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Becoming pure: identifying generational classes of admixed individuals within lesser and greater scaup populations

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Abstract

Estimating the frequency of hybridization is important to understand its evolutionary consequences and its effects on conservation efforts. In this study, we examined the extent of hybridization in two sister species of ducks that hybridize. We used mitochondrial control region sequences and 3589 double-digest restriction-associated DNA sequences (ddRADseq) to identify ADMIXTURE between wild lesser scaup (*Aythya affinis*) and greater scaup (*A. marila*). Among 111 individuals, we found one introgressed mitochondrial DNA haplotype in lesser scaup and four in greater scaup. Likewise, based on the site-frequency spectrum from autosomal DNA, gene flow was asymmetrical, with higher rates from lesser into greater scaup. However, using ddRADseq nuclear DNA, all individuals were assigned to their respective species with >0.95 posterior assignment probability. To examine the power for detecting ADMIXTURE, we simulated a breeding experiment in which empirical data were used to create F1 hybrids and nine generations (F2–F10) of backcrossing. F1 hybrids and F2, F3 and most F4 backcrosses were clearly distinguishable from pure individuals, but evidence of admixed histories was effectively lost after the fourth generation. Thus, we conclude that low interspecific assignment probabilities (0.011–0.043) for two lesser and nineteen greater scaup were consistent with admixed histories beyond the F3 generation. These results indicate that the propensity of these species to hybridize in the wild is low and largely asymmetric. When applied to species-specific cases, our approach offers powerful utility for examining concerns of hybridization in conservation efforts, especially for determining the generational time until admixed histories are effectively lost through backcrossing.

Keywords: ddRADseq, diving duck, evolution, hybridization, introgression, population genetics

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Introduction

Although interspecific gene flow can be detrimental to biodiversity (i.e. outbreeding depression, extinction via genetic swamping; Rhymer 2006; Seehausen 2006; Webb *et al.* 2011), such events can also be adaptive (i.e. adaptive introgression; Morjan & Rieseberg 2004; Whitney *et al.*

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2006; Heliconius Genome Consortium 2012; Hedrick 2013; Fontaine *et al.* 2015), and even necessary to complete the speciation process (i.e. reinforcement, speciation with gene flow; Dobzhansky 1940; Hoskin *et al.* 2005; Mallet 2005; Rundle & Nosil 2005; Schluter 2009). Thus, determining the role of gene flow in natural populations is necessary to understand its effects on the speciation process and on populations that are the focus of management and conservation. However, distinguishing signals of interspecific gene flow and incomplete lineage sorting (i.e. genetic similarity due to common ancestry) is nontrivial at stages of early divergence (Dobzhansky 1940; Coyne & Orr 2004; Carstens & Knowles 2007; Schluter 2009; Kutschera *et al.* 2014). Doing so is particularly difficult in scenarios in which hybridization is relatively rare (i.e. no hybrid zone/regions) and hybrid offspring backcross into the parental populations. For example, low interspecific assignment probabilities (e.g. <10%) might be expected as a result of incomplete lineage sorting and low power under scenarios of no gene flow (Noor & Bennett 2009; Cruickshank & Hahn 2014; Seehausen *et al.* 2014). However, even the smallest interspecific assignment probabilities can be indicative of backcrossed individuals because each generation of backcrossing diminishes the overall 'hybrid' signal. Thus, the presence/absence and number of generational backcrosses need to be considered when studying the effects and prevalence of gene flow on population structure.

The differential influence of gene flow and genetic drift can yield discordant relationships among genomic marker types, making it challenging to reconstruct lineage demographics and histories (Carstens & Knowles 2007). For example, mitochondrial DNA (mtDNA) has been a flagship marker for population geneticists and an excellent indicator for the possible occurrence of gene flow between populations (Rheindt & Edwards 2011). However, the presence of putatively introgressed mtDNA haplotypes can be misleading when attempting to determine the extent of contemporary versus historical/ancient hybridization (Liu *et al.* 2010). Specifically, being maternally inherited, similar mtDNA lineages can persist within a population long after introgression occurred and can even become 'captured' within the populations or species with little or no sign of nuclear introgression due to subsequent generations of backcrossing (Ballard & Whitlock 2004; Toews & Brelsford 2012). Alternatively, populations can have monophyletic mtDNA lineages with recently admixed nuclear genomes due to sex-biased mating events and/or incomplete lineage sorting (Choleva *et al.* 2014; Peters *et al.* 2014; Lavretsky *et al.* 2015b). Thus, determining the effects of alternative factors influencing population structure requires multiple marker types to be considered simultaneously.

In this study, we aimed to determine the extent of hybridization and test for the effects of gene flow versus incomplete lineage sorting in two sister species of ducks that are thought to hybridize (Gray 1958). First, we isolated several marker types (mtDNA, autosomal, Z chromosome linked) that differ in effective population size and inheritance to test for signatures of differential introgression (i.e. signatures of gene flow, selection and genetic drift/incomplete lineage sorting). Next, we determined the number of hybrid classes to further test whether 'admixed' signals are the result of gene flow or incomplete lineage sorting. To do so, empirical data were used to simulate an F1 hybrid and subsequent backcrosses for a number of generations, providing a virtual breeding experiment. These *in silico* results should help us understand and classify hybrid individuals from the wild. For example, if gene flow is primarily influencing empirical population structure, then we expect individuals with admixed proportions to fall into simulated hybrid classes, with 'pure' individuals assigned with >99% probability to their respective population. F1 hybrids will be rarer as rates of hybridization decrease, and thus, we might expect hybrids to primarily be represented by later stages of backcrosses. Additionally, the number of individuals falling into each hybrid class will help test between contemporary and ancient/historical hybridization. For instance, contemporary hybridization may lead to mtDNA and nuclear signatures of introgression, whereas historical hybridization may be supported with mtDNA introgression but with few individuals with admixed nuclear genomes. Alternatively, if incomplete lineage sorting is the cause of shared ancestry within the empirical data, then we expect similar ADMIXTURE proportions to persist throughout simulated backcrossed generations (i.e. average assignments <99% probability). Finally, if incomplete lineage sorting and gene flow simultaneously influence assignment probabilities, then we expect hybrid signals to be substantially elevated and distinguishable from stochastic background levels; although we acknowledge that the latter scenario may only be diagnostic for the first few hybrid generations.

