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Population genomic structure of a widespread, urban-dwelling mammal: The eastern grey squirrel (*Sciurus carolinensis*)

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Abstract

Urbanization is a persistent and widespread driver of global environmental change, potentially shaping evolutionary processes due to genetic drift and reduced gene flow in cities induced by habitat fragmentation and small population sizes. We tested this prediction for the eastern grey squirrel (*Sciurus carolinensis*), a common and conspicuous forest-dwelling rodent, by obtaining 44K SNPs using reduced representation sequencing (ddRAD) for 403 individuals sampled across the species' native range in eastern North America. We observed moderate levels of genetic diversity, low levels of inbreeding, and only a modest signal of isolation-by-distance. Clustering and migration analyses show that estimated levels of migration and genetic connectivity were higher than expected across cities and forested areas, specifically within the eastern portion of the species' range dominated by urbanization, and genetic connectivity was less than expected within the western range where the landscape is fragmented by agriculture. Landscape genetic methods revealed greater gene flow among individual squirrels in forested regions, which likely provide abundant food and shelter for squirrels. Although gene flow appears to be higher in areas with more tree cover, only slight discontinuities in gene flow suggest eastern grey squirrels have maintained connected populations across urban areas in all but the most heavily fragmented agricultural landscapes. Our results suggest urbanization shapes biological evolution in wildlife

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species depending strongly on the composition and habitability of the landscape matrix surrounding urban areas.

KEYWORDS
contemporary evolution, ddRAD, eastern grey squirrel, evolution, gene flow, habitat fragmentation, population genomics, population structure, *Sciurus carolinensis*, urbanization

1 | INTRODUCTION

Anthropogenic landscape alteration is an increasingly pervasive outcome of global environmental change. Urbanization is among the most extreme forms of landscape alteration, converting extensive vegetated areas to infrastructure to support high densities of people (e.g. buildings, transportation networks). The transformation of native vegetation to urban land cover in cities is commonly predicted to decrease population size and dispersal, ultimately increasing the strength of genetic drift within animal and plant populations and reducing gene flow between populations (i.e. urban fragmentation model; Miles et al., 2019). Under this fragmentation model, effective population size is reduced in small habitable spaces physically isolated by a network of roads and buildings, ultimately eroding genomic variation (e.g. Munshi-South et al., 2016). In support of this model, a recent review found that North American mammals tend to have lower effective population sizes and genetic diversity in urban environments compared to rural environments (Schmidt et al., 2020).

In contrast to the fragmentation paradigm, some species thrive in urban areas by exploiting novel resources and having high potential for dispersal, including human-mediated transport. For these species, the urban facilitation hypothesis proposes that urbanization decreases the strength of drift within populations and instead increases gene flow between populations (Miles et al., 2019). Large-scale tests of this model across multiple cities have focused largely on non-native species highly dependent on urban resources (Blair, 2016; Kark et al., 2007; Shochat et al., 2006). For example, Carlen and Munshi-South (2021) found extensive gene flow of pigeons (*Columba livia*) across the megacity in the eastern United States, with genetic clustering likely driven by discontinuities in urban land cover. In contrast some native and non-native species thrive in urban areas but also use non-urban landcover (Blair, 2016; Kark et al., 2007; Shochat et al., 2006). Given their ability to use resources across multiple habitat types, these species may have extensive genetic connectivity across large spatial scales, encompassing both cities and non-urban landscapes.

An intriguing model for examining the degree of genetic connectivity for species that span their range across urban and forested areas is the eastern grey squirrel (*Sciurus carolinensis*). This species is common in forested environments throughout the eastern United States and southeastern Canada, a region that has undergone dramatic deforestation and, in some areas, subsequent reforestation over the past three centuries (Leyk et al., 2020; United Nations, 2019). Although extensive landscape change often leads to genetic isolation, such an outcome may have been mitigated in *S. carolinensis* due in part to its adaptability to the novel environments presented by urbanization. Historically restricted to rural woodlands, *S. carolinensis* was introduced to city parks starting in the 19th century (Benson, 2013) or colonized them directly, in part due to the species' high potential for long-distance natal dispersal (Perlut, 2020), including episodic mass migration events (e.g. Flyger, 1969; Seton, 1920; Shorger, 1947). The species' dietary flexibility (Parker & Nilon, 2008) and high fecundity (Koprowski, 1994) also enable the grey squirrel to function as a habitat generalist using a broad range of forest types in both urban and forested areas. Today, *S. carolinensis* densities are ~2.5 times greater in urban than non-urban areas (Koprowski et al., 2016).

Despite the ability of grey squirrels to colonize new environments via both natural and human-mediated dispersal, along with the species' high intrinsic rate of population growth in rural forests and developed cityscapes, fragmentation of tree cover is known to constrain *S. carolinensis* movements and distribution (Fidino et al., 2021; Goheen et al., 2003). A previous study suggested *S. carolinensis* has limited spatial genetic structure (Moncrief et al., 2012), but this study was limited by a narrow spatial extent and focus on mitochondrial DNA, which has reduced the scope for detecting evolutionary responses to recent landscape change. Exploration across additional loci could reveal signatures of more contemporary effects due to anthropogenic landscape change.

To test whether *S. carolinensis* has limited genetic structure across urban and non-urban areas, we investigated genome-wide patterns of neutral variation throughout much of the species native geographic range. We screened ~44K single nucleotide polymorphisms (SNPs) generated through double digest reduced representation (ddRAD) across 403 individuals from 17 urban sampling sites (Figure 1) to assess genome-wide differentiation, population structure, and the effect of the landscape configuration on gene flow. Given the range-wide scale of our study and likely effect of isolation by distance, we did not expect *S. carolinensis* to comprise a single panmictic population. However, because of its ability to thrive in urban areas, we predicted any genetic clustering across the species range would include multiple urban areas separated by non-urban areas. We also predicted that gene flow among populations would be facilitated by forest land cover and limited by agriculture where forest cover is limited.

