountain–DeMarco pres. obs.) in areas where WGS have been reported (Cooper 2013; Cooper and Muchlinski 2015), however these were met with little to no success and may be indicative of the greater decline in WGS across the Los Angeles area than has been appreciated. Additional work should be conducted to assess within-population variation for viable populations of WGS, especially in impacted areas like Los Angeles.

Sites within GP showed a lower level of private allelic richness when compared to other sites. This observation indicates a lack of genetic distinctiveness for GP when compared to SMM and BP, and could also be a result of low effective population sizes (Stuart 2011). Elevated Ap is associated with a lack of gene flow and increased genetic uniqueness for a population (Slatkin 1985; Kalinowski 2005), which was observed in SMM and BP. Low Ap in GP sites could be a result of drift fixing more common alleles, and not necessarily higher connectivity among GP and other sites.

We also observed elevated F_{IS} for the BC subpopulation. A possible explanation is that mating is not random

and would thus violate the assumptions used for that model to calculate F_{IS} estimates. Two other possibilities can explain specifically why BC had an excess of heterozygotes. The first reason is known as "isolate-breaking," when two discrete subpopulations that were previously separated, interbreed (Charlesworth 1998). The second reason for the homozygosity excess is that BC experienced a bottleneck and that there is a temporary excess of homozygosity relative to heterozygosity based on the number of alleles (Roderick and Navaias 2003).

When comparing other mammal populations that are endangered or vulnerable to extirpation, WGSs in GP have similar or lower levels of allelic richness. This includes other WGS populations (Stuart 2011; allelic richness 2.737–3.670), other rodent species like Stephen's kangaroo rat (Shier and Navarro, 2016; allelic richness 3.46–7.42), and urban wildlife like southern California mountain lions (Ernest et al. 2014; allelic richness 0.1–2.4). Within the Los Angeles region, GP had significantly lower allelic richness estimates than SMM and BP. These results indicate that WGSs in GP are more likely to have low N_e and have experienced a demographic shift, such as a bottleneck (Peery et al. 2012; Ernest et al. 2014). Low microsatellite allelic variation in GP WGSs was also consistent with other southern California populations with low genetic diversity.

Bottlenecks

Tests for population bottlenecks consistently found evidence for recent bottlenecks in the BC subpopulation (BOTTLENECK, M-ratio and mode shift) and the m-ratio test suggest a recent population bottleneck in FD. These patterns hold up given a wide array of model assumptions. Low levels of genetic variation and increased within subpopulation relatedness (see below) point towards small effective population sizes for all GP sub-populations, however inconsistent signals of recent bottleneck may appear to be counter to this. The bottleneck tests used here are more sensitive to recent events and the dissipation of the bottleneck signal is related to duration of the bottleneck and the size of the bottleneck relative to the pre-bottleneck effective size (Cornuet and Luikart 1996). The lack of a consistent bottleneck signal could be due to a long period of isolation and low effective sizes for the GP population, as GP has been isolated from regional populations by multi-lane freeways for c. 50 years, and by agriculture and roads for much longer (Eberts 1996). Also, fires are a perpetual threat in GP and have burned through areas bordering BC, FD, and VC for centuries. Repeated burns in and around WGS subpopulations sites could have reduced movement and made populations more vulnerable to population declines. In 2007, a fire consumed 817 acres in GP, effectively burning through the southern portion of the park and potentially isolating FD and VC from the rest of the park.

Low Ne/relatedness

The effective population size estimate for subpopulations of WGSs in GP was low and we observed elevated within-population relatedness within each subpopulation sampled. It is unlikely that the low estimates of effective size are due to a recent bottleneck across all GP populations, with the exception of BC, based on tests for recent bottlenecks. These results suggest that the GP population has been isolated with a small effective size for a longer period. Given the current isolation and low effective size for the GP WGS, the continued loss of genetic variation due to drift is likely to be a major concern for this population. Additionally, the elevated levels of within-subpopulation relatedness indicate that these groups are comprised of highly-related individuals. With high percentages of relatedness and low effective sizes, WGSs in GP could be at risk for inbreeding depression and the extreme loss of variation due to drift (Frankham 1995; Markert et al. 2010).

Genetic divergence

Results from STRUCTURE support that GP is genetically distinct from the other populations sampled and most likely isolated from them. Coupled with results from mitochondrial DNA, this indicates that the GP population is a small, isolated set of subpopulations that has become distinct because of the strong impact of drift. More intense sampling in areas surrounding GP would provide additional evidence for the severity of isolation for the GP samples. Currently there are major highways surrounding GP that could account for the observed isolation, and the nearest consistent populations of WGS (in the SGM foothills and central SMM) are 20 + km away.

Results from F_{ST} estimates for WGS subpopulations in GP showed significant differentiation among them, reflecting the isolation of BC, FD, and VC from one another. Within GP, we also observed genetic structure with STRUCTURE suggesting that BC and FD are one sub-population, and VC a separate sub-population, however there is some shared ancestry between these two 'clusters'. Comparison of pairwise relatedness between the sampled subpopulations shows a similar trend. This result suggests that population substructure within GP is significant and may be due to recent and or continual fragmentation within the park rather than pure isolation due to dispersal capabilities. The potential consequences are that these

sub-populations may be further isolated in the future and suffer even more dramatic effects of drift and inbreeding.

Conclusion and conservation implications

Our study demonstrated that the sub-populations of WGSs in GP are isolated from one another, have small effective sizes, contain a significant proportion of related individuals, and are at risk for extinction due to these factors. Thus, one of the last lowland populations of WGS in the region, and one of the few urban populations left in the state, is at risk. Thus, there are several measures that would hopefully connect subpopulations within the park, including seasonal trail closures, habitat restoration, and translocation of family groups from elsewhere in the Los Angeles Basin. Furthermore, the situation at GP may be representative of other isolated WGS populations inhabiting habitat fragments in southern California, which has seen a near-complete invasion of a successful competitor (FS).

WGSs, a native species with a legacy in California dating back to the last ice age, have been observed in this region since the 1850′s (Eberts 1996). Our results suggest a high priority for the preservation of WGS in GP and continued investigation into the status of WGS throughout southern California. These results also stress the importance of remnant urban wild lands in the preservation of sensitive species (Ordenana et al. 2010). Globally, squirrels in urban settings are facing similar pressures from habitat loss and invasive species (Koprowski 2005; Vander Haegen et al. 2018; Jessen et al. 2018; Johnston et al. 2019). Given the ecological importance of squirrels as tree propagators, their persistence is crucial for effective ecosystem processes (Steele 2008; Blythe et al. 2015; Mendes et al. 2019). Thus, the conservation of squirrels and the preservation of wild lands are inexorably linked.

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Author contributions CD, DC and AM designed the study. CD collected samples and generated all data. CD analyzed the data with assistance from AA and ET. CD, AM and AA drafted the manuscript and DC and ET provided comments on manuscript drafts.

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Compliance with ethical standards

Conflicts of interest Not applicable.

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Consent to participate Not applicable.

Consent for publication Not applicable.

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