Study system

Avian lineages are especially prone to hybridization, even between relatively deep divergences (Grant & Grant 1997; Rheindt & Edwards 2011; Ottenburghs *et al.* 2015). Waterfowl (order Anseriformes) in particular exhibit some of the most extensive cases of hybridization (Johnsgard 1960; Livezey 1986; Randler 2002; Lijtmaer *et al.* 2003), with 40–60% of species being capable of interbreeding (Grant & Grant 1992; Aliabadian

& Nijman 2007) and about 20% producing viable hybrids (Scherer & Hilsberg 1982). The high rates of hybridization in birds are attributable to their dispersal ability (Greenwood 1980), chromosomal stasis (Ellegren 2010) and relatively low levels of reinforcement (Grant & Grant 1997). The probability for continued interspecific pairings is further perpetuated in many waterfowl species through forced copulation (McKinney *et al.* 1983) and/or brood parasitism (Lyon & Eadie 1991; Sayler 1992). Forced copulation by males has been proposed to be the cause of most interspecific hybridizations in this order (McKinney & Evarts 1998). In addition, brood parasitism has the potential to result in parental imprinting and future interspecific pairings (Slagsvold & Hansen 2001; Sorenson *et al.* 2010). Consequently, such events, although perhaps rare, may be a reason why many waterfowl species have not reached complete reproductive isolation (Gill 2014; Sangster 2014). It is important to note, however, that while reproductive isolation may never be attained, species boundaries generally remain intact in the face of continued gene flow (Tubaro & Lijtmaer 2002; Kraus *et al.* 2012). With many 'hybrids' primarily characterized in males, Haldane's rule (Haldane 1948) may be an important speciation mechanism within the order Anseriformes (Tubaro & Lijtmaer 2002; Kirby *et al.* 2004), although this can also be explained by the ease of male versus female plumage hybrid diagnosability in waterfowl (Tubaro & Lijtmaer 2002; Randler 2004).

Here, we study the genetic structure between and within two closely related sister species of scaup: lesser scaup (*Aythya affinis*) and greater scaup (*A. marila*) (Kessler & Avise 1984; Livezey 1996). Lesser scaup are endemic to North America, whereas greater scaup have a circumpolar distribution with two recognized subspecies that include the Eurasian subspecies (*A. m. marila*; also found in the Nearctic region) and the Nearctic subspecies (*A. m. nearctica*; also found in the Palearctic region) (Fig. 1; Livezey 1997; Baldassarre 2014). Scaup populations have been steadily declining since 1974—although much of the decline has been attributed to decreasing lesser scaup recruitment in boreal forest habitats (Austin *et al.* 2000; Afton & Anderson 2001; Anteau *et al.* 2007)—with current populations estimated at 1.2–1.4 million greater scaup (Delany & Scott 2006) and 4.2 million lesser scaup (U.S. Fish and Wildlife Service 2015). Morphologically, the two species are similar but differ in body size (greater scaup > lesser scaup), head shape (greater scaup = round; lesser scaup = peaked) (Ryan 1972; Austin *et al.* 1998; Baldassarre 2014) and extent of white on the wing (Wilson & Ankney 1988).

Scaup are known to engage in intra- and interspecific brood parasitism, with mixed nests identified in both species (Bengtson 1972), which increases the potential for wrongful parental imprinting and future interspecific pairings. In addition, males of both species pursue extra-pair copulation (Afton 1985), further increasing the potential for hybridization where the two species overlap geographically. However, spring and wintering habitats (Martínez-Vilalta *et al.* 1992; Austin *et al.* 1998), as well as food resource (Badzinski & Petrie 2006) partitioning, have been documented and are thought to be mechanisms by which interspecific competition is minimized. Thus, although the two species are believed to hybridize and are known to do so in captivity (Johnsgard 1965; Gillham & Gillham 1996), the propensity to do so in the wild remains unknown. To date, hybrid identification has been restricted to phenotypic characters, which have largely been inconclusive (Wilson & Ankney 1988). Nevertheless, if hybridization occurs, we expect more hybrids where the two species overlap (e.g. Interior Alaska; Fig. 1) as compared to where they are largely allopatric (Fig. 1). Furthermore, if hybridization occurs but is rare, then we expect few, if any, F1 hybrids, but rather later stages (i.e. ≥F2) of backcrossed individuals within sample sets. This is, then, an excellent system in which to develop and test methods that can provide a rigorous framework for genomic studies of lineages affected by gene flow and genetic drift, both in the context of evolutionary biology and in conservation and wildlife management.

Materials and methods

Sampling and DNA extraction

Blood and muscle tissues from 111 greater ($N = 64$) and lesser ($N = 47$) scaup were sampled across their respective ranges (Fig. 1; Table S1, Supporting information). Genomic DNA was extracted using a DNeasy Blood & Tissue kit and following the manufacturer's protocols (Qiagen, Valencia, CA, USA). Extractions were quantified using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc.) to ensure a minimum concentration of 20 ng/μL.

ddRAD-seq library preparation

Sample preparation for ddRAD sequencing followed the double-digest protocol outlined in DaCosta & Sorenson (2014). In short, ~1 μg of genomic DNA was digested using 10 U of each *Sbf*I and *Eco*RI restriction enzymes. Adapters containing sequences compatible for Illumina TruSeq reagents and barcodes for de-multiplexing reads were ligated to the sticky ends generated by the restriction enzymes. The adapter-ligated DNA fragments were then size-selected using gel elec-

