

Gene flow and pathogen transmission among bobcats (*Lynx rufus*) in a fragmented urban landscape

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Abstract

Urbanization can result in the fragmentation of once contiguous natural landscapes into a patchy habitat interspersed within a growing urban matrix. Animals living in fragmented landscapes often have reduced movement among habitat patches because of avoidance of intervening human development, which potentially leads to both reduced gene flow and pathogen transmission between patches. Mammalian carnivores with large home ranges, such as bobcats (*Lynx rufus*), may be particularly sensitive to habitat fragmentation. We performed genetic analyses on bobcats and their directly transmitted viral pathogen, feline immunodeficiency virus (FIV), to investigate the effects of urbanization on bobcat movement. We predicted that urban development, including major freeways, would limit bobcat movement and result in genetically structured host and pathogen populations. We analysed molecular markers from 106 bobcats and 19 FIV isolates from seropositive animals in urban southern California. Our findings indicate that reduced gene flow between two primary habitat patches has resulted in genetically distinct bobcat subpopulations separated by urban development including a major highway. However, the distribution of genetic diversity among FIV isolates determined through phylogenetic analyses indicates that pathogen genotypes are less spatially structured—exhibiting a more even distribution between habitat fragments. We conclude that the types of movement and contact sufficient for disease transmission occur with enough frequency to preclude structuring among the viral population, but that the bobcat population is structured owing to low levels of effective bobcat migration resulting in gene flow. We illustrate the utility in using multiple molecular markers that differentially detect movement and gene flow between subpopulations when assessing connectivity.

Keywords: carnivores, disease ecology, gene flow, habitat fragmentation, microsatellites, viral phylogenetics

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Introduction

Habitat loss and degradation are the leading causes of species declines around the world (<http://www.iucnredlist.org/>). Urbanization, an extreme form of habitat degradation, results in immediate displacement of

wildlife from developed areas, followed by the increasing isolation of groups of animals confined to shrinking natural areas (McKinney 2002; McDonald *et al.* 2008). A common effect of urbanization is the fragmentation of once contiguous landscapes into smaller patches of non-contiguous habitat.

Maintaining functional connectivity, the extent to which organisms and genetic material move between

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habitat patches, can be essential for population persistence in fragmented landscapes (Crooks & Sanjayan 2006). Reduced functional connectivity between habitat patches can result in physically and genetically isolated subpopulations prone to inbreeding and to the loss of genetic diversity through genetic drift (Frankham 2006). However, measuring functional connectivity can be difficult, especially for cryptic solitary species such as large carnivores (Crooks 2002). One commonly used method of evaluating functional connectivity involves characterizing patterns of gene flow using molecular markers to evaluate the distribution of genetic diversity within and among groups of individuals (Frankham 2006; Balkenhol & Waits 2009; Ruell *et al.* in press). Populations with high connectivity should exhibit homogeneous distributions of genetic diversity. Populations with low connectivity will be genetically structured, exhibiting localized variations in genetic diversity as a consequence of reduced gene flow among isolated groups of individuals.

Microsatellites are neutral heritable molecular markers commonly used to evaluate genetic structure, and hence connectivity, among natural populations (Avice 2004; Hedrick 2005a). These polymorphic, codominant markers provide a powerful means for assessing gene flow. However, microsatellite markers only reflect individual movement within structured populations if a migrant is sampled or if a migrant successfully reproduces and at least one offspring is sampled. Transient movements between subpopulations, or migrants that do not reproduce, may not be detected by analysing microsatellites (Riley *et al.* 2006). Therefore, while powerful, host genetic markers may not accurately characterize connectivity when individual movements do not result in gene flow.

Because directly transmitted obligate pathogens are inextricably linked to their hosts, pathogens can serve as alternative or additional markers for studies of wild-life population dynamics (Nieberding & Olivieri 2007; Liu *et al.* 2008). Feline immunodeficiency virus (FIV), a retrovirus that naturally infects many felid species (Troyer *et al.* 2005; Vandewoude & Apetrei 2006), has many characteristics that render it potentially useful as a marker of population dynamics in wild cats. First, the mutation rate of FIV is significantly faster than that of host genetic markers (approximately $\mu = 1\text{--}3\%$ every 10 years in mountain lions) (Biek *et al.* 2003). Also, infection with FIV is life-long because an obligatory step in viral replication involves the permanent insertion of a copy of the viral genome into the host's chromosomal DNA. Therefore, FIV genotypes have the capacity to serve as life-long molecular markers for each infected individual. Furthermore, the virus cannot be transmitted by insect vectors, nor is it stable in the envi-

ronment, and thus, transmission events are indicators of direct contact between individuals.

Finally, the strains of FIV, which infect domestic cats, are genetically distinct from the strains isolated from wild felids. The domestic cat strains have never been documented to infect nondomestic felids in the wild, and therefore, the genetic diversity of FIV among nondomestic felids is only influenced by the distribution, movement and contact rates among conspecifics across the landscape.

Indeed, previous studies have demonstrated that patterns of FIV relatedness closely reflect the geographical distribution of bobcats, mountain lions and African lions at various geographical scales (Biek *et al.* 2006; Franklin *et al.* 2007a; Antunes *et al.* 2008). Specifically, Franklin *et al.* (2007a) demonstrated that the FIV isolates infecting bobcats north and south of Los Angeles, CA are genetically distinct, having diverged since the isolation of the two host populations. Because of these characteristics, viral genetic analyses may provide novel and powerful techniques for assessing connectivity and population structure with improved resolution, supplementing that which is currently possible using host genetic markers.

Bobcats (*Lynx rufus*), with large home ranges and high resource requirements, are susceptible to the effects of habitat loss and fragmentation in urbanizing systems (Crooks 2002; Riley *et al.* 2003, 2006, 2010). We investigated patterns of genetic diversity among bobcats and FIV isolates from a fragmented landscape in southern California to evaluate how urbanization affects connectivity among bobcats in this region. We specifically evaluated the extent to which several large freeways, and the developed areas surrounding them, are barriers to gene flow and pathogen transmission between habitat patches. We predicted that decreases in connectivity would lead to significant genetic structure among both host and pathogen populations. We performed standard population genetics analyses using 16 unlinked microsatellite loci from 106 bobcats. We also constructed phylogenetic trees to assess patterns of relatedness among FIV isolates from 19 of these individuals infected by the virus.

Our findings, presented below, demonstrate that two spatially structured, genetically distinct bobcat subpopulations exist. However, the pathogen phylogeny revealed no association between FIV relatedness and bobcat population structure, suggesting movements, contacts and disease transmission between subpopulations continue despite very low levels of host gene flow. This finding was inconsistent with our hypothesis that decreased gene flow among bobcats would lead to similar genetic structure among the virus population. However, these results are consistent with other studies that have found major roads and urban development to

be more permeable to transient bobcat movements than to effective migration, allowing for the potential movement of pathogens in the absence of gene flow (Riley *et al.* 2006; Ruell *et al.* in press). We illustrate the utility in using multiple molecular markers, each with different determinants of movement throughout populations, to assess complex questions of connectivity.

