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The American Kestrel (*Falco sparverius*) Genoscape: Implications for Monitoring, Management, and Subspecies Boundaries

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Title: The American Kestrel (*Falco sparverius*) Genoscape: Implications for Monitoring, Management, and Subspecies Boundaries

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KCR, JAH, MDO, SS, CJWM, TBS and MB conceived of the idea design and experiment.

KCR, MB, CMB, RB, and ECA collected the genomic data and conducted the bioinformatic and population genetic analyses. KCR, MB, JAH, and CMB wrote the manuscript with contributions from all co-authors. MB, CWB, RDD, AE, CJWM, KEM, LM, JM, MDO, BR, SS, TS, JFT, and JAH contributed blood samples for the genome-wide genetic analysis. We thank Joel Pagel and Little Gap Raptor Research for contributing samples to the genomic analysis, Anjolene Hunt for coordinating sample collection for the Kestrel Genoscape Project, the many generous supporters who contributed to the American Kestrel genoscape via a crowd-sourcing campaign, and the many citizen scientists who contributed feather samples for genetic analysis through the Peregrine Fund's American Kestrel Partnership as well as other channels. This work was made possible by a generous gift from J. Ellis, a California Energy Commission grant to K. Ruegg and T. Smith (EPC-15-043), a National Geographic grant to K. Ruegg (WW-202R-17), a donation from First Solar Incorporated, the Boise State Raptor Research Center, the Raptor Research Foundation's Dean Amadon Grant, and a grant to JAH from the Strategic Environmental Research and Development Program (RC-2702). Funding for the sample collection was provided by Natural Sciences and Engineering Research Council of Canada, Canada Foundation for Innovation, British Columbia Knowledge Development Fund, and University of Northern British Columbia. We thank the DNA Technologies and Expression Analysis Cores at the UC Davis Genome Center (supported by NIH Shared Instrumentation Grant 1S10OD010786-01) for their assistance with the Next-Generation Sequencing.

1 **ABSTRACT:** Identifying population genetic structure is useful for inferring
2 evolutionary process and comparing the resulting structure with subspecies boundaries
3 can aid in species management. The American Kestrel (*Falco sparverius*) is a
4 widespread and highly diverse species with 17 total subspecies, only two of which are
5 found north of United States/ Mexico border (*F. s. paulus* is restricted to southeastern
6 United States, while *F. s. sparverius* breeds across the remainder of the United States and
7 Canadian distribution). In many parts of their US and Canadian range, American
8 Kestrels have been declining, but it has been difficult to interpret demographic trends
9 without a clearer understanding of gene flow among populations. Here we sequence the
10 first American Kestrel genome and scan the genome of 197 individuals from 12 sampling
11 locations across the US and Canada in order to identify population structure. To validate
12 signatures of population structure and fill in sampling gaps across the US and Canada
13 range, we screened 192 outlier loci in an additional 376 samples from 34 sampling
14 locations. Overall, our analyses support the existence of 5 genetically distinct
15 populations of American Kestrels—Eastern, Western, Texas, Florida, and Alaska.
16 Interestingly, we found that while our genome-wide genetic data support the existence of
17 previously described subspecies boundaries in the US and Canada, genetic differences
18 across the sampled range correlate more with putative migratory phenotypes (resident,
19 long-distance, and short-distance migrants) rather than a priori described subspecies
20 boundaries per se. Based on our results, we suggest the resulting five genetically distinct
21 populations serve as the foundation for American Kestrel conservation and management
22 in the face of future threats.

23 INTRODUCTION

24 An important application of population genetics is the identification of genetically
25 distinct populations within species that can be used to guide conservation and
26 management efforts. Depending on the context, such groups are often referred to as
27 subspecies, management units (MUs), evolutionary significant units (ESUs),
28 conservation units, or genetically distinct populations (Moritz 1994, Allendorf and
29 Luikart 2007, Funk et al. 2007). Population genetic structure below the species level has
30 frequently been used to delineate units for conservation and management (Moritz 1994;
31 Allendorf & Luikart 2007; Funk et al. 2007), but other factors including behavior and
32 morphological variations are also important, particularly in species for which genetic data
33 are absent or lacking in resolution (Mayr 1982, Waples et al. 2007). In highly mobile
34 species it has historically been difficult to identify subspecies that correlate with
35 genetically distinct populations because gene flow often homogenizes the diversifying
36 effects of local adaptation and drift (Waples 1998, Willoughby et al. 2017, Medina et al.
37 2018, Doyle et al. 2018). As a result, identifying genetically distinct populations in
38 migratory animals, such as migratory birds, remains a challenge (Larson et al. 2014, Zink
39 2014, Freer et al. 2015, Mura-Jornet et al. 2018).