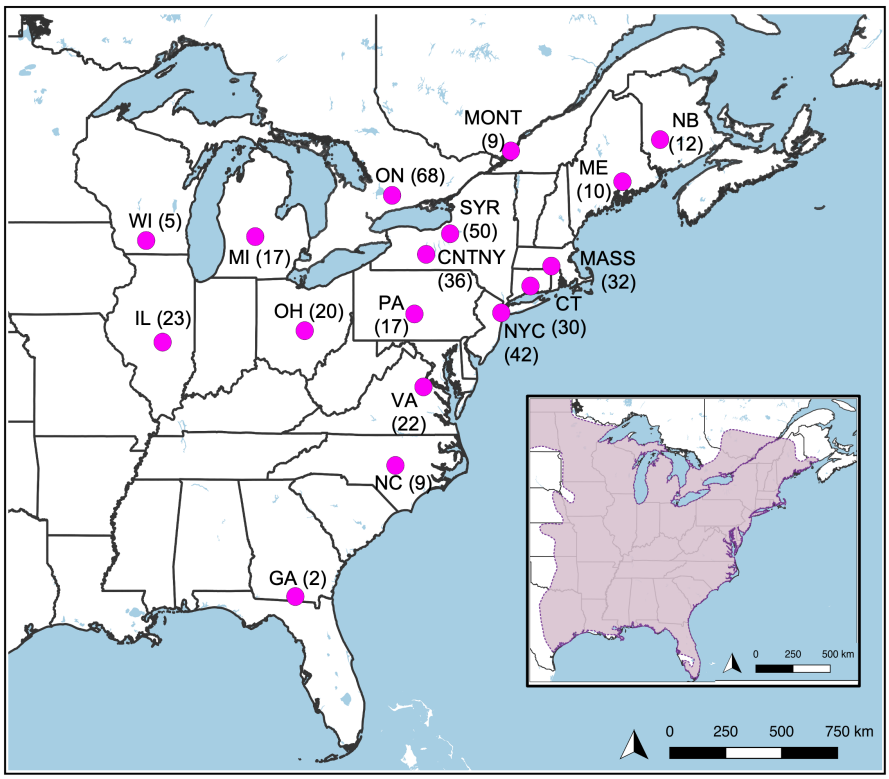


FIGURE 1 Map showing 17 geographic locations (as pink circles) sampled for eastern grey squirrels (*Sciurus carolinensis*). The map shows state-shape boundaries (black lines) for the US states and Canadian provinces and water bodies (in blue). Each a priori sample grouping is labelled with an abbreviation in capital letters and with the sample size within parentheses: WI: Wisconsin, USA, MI: Michigan, USA, IL: Illinois, USA, OH: Ohio, USA, ON: Ontario, Canada, MONT: Montreal, Quebec, ME: Maine, USA, NB: New Brunswick, Canada, CNTNY: Central, New York, USA, SYR: Syracuse, New York, USA, PA: Pennsylvania, USA, MASS: Massachusetts, USA, CT: Connecticut, USA, NYC: New York City, USA, VA: Virginia, USA, NC: North Carolina, USA, GA: Georgia, USA. The pink shading on the inset map represents the native range of *S. carolinensis*. [Colour figure can be viewed at wileyonlinelibrary.com]

2 | MATERIALS AND METHODS

2.1 | Samples

DNA was extracted from ear tissue, muscle, or blood samples of grey squirrels collected throughout the species' native range as salvaged roadkill, hunted, or live-trapped individuals (Table S1) retrieved by scientific collaborators as well as pest control agencies and wildlife rehabilitation centres (see Acknowledgements section) under required permits and IACUC approval (see Appendix S1).

2.2 | Sample processing

Genomic DNA was extracted from 1 to 2 cm pieces of tissue or 10 μL of blood from squirrel samples using the Qiagen DNEasy Blood & Tissue kit, generally following the manufacturer's protocol (Qiagen, Inc., Valencia, CA), with the addition of RNAse treatment and final elution volume of 200 μL (with modifications as found in Fusco et al., 2020). Approximately 1000 ng of genomic DNA was digested with *SpHI-HF* and *MluCI* and ligated to 48 individual barcoded adapters containing four degenerate base pairs to aid in PCR duplicate filtering during downstream analysis. Samples were pooled and purified with Serapure magnetic beads (Faircloth & Glenn, 2011). DNA fragments between 455 and 523 bp were size-selected on a 2% gel using a Sage Science Blue Pippin (Sage Science, Beverly, MA). We amplified samples by PCR using Phusion Polymerase Kit (New England Biolabs, Ipswich, MA) for 13 cycles with Illumina-specific indexing primers, and libraries were checked for quality on an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA). Library pools were sequenced at the Yale Center for Genomics using the NovaSeq 6000 resulting in paired-end 2 × 150 bp reads.

2.3 | SNP genotyping

We processed sequencing reads using the STACKS v4.3e bioinformatic pipeline (Rochette & Catchen, 2017). Reads were demultiplexed and aligned to the *S. carolinensis* genome (Mead et al., 2020) using BWA and sorted with SAMTOOLS (Li et al., 2009), then genotyped using STACKS *--ref_map* script. Additional filtering within STACKS included filtering for PCR duplicates (using *--clone_filter*) only, processing loci if present in 75% of individuals and across 75% of populations, reducing paralogs (*max-obs-het* = 0.8), and filtering for minor allele frequency (*min-maf* = 0.05). PLINK 1.9 beta (Chang et al., 2015) was then used to reduce 'missingness' by removing individuals with >5% missing genotypes (*--geno*) and only retained loci with >80% genotyping rate (*--mind*). Only the first SNP per locus was retained (*--write-single-snp*) to minimize the risk of analysing linked SNPs, and we used the filtering pipeline from Dorant et al. (2020) to retain only 'singleton' SNPs thus reducing SNPs duplicated via copy number variation. Last, we performed relatedness filtering using PLINK's *--genome* flag (PLINK 1.9 beta; Chang et al., 2015) to remove one individual per highly related pair with an identity-by-descent proportion >0.5 (full-sibling or parent-offspring relationship; Anderson & Weir, 2007).