Methods

Location and field sampling

This study was located south and west of Los Angeles, CA and included four habitat patches divided by three large freeways—Interstate-5 (I-5), the Riverside Freeway (SR-91) and the San Joaquin Hills Transportation Corridor (SR-73) (Fig. 1). I-5 through this region was constructed from 1944 to 1958 and has an average annual daily traffic volume (AADT) of ~262 000 cars per day (California DOT 2009). SR-91 was originally completed in 1971 and underwent a major expansion in 1995. The AADT of this freeway is ~264 000 cars per day (California DOT 2009). SR-73 was constructed in 1996 and has an AADT of ~73 000 cars per day (California DOT 2009). Aside from these roads and adjacent urban development, the only potential natural barrier to bobcat movement is the Santa Ana River, which flows about 100 m to the north of SR-91. No other natural barriers to gene flow (i.e. major mountain ranges) exist between these habitat patches, which are primarily characterized by chaparral, coastal scrub and grassland vegetative communities.

A total of 106 bobcats were included in this study. The majority of bobcats ($n = 75$) were live-captured between December 2002 and March 2009 using wire cage traps baited with visual and odour attractants (Lyren *et al.* 2006, 2008a,b). Animals were anesthetized, and blood samples were collected. Animals were captured, sampled and released with permission of cooperating agencies after approval by all appropriate animal care and use committees. The remaining individuals ($n = 31$) were opportunistically sampled postmortem; when possible, heart blood clots, thoracic fluid, ear punch and hair samples were collected from these bobcats. Blood and tissues were stored at USGS facilities in Irvine, CA, and aliquots were sent to Colorado State University for analysis as described below.

Putative subpopulation assignments

Bobcats were assigned to one of four putative subpopulations based on the GPS coordinates of the capture or road kill location relative to freeways I-5, SR-91 and SR-73 (Fig. 1). These three freeways were the focus of our

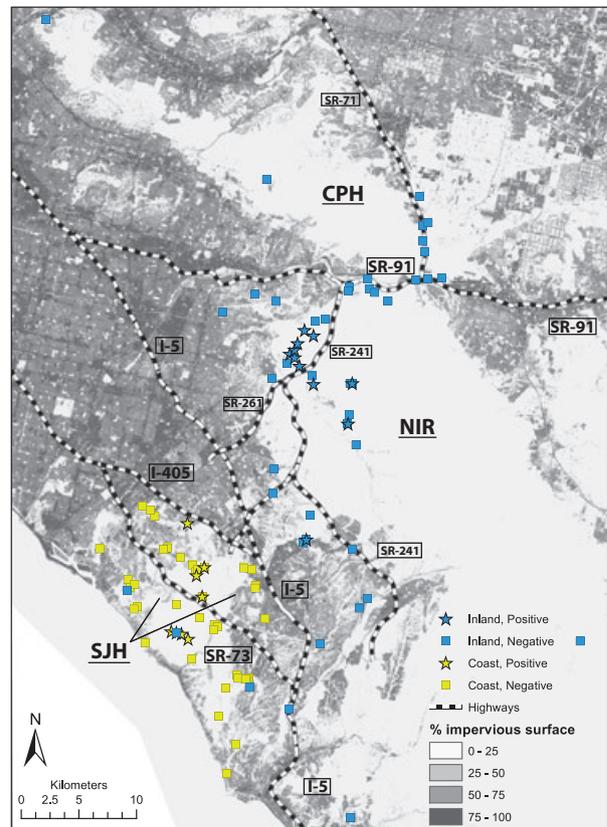


Fig. 1 Map of study locations southeast of Los Angeles, California. GPS capture locations of 105 bobcats indicated by squares [feline immunodeficiency virus (FIV) negative] or stars (FIV positive). Blue or yellow fill of each symbol represents the genetic assignment of an individual to an inland (east of I-5) or coastal (west of I-5) subpopulation, respectively (see Fig. 2). The a priori assignment of individuals to putative subpopulations (SJH-W: San Joaquin Hills West; SJH-E: San Joaquin Hills East; NIR: North Irvine Ranch; CPH: Chino Puente Hills) was based on the capture location of bobcats relative to the focal roads SR-73, I-5 and SR-91, which were investigated as potential barriers to gene flow. Degree of urban development (% impervious surface) is represented by grey shading.

investigation because they completely traverse the study area (i.e. animals cannot move between habitat fragments without crossing one of these freeways), and these roads represent a gradient of permeability to bobcat movements based on previous studies [see discussion and also Lyren *et al.* (2008a,b)]. The San Joaquin Hills west subpopulation [SJH-west ($n = 29$)] was located between the coast and SR-73, which merges with I-405 to the north and I-5 to the south. The San Joaquin Hills east subpopulation [SJH-east ($n = 20$)] included individuals sampled between SR-73 and I-5. The North Irvine Ranch [NIR ($n = 44$)] subpopulation comprised individuals captured east of I-5 and south of SR-91, whereas the Chino Puente Hills [CPH ($n = 12$)] subpopulation consisted of individuals captured east of

I-5 and north of SR-91. One individual did not have a recorded capture location, and thus, it was not included in analyses that required the above subpopulation assignments. The following population genetics analyses were, except where noted, performed using this a priori assignment of individuals to putative subpopulations and the multilocus microsatellite genotype data for each individual.

Genotyping microsatellites

Bobcat genomic DNA was extracted from whole blood, peripheral blood mononuclear cells (PBMCs) or tissue using QIAamp[®] DNeasy blood and tissue kit (Qiagen Inc., Valencia, CA, USA). Seventeen microsatellite loci (Table 1) were amplified using primer pairs for polymerase chain reaction (PCR) developed by Menotti-Raymond *et al.* (1999): FCA008, FCA023, FCA026, FCA031, FCA043, FCA045, FCA077, FCA090, FCA096, FCA132, FCA149, FCA559; Menotti-Raymond *et al.* (2005): FCA740, FCA742; and Faircloth *et al.* (2005): BCE5T, BCD8T, BCG8T. Primers BCE5T and BCG8T were modified to contain the M-13 sequence instead of the CAG sequence as published. We selected these primer pairs from the above publications based on the following criteria: longer repeat units, efficiency of amplification and maximal heterozygosity. The 5' end of the forward primer of each primer pair was modified with a 16-bp tail comprising the M-13 sequence

(5'-GTA AAA CGA CGG CCA G-3'). Reverse primers were not modified. All microsatellite PCR products were fluorescently labelled using a second forward primer consisting of the above M-13 sequence with 6-FAM on the 5' end.

PCR methodologies were adapted from Boutin-Ganache *et al.* (2001) and Riley *et al.* (2006). PCR reaction conditions included 94 °C for 3 min followed by 22 cycles of (94 °C for 30 seconds; 59 °C for 30 seconds; and 72 °C for 45 seconds), followed by 10 cycles of (94 °C for 30 seconds; 53 °C for 30 seconds; and 72 °C for 45 seconds), and a final cycle of 72 °C for 10 min. Randomly selected PCR products as well as the negative control for each reaction were visualized under UV light using gel electrophoresis with ethidium bromide in 2% agarose gel to confirm the presence of amplicons of appropriate length. Precise PCR product fragment lengths were determined using an ABI 3730xl DNA Analyzer and Peak Scanner 1.0 software (Applied Biosystems, Foster City, CA, USA). PCR and genotyping were repeated in ten per cent of randomly chosen bobcat samples for each microsatellite locus to confirm genotypes and prevent scoring errors. All duplicated genotypes were consistent with the primary analysis, demonstrating a high degree of assay reproducibility and reducing the likelihood of genotyping errors owing to false alleles or allelic dropout.