40
41 Traditionally, genetic studies focused on identifying genetically distinct populations
42 relied on a limited number of molecular markers (e.g., microsatellites, mitochondrial (mt)
43 DNA sequences, and allozymes) to make inferences about population genetic structure
44 (Ryman et al. 2006, Morin et al. 2009, Rowe et al. 2011, Mura-Jornet et al. 2018).
45 However, recent advances in sequencing technology have made it possible to screen tens

46 of thousands to millions of genetic markers and reveal patterns of population structure
47 that may have previously gone undetected (Rowe et al. 2011). For many species, greatly
48 increasing the number of loci included in population genetic analyses has improved the
49 precision of population genetic parameters (Egger et al. 2017), increased the resolution of
50 detectable population genetic structure (Ruegg et al. 2014, Benestan et al. 2015, Jahner et
51 al. 2016), and provided opportunities for fine scale investigations of genetically distinct
52 populations and their relationship to subspecies boundaries (Larson et al. 2014,
53 Fredrickson et al. 2015, Bussche et al. 2017, Mura-Jornet et al. 2018). In migratory birds,
54 Ruegg et al. (2014) coined the term *genoscapes* to refer to maps of genetic variation
55 across geographic space, but the relationship among genetically distinct populations
56 within a genoscape and previously defined subspecies boundaries has yet to be explored.
57 Here we use Next Generation Sequencing (NGS) technology to create a genoscape for
58 the American Kestrel, assess its relationship to current subspecies boundaries, and
59 provide a framework for conservation and management of this and other highly mobile
60 species with a high capacity for dispersal.

61
62 The American Kestrel is a widely distributed species that breeds throughout North and
63 South America (Smallwood and Bird 2002) and has upwards of 17 recognized subspecies
64 (Ferguson-Lees and Christie 2006). American Kestrels show highly variable migration
65 strategies across their range, including individuals that migrate long distances, short
66 distances, or do not migrate, and populations that are completely migratory, partially
67 migratory, or non-migratory (Layne 1982, Bird and Palmer 1988, Henny and Brady 1994,
68 Smallwood and Bird 2002). Here we focus on two American Kestrel subspecies found

69 north of the Mexico / United states border, the non-migratory subspecies (*F. s. paulus*)
70 found breeding in the southeastern United States and the widespread subspecies (*F. s.*
71 *sparverius*) found throughout the remainder the United States and Canada (Hoffman and
72 Collopy 1988, Smallwood 1990). In general, it is thought that populations of *F. s.*
73 *sparverius* follow a pattern of leap-frog migration, where migratory distance decreases on
74 a latitudinal gradient, with birds in the northernmost part of the breeding range migrating
75 the farthest and birds in the southernmost part of the range remaining year-round
76 residents (Heath et al. 2012). In addition, there is growing evidence that American
77 Kestrel populations are declining (Bird 2009, Farmer and Smith 2009, Smallwood et al.
78 2009, Hinnebusch et al. 2010), but estimates of demographic trends differ regionally
79 (McClure et al. 2017). One hypothesis to explain regional variation in demographic
80 trends is that genetically distinct populations with different migratory strategies are
81 exposed to different stressors across the annual cycle. As a result, identifying genetically
82 distinct populations in American Kestrels and how they correspond with previously
83 defined subspecies will improve our ability to interpret recent demographic trends and
84 appropriately focus conservation actions.

85

86 Previous American Kestrel genetic research using five microsatellite loci and one
87 mitochondrial DNA marker identified no strong signal of population genetic structure
88 across the U.S. range, with only subtle differences in allele frequencies between the two
89 recognized subspecies (Miller et al. 2012). Here we employ NGS-sequencing technology
90 to screen three times the number of samples and 10,000 times the number of genetic loci
91 relative to previous work, and use the resulting data to re-evaluate patterns of population

92 structure in American Kestrels across their U.S. and Canadian breeding distribution.
 93 Specifically, we ask the following research questions: (1) Does genome-wide SNP data
 94 provide higher resolution of population structure than previous work based on fewer
 95 markers?, (2) How does the resulting population genetic structure relate to previously
 96 defined subspecies boundaries and variation in migratory behavior across the range?, and
 97 (3) How can the resulting genoscape be used help identify genetically distinct populations
 98 and develop hypotheses to explain regional variation in demographic trends?

99

100 METHODS

101 *Sample collection and DNA extraction*

102 Genetic samples were collected from 683 breeding adult or nestling American Kestrels
 103 from across the breeding range in North America in collaboration with several non-profit
 104 organizations, state agencies, university researchers, and citizen scientists (Table 1,
 105 Appendix 1). Blood (~ 30 µl) or pin feather samples were collected from 287 individuals
 106 for the construction of Restriction Site Associated DNA sequencing (RADseq) libraries.
 107 Blood was collected via brachial or jugular venipuncture, preserved in Queen's lysis
 108 buffer (Seutin et al. 1991) and stored at -80°C or 3-4 pin feathers containing a small
 109 amount of blood in the base of the feather were collected from the breast of one nestling
 110 per brood and stored in envelopes at room temperature. The remainder of the feather
 111 samples used for high throughput SNP analyses (n=396) were collected from the breast
 112 of adult birds and stored in envelopes at room temperature. All samples were extracted
 113 using a QIAGEN DNeasy® Blood and Tissue Kits (San Francisco, CA) and blood and
 114 pin feather extractions were further quantified using Qubit dsDNA HS157 Assay kits

115 (ThermoFisher Scientific) and visually inspected via gel electrophoresis to ensure
116 selection of high quality, intact DNA for construction of RADseq libraries. Remaining
117 tissue and blood samples, as well as remaining extractions, were curated and made
118 available for future use in -20 and -80 freezers respectively in the Conservation
119 Genomics Laboratory at Colorado State University.