2.4 | Statistical analyses

2.4.1 | Range-wide genetic diversity and population differentiation

Although we collected samples throughout much of the species native range, the samples were not uniformly distributed. We chose $n = 17$ a priori population groupings based on sampling locations to explore grey squirrel population structure and named each group based on the state/province or city within which most samples were collected (Figure 1). Samples were collected from two common colour morphs, grey ($n = 329$) and melanic ($n = 74$), a polymorphism with a simple Mendelian pattern of inheritance at the melanocortin 1 receptor gene (Mc1R; McRobie et al., 2009). To ensure the two morphs could be grouped together for subsequent analyses, we tested for, and found, no genomic differences between colour morphs outside of Mc1R (see Figure S1A–D and Table S2). Thus, all analyses were completed by pooling samples from both colour morphs at each sampling location. Across these 17 sample groupings, we investigated population genetic parameters by calculating measures of observed heterozygosity (H_O), nucleotide diversity (p), and Wright's Fixation Index (F_{ST}).

2.4.2 | Population structure and genetic connectivity analyses

To evaluate spatial patterns of genetic differentiation across the study area we tested for isolation-by-distance (IBD) with a Mantel test (Smouse et al., 1986). For this analysis we used the *bed2diffs* function from Estimated Effective Migration Surface analysis (Petkova et al., 2016) to calculate the genetic difference between individuals (similar to proportion of shared alleles). Because our samples were collected over a large expanse of this species' range, and real populations are fluid and often discontinuously distributed, we chose an individual-based metric for exploring IBD to avoid bias in the estimates of genetic distance (Shirk et al., 2017). We described the spatial scale over which the IBD relationship was significant using a Mantel correlogram with the R package *ecodist* (Goslee & Urban, 2007).

To test whether genetic clustering occurred across both urban and non-urban areas, we used a clustering model in ADMIXTURE 1.23 (Alexander et al., 2009) to examine the spatial genetic structure of our 17 sampling groups (Figure 1). The clustering model uses multi-locus genotypes to estimate each individual's ancestry proportions (q-values) in each of K genetic clusters. We ran the analysis on 403 individuals for values of $K = 1$–20 for 10 iterations at each K value, and we identified the most well-supported number of genetic clusters based on the lowest cross-validation (CV) error value. To support this analysis and evaluate if similar genetic clusters were retrieved by a multivariate approach, we also performed DAPC (Discriminant Analysis of Principle Components) analysis with the R package *adegenet* (Jombart & Ahmed, 2011). We first used the package's *find.clusters* function to calculate the Bayesian information criterion (BIC) and select the most likely number of genetic clusters, as DAPC requires predefined groups. The value of K that minimized the BIC was chosen as the most likely number of genetic clusters. We then used the *optim.a.score* function to select the optimal number of principal components to retain for the DAPC.

To visualize population structure and estimate gene flow across the sampled areas we used Estimated Effective Migration Surfaces (EEMS; Petkova et al., 2016). This method uses information from a combined spatial and genetic dataset to simulate effective migration across a grid under a stepping-stone model (i.e. the local migration of individuals between demes–hexagonal neighbouring subpopulation units), providing estimates of the relative rate of effective migration between demes. We conducted analyses across a grid composed of 500 demes and used a burn-in of 2,000,000 and Markov chain Monte Carlo (MCMC) length of 8,000,000 for model convergence. Spatial visualizations of the migratory surface were generated using R scripts provided by the *EEMS.PLOT* function from the rEEMSplots package (https://github.com/dipetkov/eems).

To assess the independence of and/or connectivity between these 17 urban sampling sites we also estimated migration rates across the grey squirrel range with BayesAss3-SNPs (BA3-SNPs; Mussmann et al., 2019), a program able to handle large SNP datasets. We used its autotune program to tune the model acceptance parameters ($\Delta m = 0.100$, $\Delta a = 0.4375$, and $\Delta f = 0.0125$) to target MCMC acceptance rates between 0.35 and 0.45 (as suggested by the authors). BA3-SNPs was run on the full SNP dataset (~44K) for 10,000,000 iterations with a burn-in period of 1,000,000 at 100 sampling intervals. Tracer v.1.7.2 was used to verify the convergence of the Bayesian model and parameter values over multiple generations (Rambaut et al., 2018). The R package *circilize* was used to visualize migrations events via a chord diagram (https://jokergoo.github.io/circlize_book) and on a map.

2.5 | Landscape genetics

We used a landscape genetics approach to test for associations between gene flow and landscape features. Land cover data were acquired from the North American Land Change Monitoring System dataset (Multi-Resolution Land Characteristics Consortium – Wickham et al., 2014) at 30 m resolution and resampled to 150 m due to computational limitations encountered from evaluating such a wide geographic range. We assessed the influence of landscape features on gene flow by creating four distinct landscape models: three single-surface models to assess the effect of agriculture, urban, or forest cover alone on genetic distance, and one composite model that included all land cover types (agriculture, urban, forest, and geographic distance). We hypothesize that agriculture and urban land cover will hinder gene flow, whereas forest will aid gene flow for grey squirrels. These models