Table 1 Characterization of 17 microsatellite loci in 106 bobcats

Locus	Size range	No. of alleles	Repeat	Chromosome	H_O	H_E	PIC	References
FCA008	140–156	8	di	A1	0.71	0.77	0.73	1
FCA023	144–158	6	di	B1	0.67	0.72	0.67	1
FCA026*	138–166	13	di	D3	0.79	0.83	0.81	1
FCA031	237–255	8	di	E3	0.78	0.80	0.77	1
FCA043†	131–139	5	di	C2	0.78	0.73	0.68	1
FCA045*	147–173	7	di	A1	0.63	0.83	0.81	1
FCA077†	130–140	6	di	C2	0.77	0.74	0.70	1
FCA090*	108–126	7	di	A1	0.41	0.52	0.48	1
FCA096	189–209	8	di	A2	0.80	0.77	0.75	1
FCA132*	182–194	7	di	D3	0.66	0.78	0.74	1
FCA149	133–149	9	di	B1	0.76	0.78	0.75	1
FCA559	115–135	6	tetra	B1	0.64	0.67	0.60	1
FCA740	333–353	6	tetra	C1	0.84	0.79	0.76	2
FCA742	104–134	7	tetra	D4	0.65	0.67	0.61	2
BCD8T	156–180	5	tetra	Unknown	0.21	0.21	0.20	3
BCE5T	256–280	7	tetra	Unknown	0.70	0.75	0.71	3
BCG8T	275–299	11	di	Unknown	0.73	0.78	0.74	3

H_O , observed heterozygosity; H_E , expected heterozygosity; PIC, polymorphic information content.

*Null alleles may exist in one subpopulation.

†FCA077 and FCA 043 were found to be in linkage disequilibrium; FCA043 was not used in population genetics analyses.

References: (1) Menotti-Raymond *et al.* (1999); (2) Menotti-Raymond *et al.* (2005); and (3) Faircloth *et al.* (2005).

Validating and characterizing microsatellite data

Microsatellite data were screened for genotyping errors because of stuttering, null alleles and large allele dropout at all loci in MICROCHECKER 2.2.3 (Van Oosterhout *et al.* 2004). There was no evidence of errors because of stuttering or large allele dropout at any loci. The following three loci showed evidence of null alleles when testing across all individuals with no subpopulation information: FCA045, FCA090 and FCA132. The null alleles at these loci correspond to NIR (FCA045 and FCA090) and SJH-west (FCA132) when the same analysis was run with a priori subpopulation assignments.

Tests for linkage disequilibrium among loci were performed in GENEPOP 4.0 (Raymond & Rousset 1995; Rousset 2008). The results for linkage disequilibrium varied greatly between subpopulations with seven significant tests in SJH-west (120 total tests; $\alpha = 0.009313$), four significant tests in SJH-east (120 total tests; $\alpha = 0.009313$), two significant tests in NIR (136 total tests; $\alpha = 0.009102$) and one significant test in CPH (104 total tests; $\alpha = 0.009567$). Loci FCA077 and FCA043 were in linkage disequilibrium in all four putative subpopulations, and therefore, FCA043 was eliminated from further analyses.

Hardy-Weinberg equilibrium probabilities were calculated using GENEPOP 4.0 (Raymond & Rousset 1995; Rousset 2008). Three of four subpopulations significantly deviated from Hardy-Weinberg equilibrium at one unique locus: FCA023 in SJH-west (15 tests; $\alpha = 0.015068$), FCA045 in NIR (16 tests; $\alpha = 0.014790$) and BCE5T in CPH (16 tests; $\alpha = 0.014790$). The remaining loci in each subpopulation did not deviate from HW equilibrium, and therefore, all subpopulations were assumed to be in HW equilibrium. Observed and expected heterozygosity and the polymorphic information content (PIC) for each locus were determined using the program CERVUS 3.0 (Table 1) (Kalinowski *et al.* 2007).

Assessment of population structure

Population differentiation based on allele frequencies was calculated for each pair of putative subpopulations using GENEPOP 4.0 (96 tests; $\alpha = 0.0097$) (Raymond & Rousset 1995; Rousset 2008). Allelic richness, estimated using rarefaction to avoid bias caused by differences in sample size (Leberg 2002), was calculated for each putative subpopulation using FSTAT 2.9.3.2 (Goudet 1995). Allelic richness results were confirmed to be normally distributed using a Ryan Joiner Test in Minitab Student Version 14.11.1 (Ryan Joiner test; $P > 0.1$). Analysis of variance (ANOVA) was used to determine whether allelic richness differed significantly between subpopulations

($\alpha = 0.05$). Estimates of subpopulation differentiation (D_{est}) were calculated using the online program Software for Measurement of Genetic Diversity (Jost 2008; Crawford 2010). F_{ST} values were calculated in FSTAT (Goudet 1995). The use of F_{ST} values as measures of population differentiation has recently been criticized (Hedrick 2005b; Jost 2008). Therefore, we include them here as supplemental information only to allow a general comparison among similar, previously published studies (Table S1, Supporting information).

Bayesian clustering in program STRUCTURE 2.3.3 was used to infer the number of genetically distinct subpopulations (K) and to assign each individual to the subpopulation with which they share the highest genetic similarity. Parameters were set to include 50 000 burn-in and 500 000 Markov Chain Monte Carlo iterations (Pritchard *et al.* 2000). Data were first analysed without a priori source population information for individuals. Independent allele frequencies among subpopulations and genetic admixture were included as parameters so as not to introduce an upward bias in the estimation of K (Pritchard *et al.* 2000). This analysis was repeated five times for each K to verify the consistency of likelihood values between runs. K was varied from $K = 1$ to 5, representing a range of greater than expected K values to ensure our analysis included all ecologically plausible values of K .

This was followed by additional analyses with the data set divided into two groups: coastal animals (SJH-west and SJH-east) and inland animals (NIR and CPH) to more closely evaluate possible substructure within each of these two groups (Pritchard *et al.* 2010). The parameters of this model were the same as above, with each analysis repeated five times for each K from $K = 1$ to 3 for each group. For all of the above analyses, posterior probability values were computed for each K according to Pritchard *et al.* (2010). Additionally, ΔK values, which have been shown to accurately reflect the actual number of genetic clusters, were calculated according to Evanno *et al.* (2005).

Structure was also used to identify individuals that were captured in one subpopulation but genetically assigned to another and thus represent migrants. The parameters for this analysis were the same as described above except subpopulation assignments were included in the analysis with the migration prior set to 0.05. Individuals with a probability of assignment to their source population ≤ 0.01 were considered migrants. Individuals with ambiguous assignment probabilities were considered hybrids.

An individual pairwise relatedness test was performed after correction for null alleles using the program Maximum-likelihood (ML)-Relate (Kalinowski *et al.* 2006; Wagner *et al.* 2006; Carlsson 2008). The

average pairwise relatedness of each subpopulation was compared using a *t*-test.

Detection of FIV infection

All bobcats for which serum samples were available ($n = 91$) were screened for antibodies to FIV by Western blot as previously described (Franklin *et al.* 2007b). Of these, 24 (26.4%) were scored as 'weak positive' or 'positive' for FIV antibodies. PCR was used to confirm FIV infection in these samples (two to four PCR attempts per seropositive individual) using DNA extracted from whole blood or PBMCs. We used a set of degenerate nested primers, which was previously shown to amplify a region of the *RT-pol* gene from a diverse set of FIV isolates (Troyer *et al.* 2005). All bobcats that were not screened by Western blot ($n = 15$) were screened for FIV infection by the PCR method only. In total, 19 individual bobcat FIV isolates were amplified by PCR and included in the FIV genetic analyses.