120

121 *Genome sequencing, assembly and annotation*

122 To create a reference genome, the Illumina TruSeq DNA PCR-Free LT kit (Illumina) was
123 used to prepare a genomic DNA library from a single individual from Boise, Idaho,
124 following the adjustments made by Ruegg et al. (2018). The resulting library was
125 sequenced on two lanes of an Illumina HiSeq2500 using 250 base-pair (bp) paired-end
126 sequencing at the DNA Technologies and Expression Analysis Cores at the UC Davis
127 Genome Center (Davis, CA). Initial contigs were assembled with the Discovar DeNovo
128 assembler from the Broad Institute (<http://www.broadinstitute.org>), discarding contigs
129 less than 1000bp in length. We also sequenced mate pair libraries with two insert sizes
130 (4kb and 8kb) on one third of an Illumina HiSeq2500 2x100bp lane at the University of
131 Utah Huntsman Cancer Center. Mate pair reads were trimmed with NxTrim (O'Connell
132 et al. 2015) and scaffolds were generated with both paired end and mate pair libraries
133 with SSPACE (overlap requirement $k=3$; Boetzer et al. 2011). The assembly was then
134 broken at likely error regions using REAPR (Hunt et al. 2013) and SSPACE scaffolding was
135 repeated with $k=5$ and scaffolds less than 1000bp were discarded for the final assembly.

136

For annotation purposes, REPEATMASKER (-species birds) (Tarailo-Graovac and Chen 2009) was used to replace repetitive regions of the final genome assembly with N's. Two different ab initio gene predictions were used within the MAKER pipeline (Cantarel et al. 2008): SNAP and AUGUSTUS. The Zebra Finch (*Taeniopygia guttata*) cDNA and protein sequences were downloaded from Ensembl and used to train SNAP, and the available chicken (*Gallus gallus domesticus*) training dataset was used to train AUGUSTUS. ITERPROSCAN (Zdobnov and Apweiler 2001) was used to add Pfam protein annotation and gene ontology (GO) terms and identified 13,342 genes.

145

146 *SNP discovery and SNP filtering*

High-density RADseq was carried out on 287 individuals from 12 sampling locations following a modified version of the bestRAD library preparation protocol (SI Table 1; Ali et al. 2016). In short, DNA was normalized to a final concentration of 100ng in a 10ul volume, digested with restriction enzyme SbfI (New England Biolabs, NEB). The fragmented DNA was then ligated with SbfI specific adapters prepared with biotinylated ends and samples were pooled and cleaned using 1X Agencourt® AMPure XP beads (Beckman Coulter). Pooled and clean libraries were sheared to an average length of 400bp with 10 cycles on the Bioruptor NGS sonicator (Diagenode) to ensure appropriate length for sequencing and an Illumina NEBNext Ultra DNA Library Prep Kit (NEB) was used to repair blunt ends and ligate on NEBNext Adaptors to the resulting DNA fragments. Agencourt® AMPure XP beads (Beckman Coulter) were then used to select DNA fragments with an average length of 500bp, libraries were enriched with PCR, and cleaned again with Agencourt® AMPure XP beads. The resulting libraries were

160 sequenced on three lanes of an Illumina HiSeq 2500 at the UC Davis Genome Center
 161 using 250 base pair, paired-end sequencing, and 66 individuals with low coverage were
 162 re-sequenced on a fourth lane.

163

164 The program STACKS (Catchen et al. 2013) was used to demultiplex, filter and trim
 165 adapters from the data with the process_radtags function and remove duplicate read pairs
 166 using the clone_filter function. BOWTIE2 was used to map reads to the genome
 167 (Langmead and Salzberg 2012), and the HaplotypeCaller in the Genome Analysis Toolkit
 168 was used to identify SNPs (McKenna et al. 2010, Auwera et al. 2013). VCFTOOLS
 169 (Danecek et al. 2011) was used to remove indels, non-biallelic SNPs, and low quality and
 170 rare variants (genotype quality 20; coverage depth 10; minor allele frequency 0.05). The
 171 final number of SNPs and individuals to be retained for further analyses was assessed by
 172 visualizing the tradeoff between discarding low-coverage SNPs and discarding
 173 individuals with missing genotypes using custom scripts within the R-package
 174 GENOSCAPETOOLS (Anderson 2019). Because preliminary analyses revealed outliers in
 175 the principal components analysis and we were concerned about sample contamination
 176 among individuals during the library preparation stage, we filtered out individuals with
 177 >40% heterozygosity as heterozygosity is expected to be higher than expected in cases
 178 where multiple individuals are combined into a single well (SI Figure 2).

179

180 *Identification of outlier SNPs for Population Assignment*

181 Population genomic analyses were conducted on all SNPs that passed our filters to assess
 182 genome-wide patterns of genetic divergence and identify SNPs for population assignment

183 and assay design. Population genetic structure was assessed by calculating pairwise
 184 population level F_{ST} (with different sampling sites representing different populations)
 185 with bootstrapped confidence intervals using the R package ASSIGNER (Gosselin et al.
 186 2019), and principal components analysis (PCA) using SNPRELATE (Zheng et al. 2012).
 187 To test for isolation by distance we compared linearized F_{ST} with pairwise geographic
 188 distance calculated from the central longitudinal and latitudinal coordinates of each
 189 location using the Vincenty ellipsoid method in the R package GEOSHPERE (Hijmans
 190 2019). Because the PCA of all SNPs from the genome-wide analysis revealed five major
 191 groups, including Alaska, Texas, the west, the east and Florida (see results, Figure 1),
 192 subsequent analyses focused on developing SNPs for population assignment within and
 193 among these groups.