were tested against a null model of IBD (Table S6). We prepared raster surfaces in ArcGIS. For single-surface models, we set the cell value of the landscape feature of interest to 10 and all other cells to 1 to test the effect of each landscape feature on gene flow (Arredondo et al., 2018). For the full model we assigned arbitrary numeric values for each cell type to simply distinguish between landscape features, where the numeric values are placeholders that carry no magnitude. Thus cells containing forest were given an arbitrary value of 3, agriculture a value of 10, urban a value of 5, and all other cells a value of 1. We optimized single surface and composite surface resistance values using the genetic algorithm in *ResistanceGA* (Peterman, 2018) by calculating pairwise effective distances with commute distance (random walk commute time which represents the effective distance between points averaged over multiple pathways – Peterman et al., 2019). We fitted maximum likelihood population effects parameters using *ResistanceGA* to determine whether select landscape features (agriculture, urban, forest, or geographic distance – IBD) affect the gene flow of *S. carolinensis*. We used individual-based genetic distance measures calculated with the *bed2diffs* function from the EEMS analysis (Petkova et al., 2016) as the response variable for the Maximum Likelihood Population Effects (MLPE) models. We compared the results of model selection to assess which environmental variables were most associated with gene flow. We ranked models using the Akaike model criterion corrected for small sample size (AIC_c) by the lowest AIC_c value being the most plausible model (Burnhan & Anderson, 2002), along with those that were within $\Delta AIC_c < 7$ (Zuur et al., 2009). We also evaluated model fit using marginal R^2 (R^2m) to understand the variance explained by the highest ranking of all the models tested (Nakagawa & Schielzeth, 2013). We used the top-ranked resistance surface to visualize functional connectivity across the grey squirrel geographic range (Figure S5) by creating a cumulative current density map in CIRCUITSCAPE (McRae et al., 2008).

3 | RESULTS

3.1 | Genotyping summary

Reduced representation sequencing produced 9.8 million genotyped loci with an average effective per sample coverage of 12× for 403 individual squirrels. After filtering we created a 44,458 SNP dataset.

3.2 | Range-wide analyses

3.2.1 | Genetic diversity and population differentiation

Average genetic diversity across sampled locations (H_O; observed heterozygosity) was moderate (0.189). Estimates of inbreeding (F_{IS}; inbreeding coefficient) were also low (mean = 0.04; Table S3). Genetic differentiation among the 17 sampling locations across the species' range spanned 0.008–0.164 (Weir-Cockerham F_{ST} Index- Figure S2).

3.2.2 | Population structure and genetic connectivity

Genetic differentiation among 403 squirrels increased with geographic distance ($p < .05$) up to 63 km (Figure S3A,B), but the variance explained was very low (*adjusted* $R^2 = .01$). The most-supported clustering model from ADMIXTURE included five genetic clusters across the species' range (lowest CV error value, $K = 5$; Figure S4; Figure 2a). Cluster 1 mostly included samples from the upper Great Lakes region (Wisconsin, Chicago, Illinois, and the Michigan Upper Peninsula). Cluster 2 comprised samples from central Michigan and Ontario; with samples collected in the city of Kingston, Ontario containing pure Ontario ancestry and those from central and southern Ontario and central Michigan containing admixed ancestry. Cluster 3 included individuals from Ohio and central New York. Individuals sampled between western New York and Pennsylvania were characterized by admixed ancestry. Cluster 4 showed a distinct grouping of individuals sampled from Syracuse, New York. Last, cluster 5 comprised nearly half the squirrels in our sample set, with individuals distributed along the northeastern Atlantic coast from New York City to New Brunswick and inland to Montreal, Quebec (Figure 2a,b). The five major clusters differed in genetic admixture levels (Table S4). Clusters 2 and 3 included both individuals from specific cities with very high q-values ($q > 0.9$) and individuals with more mixed ancestries, where the majority assignment was to their respective cluster ($q > 0.40$). The greatest admixed ancestry was found for individuals sampled from the middle of the species' range, from central New York through Pennsylvania, Virginia, and the most southerly sampled US states of North Carolina and Georgia. These admixed populations assign the largest portions of their ancestry to cluster 3 (from ~17% to 90% ancestry–yellow) and/or cluster 5 (~9%–30% ancestry–teal; Figure 2a,b).

Although $K = 5$ had the lowest CV-error value, $K = 4$ was a more parsimonious model with similar support as $K = 5$ (Figure 2b, Figure S4). At $K = 4$, individuals could be distinguished among those sampled from Canada, the Midwestern United States, the north central part of the species' range, and the larger cluster along the Northeast coastal region also identified in the $K = 5$ model. The DAPC analysis separated the individuals from Montreal, Quebec, the Canadian Province New Brunswick, and Maine (axis 1) from the rest of the samples (axis 2; Figure 3a), while axis 3 separated the individuals from Canada (Kingston, Ontario, southern Ontario, and Montreal, Quebec) and Ohio from the rest of the United States samples (Figure 3b).

Estimated Effective Migration Surface analysis revealed greater than expected levels of migration, specifically a geographic area of high genetic connectivity spanning across the eastern coastal cities,