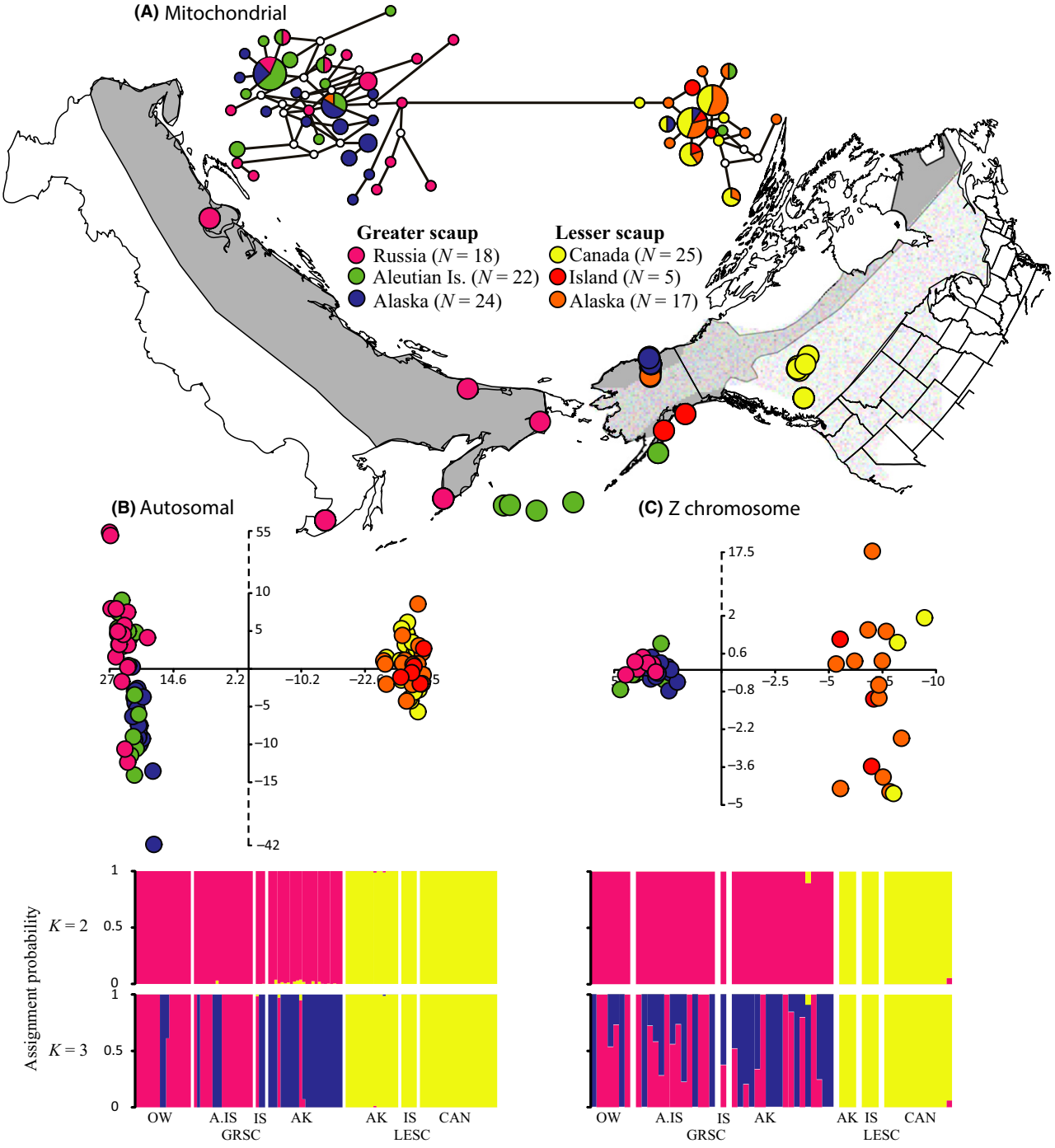

Fig. 1 Sampling locations of lesser and greater scaup with samples colour-coded by geographic region (Table S1, Supporting information; *N* = number of samples). We note that 'Interior Alaska' includes the Arctic Coastal Plain and Interior Alaska regions, and those identified as 'Island' are from any islands off the coast of Alaska that are not part of the Aleutian chain. Breeding distributions are shaded for greater (grey) and lesser (speckled) scaup. (A) The mitochondrial DNA median-joining network—size of circles corresponds to total number of individuals with that haplotype and branch lengths indicate the number of mutations separating haplotypes. The scatter plots are of the first two principal components plotted for (B) 3448 autosomal and (C) 140 Z loci (males only, because PCA does not accommodate heterogamy). Finally, the respective population assignment posterior probabilities obtained from ADMIXTURE for *K* = 2 and 3 populations reconstructed with bi-allelic SNPs from autosomal (13 532 SNPs) and Z-linked loci (254 SNPs). Colours for the mtDNA network and PCA results correspond to those shown in the sampling map.

trophoresis (2% low-melt agarose) and a MinElute Gel Extraction Kit (Qiagen). Fragments of 300–450 bp (including adapters) were selected, but fragments as small as ~155 bp are also consistently captured using this method (see DaCosta & Sorenson 2014). Size-selected fragments were then PCR amplified with Phusion high-fidelity DNA polymerase (Thermo Scientific, Pittsburgh, PA, USA), and the amplified products were cleaned using magnetic AMPure XP beads (Beckman Coulter, Inc., Indianapolis, IN, USA). Quantitative PCR using an Illumina library quantification kit (KAPA Biosystems, Wilmington, MA, USA) was used to quantify the concentration of purified PCR products, and samples with compatible barcode combinations were pooled in equimolar concentrations. A multiplexed library was sequenced as a single-end 150-base pair run on an Illumina HiSeq 2500 at the Tufts University Core Genomics Facility. Raw Illumina reads were deposited in NCBI's Sequence Read Archive (SRA; http://www.ncbi.nlm.nih.gov/sra; SRA data: SRP065086).

Bioinformatics of ddRAD-seq data

Raw Illumina reads were processed using a computational pipeline described by DaCosta & Sorenson (2014; http://github.com/BU-RAD-seq/ddRAD-seq-Pipeline). First, reads were assigned to individual samples based on barcode sequences using the *ddRADparser.py* script. Reads per sample were then collapsed into identical clusters using the *CondenseSequences.py* script with low-quality reads (i.e. sequences that failed to cluster with any other reads (−id setting of 0.90) and with an average per-base Phred score <20) filtered out with the *FilterSequences.py* script and the UCLUST function in USEARCH v.5 (Edgar 2010). Condensed and filtered reads from all samples were then concatenated and clustered with an −id setting of 0.85 in UCLUST. MUSCLE v.3 (Edgar 2004) was used to align and cluster reads, and samples within each aligned cluster were genotyped using the *RADGenotypes.py* script. Homozygotes and heterozygotes were identified based on thresholds outlined in DaCosta & Sorenson (2014; Lavretsky *et al.* 2015a), with individual genotypes falling into three categories: 'missing' (no data), 'good' (unambiguously genotyped) and 'flagged' (recovered heterozygous genotype, but with haplotype counts outside of acceptable thresholds or with >2 alleles detected). Loci with <20% missing genotypes and ≤6 flagged genotypes (of 111 individuals total) were retained for downstream analyses. Moreover, alignments with end gaps due to indels, ≥2 polymorphisms in the last five base pairs, and/or a polymorphism in the *Sbf*I restriction site were either automatically trimmed or flagged for manual inspection in Geneious (Biomatters Inc., San Francisco, CA, USA). Manual editing increased the total number of retained markers by ~7%, while reducing any bias resulting from discarding loci with indels or high levels of polymorphism. Finally, we used scripts within the DaCosta & Sorenson (2014; http://github.com/BU-RAD-seq/ddRAD-seq-Pipeline) pipeline, but included new codes to incorporate sequencing depth for all post-processing file outputs (i.e. fasta, nexus, structure-like). To limit any biases due to sequencing error and/or low depth, alleles were called 'missing' unless they met our thresholds of a minimum of 5× coverage and thus at least 10× coverage per locus for a heterozygote.