194

195 To identify SNPs useful for population assignment between the 5 genetically distinct
 196 populations, we used VCFTOOLS (Danecek et al. 2011) to calculate site-wise F_{ST} between
 197 populations and identify individual SNPs with the most power for discriminating between
 198 populations (SNPs with the biggest allele frequency differences). It is important to note
 199 that population genetic summary statistics were based on the full RADseq data set (see
 200 above) rather than downstream SNP dataset in order to avoid potential biases associated
 201 with selecting SNPs with the highest discriminatory power for population assignment.
 202 Custom R-scripts were used to evaluate which of our top-ranking SNPs would generate
 203 designable assays based on the following parameters: 1) Guanine – Cytocene content
 204 was less than 0.65, 2) there were no insertions or deletions (indels) within 30bp of the
 205 variable site, or 3) there were no ambiguous codes within 20bp of the variable site.

206 Additionally, we used BWA-MEM (Burrows-Wheeler Aligner; Li and Durbin 2009) to
207 determine which of our designable SNPs mapped uniquely to the reference genome.
208 Fluidigm SNPtype assays (Fluidigm Inc.) were then developed in the 216 top ranking
209 SNPs that passed our filters.

210

211 *Genetic screening and building the genoscape*

212 Ninety-three samples and three non-template controls were screened on the Fluidigm
213 Corporation EP1 Genotyping System (Fluidigm Inc.) and assays were ranked by
214 variability and call rate to identify the most reliable 192 SNP assays of 216 that were
215 designed. The 192 variable SNP assays with the highest call rate were used to screen an
216 additional 396 American Kestrel feather samples from 34 breeding locations in the
217 United States and Canada in order to fill in sampling gaps and refine the resulting map of
218 population genetic structure (Table 1). Following the methods described in Ruegg et al.
219 (2014), we amplified PCR products using fluorescently labelled allele-specific primers
220 and then used the EP1 Array Reader and Fluidigm's automated Genotyping Analysis
221 Software (Fluidigm Inc.) to call alleles with a confidence threshold of 90%. Each
222 genotype was also visually inspected for potential irregularities and uncertain genotype
223 calls were removed from the analysis. Samples with missing genotypes at >25% of SNP
224 assays were removed from the analyses and SNP loci with >25% missing genotypes were
225 removed, resulting in a total of 376 additional individuals at 186 SNP loci that could be
226 used to identify genetic structure across the range (Table 1).

227

228 The final analysis of population genetic structure at 186 loci was conducted on a subset
229 of the loci from the RADseq dataset combined with the SNP genotype-only dataset for a
230 total of 683 individuals. The program *structure* (version 2.3.4; Pritchard et al. 2000) was
231 used to assess how genetic variation is distributed across geographic space. The
232 admixture model with the locprior option was run with uncorrelated allele frequencies, a
233 burn-in period of 50,000, a total run length of 150,000 and assuming the number of
234 genetic clusters (K) ranged from 1 to 10 (with 5 iterations run at each assumed value of
235 K). We used the Evanno method to determine the number of genetic clusters. The
236 Evanno method (Evanno et al. 2005), implemented in POPHELPER in R (Francis 2017), is
237 an *ad hoc* method to determine the most probable number of population genetic clusters
238 based on the rate of change in the log probability of data between successive *K* values.
239 We used this algorithm to detect the uppermost hierarchical level of structure across the
240 Kestrel breeding range and visually inspected subsequent structure plots to identify
241 regions where geographic barriers to gene flow exist and/or where admixture
242 homogenizes population structure. The resulting posterior probabilities of genetic group
243 membership estimated from structure were visualized as transparency levels of different
244 colors overlaid upon a base map from Natural Earth (naturalearthdata.com) and clipped
245 to a map of the American Kestrel breeding range (NatureServe 2012), making use of the
246 R packages SP, RGDAL, and RASTER (Bivand et al. 2013, 2017; Hijmans 2017). We scaled
247 the transparency of colors within each distinguishable group, so that the highest posterior
248 probability of membership in the group according to structure is opaque and the smallest
249 is transparent. This creates a spatially-explicit map of the population structure analysis
250 that we call the genoscape of the American Kestrel (Figure 2).

251 RESULTS

252 *Genome sequencing, assembly and annotation*

253 The final American Kestrel genome assembly is 1.23 Gb in length and consists of 5,096
254 scaffolds with an N50 of 941kb.