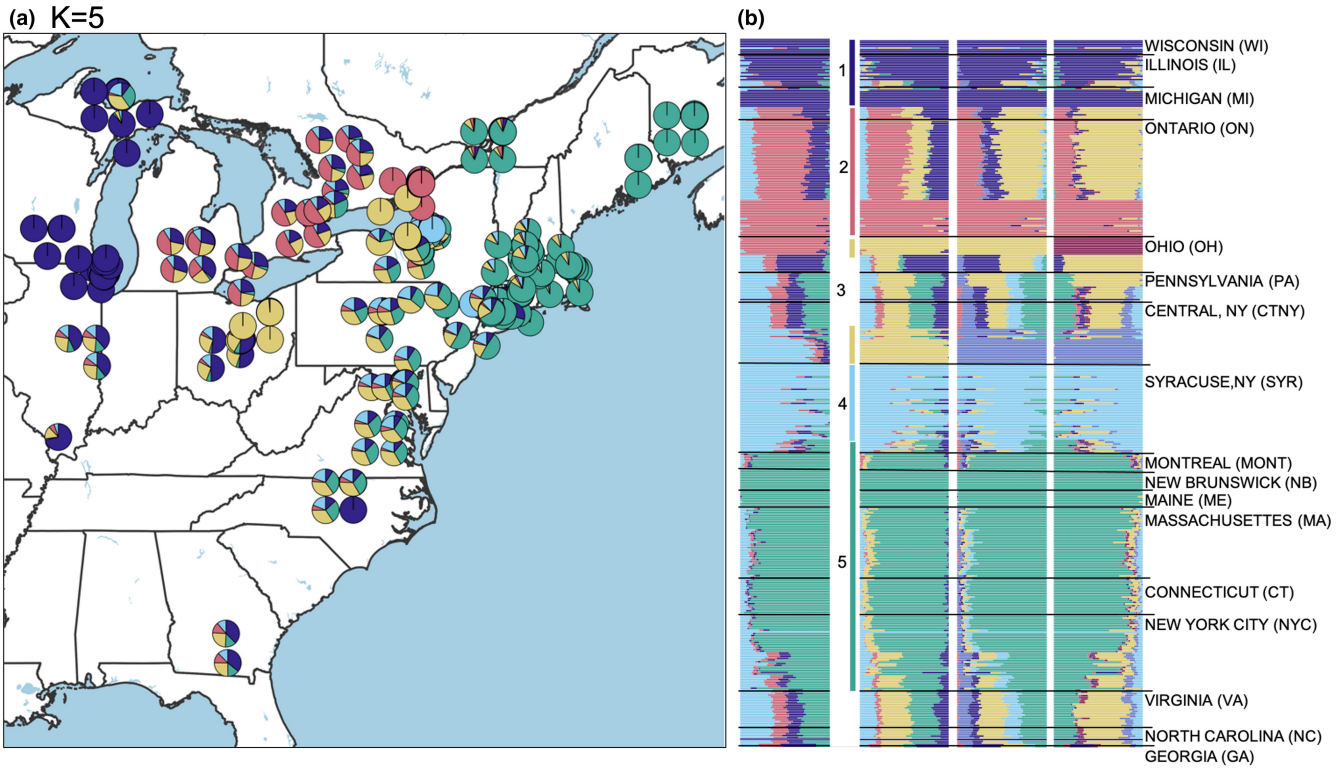


FIGURE 2 (a) ADMIXTURE analysis showing percent ancestry represented as pie charts at the geographic location for each sampled individual, shown at the most well-supported result; $K=5$ clusters. (b) Structure plots showing each individual's proportion of ancestry (horizontal lines varied by colour) for $K=4$–7 genetic clusters (all of which had similar cross-validation error values). The main clusters (for $K=5$) are labelled by a vertical colour block and number on the left side of the $K=5$ bar plot. Labels of a priori groupings for sampled individuals are shown on the right of the structure plots using the sampling group's full name and abbreviation. [Colour figure can be viewed at wileyonlinelibrary.com]

as well as an area between Pennsylvania and Ohio (Figure 4). Lower than expected migration was found within Illinois, central Ohio, at the eastern edge of Pennsylvania, and along the Canadian border with New York (Figure 4). BayesASS3-SNPs analysis revealed 14 recent migrants (Figure 5a) with greater than 50% probability of being a migrant from another sampling locality (Table S5). Most ($n=11$) were short distance migration events occurring between neighbouring localities, including eight individuals collected in Connecticut likely to be migrants from neighbouring Massachusetts and New York. All the long-distance migration events (total $n=3$) involved the movement of single individual squirrels, one east to west from New York City to Illinois, another north to south from Pennsylvania to North Carolina, and the last and farthest west to east from Illinois to Pennsylvania (Figure 5a,b).

3.2.3 | Landscape genetics

Using MLPE modelling to control for the random effect of population level differences, the most supported landscape model (with the lowest AIC_c value) included forest cover, which explained 15% of the variance in genetic distance between individuals. Since the matrix resistance value assigned by *ResistanceGA* was greater than the feature resistance value given, this suggests extent of forest cover influences genetic differentiation by acting as a conduit to gene flow among eastern grey squirrel populations across the sampled area (Table 1; Figure S5).

4 | DISCUSSION

Our results show moderate to high levels of genetic diversity are maintained within populations of eastern grey squirrels, coupled with low levels of inbreeding, as expected for a mobile, generalist species. Eastern grey squirrels experience high levels of genetic connectivity across large geographic expanses. This includes connectivity across urban and non-urban areas, particularly among cities along the Atlantic coast. While genetic connectivity among urban areas supports the urban facilitation model, some portions of the range showed limited gene flow, including between some cities and within the agricultural Midwest. This contrasts with previous findings based on mtDNA data that indicated a historical lack of spatial phylogeographic structure (Moncrief et al., 2012) albeit in a study that used different markers and sampled individuals only within the southern part of the species' range. The population structure we see across wide geographic scales is determined to a small degree by dispersal limitation given the modest but significant role of isolation-by-distance.