FIV *pol* and *env* PCR amplification and sequencing

Two gene regions were analysed to evaluate viral phylogeny using both a highly conserved region (*RT-pol*, encoding the essential viral polymerase) and a region that is less evolutionarily constrained (*env*, encoding the surface envelope protein) (Pecon-Slattery *et al.* 2008). PCR amplification of a region of the *RT-pol* gene was performed using degenerate primers as previously described (Troyer *et al.* 2005). Primers to amplify a region of the *env* gene were designed by first performing an alignment of two previously published FIV sequences: PLV-14 [GeneBank (accession no. U03982)] isolated from a Florida panther (*Puma concolor coryi*) and PLV-1695 [GenBank (accession no. DQ192583)] isolated from a puma (*Puma concolor cougar*) in British Columbia. Degenerate nested primer pairs were designed from regions of homology including first-round primers mJLenvF1 (5'-GTG CAI GTC ATI AGA TGT AGA G-3') and mPLVenvR7 (5'-GGG GTG TCA TTA TAA IIA GTA AAA TT-3'), amplifying a fragment of ~700 bp, and second-round primers mPLVenvF8 (5'-GGG TGC ATT IGT IAA AGA ICC ATT TTT AG-3') and mPLVenvR6 (5'-GGT GCI TTG AAI GGA CAC ATT CC-3'), which amplified a 570-bp product. Underlined bases indicate 5' tail sequences added to the primers to lengthen primers and increase strength of primer binding to the template DNA.

Fifty microlitre PCR reaction mixtures contained 25 μ L iQSuperMix (Qiagen), 400 nM of each primer and 10 μ L DNA. DNA concentrations varied among samples resulting in a range of ~100–500 ng template per reac-

tion. PCR reaction conditions for both rounds included a hot start at 94 °C followed by 20 cycles of melting at 94 °C for 30 seconds, touchdown annealing temperatures ranging from 55 to 46 °C decreasing by 1 °C every 2 cycles for 30 seconds, extension at 72 °C for 30 seconds, followed by 25 cycles of melting at 94 °C for 30 seconds, annealing at 52 °C for 30 seconds and extension at 72 °C for 30 seconds with a final extension at 72 °C for 3 min. This protocol successfully amplified proviral *env* fragments from three FIV-positive bobcats.

The resulting sequences were aligned, and the regions of highest homology were used to develop the following nested primer pairs that successfully amplified *env* fragments from all remaining bobcats with amplified *pol* sequences ($n = 16$). First-round primers were envfw201 (5'-TTT CTC ATG TTC CTT GAA TGG TAC-3') and envrv202 (5'-CAC ATT CCA CTT AAT TGG TAT TG-3'), resulting in approximately a 450-bp amplicon. Second-round primers were envfw202 (5'-TGG TAC ATT CTG GGT GTT TAA ATC-3') and envrv201 (5'-CTA TTT TGG TCA CTC TCT GAT GC-3'), resulting in approximately a 400-bp product. PCR reagents and reaction conditions were the same as above with the exception that touchdown annealing temperatures ranged from 58 to 49 °C and the annealing temperature for the last 25 cycles was 54 °C. PCR products were visualized under UV light using gel electrophoresis with ethidium bromide in 2% agarose gel to confirm the presence of product bands.

PCR products were purified using the QIAquick PCR Purification Kit (Qiagen Inc.) prior to sequencing. Forward and reverse sequences were aligned using BLAST (National Center for Biotechnological Information, Bethesda, MD, USA), and a single consensus FIV sequence was produced for each infected bobcat. All sequences were verified manually. All sequences are available in the NCBI GenBank under accession numbers JN383436–JN383465.

Genetic alignments and phylogenetic analyses

Sequences were trimmed at the 5' and 3' ends resulting in all sequences having the same length (*pol* = 427 bp, *env* = 347 bp). Trimmed consensus sequences for each gene fragment ($n = 19$ *pol* & *env*) were converted to coding frame using an online DNA translator tool (Swiss Institute of Bioinformatics; <http://www.isb-sib.ch/>), prior to alignment in CLUSTAL X2 (Larkin *et al.* 2007). Alignments were input into jMODELTEST (Posada 2008) to estimate the best-fit model of nucleotide substitution, which was the TPM2uf model with among-site rate variation for both gene segments (Kimura 1981). The estimated model parameters used for *pol* were [Lset base = (0.4030 0.1312 0.1592) nst = 6 rmat = (7.3755

50.7381 7.3755 1.0000 50.7381) rates = gamma shape = 0.2280 ncat = 4 pinvar = 0]. The model parameters for *env* included [Lset base = (0.3707 0.1869 0.1840) nst = 6 rmat = (4.2249 11.3706 4.2249 1.0000 11.3706) rates = gamma shape = 0.2250 ncat = 4 pinvar = 0].

Maximum-likelihood phylogenetic analyses were conducted in Phylogenetic Analysis Using Parsimony (PAUP) (Sinauer Associates, Sunderland, MA, USA) (Swofford 2003). The corresponding *pol* and *env* gene regions from an FIV isolate sequenced from a Florida panther in 1994 were included to provide a root for each tree (Langley *et al.* 1994). ML trees were constructed using an NJ starting tree, followed by a heuristic search using the tree-bisection-reconnection branch-swapping algorithm. The *pol* and *env* trees were found to be congruent, and therefore, a single *pol-env* concatenated sequence was used to construct the final phylogenetic tree for analysis. Bootstrap analyses were performed with 100 iterations for all trees. Viral isolates were divided into four 'FIV Groups' based on clusters of related isolates arising from a basal node supported with an ML bootstrap value of 70 or greater.

We estimated the number of FIV migration events between coastal and inland bobcat subpopulations by calculating the *s* statistic for the observed ML phylogenetic tree in Mesquite 2.75 (Maddison & Maddison 2011). The *s* statistic reflects the minimum number of parsimony steps that explain the discord between subpopulations as monophyletic groups on the tree (Slatkin & Maddison 1989).

To estimate the timeline of past virus transmissions within and between subpopulations, the concatenated viral sequence data were analysed in the coalescent framework as implemented by the program BEAST 1.6.2 (Drummond & Rambaut 2007). The SDR06 substitution model was used with a relaxed uncorrelated lognormal molecular clock (Drummond *et al.* 2006). The tree model included a piece-wise linear Bayesian Skyline prior with five groups and a randomly generated starting tree (Drummond *et al.* 2005). An initial run of 10 000 000 Markov chain Monte Carlo (MCMC) iterations, sampled every 1000 runs, was performed to estimate model parameter values. The first 10% of logged values were discarded as burn-in. From this analysis, the following model parameter priors were changed from default settings: (i) the relative rate parameters were set to vary from 0 to 10; (ii) the Bayesian Skyline population size was set to vary from 0 to 500; and (iii) the mean rate for the uncorrelated relaxed molecular clock was set to vary from 0 to 10.

The final analysis included the above settings with 50 000 000 MCMC iterations sampled every 1000 runs. The first ten per cent of logged values were again dis-

carded as burn-in. The estimated values and associated effective sample size (ESS) for each model parameter were viewed in TRACER 1.5 (Rambaut & Drummond 2007). ESS values for all parameters were >500. The maximum clade credibility tree was produced in TREEANNOTATOR 1.6.2 (Rambaut & Drummond 2002). The resulting tree was viewed in FIGTREE 1.3.1 (Rambaut 2006), and the mean posterior probability heights with 95% highest posterior density (HPD) intervals were labelled on internal nodes.