255

256 *SNP discovery and SNP filtering*

257 RAD-sequencing data from 287 individuals resulted in the identification of 199,705 bi-
258 allelic loci with a minor allele-frequency greater than 5%, minimum quality score greater
259 than 20, and minimum per individual sequencing depth greater than 10. After assessing
260 the tradeoff between low coverage SNPs and missing genotypes (SI Figure 1), the data
261 were further filtered to include 197 individuals, 12 populations, and 75,000 loci; 2
262 populations, one in CA and one in Idaho (Table 1), were subsequently dropped for the
263 purposes of population genetic analyses as a result of low sample size ($n < 3$). Seven
264 outlier individuals with greater than >40% heterozygosity were also subsequently
265 removed to avoid inclusion of samples potentially subject to contamination (as indicated
266 by a histogram of the distribution of heterozygosity across all individuals which showed
267 individuals above this threshold to be clear outliers, SI Figure 2). The final RADseq data
268 set consisted of 197 individuals and 72,263 SNPs.

269

270 *Population genetic structure*

271 Significant pairwise F_{ST} between the 10 sampling locations (2 were filtered out because
272 they had fewer than 4 individuals, see above) ranged from 0.0010-0.0162 (Table 2). The
273 positive correlation between F_{ST} and geographic distance ($r^2 = 0.123$; p-value = 0.01)

suggests that isolation by distance contributes to genetic differentiation across the range. Overall, F_{ST} was highest between non-migratory Florida and Texas sampling locations, while the genetic differentiation was the lowest among sampling locations within Eastern and Western breeding areas (Table 2). Principal components analysis based on 72,263 RAD-sequence loci revealed 4 main clusters with East, West, and Florida falling out separately, while Alaska and Texas overlapped (Figure 1). Principal component 1 was strongly influenced by data missingness, while PC2 and PC3 reflected differences in geography. Although there was overlap in principal component space between Texas and Alaska along PC2 and PC3, these groups were separated in the subsequent PC axes and in the search for loci representative of the observed population structure based on significant pairwise F_{ST} s and geographic distance. Overall, the first three principal components explained less than 3% of the total variation in allele frequencies.

Genoscape Construction / Structure Results

We successfully genotyped 376 samples collected from 34 breeding locations in the United States and Canada using the final panel of 192 SNP-type assays for population assignment (Table 1). Running structure with K ranging from 1 to 10 revealed the strongest support for $K = 5$, where the plateau of delta K (i.e. the greatest change in K) supports the uppermost hierarchical level of structure being $K = 3$ (SI Figure 3A; Evanno et al. 2005, Pritchard et al. 2000); however, subsequent increases of K , until $K = 5$, reduced the log likelihood of the model and the addition of the Florida ($K=4$) and Alaskan populations ($K=5$) were biologically feasible (SI Figure 4), suggesting that American Kestrel's can be separated into 5 genetically distinct populations. Ultimately,

we find genetic distinctness of the residential Texas population, the residential Florida *F. s. paulus* subspecies, as well as distinct clustering of the Eastern and Western migratory populations, and an Alaskan migratory population (Figure 2).

DISCUSSION

Identifying distinct units for conservation is an important first step in the management of declining populations (Allendorf and Luikart 2007, Funk et al. 2012). Historically, conservation biologists have used a combination of morphological, behavioral, and genetic variation to define management or conservation units within species and these have sometimes, but not always, corresponded to subspecies boundaries. Here we generate a genoscape for the American Kestrel by sequencing the first American Kestrel genome, assessing population structure at 72,263 SNP markers screened in 12 populations from across the US and Canada migratory and non-migratory range, and validating patterns of population structure at 192 SNP markers screened in 34 populations. In contrast to previous work based on a more limited number of samples and markers that detected no major signals of population structure across the breeding range (Miller et al. 2012), our genoscape supports the existence of five genetically-distinct populations within American Kestrels found breeding across Canada and the United States (East, West, Alaska, Texas and Florida), one of which correlates with the previously identified southeastern subspecies (*F. s. paulus*). Overall, the most significant genetic differences occurred between the two resident populations (Texas and Florida), followed by differences between resident and migratory populations, and regional separation of Eastern and Western breeding populations. Here we discuss the utility of

the resulting genoscape for clarifying the relationship between previously defined subspecies boundaries and genetically distinct populations identified using genome-wide genetic data, as well as for providing a framework for developing hypotheses regarding drivers of regional variation in demographic trends.

The question of whether subspecies represent defensible taxonomic units has been controversial in the past because some molecular studies have failed to identify subspecies as phylogenetically distinct (Barrowclough 1980, Mayr and Ashlock 1991, O'brien and Mayr 1991, Ball and Avise 1992, Burbrink et al. 2000). Further, discord in location of subspecies boundaries often arises when there are mismatches in the timescales over which divergence occurs in various datasets; for example, subspecies boundaries based on neutral genetic markers often diverge from subspecies boundaries identified based on genetic or morphological markers that may be under selection (Haig and Winker 2010). Using genome-wide sequencing, we found support for genetic differentiation between the two US and Canadian subspecies of American Kestrels breeding North of Mexico, *F. s. paulus* (southeastern) and *F. s. sparverius* (remainder of the US and Canadian breeding range), but also found that divergence between the subspecies is similar in magnitude to the degree of divergence detected between resident *F. s. sparverius* in Texas and their migratory counterparts to the North. In general, levels of genetic differentiation across the range were low and pairwise genetic distance versus geographic distance suggest that patterns of divergence are in large part explained by isolation by distance. Such low levels of differentiation are consistent with past studies of American Kestrels based on fewer loci (Miller et al. 2012) and suggest that gene flow

may homogenize the diversifying effects of local adaptation and drift in high dispersal species like Kestrels (Willoughby et al. 2017; Doyle et al. 2018; Medina et al. 2018). While our genome-wide genetic analysis supports the existence of *F. s. paulus* as a genetically distinct subspecies, it also suggests that weak population structure within American Kestrels relates as much to migratory phenotype as it does to subspecies boundaries per se.

