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RESEARCH ARTICLE

Interspecific hybridization contributes to high genetic diversity and apparent effective population size in an endemic population of mottled ducks (*Anas fulvigula maculosa*)

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Abstract Under drift-mutation equilibrium, genetic diversity is expected to be correlated with effective population size (N_e). Changes in population size and gene flow are two important processes that can cause populations to deviate from this expected relationship. In this study, we used DNA sequences from six independent loci to examine the influence of these processes on standing genetic diversity in endemic mottled ducks (*Anas fulvigula*) and geographically widespread mallards (*A. platyrhynchos*), two species known to hybridize. Mottled ducks have an estimated census size that is about two orders-of-magnitude smaller than that of mallards, yet these two species have similar levels of genetic diversity, especially at nuclear DNA. Coalescent analyses suggest that a population expansion in the mallard at least partly explains this discrepancy, but the mottled duck harbors higher genetic diversity and apparent N_e than expected for its census size even after accounting for a population decline. Incorporating gene flow into the model, however, reduced the estimated N_e of mottled ducks to 33 % of the equilibrium N_e and yielded an estimated N_e consistent with census size. We also examined the utility of these loci to distinguish among mallards, mottled ducks, and their hybrids. Most putatively pure individuals were correctly assigned to species, but the power for detecting hybrids was low. Although hybridization with mallards potentially poses a conservation threat to mottled ducks by creating a risk of extinction by hybridization, introgression of mallard alleles has helped maintain high genetic diversity in mottled ducks and might be important for the adaptability and survival of this species.

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Introduction

Genetic diversity is a fundamental parameter in conservation, evolution, and ecology. Species with high genetic diversity have greater potential for adapting to new or changing environments, whereas species with low genetic diversity have a greater risk of extinction (Reed and Frankham 2003; Frankham 2005). The amount of genetic diversity within a population is primarily a function of effective population size (N_e); because more mutations occur and genetic drift is less efficient in larger populations, genetic diversity should be positively correlated with population size (Wright 1931). This theoretical relationship has been supported, both within species and among species, by numerous empirical studies (reviewed in Frankham 1996; Frankham 2012). However, genetic diversity can deviate from expectations based on N_e if the population is out of drift-mutation equilibrium due to changes in population size, a recent divergence, selection, or immigration of novel alleles through gene flow. In this study, we compare genetic diversity between two species of ducks (genus *Anas*) that have been shown to hybridize and have orders-of-magnitude differences in census population sizes. Using a panel of six independent loci we quantitatively evaluate the influence of these evolutionary forces on standing genetic diversity.

The mallard (*Anas platyrhynchos*) and mottled duck (*Anas fulvigula*) are two closely related species that differ substantially in census population sizes (hereafter census size). The mallard has a census size of nearly 20,000,000 individuals distributed across North America (~9,300,000 individuals), Europe and Asia (~10,000,000 individuals, Delany and Scott 2006), and several other locations throughout the world where it is invasive. North American and Eurasian populations of mallards are well differentiated in mitochondrial (mt) DNA (Kulikova et al. 2005; Kraus et al. 2011), but nuclear (nu) DNA supports strong connectivity with high levels of gene flow across their Holarctic distribution (Kraus et al. 2013). In contrast, the mottled duck has a much smaller census size, with perhaps fewer than 200,000 individuals, and is endemic to two disjunct regions in southeastern North America: Florida (~35,000 individuals) and the Western Gulf Coast (WGC) comprising Louisiana, Texas, and the coastal Mexican states of Tamaulipas and Veracruz (~135,000 individuals, Delany and Scott 2006). Although debatable, some authorities consider these two populations to be different subspecies (Bielefeld et al. 2010), with *A. f. fulvigula* endemic to Florida and *A. f. maculosa* endemic to the WGC. Both mtDNA sequences and microsatellite genotypes support the genetic distinctiveness of these populations (McCracken et al. 2001; Williams et al. 2005a).

Based on both mtDNA and nuDNA sequences, mallards and mottled ducks are part of a closely related clade of ducks (subfamily Anatinae) that likely radiated during the past 100,000 years or so (Johnson and Sorenson 1999, McCracken et al. 2001; Kulikova et al. 2004; Lavretsky et al. 2014). Given this recent, shared ancestry, differences in genetic diversity might not reflect differences in N_e as a result of one or both species being out of drift-mutation equilibrium. Nonequilibrium conditions will especially be likely if either population declined or expanded following divergence (Kuhner et al. 1998), and estimates of genetic diversity might better reflect ancestral population sizes rather than current sizes.

