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Subspecies limits based on morphometry and mitochondrial DNA genomics in a polytypic species, the common grackle, *Quiscalus quiscula*

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Nearctic migratory songbirds have demonstrated low levels of genetic differentiation and weak phylogeographical structure in mitochondrial DNA lineages compared with resident species. The common grackle, *Quiscalus quiscula*, is a widespread, partially migratory, North American icterid composed of three currently recognized subspecies. In this study, mensural characters (external and skeletal measurements) and the complete mitochondrial genome together with two mitochondrial genes, *Cytb* and *ND2*, were used to investigate subspecific differentiation and demographic history of the common grackle. The results showed substantial variation in body size among subspecies, mostly distributed between the 'Florida grackle', *Quiscalus quiscula quiscula*, and the two other subspecies. Analysis of mitochondrial DNA indicated low levels of genetic variation, but we found distinct haplotypes in Florida that form a clade in the phylogenetic tree. This suggests that the nominate subspecies in Florida is a distinct evolutionary unit. The sharing of haplotypes among the other subspecies (*Quiscalus quiscula versicolor* and *Quiscalus quiscula stonei*) in the north suggests high levels of gene flow, making the status of these two subspecies equivocal. Gene flow between nominate *Q. q. quiscula*, *Q. q. versicolor* and putative *Q. q. stonei* is probably attributable to historical changes in distribution and abundance following climate change events. We therefore recognize only two subspecies in the common grackle complex.

ADDITIONAL KEYWORDS: climate change – *Cytb* – demographic history – mitochondrial DNA – *ND2* – Nearctic songbirds – North America – phylogeography.

INTRODUCTION

Most phylogeographical studies of Nearctic migratory songbirds conducted to date have demonstrated low levels of genetic differentiation and weak phylogeographical structure in mitochondrial DNA (mtDNA) lineages compared with resident species studied in the same area (Avise & Nelson, 1989; Zink, 1994; Milà *et al*., 2000). Factors leading to this pattern might include gene flow, and population expansions from bottlenecked populations after the Last Glacial Maximum (LGM). For example, Capainolo *et al*. (2020) provide evidence for the role played by Pleistocene postglacial population expansions in the phylogeography of the common grackle, *Quiscalus quiscula*, a widespread, long-distance and partially migratory bird.

The common grackle is a single species of icterid. Three subspecies are currently recognized (Fig. 1). Nominate *Quiscalus quiscula quiscula*, the 'Florida grackle', is a non-migratory resident found from Florida (including the Florida Keys in spring and summer) to coastal Louisiana, southern Mississippi and Alabama to the coast of Georgia and South Carolina. The 'bronzed grackle', *Quiscalus quiscula versicolor*, breeds

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Figure 1. The common grackle is a single species of icterid composed of three currently recognized subspecies, nominate *Quiscalus quiscula quiscula,* the 'Florida grackle' (bottom; AMNH 841935), *Quiscalus quiscula stonei*, the 'purple grackle' (middle; AMNH 844170), and *Quiscalus quiscula versicolor*, the 'bronzed grackle' (top; AMNH 844130). Painting by Dale Dyer.

from north-east British Columbia east through central Saskatchewan and northern Manitoba and Ontario to southern Quebec, south-western Newfoundland, New England, northern New York and west of the Eastern Continental Divide to the Rocky Mountains, central Texas, south-west Louisiana and western Mississippi. The third subspecies, the 'purple grackle', *Quiscalus quiscula stonei*, breeds from New Jersey to Louisiana in a diagonal distribution along the Appalachian Mountain range east through north-west Georgia, east Tennessee and west North Carolina, along the boundary between Virginia and West Virginia, east Pennsylvania to southern New York and New Jersey to South Carolina and central Alabama (Fig. 2). Most of the taxonomic and nomenclatural work on this species was done by Chapman and others between 1892 and 1940 (Chapman, 1892, 1935a, b, 1936, 1939a, b, 1940; Oberholser, 1919; Wetmore, 1939). Subspecies show clinal variation in size and colour of plumage. Chapman developed a 'colour phase scoring system' consisting of 'intermediates' linking the phenotypically stable forms (Figs 3, 4). Florida and bronzed grackles are the most phenotypically stable forms, whereas purple grackles are extremely variable in colour (Braislin, 1904; Griscom 1923a; Griscom 1923b ; Bent, 1958; Peer & Bollinger, 1997; Jamarillo & Burke, 1999; Sibley, 2022). Florida, purple and bronzed grackles are sympatric along portions of the Eastern Divide and produce hybrid offspring during the spring breeding season; introgression accounts for some variation observed (Yang & Selander, 1968). Hybrid specimens are often referred to as 'Ridgway's grackle'

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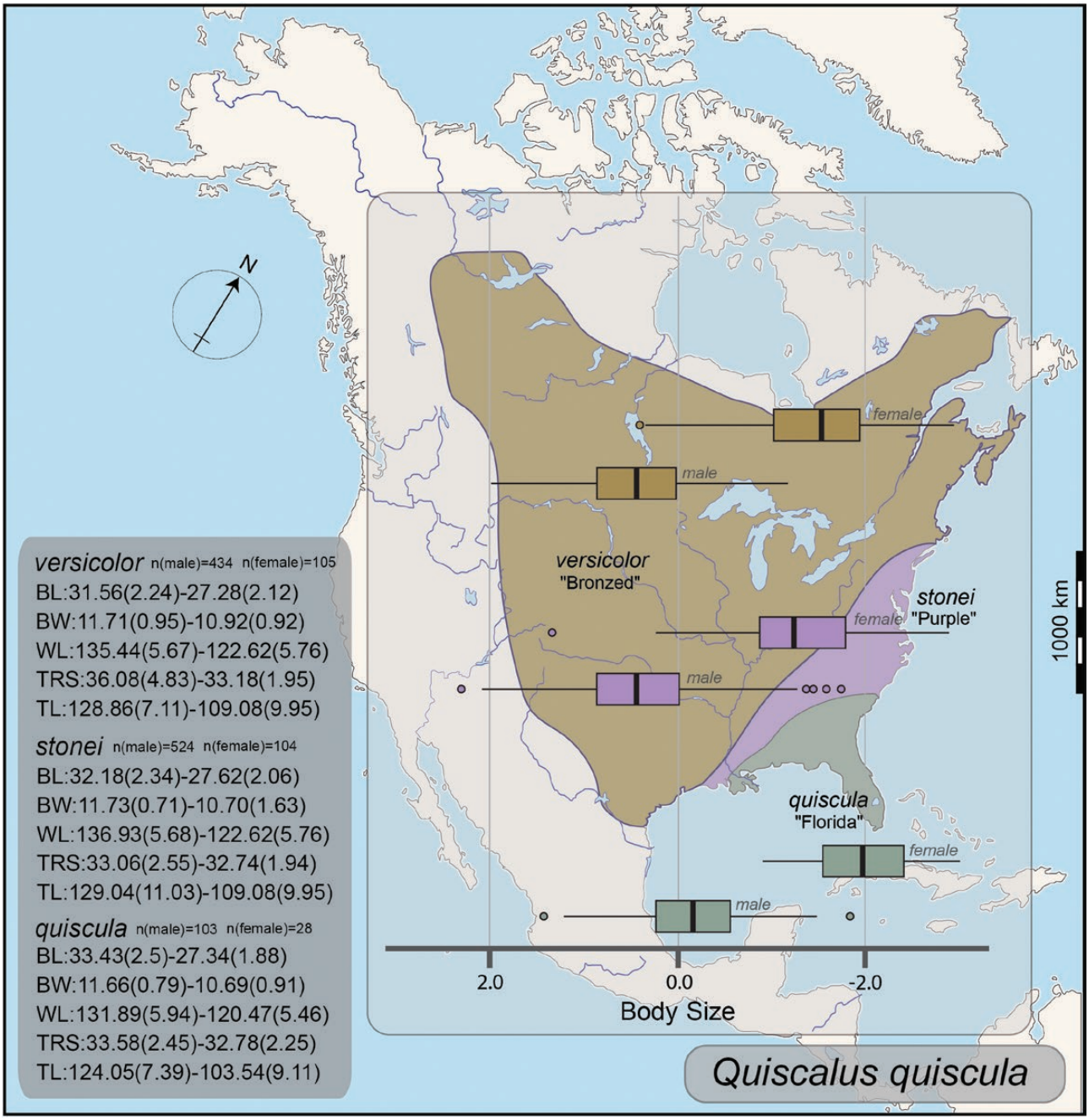

Figure 2. Subspecies distribution of the common grackle in North America. Descriptive statistics of external measurements are given in the box; body size differences among subspecies based on external measurements are given as a boxplot graph. Colours correspond to the subspecies distribution on the map.