One possible explanation for the putative relationship between genetic differentiation and migratory phenotype in American Kestrels is that dispersal is limited between distinct migratory phenotypes, as has been found in other migratory systems (reviewed within Turbek et al. 2018). Like most raptors, Kestrels in Eastern and Western North America follow a strong north-to-south pattern of migration, with little longitudinal drift (Mueller and Berger 1967, Evans and Rosenfield 1985, Goodrich and Smith 2008). Additionally, the frequency of long-distance migration into Mexico is thought to increase from east to the west (Mueller & Berger 1967; Evans & Rosenfield 1985; Goodrich & Smith 2008), supporting the idea that Eastern and Western populations have different overwintering locations. One explanation for the observed genetic break in central North America is that separate Eastern and Western migratory routes and overwintering locations have resulted in the evolution of a weak migratory divide where gene flow is limited as a result of reproductive isolation between distinct migratory phenotypes, as has been documented across migratory divides in other avian taxa (reviewed within Turbek et al. 2018). Alternatively, the observed genetic break between Eastern and Western populations may have nothing to do with migratory phenotype per se, but instead may result from low

366 population density in Central North America (an additive effect of Isolation-by-Distance)
 367 limiting gene flow between eastern and western groups (Winker et al 2010). Future work
 368 will focus on quantifying migratory phenotypes in American Kestrels across their North
 369 American range and assessing the relative contribution of migration and isolation by
 370 distance to patterns of genetic divergence in this and other species with similar variation
 371 in migratory phenotypes.

372

373 An alternative explanation for higher levels of divergence between resident and
 374 migratory *F. sparverius* populations in our study is that divergence is not caused by
 375 differences in migratory phenotype, but instead results from gene flow between resident
 376 forms in Texas and Florida (*F. s. Paulus*) and resident subspecies further to the south (*F.*
 377 *s. peninsularis* from Baja and W. Mexico, and *F. s. sparverioides* from Cuba). Previous
 378 work suggests *F. s. sparverius* may hybridize with *F. s. peninsularis* in northern and
 379 eastern Arizona (Bond 1943), but here we successfully genotyped 15 samples from
 380 breeding migratory *F. s. sparverius* from Arizona and found that they all assigned clearly
 381 to the western migratory group of *F. s. sparverius* rather than to the resident Texas
 382 group. This result is opposite of what we would expect if high levels of divergence
 383 between Texas residents and their migratory counterparts to the North was due to
 384 hybridization between Texas birds and the *F. s. peninsularis* subspecies to the
 385 South. While to the best of our knowledge, there are no known records of hybridization
 386 between *F. s. sparverioides* from Cuba and *F. s. paulus* from Florida, eBird records of
 387 kestrels from extreme southeastern Florida during the summer support the possibility that
 388 *F. s. sparverioides* vagrants may occur within the same region. However, gene flow is

389 unlikely, given the infrequent (<1 per year) sightings of kestrels in southeastern Florida
 390 and the fact that the nearest breeding population of *F. s. paulus* is >150 km to the north
 391 (FWC 2003). Thus, while on-going gene flow with resident subspecies to the south seems
 392 like an unlikely explanation for the observed patterns of divergence between resident and
 393 migratory populations north of Mexico, more extensive sampling south of the United
 394 States border is needed to fully test all alternative hypotheses.

395

396 Heterogeneity in patterns of American Kestrel population decline across North America
 397 suggest that regional populations are experiencing different threats and / or are
 398 responding to the same threats differently (Butcher 1990, Smallwood et al. 2009, Sauer et
 399 al. 2014, McClure et al. 2017), but past analyses have been limited by the lack of
 400 genetically distinct populations. The results presented herein demonstrate the utility of
 401 the genoscape approach for identifying five genetically distinct populations of American
 402 Kestrels – East, West, Alaska, Texas and Florida, which can serve as the foundation for
 403 the development of hypotheses to explain regional variation in demographic trends. For
 404 example, while interpreting patterns of population decline from existing datasets is
 405 complicated by known northward shifts in distribution (Paprocki et al. 2014), migration
 406 count data from the Raptor Population Index project between 2006-2016 support the idea
 407 that western populations have largely remained stable or are increasing, while eastern
 408 populations are largely declining (SI Figure 3; Brandes et al. 2016, Crewe et al. 2016). In
 409 addition, work focused specifically on understanding responses of American Kestrels to
 410 climate change in the last decade supports the hypothesis that western populations are
 411 migrating shorter distances and breeding earlier (Heath et al. 2012), while corresponding

412 changes in the east have not been documented. In light of the genoscape results
 413 presented herein, one hypothesis that warrants further exploration is that genetically-
 414 based differences in phenology between Eastern and Western groups affect population-
 415 specific responses to changing climate conditions, resulting in population decreases in the
 416 east, but not the west.