Results

Bobcat population structure

Distribution of alleles. An analysis of population differentiation performed in GENEPOP indicated that the distribution of alleles among bobcats from the two coastal subpopulations, SJH-west and SJH-east, differed significantly ($P = 0.0064$) at only the FCA008 microsatellite locus. Therefore, the coastal bobcats, regardless of whether they were captured east or west of SR-73, had a similar distribution of alleles at 15 of the 16 microsatellite loci examined. Similarly, the two inland subpopulations, NIR and CPH, significantly differed in allelic distribution at only FCA026 ($P = 0.0025$) and FCA077 ($P = 0.0048$). This finding demonstrates that bobcats captured north and south of SR-91 had a similar distribution of alleles at 14 of the 16 microsatellite loci we analysed. However, the distribution of alleles differed greatly between the coastal and inland subpopulations. SJH-west bobcats significantly differed from NIR and CPH bobcats at 13 and 10 microsatellite loci, respectively, and SJH-east bobcats differed from both NIR and CPH bobcats at 13 loci ($P < 0.01$ all significant pairwise tests). These results indicate a high degree of genetic differentiation exists between bobcats separated by I-5 and its associated urban development.

Genetic diversity. No pairwise difference existed in allelic richness (Table S2, Supporting information) when comparing the two coastal subpopulations ($F_{1,29} = 2.59$, $P = 0.118$) or the two inland subpopulations ($P = 0.982$, $F_{1,30} = 0.00$). However, the coastal bobcats (combined SJH-W and SJH-E) had significantly lower allelic richness than the inland bobcats (combined NIR and CPH) ($P < 0.001$, $F_{1,62} = 12.62$). This further suggests that coastal and inland bobcats are genetically distinct and indicates that coastal bobcats have lower genetic diversity than inland bobcats. Interestingly, the BCD8T locus appears to have drifted to fixation in coastal bobcats as only one allele was sampled at this locus from all 49 coastal bobcats. Although the frequency of this allele was also high in NIR (0.82) and CPH (0.67), four and

five alleles, respectively, were present at this locus in these subpopulations.

Departures from random mating. The overall estimate of genetic differentiation among the four putative subpopulations, $D_{est} = 0.11$, indicates a moderate amount of genetic structure exists within this population. Pairwise D_{est} values were lowest when comparing the two coastal subpopulations ($D_{est} = 0.008$, SJH-west:SJH-east) or the two inland subpopulations ($D_{est} = 0.014$, NIR:CPH) and highest between the coastal and inland subpopulations ($D_{est} = 0.11$, SJH-west:NIR; $D_{est} = 0.13$, SJH-west:CPH; $D_{est} = 0.14$, SJH-east:NIR; $D_{est} = 0.19$, SJH-east:CPH). The estimated differentiation between the coastal bobcats (combined SJH-W and SJH-E) and the inland bobcats (combined NIR and CPH) was $D_{est} = 0.14$. Locus-specific D_{est} values are reported in Table S2 (Supporting information).

We found no evidence of inbreeding within the overall population ($F_{IS} = 0.013$; 95% CI: -0.29 – 0.065), nor in any of the putative subpopulations [$F_{IS} = (0.011, \text{SJH-W}; -0.045, \text{SJH-E}; 0.029, \text{NIR}; 0.034, \text{CPH})$].

Individual assignment tests. Bayesian clustering indicated the assumption of two genetically distinct subpopulations ($K = 2$) best explained the variation in our microsatellite data (Fig. S1, Supporting information). This result was the same regardless of whether or not source population information was used as a prior in the anal-

ysis. All bobcats caught east of I-5 (NIR and CPH bobcats $n = 56$) were assigned to one 'inland' subpopulation, while 46 of 49 bobcats caught west of I-5 (SJH-west and SJH-east bobcats) were assigned to a 'coastal' subpopulation (Fig. 2). Three individuals captured west of I-5 were genetically assigned to the inland subpopulation and thus represent possible migrants. However, no bobcats captured east of I-5 were genetically assigned to the coastal subpopulation. If any additional human development and/or freeways in this region (i.e. SR-91, SR-73, SR-241, SR-261) were causing genetic structure, a population model with $K > 2$ should have had the highest support. Therefore, the I-5 corridor is the only human development in this region implicated as a cause of genetic structure among bobcats during our period of sample collection.

Three individuals were identified as first-generation migrants as they were captured in the coastal area but had assignment probabilities of 1.00 to the inland population when capture locations were included in the Structure analysis (denoted by * in Fig. 2b). Three other bobcats captured in the coastal area had the genetic profile of hybrids with partial assignment to both subpopulations (denoted by # in Fig. 2b). No individuals sampled from the inland area were identified as migrants or hybrids from the coastal area.

Relatedness of individuals. The average relatedness of bobcats in the coastal population ($R = 0.096$; $SE = 0.0037$)

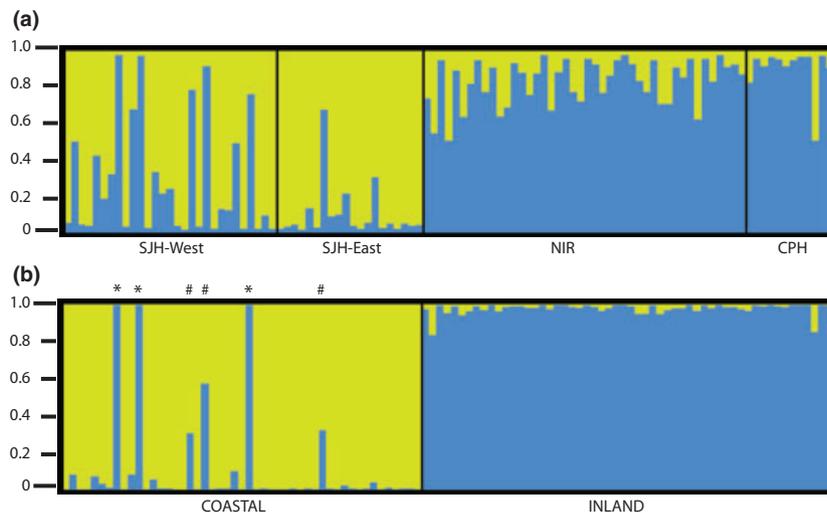


Fig. 2 Individual bobcat genetic assignments to each of two distinct subpopulations. Each vertical bar represents one individual. Values on the y -axis are the probability of assignment of each individual to one of the two genetic groups identified (Fig. S1). The shading of each bar corresponds to the probability of genetic assignment to either the coastal group (yellow) or the inland group (blue) of bobcats. (a) Results for simulation of $K = 2$ without including a priori capture locations. The majority of individuals captured west of I-5 had strong assignment to one subpopulation (coastal—yellow), while all of the individuals captured east of I-5 had a high probability of assignment to a second subpopulation (inland—blue). (b) Three migrants (*) and three hybrids (#) were identified in Structure using a priori assignments to coast or inland subpopulations based on capture locations. All gene flow was detected from the inland to the coastal subpopulation.

was significantly higher ($t_{5634} = 7.23$; $P < 0.001$) than in the inland population ($R = 0.064$; $SE = 0.0026$).