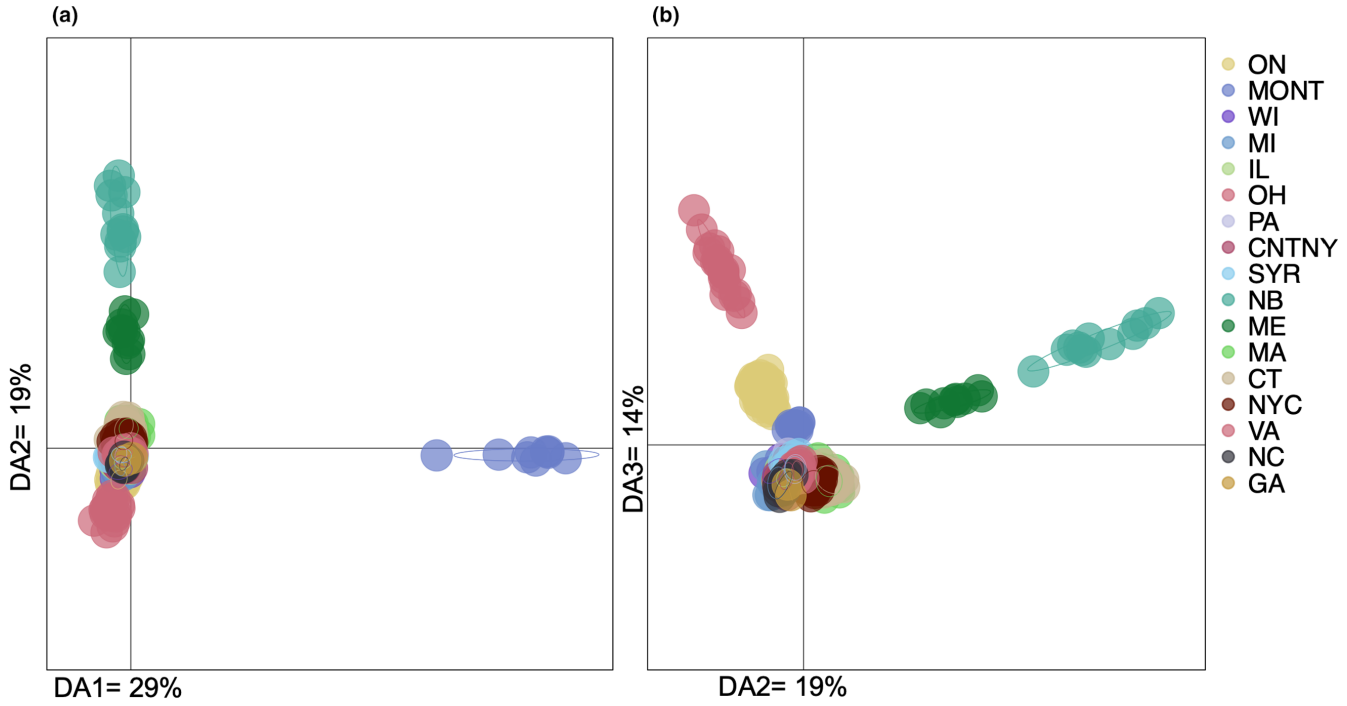
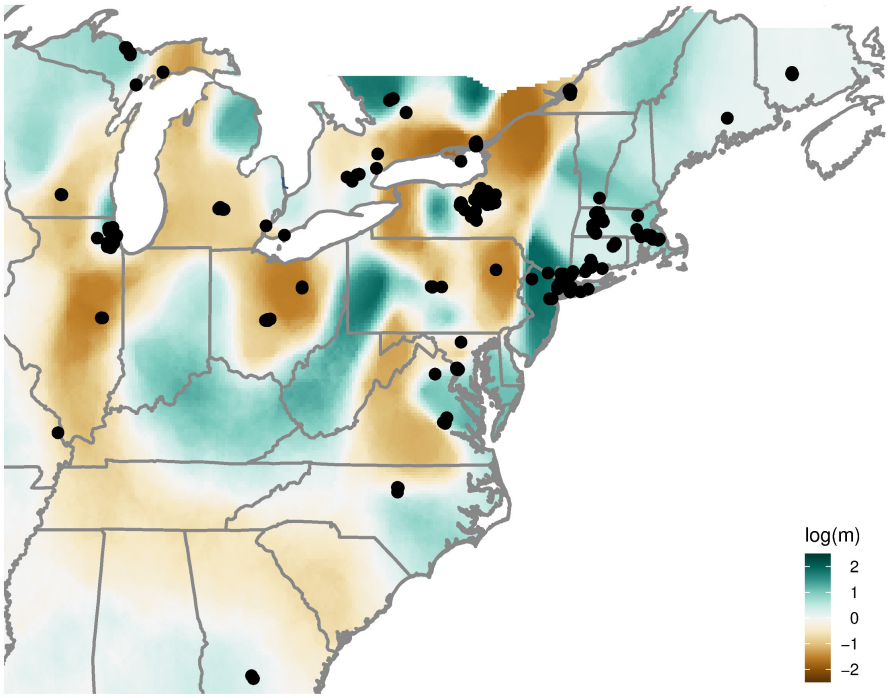


FIGURE 3 Discriminant analysis of principal components (DAPC) using ~44K SNPs for eastern grey squirrels sampled ($n = 403$) displaying genetic differentiation based on sample groupings (different coloured points are individuals and ellipses signify the sample grouping). Results are displayed across, (a) DA axis 1 (horizontal line) and DA2 (vertical line) and (b) DA2 (horizontal line) and DA3 (vertical line). Sample points farther apart signify greater genetic differentiation and sample points closer together signify greater genetic similarity between individuals and groups. [Colour figure can be viewed at wileyonlinelibrary.com]

FIGURE 4 Estimated Effective Migration Surface (EEMS) across the geographic range of the eastern grey squirrel. Collection location for each individual is represented as a black dot, and the colours signify either greater than expected migration (*darker green* – the probability of m is statistically significantly greater than the mean rate of migration), areas with uniform migration (*white* – isolation by distance) and areas of less than expected migration (*darker brown* – the probability of m is statistically significantly less than the mean rate of migration). [Colour figure can be viewed at wileyonlinelibrary.com]