Introgression of mallard alleles into the mottled duck gene pool could also contribute to genetic diversity deviating from expectations based on population sizes. Hybridization with mallards poses a conservation threat to several closely related mallard-like species worldwide, including mottled ducks, by creating a risk of extinction by hybridization (Rhymer and Simberloff 1996). Introgressive hybridization is perhaps the biggest threat to the conservation of Florida mottled ducks (Bielefeld et al. 2010), where 10 % of sampled individuals were inferred to have had a hybrid ancestry (Williams et al. 2005b). Although of lesser concern for the WGC population (Bielefeld et al. 2010), the frequency of hybridization may be increasing (Paulus 1988; McCracken et al. 2001). Currently estimates of introgression rates, and the contribution of these rates to extant genetic diversity, are lacking for WGC mottled ducks.

Here, our objectives are to use multilocus DNA sequences to examine genetic diversity within North American mallards and WGC mottled ducks in relation to census sizes and to test the contribution of population demography and gene flow to standing genetic diversity. We also test the ability of our markers to distinguish between species and to identify putative hybrids as a potential tool for conservation efforts.

Methods

Sampling and DNA sequencing

We sampled 190 mottled ducks from eight locations in Louisiana and Texas, and 99 mallards from six locations in North America (Fig. 1). Sampling details are described in McCracken et al. (2001) for mottled ducks; mallard samples included muscle sampled from wings collected at the

Canadian Wildlife Service Parts Collection Survey or from frozen tissue collections (University of Alaska Museum, Fairbanks, AK, USA; Burke Museum, University of Washington, Seattle, WA, USA; Museum of Natural History, Baton Rouge, LA, USA). Most individuals were harvested during early fall migration, and therefore, our sampling likely consists of mallards representative of local breeding populations and migrating populations. Because mottled ducks are nonmigratory, we assume that all samples are representative of regional breeding populations. We sampled an additional 78 mottled ducks and putative hybrids that were scored for wing-plumage characteristics; unless otherwise stated, these samples were excluded from analyses of genetic diversity and differentiation.

We obtained 666–667 bp of previously published mtDNA control region sequences for 186 mottled ducks (GenBank accessions AF382464–382649; McCracken et al. 2001) and 39 mallards (AF382411–382513, AY928870–928900, KF608514–608518; McCracken et al. 2001; Kulikova et al. 2005; Lavretsky et al. 2014), and we sequenced the same region of mtDNA for an additional 61 mallards using the primers and protocols described therein. In addition, we sequenced five nuclear introns for each individual using previously published primers and protocols, including the autosomal loci fibrinogen beta chain (FGB, intron 7, McCracken et al. 2009), T cell surface glycoprotein CD4 (CD4, intron 4, Bulgarella et al. 2010), alpha enolase 1 (ENO1, intron 8, Peters et al. 2008), and ornithine decarboxylase 1 (ODC1, intron 4, Kulikova et al. 2004, note reported as intron 6), and the sex-linked chromo-helicase-DNA binding protein gene 1 from the Z-chromosome (CHD1Z, intron 19, Peters et al. 2007, note reported as intron b). PCR and DNA sequencing protocols followed standard methods (e.g., McCracken et al. 2009), with an annealing temperature of 58 °C. Sex was determined by PCR and gel electrophoresis using primers that simultaneously amplified CHD1 from both sex chromosomes (two bands in females, ZW, and one band in males, ZZ; CHDaF, TTCTCTCAGATGGTGAGGATG; CHDaR, TCCTCAATTCYCCTTTTATTGA). Sequences for five mallards were previously published for CHD1Z ($N = 5$ sequences, KF609022–609026), ENO1 ($N = 5$ sequences, KF609086–609090), and FGB ($N = 5$ sequences, KF608958–608962) (Lavretsky et al. 2014). All new sequences have been archived in GenBank (Accession numbers KF857589–KF859542).