[*Quiscalus quiscula ridgwayi* of Oberholser (1919) is of no taxonomic import] when they cannot be assigned easily to one of the three described subspecies. Bronzed and purple grackles have evolved migratory behaviour; bronzed grackles are the most migratory subspecies and spend the non-breeding season in points south to the central gulf states (Bent, 1958; Peer & Bollinger, 1997; Jamarillo & Burke, 1999; Capainolo *et al*., 2020; Sibley 2022). Owing to misidentification of specimens, confusing synonymies and the impulse of late 18th and early 19th century taxonomists to 'split' taxa (Coues, 1894; Barrow, 2004; Winker, 2010), the nomenclatural history of the common grackle is one of the more confusing in the annals of avian taxonomy (Maxwell,

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Figure 3. Dorsal view of breeding adult male common grackles illustrating Chapman's system of scoring colour 'phases' linking phenotypically stable forms 1 and 4 through intermediates from the south-east to north-east. Left to right: phase 1, *Quiscalus quiscula quiscula*, 'Florida grackle' (AMNH 386969), Brevard County, FL, USA; intermediate (1½) (AMNH 59608), Hale County, AL, USA; phase 2, *Quiscalus quiscula stonei*, 'purple grackle' (AMNH 325287), Burlington County, NJ, ISA; intermediate (2½) (AMNH 322804), Nassau County, NY, USA; phase 3, 'Ridgway's grackle', hybrid between 'bronze', 'purple' and 'Florida' but given the subspecific rank '*Quiscalus quiscula ridgwayi*' (AMNH 322793), Nassau County, NY, USA; intermediate (3½) (AMNH 387020), Kings County, NY, USA; phase 4, *Quiscalus quiscula versicolor*, 'bronzed grackle' (AMNH 387058), Cook County, IL, USA.

1965), and the taxonomic identity of common grackle subspecies remains confusing.

Previous morphometric and phylogeographical studies of the common grackle have analysed morphological and mtDNA variation in North America and discussed the variation in terms of ecogeographical rules, gene flow among subspecies and the demographic history of the species (for a morphometric analysis, see Huntington, 1952; for a phylogeographical study, see Zink *et al*., 1991) but have not focused on taxonomic problems. Since the publication of the fifth edition of the *Checklist of North American Birds* (American Ornithologists' Union, 1957), scientific names of the common grackle have remained unmodified and unchallenged. Patterns of geographical differentiation at the morphometric and genetic level of bird species can reveal valuable information about underlying evolutionary differentiation processes, past demographic events and taxonomic inferences. When it comes to taxonomic questions and implications, a myriad of ideas, hypotheses and philosophies are aroused (Fitzpatrick, 2010). Multiple interpretations of data and questions regarding species and subspecies have resulted in long-standing debates about the taxonomy and nomenclature of a host of bird species. In this study, we aimed to shed light on the evolutionary history and taxonomic issues of the common grackle and designed the perspective of the study with the following questions:

1. Does the geographical distribution of morphometric variation of the common grackle match its three known subspecies?
2. Is there a match between the intraspecific genetic diversity of the common grackle and its known subspecies?
3. Does the demographic history of the common grackle explain its intraspecies taxonomic diversity?

Integrating genetic-level studies with morphological analyses offers new opportunities to understand complex patterns of taxonomic problems (Perktaş *et al*.,

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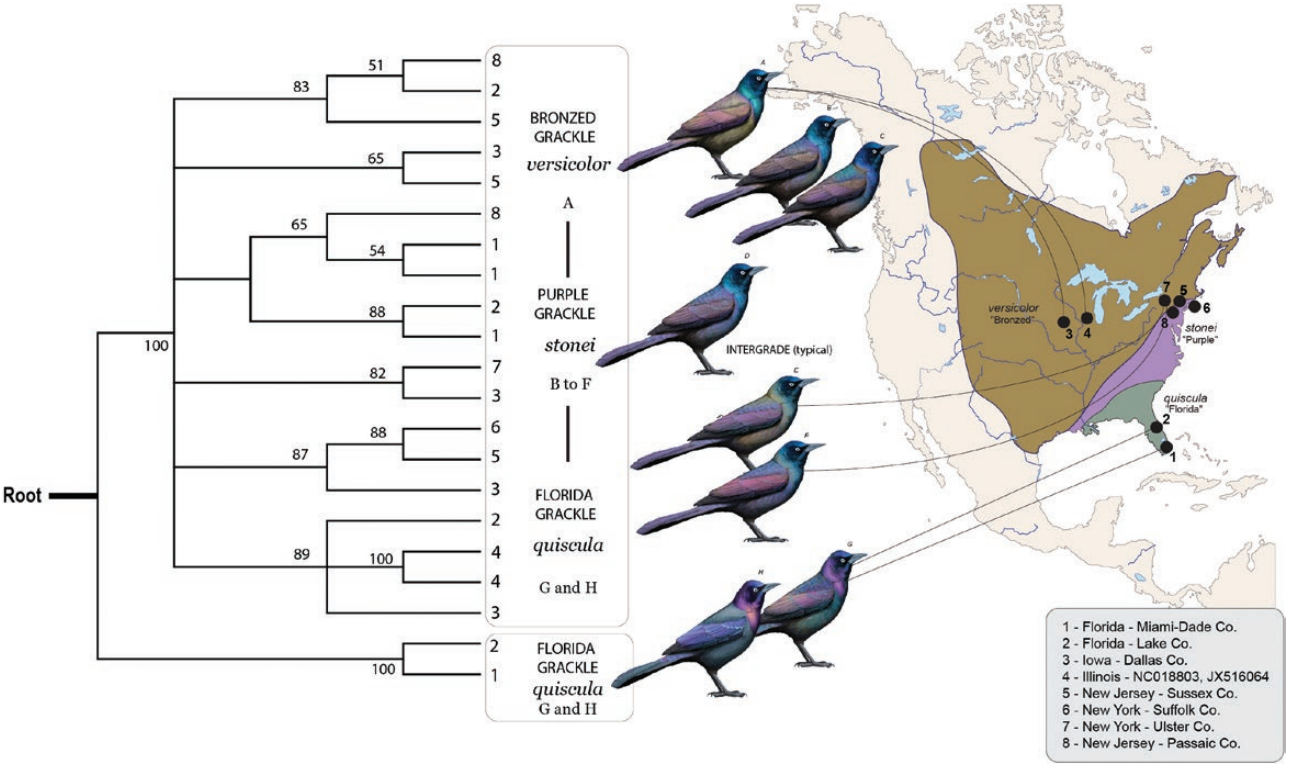

Figure 4. Strict consensus mitochondrial DNA tree for common grackle haplotypes. Bootstrap percentages are indicated for basal nodes. Illustrations show subspecies plumage variation and are used with permission of David Sibley.

2017). Although different methodological approaches have been used to reduce taxonomic uncertainty, both within and between species (Tobias *et al.*, 2010; Braby *et al.*, 2012), no standard method has been put forward on how to define a diagnostic evolutionary unit. Within the limits of the questions stated and the specimen material at hand, our perspective is to use evolutionary, ecological (i.e. historical demography) and genetic information (Crandall *et al.*, 2000) to develop a comprehensive survey of patterns of morphometric and genetic variation among common grackle populations to evaluate population history, and in particular, their subspecies identity.