FIV phylogenetic analyses

We amplified a 427-bp region of *pol* and 347-bp region of *env* by PCR from 19 bobcats (four in SJH-west, four in SJH-east and 11 in NIR) bobcats. These included 17 of the 24 (70.8%) bobcats putatively seropositive by Western blot, one bobcat that was negative by Western blot and one bobcat that was screened by PCR only. The inability to amplify FIV sequences from a subset of putatively seropositive bobcats is similar to the findings of previous studies (Troyer *et al.* 2005; Franklin *et al.* 2007b). This is probably the result of a difference in the sensitivity and specificity of these two assays and/or a relatively low FIV proviral load present in a subset of infected animals.

The demographic information for 18 of the 19 FIV-positive bobcats was known: 13 were adult males, one was a yearling male, two were adult females and two were yearling females. The age-specific prevalence of FIV was 14.2% (3/21) for yearlings and 23.8% (15/63) among adults.

The ML phylogenetic tree built from *pol-env* concatenated gene sequences (Fig. 3) shows no evidence of association between capture location (coastal vs. inland) and FIV relatedness (see also Fig. 4). The basal nodes have low bootstrap support, suggesting little overall genetic structuring within the virus population. The number of parsimony steps (*s* statistic) that best explains the discord of FIV relatedness between subpopulations indicates a minimum of three FIV transmission events have occurred between the coastal and inland subpopulations (Slatkin & Maddison 1989).

We estimated the past timeline of virus movement within and between bobcat subpopulations using a coalescent Bayesian model (Drummond *et al.* 2005; Drummond & Rambaut 2007). Figure 5 illustrates the maximum clade credibility tree with labels representing the estimated year of coalescence (mean posterior node ages) for each internal node. The age of the basal nodes is difficult to interpret because of large 95% HPD intervals (analogous to 95% confidence intervals). Therefore, it is impossible to reconstruct a precise timeline of ancestral divergence into the observed FIV groups. However, the more terminal nodes are insightful as they depict the estimated year of recent FIV coalescent events. FIV Group 4, the largest supported group of isolates, comprises six isolates from inland bobcats (x23, x24, x27, x31, x37 and x55) and two isolates from coastal bobcats (x240 and x46). The two coastal isolates in this group share a common ancestor with two of the

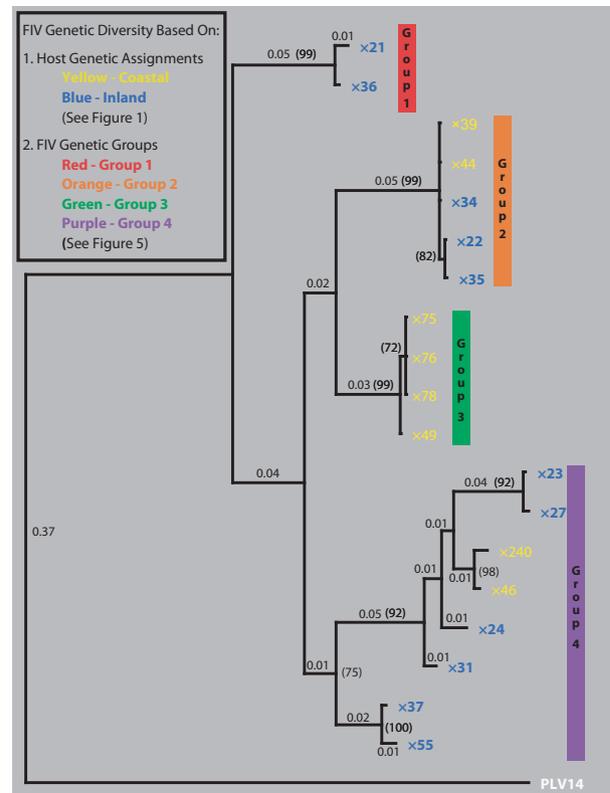


Fig. 3 Maximum-likelihood phylogenetic tree of feline immunodeficiency virus (FIV) concatenated *pol-env* sequences from 19 infected bobcats. The tree was constructed using a single 774-bp sequence (427 bp from *pol*, 347 bp from *env*) of proviral DNA from each individual. All sequences are in coding frame. Bootstrap values >70 are indicated in parentheses. Branch lengths ≥ 0.01 are also indicated. Individuals have been coloured to represent their genetic assignment based on the results of the *Structure* analysis (see Figs 1 and 2). Because no migrants were infected with FIV, the colour of an individual also represents whether an individual was captured west (yellow) or east (blue) of I-5. FIV isolates were assigned to groups based on the four most basal supported nodes to illustrate the distribution of FIV genetic diversity across the landscape (See Fig. 4). The tree is rooted with PLV-14, a viral sequence from the same FIV clade, which was sequenced from a Florida panther in 1994.

inland isolates (x23 and x27) in *c.* 1990 (Fig. 5; 95% HPD: 1967–2003). Similarly, FIV Group 2, with 99% bootstrap support, contains nearly identical sequences from three inland bobcats (x22, x34 and x35) and two coastal bobcats (x39 and x44), which share a common ancestor in approximately the year 2000 (Fig. 5; 95% HPD: 1995–2003).

Feline immunodeficiency virus Group 3 contains four closely related viral isolates (Fig. 3; x75, x76, x78 and x49) from related coastal bobcats ($r > 0.25$). To our knowledge, this is the first evidence of FIV familial transmission among bobcats. Familial transmission of

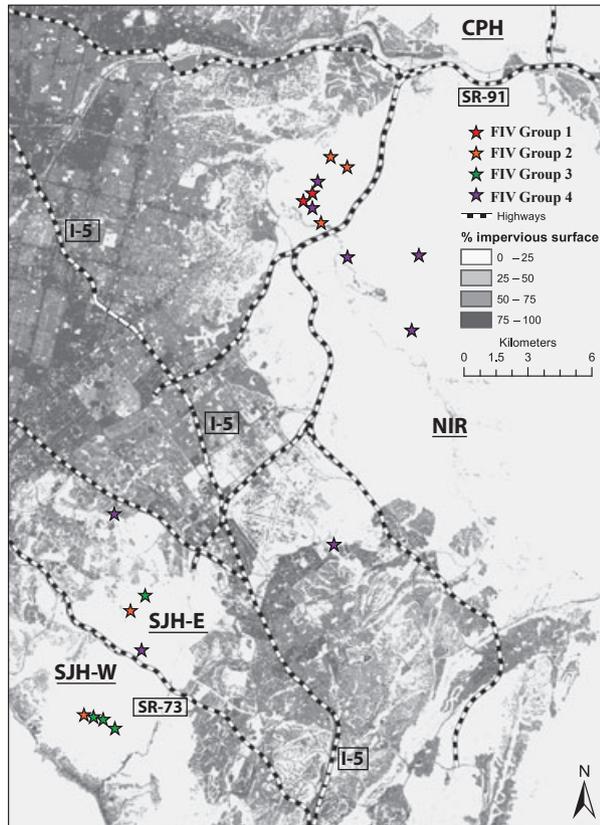


Fig. 4 Geographical distribution of feline immunodeficiency virus (FIV) genetic diversity among inland and coastal bobcats. Each star indicates the capture location of one FIV-positive bobcat. The stars are coloured corresponding to which of the four groups of related FIV isolates was sampled from each bobcat (See Fig. 3). FIV Groups 1 (red) and 3 (green) contain isolates sampled only from inland or coastal bobcats, respectively. FIV Groups 2 (orange) and 4 (purple), containing both inland and coastal bobcats, resulted from the movement of FIV across Interstate-5. Degree of urban development (% impervious surface) is represented by grey shading.