Finally, autosomal and Z chromosome linked loci were identified as described in Lavretsky *et al.* (2015a), with assignments based on differences in sequencing depth and homozygosity between males and females (Fig. S1, Supporting information). Because females have only one Z chromosome, Z-linked markers in females should appear homozygous and be recovered at about one half the sequencing depth of males.

Mitochondrial DNA

Primers L78 and H774 were used to amplify and sequence 635 bp of the mtDNA control region (Sorenson & Fleischer 1996; Sorenson *et al.* 1999) following methods described in Lavretsky *et al.* (2014). Final forward and reverse products were sequenced on an ABI 3730 at the Yale University DNA Analysis Facility. Sequences were aligned and edited using SEQUENCHER v.4.8 (Gene Codes, Inc). All sequences have been submitted to GenBank (*Accession nos. KT934839–KT934941*). A median-joining haplotype network was constructed using the program NETWORK (Fluxus Technology) (Bandelt *et al.* 1999).

Population genetics

Pairwise Φ_{ST} estimates (i.e. 'nuc.F_ST'), as well as nucleotide (i.e. 'nuc.div.within') and haplotype (i.e. 'hap.div.within') diversity estimates for mtDNA, autosomal, and Z-linked ddRAD-seq loci were calculated in the R (http://cran.r-project.org/) program POPGENOME (Pfeifer *et al.* 2014); indel positions were excluded from analyses.

Population structure was analysed using two methods. First, a principal component analysis (PCA) as implemented in the adegenet R program (i.e. 'dudi.pca'; Dray & Dufour 2007; also see Jombart 2008) was used. For PCAs, we plotted the first two principal components. We note that because PCAs require individuals to be either diploid or haploid, only males (the sex with two copies of the Z chromosome) were included in the analysis of the Z chromosome loci (Jombart 2008).

Next, maximum-likelihood-based individual assignment probabilities were calculated in the program ADMIXTURE (Alexander *et al.* 2009; Alexander & Lange 2011). To do so, bi-allelic single nucleotide polymorphisms (SNPs) for each autosomal and Z-linked (males only) cluster were formatted for ADMIXTURE analysis, then processed through plink (Purcell *et al.* 2007) following steps outlined in Alexander *et al.* (2012). For each ADMIXTURE analysis, a 10-fold cross-validation was performed, with a quasi-Newton algorithm employed to accelerate convergence (Zhou *et al.* 2011). For each number of populations (K = 1–10) tested, we used a block-relaxation algorithm for the point estimation, with analyses terminated once the change (i.e. delta) in the log likelihood of the point estimations increased by <0.0001. Final outputs were based on ADMIXTURE proportions (Q estimates; the log likelihood of group assignment) per individual. All analyses were carried out without *a priori* assignments.

Testing for generational hybrids

To determine expected assignment probabilities of hybrid and backcrossed individuals, we independently simulated 10 generations of hybridization and backcrossing for nuclear markers. To minimize the carryover of missing data across simulated generations, we replaced missing data by randomly choosing an allele from the pool of alleles for each respective species using a custom R script *ImputeSim.r* (Dryad: doi:10.5061/dryad.g3g65). Next, first generation lesser × greater scaup hybrids were created by randomly choosing an allele from a lesser scaup and one from a greater scaup across markers. Similarly, we then 'backcrossed' each subsequent hybrid generation to a lesser or greater parental population by randomly choosing one allele from each population across markers (i.e. F2 LEGR hybrid × LESC) for nine generations of backcrossing. Doing so provides estimates of the number of generations required to attain what is essentially, for detectability's sake, a genetically 'pure' individual in which backcrossed and parental individuals are genetically indistinguishable based on an assignment probability of ≥99%. All simulations were generated with a custom R script *HybSim.r* (Dryad: doi:10.5061/dryad.g3g65) and replicated 25 times. Subsequently, assignment probabilities were estimated in the program ADMIXTURE at the optimum K obtained with empirical data and following similar steps as described above. Final results are represented as the average and range for each simulated generation overlapped by the assignment probability for empirical data averaged across the 25 replicates.

We further tested for and estimated rates and directionality of gene flow with the program *∂a∂i* (Gutenkunst *et al.* 2009, 2010). *∂a∂i* implements an efficient diffusion-based approach to test empirical data against specified evolutionary models (e.g. isolation-with-migration) with the best-fit model determined using a log-likelihood-based multinomial approach. Using *∂a∂i*, a site-frequency spectrum was derived from all bi-allelic SNPs—loci were concatenated and SNPs extracted and formatted for *∂a∂i* using custom python scripts. Because we lacked an out-group, data sets were folded, with only minor alleles considered in the frequency spectrum. Variants observed in zero or in all samples were ignored ('masked'), as described by Gutenkunst *et al.* (2010). Finally, for *∂a∂I* to accommodate missing data and differences in sample sizes between lesser (N = 47 individuals or 94 alleles) and greater (N = 64 individuals and 128 alleles) scaup, data sets were projected down to a total of 76 alleles per species. We tested the empirical data against isolation-with-migration, splitmigration and neutral-no-divergence evolutionary models that are included in *∂a∂i* (Gutenkunst *et al.* 2009, 2010). In addition, we tested an isolation model by setting migration parameters in the isolation-with-migration model to zero. The best-fit model was based on the log likelihood using a multinomial approach, where model parameters were optimized prior to calculating the likelihood, which is the product of Poisson likelihoods for each parameter given an expected value from the model frequency spectrum. Depending on the evolutionary model, different demographic parameters were estimated, including population sizes ($n_i = (N_i/N_{ref}) \times N_{Anc}$; N_{ref} = reference effective population size; N_{Anc} = Ancestral effective populations size), migration rates ($M_{i \leftarrow j} = 2N_{Anc}m_{i \leftarrow j}$) and divergence times ($t = T/2N_{ref}$; T = time since divergence in generations).