MATERIAL AND METHODS

Morphometry

Metadata for all specimens used in all analyses in this study are available upon reasonable request to the corresponding author (specimen numbers and location data are available in the Supporting Information, Tables S1 and S2). We examined and measured museum specimens of male and female common grackles for morphometric analysis to gain a better understanding of variation between subspecies. Recently collected grackles were prepared using an atypical protocol that greatly increases the scientific information content of the specimen. Groth (1990) developed this method of preparing study skins, whereby the bill remains on the skin, and most of the axial and appendicular skeleton (minus the carpometacarpi, tarsometatatarsi and caudal vertebrae and pygostyle) is retained for cleaning and measuring. This results in a traditional-style study skin, many of the skeletal elements and various tissue types for analysis (for an instructional video demonstrating this method, see https://www.youtube.com/watch?v=Ev2ArqSe7xA).

External measurements

Study skins (N = 1759) of common grackles held by the American Museum of Natural History (AMNH) and 119 skins borrowed from seven sister institutions were measured for morphometric analysis (total N = 1878). Over several field seasons, between 2013 and 2020, P. Capainolo collected 217 common grackles, bringing the total number of specimens to 2095. Specimens were grouped by subspecies based on their collection localities and identifiable phenotype. Only birds determined to be breeding adults were measured. Non-breeding or damaged specimens were filtered out (N = 797), and a final tally of 1298 study skins were included in the external morphometric

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data set. Six standard external measurements were taken: culmen (in millimetres); anterior nares to tip of bill (in millimetres); bill width at base, ventral (in millimetres); wing chord (not flattened) (in millimetres); tarsus with podotheca (in millimetres); and tail length (in millimetres). A Fowler 74-101-150-2 electronic digital calliper and a 30 cm Avinet wing and tail ruler were used for measurements in millimetres.

Internal measurements

Skeletons of common grackles in the AMNH collections (N = 21) were used in this study, and 19 were borrowed from six sister institutions (total N = 40). Common grackle skeletons (N = 156) were prepared from specimens collected by P. Capainolo, bringing the grand total to 196. Forty damaged or otherwise unusable specimens were filtered out, and a total of 156 common grackle skeletons were used for the internal morphometric data set. Ten standard measurements were taken using a Fowler 74-101-150-2 electronic digital calliper (in millimetres) as follows: post-orbital width, inter-orbital width at narrowest point, length of sternum from middle of anterior notch, length of carina, dorsal synsacrum mid-width, length of scapula, length of humerus, length of ulna, length of femur and length of tibiotarsus (Fig. 5).

Body mass

A total of 121 freshly collected common grackles were weighed (in grams) using a Brecknell EPB-3000G series digital scale. Fifty-five males and 47 females (N = 102) made up the total data set for body mass analysis after filtering out 19 unsuitable specimens.

We used raw measurements (Rising & Somers, 1989; Perktaş & Gosler, 2010) and principal component analysis (PCA) to explore morphological variation among three currently recognized subspecies of common grackle. Principal component analysis reduces dimensionality and complexity of correlated data; we based analyses on the correlation matrix among three standard external morphological characters (tarsus, wing length and tail length) and ten skeletal characters to derive variables related to size (Robins & Schnell, 1971). Bill measurements show disparate size and shape patterns among subspecies. The Florida grackle has a longer bill than the other two subspecies (Bent, 1958); bill measurements were not included to quantify body size.

Based on external and skeletal morphological measurements, the first component (PC1) is interpreted as a variable summarizing overall size if all the characters have positive loadings (Bookstein, 1989; Rising & Somers, 1989). We used one-way ANOVA to assess geographical patterns and mean differences of PC1 among subspecies based on both types of morphological measurements. Body mass (weight in grams) from 102 freshly collected common grackles was used as our measure of body size. Although recommended (Gaston & Blackburn, 1995), we did not transform mass data, because raw data were used for both external and skeletal measurements. We checked normality and homogeneity of variances before the analyses. For this, we used the Kolmogorov–Smirnov test for normality and Levene statistic for homogeneity of variances. For multiple comparisons, we used Hochberg's GT2 method, which can manage unequal sample sizes (Quinn & Keough, 2002). We performed all analyses using IBM SPSS Statistics for Windows, v.28.0 (IBM Corp. Released 2021).

PHYLOGEOGRAPHY

DNA extraction and analysis

DNA was extracted from fresh muscle tissue of recently collected breeding grackles, kept in 100% ethanol in the field and laboratory and subsequently accessioned into the Ambrose Monell Cryo Collection (AMCC) at the AMNH. All sequences have been deposited in GenBank with associated museum voucher numbers (GenBank accession numbers OP609716–OP609734). Complete mtDNA (16.768 bp) from 22 individual common grackles collected from seven breeding populations in North America (see Fig. 4) was analysed. Six additional mtDNA gene sequences (*ND2* and *Cytb*) were obtained from GenBank and combined with our data (N = 28). GenBank accession numbers for *ND2* and *Cytb*, are as follows: AF109956, NC018803, JX516064, AF089058, AY509636 and AY509635.

DNeasy tissue kits (Qiagen) were used to extract total genomic DNA from fresh muscle tissue. Paired-end DNA sequencing was performed by Rapid Genomics (Gainesville, FL, USA). After assessing the quality of the sequences, FASTQC (v.0.11.8; Andrews, 2010) was used to detect overrepresented sequences. Using TRIMMOMATIC (v.0.39; Bolger *et al.*, 2014), sequences were trimmed and filtered. Parameters used for the TRIMMOMATIC run were PE-phred33 ILLUMINACLIP: adapters. fa: 2:30:10 MAXINFO: 40:0.5 LEADING:28 MINLEN:30. All DNA sequence reads were mapped to a reference genome (NC018803) with BWA MEM (v.0.7.17; Li, 2013). Derived sam files were converted to bam files and were sorted using SAMTOOLS (v.1.11; Li *et al.*, 2009). To call single-nucleotide polymorphisms (SNPs), we used BCFTOOLS MPILEUP (v.1.11; Li, 2011). To filter SNPs, we used VCFUTILS.PL VARFILTER (in SAMTOOLS). Using BCFTOOLS consensus, we created consensus sequences for all individuals sampled. The number of haplotypes, haplotype diversity and nucleotide diversity were estimated for the sequence data. Departure from neutrality, Tajima's D (Tajima