FIV has been previously documented in mountain lions (Poss *et al.* 2008).

Discussion

Bobcat population structure

Understanding the degree of connectivity among populations in heterogeneous landscapes is an important goal of ecology, population genetics and conservation biology (Taylor *et al.* 1993; Crooks & Sanjayan 2006; Fischer & Lindenmayer 2007). We evaluated host and pathogen genetic markers to investigate connectivity among bobcats throughout a fragmented urban landscape in southern California. Our results indicate that two genetically distinct groups of bobcats existed in our

study area, defined as coastal and inland subpopulations, separated by urban development including Interstate-5 (Figs 1 and 3). This finding is in agreement with other analyses performed previously with a small subset of these bobcats and four of the 16 microsatellite loci utilized in this report (Ruell *et al.* in press).

Our results indicate that the coastal and inland bobcat subpopulations had a different distribution of alleles at most of the microsatellite loci examined and a low, unidirectional pattern of migration from the inland to the coastal area. We also observed reduced genetic diversity and increased relatedness among individuals in the coastal population. These findings suggest the observed genetic differentiation is because of decreased migration through the urban matrix between the inland and coastal habitat patches. We therefore conclude that urban development, including I-5, has been a physical barrier that has reduced bobcat movement and gene flow between isolated groups of individuals.

We did not detect substructure among bobcats separated by the two other freeways that we specifically evaluated, SR-91 and SR-73 and nor did we find evidence of genetic structure because of any other freeways (i.e. SR-241, SR-261) or human development in the region. The maintenance of gene flow across SR-91 and SR-73 is probably explained by the fact that these roadways are perforated by more functional wildlife underpasses and are bordered by more natural habitat than I-5. Therefore, the distance between habitat patches is shorter across SR-91 and SR-73 than across I-5, a factor that has been shown to be an important determinant in carnivore movement among habitat fragments in this region (Crooks 2002). It is possible that because SR-91 and SR-73 are newer roads, any isolation these may be causing has not yet resulted in detectable genetic structure.

Our findings, revealed by analyses of empirical genetic data, are supported by observations from remotely triggered cameras placed near underpasses of all three focal roadways. Cameras placed near the only potential wildlife corridor under I-5 that directly links SJH-E to NIR did not document any movement of bobcats between these habitat patches during 204 consecutive days of observation (Lyren *et al.* 2008a). Cameras, however, did not monitor another potential path across I-5 connecting NIR to SJH-W to the south; road kill carcasses and models of connectivity both suggest this path may be utilized by bobcats (Lyren *et al.* 2008a). In contrast to the lack of remote camera observations of bobcat movement across I-5, a similar duration of camera monitoring of potential wildlife corridors under SR-91 documented many successful movements between NIR and CPH (E. E. Boydston, unpublished data). Likewise, multiple bobcat movements between

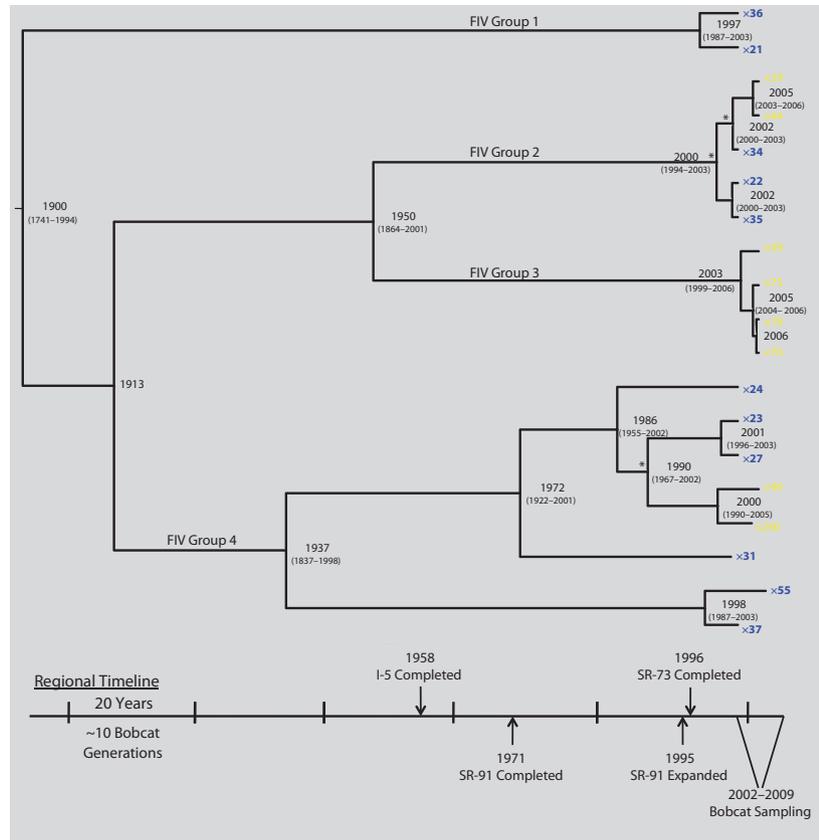


Fig. 5 The estimated timeline of viral coalescent events. Maximum clade credibility Bayesian phylogenetic tree of *pol-env* concatenated sequences constructed using dated tips. Internal node labels correspond to the highest mean posterior probability estimate [with 95% highest posterior density (HPD) interval] for the year of coalescence. The estimated dates on basal nodes lack precision; therefore, it is impossible to accurately reconstruct the timeline of ancestral diversification into the current four-group structure of feline immunodeficiency virus (FIV). Estimates of recent coalescent events are more precise and depict contemporary FIV transmission within and between bobcat subpopulations. *Indicates common ancestor to contemporary isolates sequenced from both inland and coastal bobcat isolates. Viral movement across I-5 has probably occurred since the estimated date of these coalescent events, resulting in the presence of related viral strains sampled in both subpopulations (See Fig. 4).

SJH-W and SJH-E were also recorded during 358 days of camera observations near SR-73 (Lyren *et al.* 2008b).

Interstate-5 and the surrounding urban matrix have greatly increased in size over time since the original construction was completed in 1958. While it is difficult to know when I-5 became a 'barrier' to bobcat movement, Crooks (2002) estimated that the coastal San Joaquin Hills might have become effectively isolated from inland natural areas around 1980. Given the generation time of bobcats (*c.* 2 years) (Knick *et al.* 1985), between 12 (*c.* 1980) and 25 (*c.* 1958) generations have passed because the inland and coastal bobcat subpopulations became physically isolated.

FIV phylogenetic analyses

Directly transmitted pathogens are inextricably linked to their hosts, and therefore, the geographical distribu-

tion of pathogens reflects the movement and contacts of their hosts throughout the landscape. We analysed two gene sequences from FIV, a retroviral pathogen of bobcats, to evaluate whether the gene flow (transmission) of the virus revealed information about bobcat movement not discernable from traditional host genetic analyses. We hypothesized that urban development, which limits bobcat gene flow, would also limit viral transmission between bobcat subpopulations. Given that only one in five bobcats are infected with FIV, the expected virus migration rate is five times lower than the host migration rate. Thus, we expected each of the two bobcat subpopulations would be infected with a genetically distinct viral strain, divergent from one another owing to years of isolation, low viral migration rates and the high mutation rate of FIV.