To convert the parameter estimates from *∂a∂i* to biologically informative values, we estimated generation time (G) and mutation rates (μ, per locus). First, generation time (G) was calculated as $G = \alpha + (s/(1-s))$, where α is the age of maturity and s is the expected adult survival rate (Sæther *et al.* 2005). Although sexually active by the first generation, both scaup species reach sexual maturity in their second year (α = 2) with an average adult survival rate of 0.65 (range: 0.44–0.87) and 0.72 (range: 0.6–0.83) for lesser and greater scaup, respectively (Austin *et al.* 2000). Using an overall survival rate average of 0.67 between the two scaup species, we estimated a generation time to be 4.03 years. Next, to obtain a mutation rate for nuclear genes, we multiplied a rate of 1.2×10^{-9} substitutions/site/year—previously calculated for nuclear genes in other ducks (Peters *et al.* 2008)—by generation time to attain a rate of 4.8×10^{-9} substitutions/site/generation (s/s/g). A final mutation rate was calculated as the product of the above mutation rate and the total number of base pairs.

Results

A total of 75 501 452 raw reads were recovered from the Illumina run. After de-multiplexing and quality filtering, we recovered 3589 ddRAD-seq loci that met our thresholds, with 3448 loci (414 540 base pairs) assigned to autosomes and 140 loci to the Z chromosome (17 940 base pairs); a single gametolog was also identified and excluded from analyses (Fig. S1, Supporting information). On average, there was a median depth of 64 sequences per individual per locus (range = 26–218 sequences/individual/locus). Although both scaup species had similar nucleotide (*t stat* = 2.32; $P = 0.15$) and haplotype (*t* stat = 5.26; $P = 0.034$) diversity estimates for autosomal markers, greater scaup had significantly lower Z chromosome nucleotide (*t* stat = 7.86; $P = 0.0043$) and haplotype (*t* stat = 13.25; $P = 0.00093$) diversity (Table 1). In contrast, lesser scaup had lower mtDNA nucleotide (*t* stat = −4.27; $P = 0.051$) and haplotype (*t* stat = −2.28; $P = 0.085$) diversity, although differences were not significant (Table 1).

Population structure

Between lesser and greater scaup, the overall Φ_{ST} across all ddRAD-seq loci was 0.23 (Fig. 2), with slightly higher estimates at Z-linked ($\Phi_{ST} = 0.32$) than for autosomal ($\Phi_{ST} = 0.23$) markers (Fig. 2). The overall Φ_{ST} estimate for mtDNA ($\Phi_{ST} = 0.77$) suggested nearly fixed differences between two distinct mtDNA clades that were consistent with species designation (Fig. 1). Excluding putative hybrid individuals with mtDNA haplotypes from the other species (i.e. one lesser scaup & four greater scaup) from mtDNA analyses elevated the overall Φ_{ST} estimate to 0.85.

Within lesser scaup, pairwise estimates of relative divergence among the three populations (Fig. 2) for autosomal (avg. pairwise $\Phi_{ST} = 0.0050$), Z-linked (avg. pairwise $\Phi_{ST} = -0.0051$) and mtDNA (avg. pairwise $\Phi_{ST} = 0.022$) were relatively low and supported a single continuous population. Conversely, Φ_{ST} estimates among greater scaup populations (Fig. 2) were an order of magnitude higher, supporting population structuring corresponding with current subspecies designations. Specifically, across markers, greater scaup from Interior Alaska were similarly diverged from Eurasian ($\Phi_{ST\ aut} = 0.011$; $\Phi_{ST\ z} = 0.037$; $\Phi_{ST\ mt} = 0.021$) and Aleutian Island ($\Phi_{ST\ aut} = 0.0078$; $\Phi_{ST\ z} = 0.020$; $\Phi_{ST\ mt} = 0.018$) individuals. Although Aleutian Island greater scaup were more similar to Eurasian individuals at autosomal ($\Phi_{ST} = 0.0026$) and Z-linked ($\Phi_{ST} = -0.0062$) markers, they were more similar to Interior Alaska individuals ($\Phi_{ST} = 0.018$) than Eurasian individuals ($\Phi_{ST} = 0.063$) at mtDNA (Fig. 2).

A total of 13 532 and 254 bi-allelic autosomal and Z-linked SNPs, respectively, were used for ADMIXTURE analyses. The optimal *K* was two for both autosomal and Z-linked markers (Fig. S2, Supporting information), and ADMIXTURE results largely distinguished between the two species and were concordant with respective PCA results (Fig. 1B, C). ADMIXTURE analysis of $K = 3$ for autosomal markers, and less-so for Z-linked markers, distinguished Interior Alaska greater scaup from Eurasian greater scaup (Fig. 1B, C). Moreover, at $K = 3$ for autosomal markers, six Aleutian greater scaup were assigned to Interior Alaska greater scaup with ≥99% probability and sixteen others assigned to the Eurasian greater scaup group with ≥99% probability (Fig. 1B).

Hybrid simulations and gene flow

Interspecific mtDNA haplotypes were recovered from four greater scaup and one lesser scaup (Fig. 1A), indicating that gene flow between the two scaup species occurs. However, on the basis of ADMIXTURE, all individuals were assigned to their respective species with

Table 1 Nucleotide and haplotype diversity for the mitochondrial (mtDNA) control region, 140 Z chromosome loci and 3448 autosomal loci for greater (GRSC) and lesser (LESC) scaup sampling locations (Fig. 1)

	Nucleotide diversity			Haplotype diversity		
	Mitochondrial	Autosomal	Z chromosome	Mitochondrial	Autosomal	Z chromosome
GRSC						
Russia	0.0087	0.0031	0.00090	0.95	0.20	0.091
Aleutian Is.	0.0087	0.0031	0.00089	0.90	0.21	0.090
Alaska	0.0082	0.0032	0.00097	0.93	0.21	0.087
LESC						
Canada	0.0025	0.0033	0.0011	0.88	0.22	0.12
Island	0.0025	0.0032	0.0012	0.90	0.22	0.12
Alaska	0.0059	0.0033	0.0012	0.85	0.22	0.12

>0.95 posterior probabilities (Fig. 1B, C). Nevertheless, several individuals had a relatively small probability (range = 1–5%) of assignment to the interspecific group, which included four of the five individuals possessing haplotypes of the opposite species.