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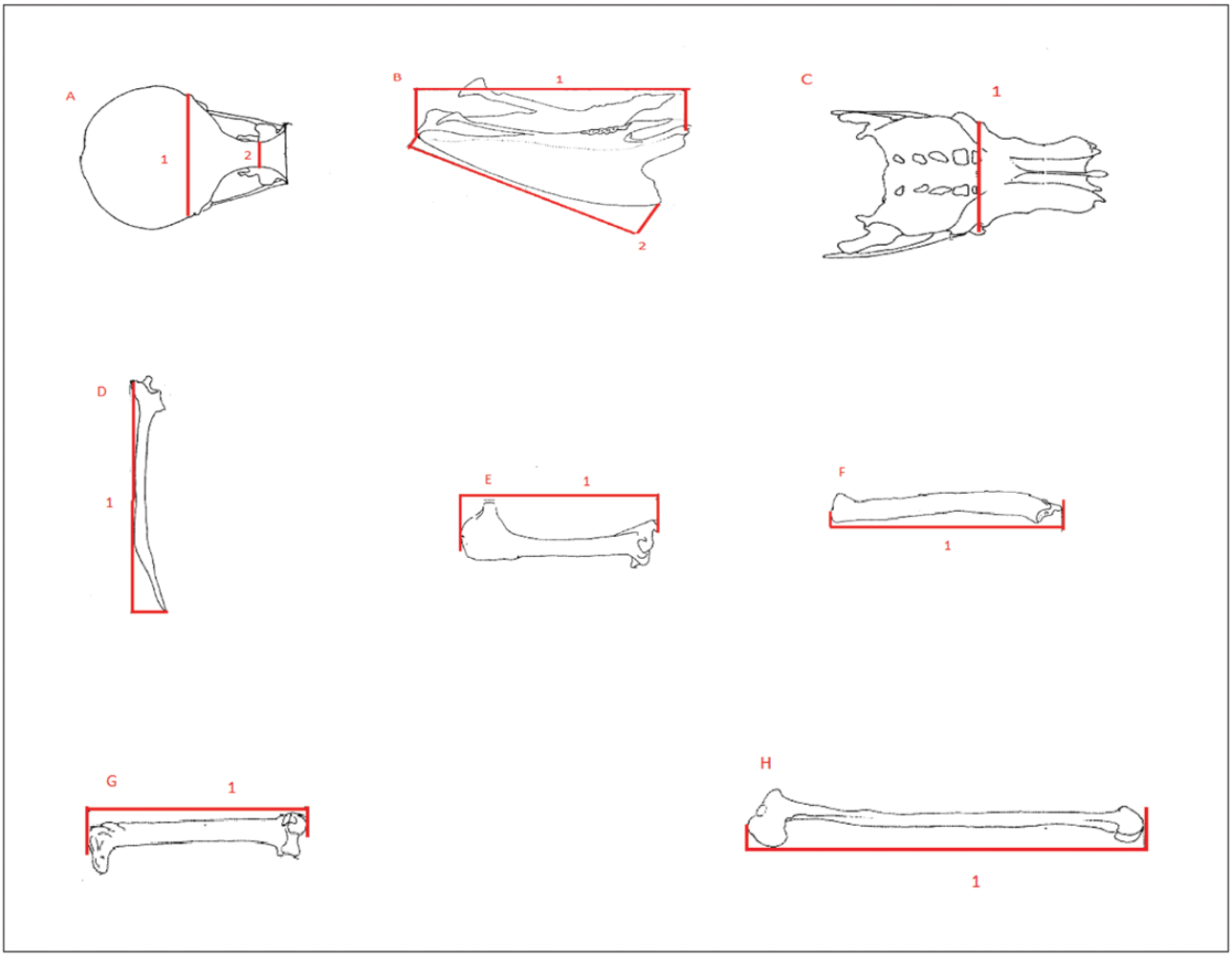

Figure 5. Description of skeletal characters measured. A1, post-orbital width. A2, inter-orbital width at narrowest point. B1, length of sternum from middle of anterior notch. B2, length of carina. C1, dorsal synsacrum mid-width. D1, length of scapula. E1, length of humerus. F1, length of ulna. G1, length of femur. H1, length of tibiotarsus. After Robins & Schnell (1971).

1989), was calculated using POPART v.1.7 (Leigh & Bryant, 2015). All phylogenetic analyses included three individuals of Brewer's blackbird, *Euphagus cyanocephalus*, as an outgroup (GenBank accession numbers: NC018827, JX516072 and AF109951).

We estimated phylogenetic relationships via maximum parsimony using PAUP 4.0b10 (Swofford 2001) under the heuristic search option, using the tree-bisection and reconnection branch-swapping algorithm. Next, we computed strict consensus trees for all equally most-parsimonious trees. Support for nodes was estimated using 1000 replicates of a non-parametric bootstrap using the same search parameters. A parsimony network was also built for *Cytb* and *ND2* using the TCS algorithm via the software POPART v.1.7 (Leigh & Bryant, 2015), because underlying assumptions of tree-building methods (e.g. complete lineage sorting) could be violated in intraspecific studies (Posada & Crandall, 2001).

To examine the demographic history of common grackles, we used an extended Bayesian skyline plot (EBSP) analysis implemented in BEAST (v.1.8.3; Drummond *et al.*, 2012). We used MEGA v.11 (Tamura *et al.*, 2021) to identify mutation models for *Cytb* and *ND2* using corrected Akaike information criteria (AICc). Fifty million generations were run for analysis and were sampled every 5000 generations. TRACER (v.1.7.3) assessed convergence. For each run, effective sample size values of the parameters were > 200.

RESULTS

MORPHOMETRY

Descriptive statistics for individual external and skeletal measurements are shown in Figure 2 and Table 1, respectively. Correlations among external

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Table 1. Descriptive statistics for skeletal measurements

Skeletal measurements		*N*	Mean	SD	SEM	95% Confidence interval for mean	
						Lower bound	Upper bound
A1 Skull post-orbital width	Male	127	18.99	0.67	0.06	18.87	19.11
	Female	68	18.38	0.58	0.07	18.24	18.52
	Total	195	18.78	0.70	0.05	18.68	18.88
A2 Skull mid-orbital width	Male	126	7.55	0.67	0.06	7.43	7.67
	Female	64	6.82	0.59	0.07	6.68	6.97
	Total	190	7.31	0.73	0.05	7.20	7.41
B1 Sternum length	Male	126	37.69	1.87	0.17	37.36	38.02
	Female	65	34.35	1.36	0.17	34.01	34.69
	Total	191	36.56	2.34	0.17	36.22	36.89
B2 Carina length	Male	126	36.40	2.16	0.19	36.02	36.78
	Female	68	32.68	1.63	0.20	32.29	33.08
	Total	194	35.10	2.66	0.19	34.72	35.47
C1 Synsacrum mid-point width	Male	128	14.02	1.40	0.12	13.78	14.27
	Female	68	13.41	1.30	0.16	13.10	13.73
	Total	196	13.81	1.39	0.10	13.62	14.01
D1 Scapula length	Male	126	34.16	1.57	0.14	33.88	34.44
	Female	62	31.49	1.19	0.15	31.19	31.79
	Total	188	33.28	1.92	0.14	33.00	33.55
E1 Humerus length	Male	125	33.17	1.45	0.13	32.92	33.43
	Female	67	30.29	1.10	0.13	30.03	30.56
	Total	192	32.17	1.92	0.14	31.90	32.44
F1 Ulna length	Male	125	38.61	1.75	0.16	38.30	38.92
	Female	66	34.53	4.59	0.57	33.40	35.66
	Total	191	37.20	3.60	0.26	36.68	37.71
G1 Femur length	Male	124	30.91	1.49	0.13	30.64	31.17
	Female	68	28.68	1.38	0.17	28.35	29.02
	Total	192	30.12	1.80	0.13	29.86	30.38
H1 Tibiotarsus length	Male	118	51.90	1.90	0.17	51.56	52.25
	Female	65	47.43	6.20	0.77	45.89	48.96
	Total	183	50.31	4.52	0.33	49.65	50.97

morphometric characters of common grackles on PC1 were all high and positive, especially for wing and tail lengths (loadings on PC1: tarsus length = 0.195, wing length = 0.868 and tail length = 0.878), hence PC1 was interpreted as a measure of overall size. Principal component 2 showed mixed positive and negative loadings. Principal component 1 and PC2 together accounted for 52.1% of the total variance among individuals including all subspecies. Correlations among skeletal morphometric characters of common grackles on PC1 were also all high and positive (loadings on PC1: skull post-orbital width = 0.645, skull mid-orbital width = 0.601, sternum length = 0.936, carina length = 0.884, synsacrum mid-point width = 0.222, scapula length = 0.898, humerus length = 0.906, ulna length = 0.874, femur length = 0.845 and tibiotarsus length = 0.540). Again, PC1 was interpreted as a measure of overall size. Principal component 2 also showed mixed positive and negative loadings. Principal component 1 and PC2 together accounted for 69.8% of the total variance among individuals including all subspecies. Therefore, both PC1 scores based on external and skeletal measurements had similar patterns and demonstrated size variability in the common grackle.