Comparable studies of the neutral genetic evolution of grey squirrels in areas where the species has been translocated outside their native range have been conducted using microsatellite loci (Dominguez McLaughlin et al., 2022; Signorile et al., 2014). Invasive grey squirrel populations across Europe (UK, Ireland, and Italy) show low levels of genetic diversity possibly due to founder effects, with a few isolated populations (specifically in central Ireland; Dominguez McLaughlin et al., 2022). We found moderate genetic diversity

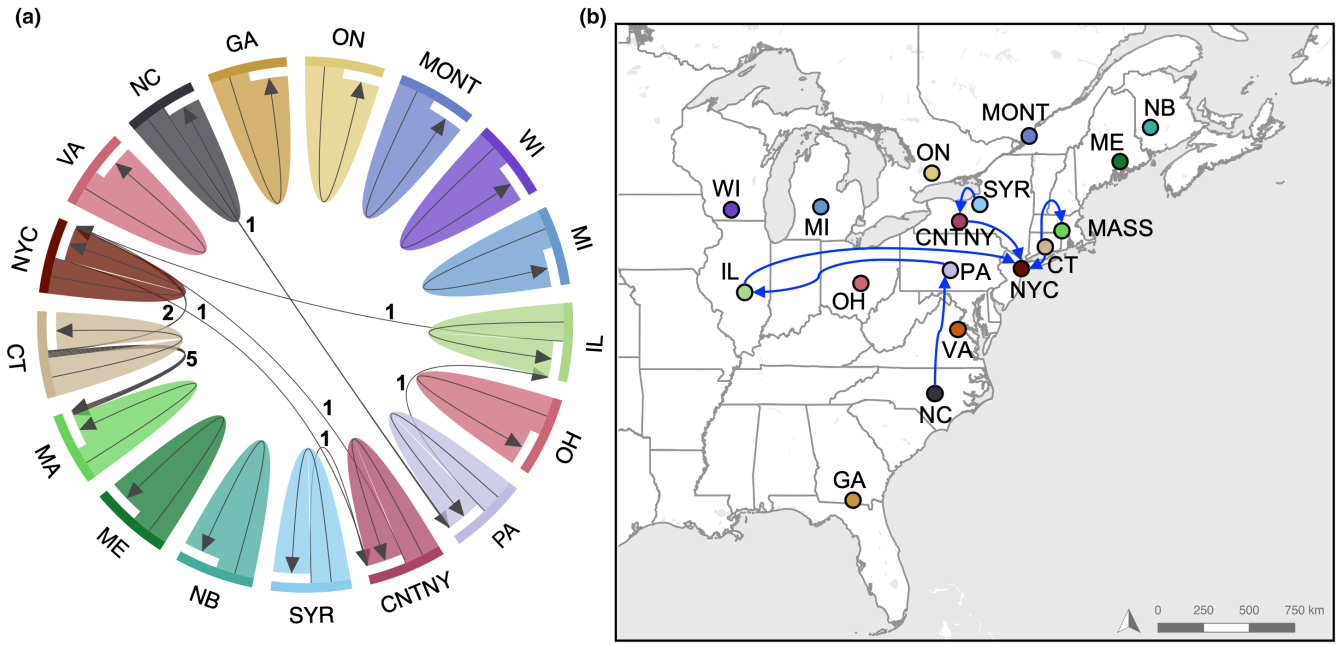


FIGURE 5 The results of BayesAss3-SNPs migration events based on ~44K SNP ($n = 403$ individual grey squirrels). (a) Depicted by a chord diagram and (b) on a map. Colour indicates a priori sample grouping (labelled with group abbreviation), and arrows indicate direction of migration; either out to another location (labelled with number of migrants), or internally within their group (arrow within the coloured area). AIC_c, Akaike information criterion value corrected for small sample size; R^2m, marginal R^2; ΔAIC_c, the calculated difference between that model and the most well-supported model. [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Landscape genetic results output table for the MLPE models run in *ResistanceGA* for the range-wide dataset ($n = 403$ individuals) using five landscape resistance models. [Colour table can be viewed at wileyonlinelibrary.com]

Landscape feature	Feature resistance	Matrix resistance	Direction of relationship to gene flow	AIC_c	ΔAIC_c	R^2m
Forest	1.00	1.57	Conduit	−517261.62	0	0.15
Agriculture	1.19	1.00	Barrier	−516922.06	339.56	0.15
Null IBD	–	–		−516863.64	58.42	0.15
Urban	1.02	1.00	Barrier	−516862.03	1.60	0.15
Full model	–	–		−498483.10	18,378.93	0.19

Note: These landscape resistance models include Agriculture, Forest, Null IBD, Urban, and the Full model; a composite model including forest, urban, agriculture. The results of model selection are ordered as lowest to highest for AIC_c value, with models highlighted in blue ($\Delta AIC_c < 7$) considered having an effect on genetic differentiation between individual grey squirrels. Features that acted as conduits to gene flow had a lower resistance value compared to the surrounding landscape matrix resistance. Opposingly, those that had a matrix resistance value lower than the feature resistance acted as barriers to gene flow.

(Table S3), suggesting sustained genetic connectivity within the species' native range (Figures 2a,b and 4). European studies of the eastern grey squirrel as an invasive species showed ample gene flow across similar geographic expanses (Ireland and England) compared to what we found within the northeastern genetic cluster (cluster 5- Eastern US). Our clustering analyses support high levels of gene flow across grey squirrel populations, especially along the Atlantic coast of northeastern United States, mainly with highly connected areas across the Northeastern megacity corridor extending north towards Canada across a vast afforested landscape. Our results parallel a similar analysis carried out on non-native but well-established pigeon populations (*C. livia*) across the same Northeastern corridor of the United States (Carlen & Munshi-South, 2021) in which high levels of genetic connectivity were observed from Boston, MA to Washington, DC. This similarity is not surprising as syn-urbanized taxa, such as eastern grey squirrels (Engel et al., 2020) and pigeons (Hensley et al., 2019), possess biological attributes that facilitate occupying human-altered landscapes (Hulme-Beaman et al., 2016) and assist in sustaining populations and maintaining gene flow despite habitat fragmentation.