To determine whether the small ADMIXTURE proportions recovered with nuclear markers were due to shared ancestral diversity or gene flow, we simulated hybridization and backcrossing for 10 generations. We note that because overall population structure was similar between autosomal and Z-linked, simulations were restricted to autosomal markers to limit computational issues due to heterogamy in Z-linked markers when running ADMIXTURE with bi-allelic SNPs. Simulating a hybridization event (F1) and nine generations (F2–F10) of backcrossing for autosomal markers identified F1 hybrids with close to the expected 50:50 assignment probability, but the ratio diverged quickly with each generation of backcrossing. Specifically, most individuals became indistinguishable (≥99% probability) from the parental population by the fifth generation of backcrossing to either of the parental populations (Fig. 3). Evidence from our simulation that shared ancestry was rather quickly lost suggests that any 'admixed' probabilities within the empirical data set are unlikely to be explained by incomplete lineage sorting and are more likely the result of hybridization. Overall, we recovered two lesser and nineteen greater scaup, most from Interior Alaska (lesser scaup = 2; greater scaup = 17), having a relatively small probability (range = 0.011–0.043) of assignment to the interspecific group that were consistent with the F4 or F5 generation in our simulation (Fig. 3). Of the remaining two greater scaup with >1% interspecific assignment probabilities, one was from the Aleutian Islands (Little Kiska I.) and one was from Chirikof Island. All greater scaup from Eurasia had ≥99% assignment probability to the intraspecific group.

Finally, as with ADMIXTURE analyses, only bi-allelic SNPs from autosomal markers were used in *∂a∂i* analyses. Likelihood estimates suggested that the best-fit model was the isolation-with-migration model (Estimated Likelihoods: neutral-no-divergence = −39336.19; split-migration = −3755.33; isolation-with-migration = −3445.29; and strict isolation = −6741.40). Using autosomal markers, we estimated an average mutation rate of 1.99×10^{-3} s/s/g (4.8×10^{-9} s/s/g × 414 540 base pairs) that was used to convert *∂a∂i* results. Given the IM model, parameter estimates suggested similar effective population sizes for lesser and greater scaup (N_e = 350 000 and 320 000, respectively). Moreover, *∂a∂i* results supported significant, yet asymmetric gene flow into greater scaup from lesser ($2Nm_{21}$ = 1.34 migrants/

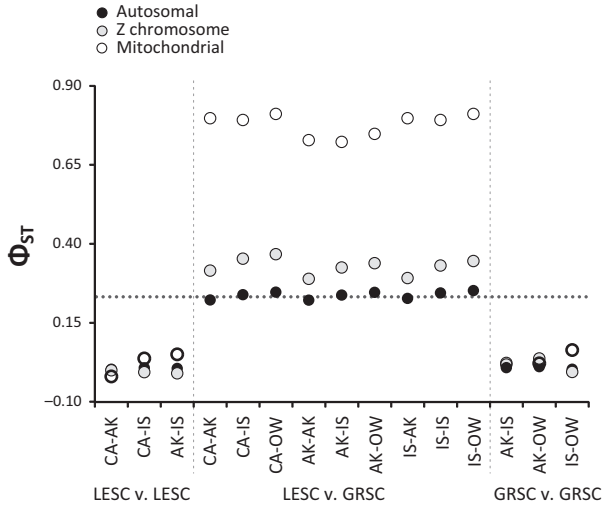

Fig. 2 Overall pairwise Φ_{ST} estimates for the mtDNA control region, 3448 autosomal loci, and 140 Z-linked loci for three lesser and three greater scaup sampling groups (see Fig. 1). The dotted line denotes the overall Φ_{ST} (0.23) across all 3589 ddRAD-seq loci between lesser and greater scaup.

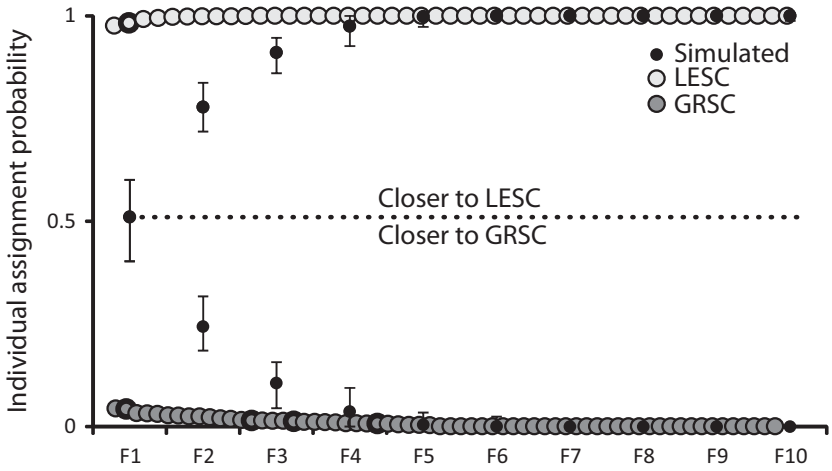

Fig. 3 The average and range of assignment probabilities from ADMIXTURE results across 25 simulated replications of hybridization (F1) and nine generations of backcrossing (F2–F10). Empirical data for lesser (LESC) and greater (GRSC) scaup are ordered by assignment probability. Individuals with interspecific mtDNA haplotypes (N = 5; Fig. 1A) are shown with a bold circle outline.

generations) as compared to gene flow from greater into lesser scaup ($2Nm_{12} = 0.31$ migrants/generation). Finally, we estimated that the two scaup species likely split around 350 000 years ago. We acknowledge that methods like ddRAD-seq can result in biased parameter estimates due to allelic dropouts of more divergent alleles, and the overexpression of low-frequency alleles (Arnold *et al.* 2013; Gautier *et al.* 2013). For *∂a∂i* results, the overexpression of the low-frequency variants can potentially bias estimated population size and time, which should be carefully considered. However, estimated migration rates should not be impacted, particularly, when looking at relative rates of migration as we do here, as any biases should affect all samples.