The ANOVA comparing subspecies for size based on external measurements showed significant differences (PC1, $F = 26.39$, d.f. = 2, $P < 0.001$), with differences being significant between *Q. q. quiscula* and *Q. q. versicolor* and *Q. q. stonei*, but not between the two northern subspecies (for multiple comparisons, see Table 2; Fig. 6). The ANOVA comparing subspecies for size based on skeletal measurements showed significant differences (PC1, $F = 8.065$, d.f. = 2, $P < 0.001$), with differences being significant between *Q. q. quiscula* and *Q. q. stonei* and between *Q. q. quiscula* and *Q. q. versicolor* but

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Table 2. Multiple comparisons of body size (external measurements) of the common grackle, *Quiscalus quiscula*, based on Hochberg GT2

Subspecies	Comparison	Mean difference	SEM	Significance	95% Confidence interval	
					Lower bound	Upper bound
Q. q. versicolor	*Q. q. stonei*	–0.109	0.058	0.165	–0.247	0.029
	Q. q. quiscula	0.575*	0.095	0.000	0.347	0.804
Q. q. stonei	*Q. q. versicolor*	0.109	0.058	0.165	–0.029	0.247
	Q. q. quiscula	0.684*	0.094	0.000	0.459	0.910
Q. q. quiscula	*Q. q. versicolor*	–0.575*	0.096	0.000	–0.804	–0.347
	Q. q. stonei	–0.684*	0.094	0.000	–0.910	–0.459

*The mean difference is significant at the 0.05 level.

not between *Q. q. versicolor* and *Q. q. stonei*, the two northern subspecies (for multiple comparisons, see Table 3; Fig. 6). In addition to these results, body mass data showed a similar pattern (Fig. 7).

PHYLOGEOGRAPHY

Descriptive statistics and haplotype relationships

Nucleotide diversity (π) equals 0.002, and the number of segregating sites is 230 for a complete data set. The common grackle populations depart from neutral expectations based on Tajima's D [Tajima's D, –1.89596, $p(D < -1.89596) = 0.04$]. A negative Tajima's D reveals an excess of low-frequency polymorphisms relative to expectation, and a population size expansion (e.g. after a bottleneck or a selective sweep). Analysing the 21 ingroup plus two outgroup sequences in a maximum parsimony analysis, we obtained 25 trees of length 1154 [consistency index (CI) = 0.92, retention index (RI) = 0.92]; to aid interpretation, one of these trees is shown as a phylogram available from the corresponding author based on a reasonable request. One hundred and twenty-five variable characters were parsimony uninformative. Nine hundred and twenty-eight characters were parsimony informative. The strict consensus tree (Fig. 4) indicated two clades. Two haplotypes from Florida populations occurred in only one clade and were not shared with any other populations. However, some haplotypes from Florida were present in the other clade among other haplotypes.

Demography

The parsimony-based haplotype networks of the two gene regions (*Cytb* and *ND2*) showed a star-shaped structure (Fig. 8). The ancestral haplotype is central and occurs in various geographical regions, including Florida. The mutation models for *Cytb* and *ND2* gene regions were TN93+G (AICc = 3989.143) and HKY (AICc = 3688.102), respectively. The EBSP indicated a clear pattern of population expansion of the common grackle populations over the Late Pleistocene and Holocene (Fig. 9). Analyses across a wide range of plausible mutation rates for mtDNA control regions (2–4%/Myr), and considering a generation time of 1 year, uniformly indicated that population expansion began after the LGM.

DISCUSSION

Coupling phylogeography with phenotypic (Zamudio *et al.*, 2016), morphometric and ecological studies has shown how climate change affects biogeographical patterns of species (Rissler & Apodaca, 2007; Waltari *et al.*, 2007; Perktaş *et al.*, 2017, 2019). Genetic diversity for populations is very important because it encourages future adaptations and requires sufficient genetic diversity to maintain population continuity by preventing low population fitness (Frankham *et al.*, 2010; Hedrick, 2013). Our strategy in this study was to bring together the morphometric variation and genetic diversity of the common grackle, a common bird species for North America, in addition to accurate taxonomic inferences based on genetic diversity patterns, and to evaluate the ecological processes related to these variations correctly. To date, no study has combined morphometry and phylogeography to elucidate subspecies limits in the common grackle. In the present study, we took two approaches to understand the evolutionary history of the common grackle. Huntington (1952) determined that variation in body size between populations of this species is clinal. Our analysis of external and skeletal morphology and body mass also revealed significant variation in body size. We also evaluated molecular data among common grackles; subspecies showed significant variation in body size, but not in their mitochondrial genome.

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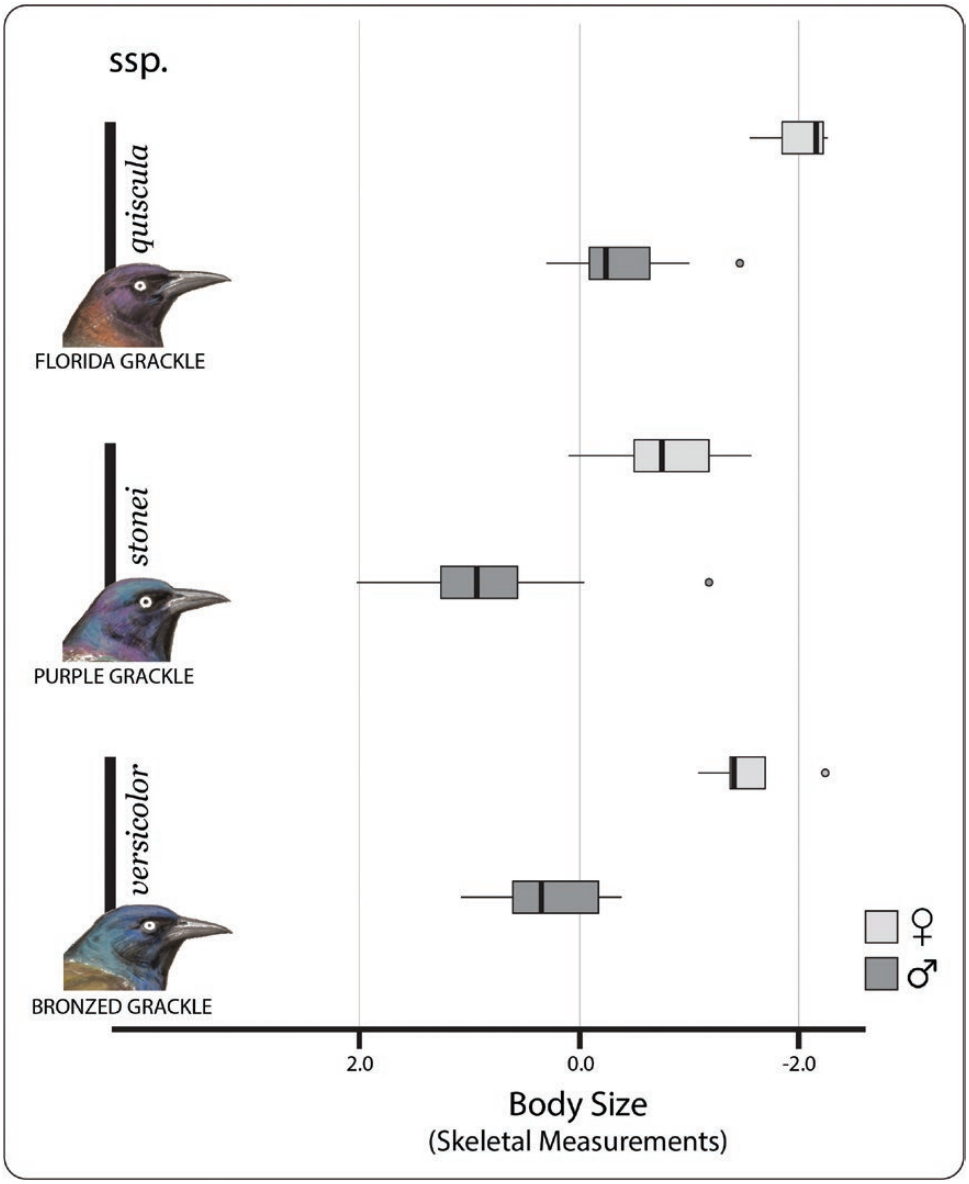

Figure 6. Differences in body size among subspecies of the common grackle based on skeletal measurements.