Yet, our results also showed that some areas of the grey squirrel's native range, despite the potential for genetic mixing, harbour genetically distinct population clusters. For example, there is limited connectivity between two cities: Columbus and Wooster, Ohio. Squirrels in Columbus, Ohio, were more closely related to squirrels in the Midwest (Michigan, Wisconsin, Illinois- cluster 1) >600 km away,

than to squirrels in Wooster, Ohio (cluster 3) only ~160 km away (Figure 2a,b). Perhaps these populations genetically differ due to different founding populations followed by a subsequent lack of gene flow between cities. Known introductions of melanic squirrels have occurred across the range, such as Kent State University grounds in Kent, Ohio, in 1961 (*Kent State Historical Society*). Notably, there is also a very high prevalence of melanism in Wooster, Ohio, but not in Columbus (B. J. Cosentino, unpublished data), suggesting potential differential introduction history between Wooster and Columbus. Also, the absence of major dispersal barriers (e.g. road or river) suggests that forest fragmentation driven by increasing agricultural land use in central Ohio (relative to the eastern United States) may be limiting gene flow. A closer examination of genetic differentiation between such cities may help us understand what is causing these differences. We also found non-urban areas across the Midwestern states had lower than expected migration rates between sampled locations than in other areas of the species' range (Figure 4). This could be due to the high levels of uninterrupted agricultural land found specifically in this area which may constrain squirrel dispersal. Studies have found that agricultural land reduces dispersal for grey squirrels between habitat patches (Goheen et al., 2003), where larger connected woodlots are necessary to maintain connectivity (Nupp & Swihart, 2000).

At the range-wide scale presence of forest was the most important landscape factor (among those measured) mediating genetic differentiation between individual eastern grey squirrels across the range. As hypothesized, the presence of forest cover was a conduit to gene flow between populations, potentially because this landscape feature is the species' primary habitat for food and shelter. Likely, urban and agricultural landscapes act as a barrier to gene flow in *S. carolinensis* given they both contribute to the reduction and fragmentation of forest cover (Figure S5). Land cover effects on gene flow can be scale-dependent (e.g. Burgess & Garrick, 2021), and additional work is needed to reveal if results will differ at the finer spatial scale across this range. Including more variables and performing landscape genetic analyses across cities that showed limited genetic connectivity with their nearby suburban and rural populations could reveal how specific landscape features affect gene flow.

Altogether we found areas of both high and low connectivity among cities across a wide spatial scale within the native range of eastern grey squirrels, underlying the complexity of the evolutionary and ecological factors shaping the spatial genomic patterns for species that thrive in both urban and non-urban habitats. In a recent review, Miles et al. (2019) provided evidence that wildlife responses to urbanization are species-specific, where urbanization tends to either facilitate or hinder movement between populations, with responses highly dependent on both variation in the species life-history traits and the variability in the heterogeneous landscapes. Our range-wide analysis of eastern grey squirrels demonstrates how spatial genomic structure can be context dependent, with strong genetic connectivity among cities in some regions and strong fragmentation in others. In particular, our results suggest that introduction history and the landscape matrix surrounding cities can be important drivers of intercity genetic connectivity for urban dwelling species. In regions with extensive habitat fragmentation between cities, patterns of genetic connectivity may align more closely with predictions of the urban fragmentation model even for grey squirrels thriving in cities. Uncovering how human-induced landscape change affects contemporary levels of neutral evolution for common species can help us understand how humans influence population level dynamics of organisms we interact with in our everyday lives.

AUTHOR CONTRIBUTIONS

The authors confirm contribution to the paper as follows: *study conception and design*: NA Fusco, A Caccone, BJ Cosentino, and JP Gibbs; *data collection*: ML Allen, AJ Blumenfeld, GH Boettner, EJ Carlen, M Collins, C Dennison, D DiGiacopo, AP Drapeau Picard, J Edmonson, MC Fisher-Reid, R Fyffe, T Gallo, A Grant, W Harbold, SB Heard, DJR Lafferty, RM Lehtinen, S Marino, JE McDonald, A Mortelliti, M Murray, A Newman, KN Oswald, C Ott-Conn, JL Richardson, R Rimbach, P Salaman, M Steele, MR Stothart, MC Urban, K Vandegrift, JP Vanek, SN Vanderluit, and L Vezina; *analysis and interpretation of results*: NA Fusco, A Caccone; *writing of the draft manuscript*: NA Fusco; *major comments and edits provided for the draft manuscript*: NA Fusco, A Caccone, BJ Cosentino, JP Gibbs, JP Vanek, and AJ Blumenfeld. All authors reviewed the results and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest.

BENEFIT-SHARING STATEMENT

This research collaboration was developed with professional, student, and community scientists from the states across the eastern United States and provinces of Canada who provided tissue samples for genetic analysis, and all collaborators who contributed amply are included as co-authors. The results of this research have been shared with communities that provided samples, many of whom are co-authors of this manuscript, and shared with the broader scientific community. This research addresses an understanding of local wildlife population dynamics and evolution within a changing human-altered world. Our group is committed to forming collaborations and scientific partnerships to enable the understanding of the ecology and evolution of species within urban environments.

DATA AVAILABILITY STATEMENT

Raw sequence reads are deposited on NCBI SRA (BioProject PRJNA1041891). Related metadata can be found on osf.io at DOI 10.17605/OSF.IO/2UXDC (including geo-tag references and date/month/year of sampling event) and unique sample identifier tags that can be matched to both the deposited genetic data and deposited metadata.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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