Discussion

Hybridization is common not just in birds (Grant & Grant 1997; Rheindt & Edwards 2011; Ottenburghs *et al.* 2015) but in other animals (Seehausen 2004; Arnold & Meyer 2006; Goedbloed *et al.* 2013; Nussberger *et al.* 2013; Combosch & Vollmer 2015). Comparing empirical and simulated data and attempting to tease apart the effects of drift and gene flow, as we do here, can be a powerful approach to understanding hybridization in the context of management, conservation and evolution (Seehausen *et al.* 2014). To test for effects of gene flow and incomplete lineage sorting, we implement novel techniques using empirical data to simulate hybridization and backcrossing events to generate expected assignment probabilities for each generational hybrid/backcross class. By doing so, we identified only late-stage backcrosses within our data set, supporting low levels of gene flow. Furthermore, simulated results suggested that signals from geneflow events were effectively lost within a few generations of backcrossing. In general, our methods provide a way to test between alternative hypotheses using empirical data to establish study-specific simulations of hybridization and backcrossing that would otherwise be difficult, if not impossible, for wild and/or nonmodel systems. Moreover, establishing study-specific admixed classes (i.e. F1, F2, F3) via simulations will allow researchers to determine the frequency of hybridization for their taxa. Such information is invaluable for understanding the consequences of gene flow on population conservation and evolutionary trajectories.

Across our sampled individuals, population assignments based on the nuclear genome corresponded with their respective species with high probability (>95%; Fig. 1). Although such high assignments are strong indicators of 'purity', simulated results suggest that assignments between 0.95 and 0.99 likely represented individuals with relatively recent admixed histories (Fig. 3). First, 95% of individuals with admixed signals were from Interior Alaska or nearby islands where both lesser and greater scaup overlap geographically and would be predicted to interact. In contrast, assignment probabilities were ≥99% to their respective species for individuals in allopatric regions (i.e. Canada and Russia). Next, four of the five individuals with interspecific mtDNA lineages had <99% assignment probability using nuclear genes (Fig. 3). If incomplete lineage sorting was a confounding factor between greater and lesser scaup, then we would predict that simulated backcrosses would retain some overlap in assignment regardless of number of generations. However, the 'offspring' of simulated hybrids became genetically indistinguishable from the parental population within five generations since hybridization (Fig. 3), and thus, our empirical results were more consistent with expectations under a scenario of gene flow. These results suggest that the relatively small ADMIXTURE proportions recovered for 21 individuals (two lesser scaup and 19 greater scaup) are more likely due to gene flow, rather than incomplete lineage sorting.

Comparing empirical and simulated data suggest that even the smallest ADMIXTURE assignments can be indicative of individuals with hybrid ancestry. This is particularly important if hybridization is rare, and where the recovery of backcrossed individuals within wild sample sets is more likely than sampling F1 hybrids. In systems lacking clear morphological indicators and/or knowledge of hybrid ancestry, the latter of which is rarely obtainable for wild populations, population geneticists are forced to make inferences based solely on molecular evidence. Consequently, as in this study, attaining high assignment probabilities (e.g. >90%) could be interpreted as evidence of no gene flow. Also, the mito-nuclear discord represented by high intraspecific nuclear-based assignment probabilities and an interspecific mitochondrial haplotype would support historic/ancient introgression (Liu *et al.* 2010; Lavretsky *et al.* 2015b). However, simulated results suggest that an individual's admixed nuclear history dissolves within a relatively short time period. Specifically, for scaup, signals from an admixed nuclear genome subside by the third generation of backcrossing, and become indistinguishable from the parental population by the fifth generation of backcrossing (Fig. 3). Given an average generational time of 4 years for scaup, much of the admixed nuclear signal can potentially be lost within 12 years and 'purity' restored by 30 years. Thus, the combined nuclear assignments and mtDNA results support contemporary hybridization with an exponentially decreasing hybrid nuclear signal within a few subsequent backcrosses (Fig. 3). Such a scenario stands in contrast to how interspecific mtDNA lineages can per-

sist long after the nuclear genome no longer appears admixed, and studies need to consider this when testing for causes of marker discordances.

Teasing apart whether drift or gene flow is having the stronger effect can be accomplished for specific studies by applying the simulation methods described here, in which researchers can first probabilistically determine whether any admixed assignments are best described by gene flow or incomplete lineage sorting. If gene flow is the more likely cause of admixed signals, then the simulated 'breeding' experiments can help determine the number of hybrid classes and assign individuals to those classes. For example, for scaup simulations, there are a total of six hybrid generations, of which three show little or no overlap in assignment (i.e. F1, F2 and F3/F4), and thus, individuals within these classes are diagnosable from one another and 'pure' individuals. We note that the difference in averages makes individuals in the F3/F4 and F5/F6 classes further distinguishable, although backcrossed individuals of >F4 generations become increasingly difficult to distinguish from 'pure' individuals (i.e. ≥F7 class). Moreover, the proportion of individuals falling into each hybrid class provides an estimate of the relative rate of hybridization. Given that scaup individuals with admixed histories fell into the simulated F4/F5 class, with little evidence of F1–F3 classes, we can infer a relatively low rate of hybridization that is primarily confined to regions of sympatry (i.e. Interior Alaska; Fig. 1). On the basis of these criteria, 10% of lesser scaup and 70% of greater scaup individuals sampled from interior Alaska putatively have a hybrid ancestry. These results are consistent with the proportional difference in the presence of interspecific mtDNA haplotypes identified in lesser versus greater scaup and the 4.5-fold higher migration rate from lesser into greater scaup estimated using *∂a∂i*. Both results suggest asymmetric gene flow from lesser scaup into greater scaup. Although additional work will need to be done to conclusively identify the cause of the asymmetric hybridization, we hypothesize that the disproportionate number of lesser scaup vs. greater scaup (i.e. 1:8.5; U.S. Fish and Wildlife Service 2015) in North America, and in regions where the two overlap (i.e. Alaska), specifically, is likely driving these patterns.