We analysed the entire mtDNA genome from a phylogenetic perspective. Parsimony analysis showed that some haplotypes from Florida (*quiscula*) were different from haplotypes of the northern subspecies (*versicolor* and *stonei*) and formed a separate cluster. However, Florida haplotypes did occur within populations of *versicolor* and *stonei*. Such mtDNA paraphyly is probably attributable to hybridization and/or introgression (Funk & Omland, 2003; Vázquez-Miranda *et al.*, 2009) or an event of incomplete lineage sorting between Florida and non-Florida populations (Joseph & Omland, 2009). Distinguishing between these possibilities would require multilocus data sets that are not currently available. Our analysis of genetic demography showed that the most common haplotypes were shared by Florida and the northern populations of the common grackle. This result together with the phylogenetic tree pattern suggests ancestral allelic diversity, and paraphyly of northern common grackles with respect to common grackles in Florida. These lineages might not have achieved reciprocal monophyly, probably a strong indication of incomplete lineage sorting (McKay & Zink, 2010).

The assessment of the demographic history of the species suggested that the effective population size of the common grackle started to increase after the LGM, and the most reasonable explanation of this increase might be short-term isolation within a glacial refugium

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Table 3. Multiple comparisons of body size (skeletal measurements) of the common grackle, *Quiscalus quiscula*, based on Hochberg GT2

Subspecies	Comparison	Mean difference	SEM	Significance	95% Confidence interval	
					Lower bound	Upper bound
Q. q. versicolor	*Q. q. stonei*	–0.435	0.227	0.161	–0.983	0.113
	Q. q. quiscula	0.458	0.305	0.352	–0.278	1.194
Q. q. stonei	*Q. q. versicolor*	0.435	0.227	0.161	–0.113	0.983
	Q. q. quiscula	0.893*	0.237	0.001	0.320	1.467
Q. q. quiscula	*Q. q. versicolor*	–0.458	0.305	0.352	–1.194	0.278
	Q. q. stonei	–0.893*	0.237	0.001	–1.467	–0.320

*The mean difference is significant at the 0.05 level.

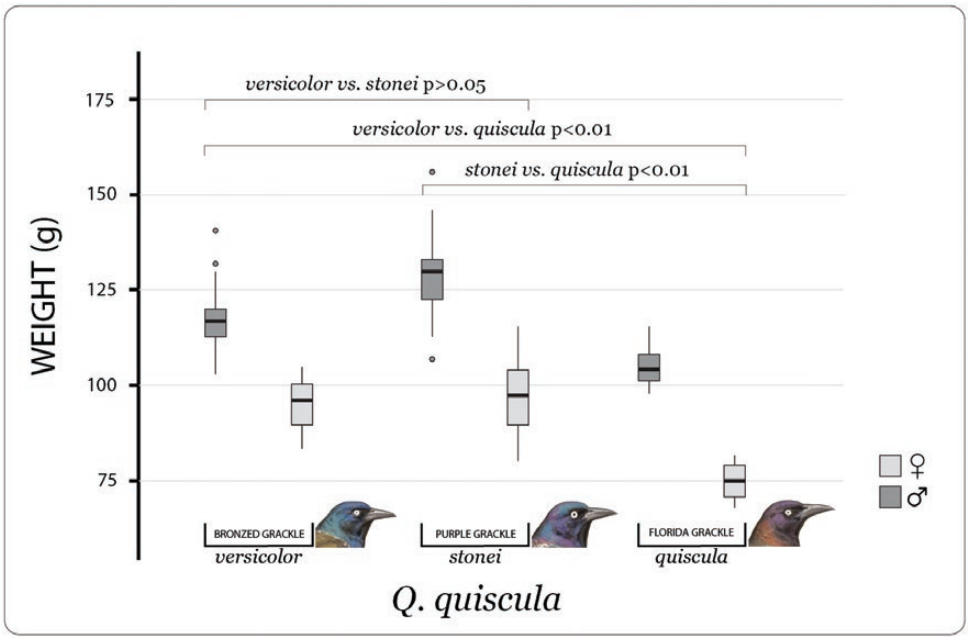

Figure 7. Differences in body mass among subspecies of the common grackle. *P*-values show differences between subspecies based on multiple comparisons.

located in the southern part of North America. This is probably attributable to high genetic diversity in the samplings from Florida and long-term climatic stability in Florida and its environs (Capainolo *et al.*, 2020). Gene-based haplotype networks together with phylogenetic relationships based on complete mtDNA data indicated a category IV phylogeographical pattern, which shows extensive gene flow in a species not sub-divided by persistent geographical barriers (Avise *et al.*, 1987).

The use of mtDNA as a single marker to explain intraspecific genetic differentiation patterns (Perktaş *et al.*, 2020) is the subject of considerable debate (Zink & Barrowclough, 2008; Edwards & Bensch, 2009; Bohonak & Vandergast, 2011). There is now a consensus among proponents and opponents of various uses of mtDNA that it is still useful for inferring patterns (phylogenies). However, by itself, it is often not sufficient to reveal demographic histories (Zink & Barrowclough, 2008; Edwards & Bensch, 2009; Lucas *et al.*, 2022). Comprehensive studies at the whole-genome level in the future will be useful to reveal the deeper evolutionary history of the common grackle.

SUBSPECIES LIMITS

Owing to the contentious nature and multiplicity of species concepts (Hey, 2001; Phillimore & Owens, 2006; Reydon & Kunz, 2021), integrative taxonomy

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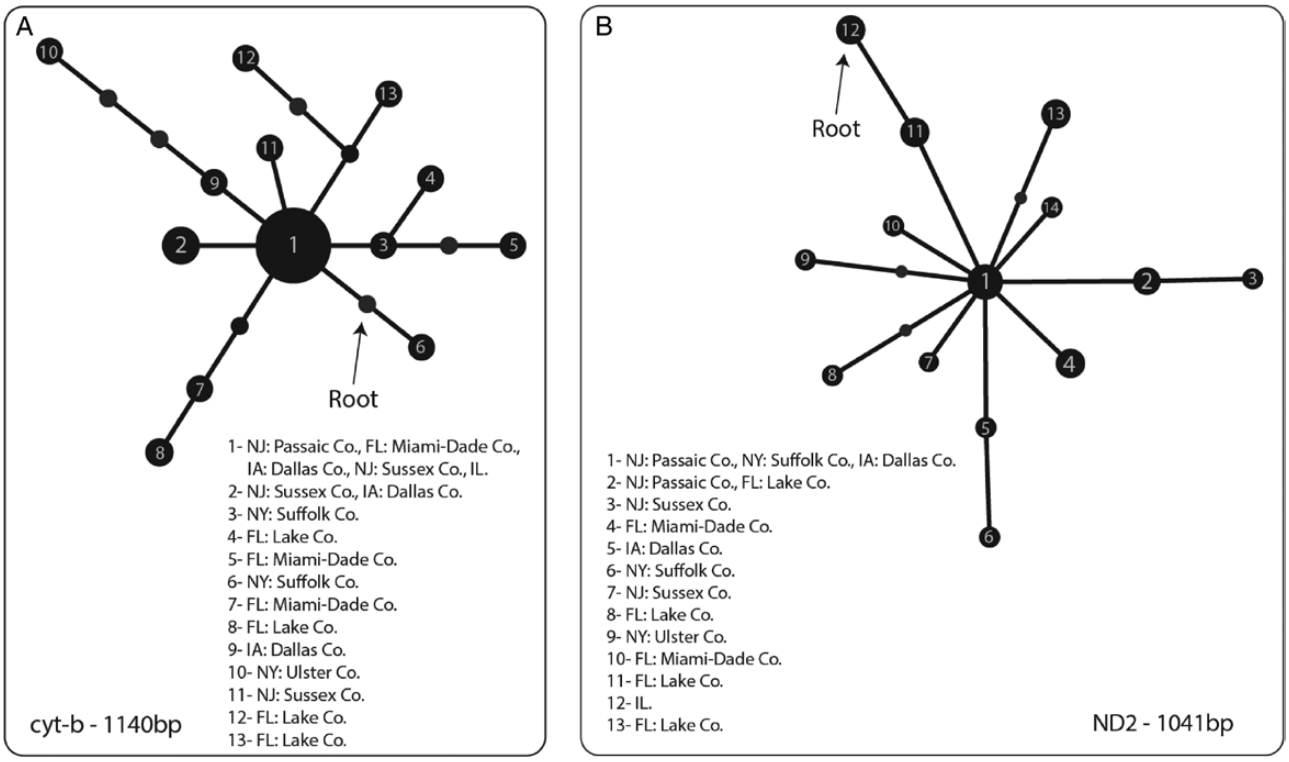

Figure 8. Parsimony-based haplotype networks of two gene regions (*Cytb* and *ND2*).