417

418 In conclusion, the American Kestrel genoscape reveals previously undetected levels of
 419 population structure among Eastern, Western, Alaska, Texas and Florida populations.
 420 While our data support the existence *F. s. sparverius* and *F. s. paulus* subspecies as
 421 genetically distinct groups, it also suggests that genetic differentiation is more closely
 422 tied to migratory phenotype (resident, long-distance, and short-distance migrants) than to
 423 previously defined subspecies boundaries. Based on our results, we suggest it would be
 424 ecologically appropriate to establish five management areas corresponding to the five
 425 genetically unique populations identified by our genoscape. More importantly, when the
 426 resulting genetically distinct populations are paired with data from existing long-term
 427 monitoring efforts, such as the Raptor Population Index, the results can be used to test
 428 hypotheses regarding drivers of observed population-specific responses to climate change
 429 and other stressors.

430

431

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Table 1. Sampling locations and sample type used in the construction of the genoscape map for American Kestrels. The samples are broken down into those used for the RADsequencing analysis (pre and post filters) and the SNP genotyping (pre and post filtering). State and Province location codes correspond to Figure 1, while letter location codes correspond to the locations on Figure 2.

STUDY SITE	LATITUDE	LONGITUDE	SAMPLE TYPE	N _{RAD_NoFilter}	N _{RAD_Filter}	N _{SNP_NoFilter}	N _{SNP_NoFilter}	LOCATION CODE Fig 1 / Fig 2
ALBERTA, CANADA	56.16262	-117.34	Feather			12	12	E
ALASKA, USA	63.96041	-145.136	Blood, feather	25	18	7	7	AK / A
ARIZONA, USA	33.45007	-111.943	Feather			20	15	G
CALIFORNIA (1), USA	37.23551	-120.244	Blood, feather	26	10	22	22	CA / D1
CALIFORNIA (2), USA	33.7315	-117.878	Blood, feather	4	1	3	3	CA / D2
CONNECTICUT (1), USA	41.95001	-73.364	Feather			10	10	Y1
CONNECTICUT (2), USA	41.94374	-72.5072	Feather			10	7	Y2
DELAWARE, USA	39.75047	-75.6969	Feather			4	4	W
FLORIDA, USA	29.54151	-82.2334	Blood, feather	18	16	20	20	FL / AA
IDAHO (1), USA	43.51209	-116.377	Blood, feather	33	29	18	11	ID / F1
IDAHO (2), USA	43.27597	-115.003	Blood, feather	9	2	6	5	ID / F2
ILLINOIS, USA	38.30563	-89.7969	Feather			5	3	P
KANSAS, USA	38.77533	-95.2486	Feather			4	4	M
MASSACHUSETTS, USA	42.15064	-71.8496	Feather			20	18	Z
MICHIGAN (1), USA	42.3216062	-86.182447	Feather			6	6	R1
MICHIGAN (2), USA	44.9774725	-85.712026	Feather			6	6	R2
MICHIGAN (3), USA	44.9034875	-83.4873	Feather			8	8	R3
MISSOURI (1), USA	39.04824	-94.3663	Feather			8	5	N1
MISSOURI (2), USA	38.3804	-90.925	Feather			1	1	N2
MONTANA, USA	45.76764	-111.154	Feather			19	15	I
NORTH CAROLINA, USA	35.73285	-82.6776	Feather			17	16	S
NEBRASKA, USA	40.99115	-96.8957	Feather			6	6	L
NEW JERSEY, USA	40.4297	-74.9271	Feather			30	28	X
NEW YORK, USA	41.8773	-74.7309	Feather			4	3	U
OREGON (1), USA	45.57362	-123.048	Blood, feather	14	11	3	3	OR / C1
OREGON (2), USA	44.36466	-121.412	Feather			25	24	C2
PENNSYLVANIA, USA	40.60183	-75.5961	Blood, feather	37	23	13	13	PA / V
SASKATCHEWAN, CANADA	55.01968	-105.893	Blood, feather	40	28	12	11	SK / K
TENNESSEE, USA	35.14944	-90.0489	Feather			5	5	O
TEXAS, USA	33.63184	-101.889	Blood, feather	13	12	1	1	TX / B
UTAH, USA	40.62255	-111.987	Feather			30	28	H
VIRGINIA, USA	38.6457	-78.7522	Blood, feather	38	29	17	17	VA / T
WISCONSIN, USA	43.77114	-89.4059	Blood, feather	30	18	24	24	WI / Q
WYOMING, USA	43.60488	-110.658	Feather			17	15	J
TOTAL				287	197	396	376	

Table 2. Weighted mean pairwise Weir and Cockerham (1984) F_{ST} values (upper triangle) and confidence intervals (lower triangle) between all American kestrel sampling locations with greater than five individuals. Sampling locations correspond to those listed in Figure 1 and Table 1 and are organized by approximate migration strategy. Color coding indicates degree of differentiation between locations with the greatest values of genetic divergence between nonmigratory populations in Florida and Texas.