Similar signals of divergence are found across the genomes of two scaup species

Under a scenario of equal reproductive success between sexes, the Z chromosome has ¾ the effective population size of autosomal markers, resulting in an expected Z:autosomal Φ_{ST} ratio of ≤1.33 (Caballero 1995; Whitlock & McCauley 1999; Dean *et al*. 2015). Our observed mean ratio of 1.4 (Fig. 2; range = 1.28–1.48) is close to neutral expectations and consistent with previous work finding that genomic patterns are largely explained by genetic drift and demographic differences of the two marker types (Mank *et al.* 2010; Wright *et al.* 2015). Similarly, the mtDNA:autosomal Φ_{ST} ratio (overall = 3.32; range = 3.04–3.60) was once again consistent with neutral expectations (expected mtDNA:autosomal Φ_{ST} ratio ≤4; Moore 1995; Zink & Barrowclough 2008). Excluding individuals with an interspecific mtDNA haplotype elevated the overall Φ_{ST} estimate to 0.85 and the mtDNA:autosomal Φ_{ST} ratio to 3.69. Consequently, although overall Φ_{ST} estimates across marker types differed between greater and lesser scaup (Fig. 2), the Φ_{ST} ratios (Z:Aut = 1.4; mtDNA:Aut = 3.69) among them were consistent with expectations under a scenario of genetic drift primarily acting on markers with differing effective population sizes (Caballero 1995; Moore 1995; Zink & Barrowclough 2008; Dean *et al.* 2015). These results demonstrate the need to carefully consider whether markers that appear discordant in estimates of differentiation show this pattern due to genetic drift rather than selection.

We acknowledge that the influence of selection across markers cannot be rejected; however, selection would need to equally influence marker types to attain the observed Φ_{ST} ratios. For example, these results are in contrast to those obtained between two other duck taxa, mallards (*A. platyrhynchos*) and Mexican ducks (*A.[p.] diazi*), in which elevated Φ_{ST} ratios were best explained by selection on the Z chromosome (Z:Aut = 5.4), but are similar to results obtained when comparing Φ_{ST} ratios among Mexican duck populations (Z:Aut = 1; Lavretsky *et al.* 2015a). Although recent work suggests that in wild bird populations, the effective population size (N_E) of sex chromosomes likely deviates from neutral expectations due to differences in the reproductive success of the sexes (Wang *et al.* 2014), scaup, and perhaps most waterfowl species (see Lavretsky *et al.* 2015a), may represent study systems in which these do not deviate from expectations. Specifically, the life-history traits of ducks (Baldassarre 2014), which include seasonal monogamy (e.g. expected $N_{E\ Z:AUT} = 0.75$), male extra-pair copulation (e.g. expected $N_{E\ Z:AUT} > 0.75$) and higher female mortality during nesting (e.g. expected $N_{E\ Z:AUT} < 0.75$) suggest that the variance in reproductive success is likely to be similar between sexes and unlikely to cause extreme deviations in the effective population size of markers. Thus, these factors suggest that deviations from the expected ratios, at least for ducks and other species with similar life-history traits, likely demarcate genetic markers that are under different evolutionary forces (i.e. selection, genetic drift,

gene flow; Lavretsky *et al.* 2015a). Future work will benefit from additional taxonomic comparisons.

Consequences of hybridization on evolutionary trajectories and conservation

Although hybridization is prevalent in birds, and ducks especially (Cade 1983; Rhymer 2006), species extinction due to complete genetic swamping, although concerning (Rhymer & Simberloff 1996; Buerkle *et al.* 2003), has been identified in few systems (Rhymer & Simberloff 1996; Salzburger *et al.* 2002). Our simulated models suggest that identifying individuals with hybrid ancestry can become increasingly difficult past the first three generations with current methods. Interestingly, hybrid studies of other waterfowl species have also noted that individuals backcrossed past the F3 stage are phenotypically and genetically indistinguishable from their parental population (Kirby *et al.* 2000, 2004). To an extent, there are advantages to maintaining a porous genome in which the effects of gene flow are relatively low. For example, maintaining the possibility for genetic exchange provides the opportunity for adaptive introgression that may help with competition/survival in changing environments and/or novel niche space (Morjan & Rieseberg 2004; Castric *et al.* 2008; Whitney *et al.* 2010; Kraus *et al.* 2012; Hedrick 2013).

Understanding the relative propensity for hybridization between groups of interest is an important aspect of conservation planning (Allendorf *et al.* 2001; Jackiw *et al.* 2015). Consequently, determining the number of hybrid classes, and subsequently estimating the generational time until admixed histories are effectively lost within individuals, and thus 'purity' regained, can be especially informative for conservation efforts. For example, if gene flow occurs, but the genetic signal from such events is effectively lost within a few generations, then conservation efforts may include those that minimize such interactions and to allow time to reverse any negative impacts due to gene flow. For lesser and greater scaup that are known to produce viable hybrids (Johnsgard 1965; Gillham & Gillham 1996), we identified only late-stage backcrosses (i.e. >F3 backcross; Fig. 3), suggesting low levels of gene flow between the two species. The low propensity for hybridization, and thus difficulty of diagnosing hybrids that are at later stages of backcrosses, might explain why previous studies attempting to identify hybrids morphologically were inconclusive (Wilson & Ankney 1988). Thus, although scaup populations, and specifically lesser scaup, have been a species of management concern since population declines in the early 1970s (Austin *et al.* 2000; Afton & Anderson 2001; Anteau *et al.* 2007), we conclude that the low levels of gene flow between the two do not warrant conservation concern.

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P.L., J.L.P., C.B. and K.G.M. conceptualized this study. I.K., Y.N.Z., R.E.W., C.B., K.W., K.G. and K.G.M. collected samples. P.L. collected and analysed molecular data, and J.L.P. contributed to data analysis. V.B. wrote scripts for hybrid simulations. All authors contributed to the writing and approval of the manuscript.

Data accessibility

Mitochondrial DNA sequences: GenBank Accession nos. KT934839–KT934941.

Raw Illumina Reads: NCBI's Sequence Read (SRA) Archive: SRP065086.

Other data files (e.g. FASTA, PCA input, ADMIXTURE input, $\partial a \partial i$ input, custom R simulation script): Dryad: doi:10.5061/dryad.g3g65.

Supporting information

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

Fig. S1 Marker assignments to autosomal or the Z chromosome.

Fig. S2 The optimum population (K) number recovered for ADMIXTURE analyses.

Table S1 Sample information.