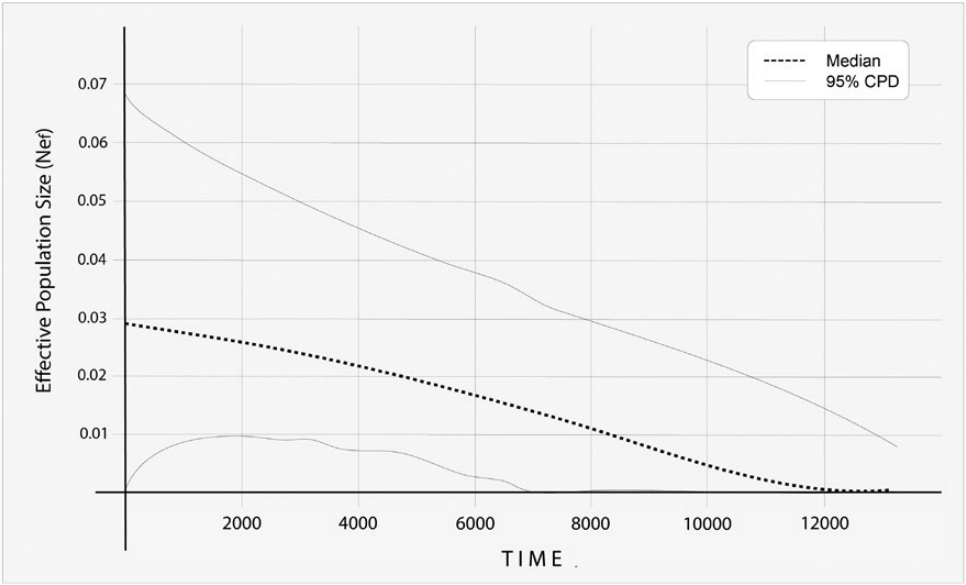

Figure 9. Extended Bayesian skyline plot indicating a clear pattern of population expansion of the common grackle populations over time.

requires careful evaluation of genetic, morphological, ecological and behavioural variation both within and among populations (Mayr, 1969; Bock, 1974; Dayrat, 2005; Will *et al.*, 2005, Padial *et al.*, 2010, Perktaş *et al.*, 2017; Sangster, 2018; Cicero *et al.*, 2021). Likewise, an understanding of the ecological niche characteristics of these taxa is also necessary (Raxworthy *et al.*, 2007), although the utility of such characteristics in decisions

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about species limits has been debated (Tocchio *et al.*, 2015). We also published ecological niche modelling predictions of the common grackle for the past and the future (Capainolo *et al.*, 2020, 2021). Past predictions demonstrated a single refugium in the south; this part of the current range was an ancestral part of the distribution of the common grackle. Based on our genetic analysis, the Florida grackle appears to be the ancestral form occupying an ancestral range. We conclude that the diagnosable genetic structure of Florida grackles remains questionable owing to lack of monophyly and incomplete lineage sorting, but the other two subspecies demonstrate high gene flow, indicating a single subspecies in the north.

A reassessment of the taxonomy and nomenclature of the common grackle is therefore advised based on the findings in this analysis. Resident populations of the Florida grackle form a clade separate from that of northern populations of migratory bronzed and purple grackles. The latter forms, hybrids between the two (i.e. *Q. q. ridgwayi*) and introgressed populations do not form several clades but comprise one large northern clade. It seems, therefore, as presciently stated by Yang & Selander (1968), that the common grackle can be divided into two subspecies, whereby nominate *Q. q. quiscula* remains, but *Q. q. stonei*, hybrid *Q. q. ridgwayi* and introgressed forms are subsumed into *Q. q. versicolor*, as prescribed under the regulations of The International Commission on Zoological Nomenclature (ICZN).

There are reservations about the approach taken here and our philosophy regarding subspecies. It is generally accepted that divergence between well-differentiated subspecies in avian species and recently separated sister species can be attributed to geographical isolation during the mid–late Pleistocene (Avise & Walker, 1998; Lovette, 2005). Climate change events during the late Pleistocene probably precipitated evolutionary differentiation between the nominate subspecies and subsequent forms of common grackle. As indicated by some palaeoecological data (Pielou, 1991), suitable songbird habitat south of the edge of the glacier in eastern and western North America existed, but the centre of the country was occupied by tundra and desert, habitat not suitable for many songbird species. Genetic data for the common grackle, combined with ecological niche model results, show that the distribution limits of the nominate subspecies (*Q. q. quiscula*) formed the range of the entire species during the LGM. Considering the hypothetical distribution at the LGM (Capainolo *et al.*, 2020), the parapatry zones between *Q. q. quiscula*, *Q. q. versicolor* and *Q. q. stonei* probably represent secondary contact zones following postglacial expansion to the north and west. Therefore, understanding the demographic history of bird species, particularly migratory species, is especially important; the biological species concept of Dobzhansky (1937) and Mayr (1942), states that 'species are systems of populations: the gene exchange between these systems is limited or prevented by a reproductive isolating mechanism or perhaps by a combination of several mechanism'. The grey area of the biological species concept is hybridization that allows gene flow before two groups are considered subspecies rather than sister taxa. The situation in the common grackle is comparable to that in the yellow-rumped warbler, *Dendroica coronata* (Barrowclough, 1980; Milá *et al.*, 2011), which suggests recent isolation followed by colonization of mixed populations to North America. We show shared haplotypes between nominate *Q. q. quiscula* and the other two subspecies of the common grackle. This makes separating subspecies difficult, but nominate *Q. q. qusicula* is clearly diagnosable according to morphometry (e.g. body size) and colour characters (the phenotypically stable 'phase 1' of Chapman), although it is known to hybridize extensively in a hybrid zone suggested by Zink *et al.* (1991) that confirmed historical isolation. In this sense, understanding the historical demographics for any taxon is important for interpreting its current taxonomic position.

Hybrid zones for the common grackle are wide in relationship to estimates of dispersal, and there is no strong reproductive isolation between *Q. q. versicolor* and *Q. q. stonei*. However, there are substantial morphometric differences between *Q. q. quiscula*, *Q. q. versicolor* and *Q. q. stonei*; hybridization does occur between *Q. q. quiscula* and the other subspecies where their ranges meet in the south-central Atlantic and gulf states. Demographic history together with body size differentiation strongly indicate that the two northern subspecies diversified recently (after the LGM), and high genetic diversity within *Q. q. quiscula* together with ecological niche modelling results (Capainolo *et al.*, 2020) indicate that this subspecies is an ancestral form of the common grackle. Based on this inference, body size, rather than genetics, is a key character for assessing taxonomic questions regarding common grackles.