This prediction is consistent with previous literature demonstrating that geographical or social barriers,

which prevent mixing between neighbouring host subpopulations, result in genetically structured retrovirus populations (Franklin *et al.* 2007a; Liu *et al.* 2008; see also Fig. S2, Supporting information—demonstrating clear divergence between FIVs isolated from bobcats north or south of Los Angeles). However, the phylogenetic trees constructed in this study from FIV gene segments demonstrated a pattern that differs from these previously published findings. The relatedness of FIV isolates is mixed among coastal and inland bobcat subpopulations, indicating that there is no association between FIV relatedness and the geographical/genetic structure of its host (Figs 3 and 4). While this finding differs from our prediction based on the ecology of FIV, it is consistent with previous studies of urban bobcats in southern California, which have found that individual movements (and thus opportunities for disease transmission) between fragmented habitat patches occur more frequently than predicted by observed levels of gene flow (Riley *et al.* 2006; Ruell *et al.* in press).

The topology of the ML phylogenetic tree (Fig. 3) illustrates that neither the coastal nor the inland bobcats are infected with a monophyletic cluster of viruses. Instead, we identified four groups of related FIV isolates but found no evidence to suggest these groups developed because of the population structure of bobcats. While two of the four groups contained only coastal or inland isolates (FIV Groups 1 and 3), these were the smallest groups sampled and the other two FIV groups did not follow this pattern. Instead, FIV Groups 2 and 4 contained closely related viruses arising from both bobcat subpopulations. The coastal isolates within each of these two groups shared recent common ancestry with inland viruses (Fig. 5), and thus, we conclude that FIV-infected bobcat migration events across I-5 are responsible for the observed mixing of isolates. Half of the viruses infecting coastal individuals (4/8) recently originated from, or were transmitted to, inland bobcats. The former is likely to have occurred in FIV Group 2 as suggested by the presence of multiple inland isolates basal to the two coastal isolates and evidence of a long Group 2 residence time within the inland subpopulation.

Utilizing host and pathogen genetics

The presence of related FIV strains on both sides of I-5 suggests that the intervening urban development is somewhat permeable to bobcat movement and disease transmission, despite the presence of distinct genetic structure among the host population. There may be several explanations for the discrepancy between the population structure of FIV and its host. One hypothesis is that a recent increase in bobcat migration across I-5 has

led to the observed mixing of viral genotypes, but not enough time has passed for this recent increase in migration to counteract previously established genetic structure among the bobcats. While possible, we consider this unlikely as human development along the I-5 corridor has increased over time, and no notable changes have been made in the area (e.g. underpasses, culverts) that would account for a recent increase in connectivity between the two subpopulations.

An alternative hypothesis is that FIV exchange between the two subpopulations is not necessarily linked to bobcat gene flow. Transient movements of individuals across a semi-permeable barrier such as I-5 may not result in the exchange of genetic material, but may involve sufficient contact between individuals to allow for disease transmission. Under this hypothesis, the contrasting patterns of population structure may reflect differences in the underlying ecology of the two molecular markers.

Microsatellite markers are useful for detecting host gene flow, the specific process involving animal movement which results in the exchange of genetic material from one group of individuals to another (Endler 1977). According to this definition, gene flow is dependent upon successful mating after migration. FIV transmission, however, can occur both vertically *and* horizontally, allowing FIV isolates to move between individuals and subpopulations in the absence of gene flow. For example, Biek *et al.* (2003) reported that for one population of mountain lions, horizontal transmission among adults resulted in the majority of new FIV infections and accounted for the observed increasing prevalence of FIV with age. Vertical transmission, resulting in a cohort of young individuals infected prior to adolescence, was equally important in explaining the dynamics of FIV in the population.

Given that bobcats and mountain lions share many life history characteristics, it is probably that similar FIV transmission dynamics occur in bobcats. Indeed, the relative prevalence of FIV in yearlings (14%) vs. adults (24%) in this study is similar to the age-prevalence relationship described in Biek *et al.* (2003). Young infected individuals may therefore play an important role in the maintenance and spread of FIV in this population. At adolescence, juvenile (usually male) bobcats often make transient movements during dispersal from their natal range while attempting to establish a new home range (Kitchings & Story 1984; Knick 1990; Hansen 2007). Young bobcats infected prior to dispersal therefore represent a potentially important mode of virus movement within and between subpopulations.

In fragmented landscapes such as southern California, where urban development and freeways act as boundaries limiting animal movement, bobcat home

ranges may shrink, and the amount of overlap between neighbouring home ranges may increase (Riley 2006; Riley *et al.* 2006). This pattern of 'home-range pile-up' has been described in other bobcat populations in California (Riley *et al.* 2006). This phenomenon decreases the probability that juveniles dispersing to a neighbouring subpopulation will successfully mate. Under these conditions, the actual rate of bobcat movements, contacts and opportunities for disease transmission between subpopulations would be higher than expected based on migration rates estimated from gene flow.

The low level of gene flow we detected across I-5 occurred in a unidirectional pattern from the inland area towards the coast. The coastal population, while reduced in overall genetic diversity, contains both migrants and hybrids from the inland population. This suggests that, while rare, inland bobcats can successfully migrate to and breed in the coastal population, while the reverse was not seen. However, inland FIV isolates do not form a monophyletic group; two of the three FIV groups infecting coastal bobcats also infect inland bobcats. There are two possible explanations to this pattern. One is that movement of FIV has occurred repeatedly one-way from the inland subpopulation to the coast, causing the diversity among coastal isolates to closely mirror that observed inland. This hypothesis is consistent with the one-way pattern of bobcat movement we detected from the microsatellite analysis.

The other possibility is that a coastal virus population diverged from inland viruses after the two groups were isolated by urban development and has since been transmitted back into the inland subpopulation multiple times via transient movements and contacts resulting in disease transmission but not gene flow. Tracking the movements of individual bobcats, evaluating FIV diversity in other neighbouring bobcat populations and utilizing spatially explicit phylogeographic analyses may help to distinguish between these alternatives.

In conclusion, our findings indicate that bobcats inhabiting this fragmented landscape in southern California are physically isolated and genetically structured. This pattern is consistent with decreased connectivity across urban development, resulting in low levels of migration and/or a low probability that migrants establish a home range and successfully mate. However, movements are apparently made, allowing for disease transmission between these habitat fragments. This conclusion carries with it conservation implications as populations with these characteristics are susceptible to decline as a result of a continued loss of genetic diversity from genetic drift and decreased individual fitness because of inbreeding depression (Frankham 2006). In addition, both subpopulations should be managed as a

unit when considering treatment and prevention interventions during future disease outbreaks. Habitat conservation and restoration as well as connectivity enhancements such as functional underpasses to better facilitate movement of bobcats under roadways may help safeguard their persistence in the face of substantial ongoing threats posed by humans in this region.

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The authors are interested in research involving ecology, conservation, and/or infectious diseases of carnivores. They share particular interest for topics relating to the effects of human activities on carnivores.

Data accessibility

DNA sequences—GenBank accession numbers JN383436–JN383465. Microsatellite data—DRYAD entry doi:10.5061/dryad.mmm317th7.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 F_{ST} values calculated from 16 bobcat microsatellite loci.

Table S2 Measures of genetic structure among bobcats calculated from 16 microsatellite loci.

Fig. S1 Estimating the number of genetic subpopulations (K) of bobcats.

Fig. S2 Maximum-likelihood phylogenetic tree built from *pol* sequences using the same model parameters as those described to produce the Maximum-likelihood tree in Fig. 4 (see Methods).

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