		Complete Migrant		Partial Migrant						Resident	
		AK1	SK1	OR1	ID1	CA1	WI1	PA1	VA1	TX1	FL1
Complete Migrant	AK1	-	0.0031	0.0041	0.0048	0.0053	0.0060	0.0062	0.0086	0.0096	0.0124
	SK1	0.0028 - 0.0034	-	0.0019	0.0016	0.0014	0.0015	0.0024	0.0046	0.0078	0.0103
Partial Migrant	OR1	0.0036 - 0.0046	0.0015 - 0.0023	-	0.0025	0.0031	0.0046	0.0051	0.0073	0.0077	0.0112
	ID1	0.0044 - 0.0050	0.0014 - 0.0019	0.0021 - 0.0030	-	0.0019	0.0040	0.0051	0.0072	0.0088	0.0125
	CA1	0.0048 - 0.0058	0.0010 - 0.0019	0.0025 - 0.0037	0.0015 - 0.0023	-	0.0039	0.0051	0.0070	0.0099	0.0128
	WI1	0.0056 - 0.0065	0.0012 - 0.0018	0.0040 - 0.0052	0.0037 - 0.0043	0.0035 - 0.0044	-	0.0010	0.0029	0.0100	0.0082
	PA1	0.0058 - 0.0065	0.0021 - 0.0027	0.0046 - 0.0056	0.0048 - 0.0054	0.0047 - 0.0057	0.0006 - 0.0012	-	0.0027	0.0103	0.0076
	VA1	0.0082 - 0.0090	0.0043 - 0.0048	0.0068 - 0.0079	0.0069 - 0.0075	0.0065 - 0.0074	0.0025 - 0.0032	0.0025 - 0.0030	-	0.0127	0.0093
Resident	TX1	0.0091 - 0.0102	0.0074 - 0.0083	0.0070 - 0.0082	0.0083 - 0.0092	0.0092 - 0.0106	0.0094 - 0.0105	0.0099 - 0.0108	0.0121 - 0.0132	-	0.0162
	FL1	0.0120 - 0.0129	0.0099 - 0.0106	0.0105 - 0.0116	0.0121 - 0.0129	0.0122 - 0.0134	0.0076 - 0.0088	0.0072 - 0.0081	0.0089 - 0.0098	0.0156 - 0.0168	-

Key	
	$F_{ST} < 0.005$
	$0.005 \leq F_{ST} < 0.01$
	$0.01 \leq F_{ST} < 0.015$
	$F_{ST} \geq 0.015$

Figure 1. Principal components analysis of 72,263 Single Nucleotide Polymorphism markers from across the US and Canadian breeding range of American Kestrels showing separation among Eastern, Western, Alaska, and Texas genetically distinct populations, and the Florida subspecies. Each genetically distinct population is encircled by an ellipse. Separation between Alaska and Texas conservation units occurred along Principle Components axes not shown and was also evident in pairwise F_{ST} calculations.

Figure 2. The American Kestrel genoscape. A) Structure plot showing support for K= 5 genetic groups. Letters correspond to population locations on the map as well as sample numbers listed in Table 1. B) A spatially explicit representation of the population structure results showing the biggest genetic differences among eastern, western, Texas, Florida and Alaska genetic groups. Dots with circles around them indicate sampling locations where both Restriction-site associated-DNA sequencing and Single Nucleotide Polymorphism genotyping was conducted. The dashed line indicates the hypothesized southern boundary of *F. s. sparvarius* (Lane & Fischer 1997).

Figure 3. The relationship between pairwise linearized F_{ST} and geographic distance for American Kestrel based on analysis of Restriction-Site-Associated DNA sequencing data. The significant r^2 supports the idea that genetic divergence across the range is largely a result of Isolation-by-Distance.

Figure SI 1. Missingness data per individual and per locus. We determined the final number of single nucleotide polymorphisms and individuals to be retained in analyses of population genetic structure of American Kestrels by visualizing the tradeoff between discarding low coverage single nucleotide polymorphisms (y-axis) and individuals with missing genotypes (x-axis). Based on this analysis, we retained 75,000 SNPs and 204 individuals for further filtering using percent heterozygosity per individual and per locus.

Figure SI 2. Histogram of heterozygosity per individual. The above plot was used to identify high heterozygosity outliers above 0.40 which we subsequently removed from the analysis due to suspected contamination of samples.

SI Figure 3. Population Structure in American Kestrels assuming different numbers of populations. Ancestry plots generated in *structure* corresponding to K values of 2 – 6, illustrating support for 5 genetically distinct lineages within American Kestrels. The sampling locations along the top bar correspond to the locations illustrated in Figure 2 and Table 1.

SI Figure 4. The Evanno method for determining the appropriate number of genetic clusters within the data (K). A) The change in K versus the number of clusters demonstrates K= 3 is the uppermost structure in the data. B) Visual inspection of the log likelihood values illustrate that K=5 represents the best likelihood that is also a biologically reasonable value of K for the number of populations within the American Kestrel data.

Figure SI 5. Population trends for American Kestrels from Raptor Population Index. A summary of population trend estimates based on migration count data from the Raptor Population Index (RPI) project (Crewe et al. 2016; Brandes et al. 2016). Green arrows represent areas of significant increase, blue arrows represent areas of slight increase (pointing up) or decrease (pointing down), red arrows represent areas of significant decrease, and blue circles signify areas with stable population trends. These data demonstrate the variability of population trend estimates of American Kestrels in North America, with the greatest declines occurring in the east. No trends are available for Alaska or Canada.