Theoretical approaches, such as the 75% rule, should be managed carefully in the evaluation of subspecies (Remsen, 2010). Amadon's (1949) 75% rule of subspecies has a long history in ornithology, but its application has been sporadic. This rule is no longer used in recently published bird checklists, and it is not possible to say how many existing subspecies comply with this rule (see American Ornithologists' Union, 1957; Howard *et al.* 2003; Hoyo *et al.*, 1992–2008), because defining subspecies on the basis of statistically significant differences in populations means that any character might lead to misinterpretation of the definition of a subspecies (Patten & Unitt, 2002).

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When sufficient sample sizes are obtained in subspecies evaluations, a significant overlap in terms of the normally distributed values of the character in question becomes likely, which does not comply with the 75% rule, although there is a significant difference in terms of the character in question. This makes it debatable to what extent the examined character indicates a taxonomically correct assessment. Therefore, in evaluating the taxonomy of the common grackle, we consider it appropriate to subsume *Q. q. stonei* under *Q. q. versicolor*. There is high gene flow among them based on mtDNA analysis, and our analysis of historical demography indicates that they are recently diversified temporally, without clear geographical barriers between them. We evaluated nominate *Q. q. quiscula*, distributed south of the range of *Q. q. versicolor* and *Q. q. stonei*, differently. This explanation confirms the situation stated by Zink (2004); nominate *Q. q. quiscula* points to a different evolutionary history (even with the problem of 'incomplete lineage sorting' in the phylogeny). We believe that the high genetic diversity of the subspecies distributed, in the south, along with its morphological differences, support its rank as the nominate, ancestral form, and the two northern subspecies (including the hybrid *Q. q. ridgwayi*) should be treated as a single subspecies, *Q. q. versicolor*.

Moreover, songs of Florida grackles seem to differ from northern populations in key features, such as note composition and tempo within note complexes. Some male songs of the Florida grackle and the other two subspecies, *Q. q. versicolor* and *Q. q. stonei*, recorded and available (XenoCanto XC316288, XC131091 and XC565482), demonstrate variations that should be studied extensively, but are currently beyond the scope of the present study. Although these behavioural and morphological differences are probably characteristic of distinct species diverging on separate evolutionary histories, lack of reciprocal monophyly in the phylogenetic tree makes it difficult to reach concrete taxonomic recommendations at present. Considering the results of our analyses *in toto*, we suggest recognizing only two subspecies of common grackle using the 75% rule of subspecies and suggest lumping *Q. q. versicolor* with *Q. q. stonei*. Therefore, we recognize two subspecies of the common grackle, *Q. q. qusicula* (southern common grackle) and *Q. q. versicolor* (northern common grackle). Additional study and further analysis of common grackle hybrid zones is clearly needed and will provide valuable information concerning the biogeography of North America.

Simplifying common names might be welcomed by ornithologists, birders and citizen scientists. Online bird identification listservers, such as eBird (https://ebird.org/home), sometimes use confusing taxonomic terms when attempts are made to identify subspecies of common grackle, such as '*stonei*/*ridgwayi*' for some of the myriad colour phenotypes of purple grackle north of the range of *Q. q. quiscula*. Referring to nominate, resident Florida grackle, *Quiscalus quiscula*, as the 'southern common grackle' and all other forms to the north and west of its range as the 'northern common grackle' (now all *Q. q. versicolor*) provides a solution.

Re-evaluation and modification of current subspecies status might also be useful for conservation and preservation strategies. For example, the 'southern common grackle', *Q. q. quiscula*, might be particularly vulnerable to population decline, being non-migratory and occupying a limited range in regions of immense agricultural diversity and activity. Long-standing lethal control methods aimed at protecting important agricultural products, such as corn and sunflower, from seasonal ravages of large flocks of common grackles might be responsible for a reduction in population of > 50%, beginning in 1970 (Peer & Bollinger, 2017). This prompted the International Union for Conservation of Nature (IUCN) Red List for birds (https://www.iucnredlist.org/species/22724320/131484290) to list the common grackle as a 'Near Threatened' species (Bird Life International, 2020). A resident subspecies, although still abundant, might not be able to withstand a continued onslaught such as this for long, perhaps prompting federal and state fish and wildlife agencies to evaluate carefully the protocols for issuing lethal take depredation permits.

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gave generously of their time and knowledge with respect to the mtDNA aspects of the project. Common grackles are a protected species in the USA and Canada under the Migratory Bird Treaty Act (MBTA). Provincial, federal and state permits are required to collect specimens for scientific studies. We thank the following people for help in applying for and granting the proper necessary permits: Jonathan Ratcliffe and Artie McCollum (USFWS), Teresa Ruiz (New York), David Golden (New Jersey), Brian Garrett, Heather Ellison and Thomas Scott (Florida) and Karmin Klingenberg (Iowa). Permit numbers are available upon request from the corresponding author. For help in finding and collecting specimens, allowing access to private properties or giving us shelter and food during field seasons, we acknowledge and thank: in New York, Howie and Eric Lewin, the Bill Parr family, the Gantz family, Bill Robinson, Stuart Rossell, Jeff Groth, Lucas Musher, Lisa Marie Parker and Neil Duncan, Craig Eccleston, Michael Farley, Monica Thomas, Courtenay Thomas, Donovan Morant, Amethyst LaRosa, Slade Rivera, Dawn Satterly and Fabiola Marie LeCorps-Villareal; in New Jersey, Steven Polanish (Hudson Farm Club) and George Dante, Jr; in Florida, Thomas Scott (US Forest Service), Sal Finocchiaro and Leo Talarico (SNL Beans), Ashley Cornelius, Dennis Berry, Jose Guillen (Don's Bait and Tackle), John and Tara Boschell, Ronald M. Chase and Wendy Paradise; and in Iowa, Aaron Nelson (Doc's Hunt Club) and Justin and Stephanie Sipla. Robert Moyle and Mark Robbins granted permission to Lucas DeCicco and Fernando Machado-Stredel to collect common grackles for us in Kansas. These gentlemen, based at The University of Kansas Biodiversity Institute & Natural History Museum, are consummate professionals; we deeply appreciate their contribution to this study. In addition to specimens in the collections of the American Museum of Natural History (AMNH), we borrowed material from seven sister institutions. The curators and collections managers smoothed the process of borrowing and returning common grackle study skins and skeletons during a particularly challenging time, while workplace and mail delivery protocols struggled under the coronavirus disease 2019 pandemic. We gratefully acknowledge Nate Rice (Academy of Natural Sciences of Drexel University), Jeremy Kirchman and Joseph Bopp (New York State Museum), Brian O'Shea (North Carolina State Museum), Christopher Milensky (United States National Museum), Brett Benz (University of Michigan Museum of Zoology), Jean Woods (Delaware Museum of Natural History) and Mark Adams and Alex Bond (British Museum of Natural History-Tring). Dale Dyer produced the magnificent illustration of common grackle subspecies especially for this study, and we are grateful. David A. Sibley kindly gave us permission to modify and use his illustrations of common grackle phenotype varieties and range map. Matt Shanley kindly produced the excellent photograph of study skins, and Eric Cortes, Scott Unger and David Seligman produced the YouTube video demonstrating our study skin preparation technique. Two anonymous reviewers greatly improved the manuscript by suggesting edits and modifications that we incorporated into our revisions.

P.C., U.P. and M.D.E.F. conceived the study; P.C. collected and prepared most of the fresh specimens over several field seasons; U.P. developed methods, and P.C. prepared morphometric data and made all DNA extractions; C.E. aligned and prepared DNA data; P.C. and U.P. analysed all phenotypic and genotypic data; P.C. and U.P. wrote the paper, with discussion with C.E. and M.D.E.F. All authors read and approved the final manuscript. We have no conflicts of interest to declare.

DATA AVAILABILITY

The genetic data underlying this article are available in the GenBank Nucleotide Database. The morphometric data underlying this article will be shared upon reasonable request to the corresponding author.

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Table S1. Details of studied skins (institution, collection number, sex and geographical location).
Table S2. Details of studied skeletons (institution, collection number, sex and geographical location).

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