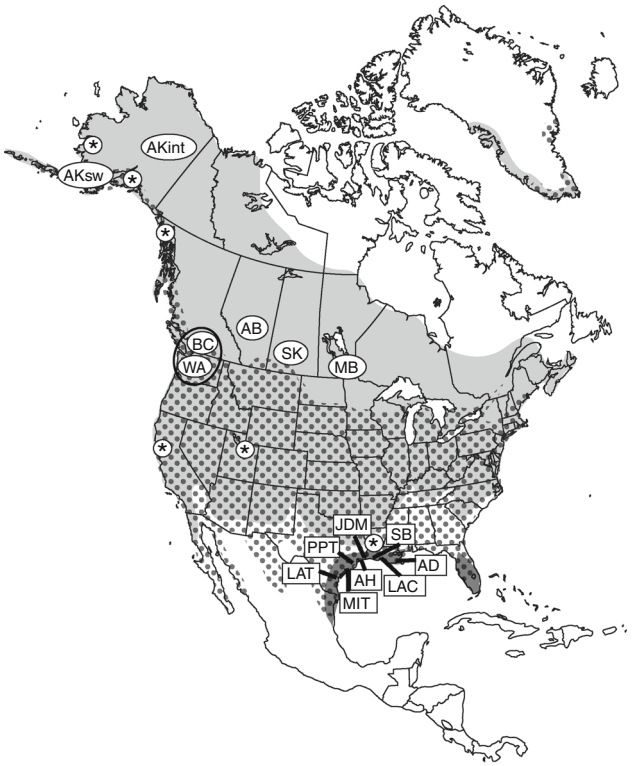

Fig. 1 Geographic distributions and sampling locations of mottled ducks (*dark gray*) and mallards (*light gray* for breeding; stippled for wintering) in North America. Mottled ducks *LAT* Laguna Atascosa National Wildlife Refuge (NWR), Texas (TX); *MIT* Mad Island Wildlife Management Area (WMA), TX; *PPT* Peach Point WMA, TX; *AH* Anahuac NWR, TX; *JDM* J. D. Murphree WMA, TX; *SB* Sabine NWR, Louisiana (LA); *LAC* Lacassine NWR, LA; *AD* Atchafalaya Delta WMA, LA. Mallards *AKsw*, southwestern Alaska; *AKint* interior Alaska; *WA-BC* Washington and British Columbia; AB, Alberta; *SK* Saskatchewan; *MB* Manitoba; *Asterisks* (*) locations of mallards that were not grouped into populations ($N \leq 6$ samples)

Sequences were aligned and reconciled using Sequencher v.5.0.1 (Gene Codes, Ann Arbor, MI). Gametic phases of alleles were resolved using three methods. First, gametic phases for sequences that were heterozygous for an insertion-deletion (indel) were resolved by comparing the ambiguous 3′-end with the unambiguous 5′-end of forward and reverse sequences following methods described in Peters et al. (2007). Because gaps cause a shift in peak locations within the chromatograms, it was possible to determine which polymorphisms and indels were linked on the same allele. Second, we used the software PHASE v.2.1.1 to algorithmically infer the most likely gametic phases (Stephens et al. 2001); alleles resolved on the basis of indels were included in the analysis as known alleles. For CHD1Z, which is hemizygous in females, we randomly paired sequences from two females to create diploid sequences and treated them as known alleles in the PHASE analysis. Finally, sequences resolved with <0.95 probability were targeted with allele specific primers to amplify and sequence one of the two alleles individually, and this allele was subtracted from the polymorphic sequence (consensus of both alleles) to resolve the second allele (Bottema et al. 1993). PHASE was rerun with these alleles defined as known alleles to verify that all sequences were resolved with ≥0.95 probability.

Genetic diversity and population structure

We used ARLEQUIN v. 3.5.1.2 (Excoffier and Lischer 2010) to calculate nucleotide diversity (π, the average pairwise difference among all copies of a locus within each species) and gene diversity (G the probability of sampling two different alleles, which is analogous to expected heterozygosity for diploid loci). We also calculated allelic richness (A the number of different alleles sampled per locus) and private alleles (P the number of alleles that are unique to each species) using rarefaction to standardize values for unequal samples size in the program HP-RARE v.1.0 (Kalinowski 2005). Each measure of genetic diversity was compared between mallards and mottled ducks using a paired t test treating each locus as an independent estimate of diversity (locus was the paired variable) as implemented in Microsoft Excel.

To examine population differentiation between mottled ducks and mallards, we performed a three-level, hierarchical analysis of molecular variance (AMOVA) in ARLEQUIN: between species, among sampling locations within species (Fig. 1), and within sampling locations. In addition, we computed pairwise Φ_{ST} (a measure of the proportion of genetic diversity partitioned between pairs of populations) among all sampling locations. Seven mallards were not grouped within sampling locations, and therefore, were excluded from the AMOVA and pairwise Φ_{ST} calculations. Each locus was analyzed separately.

We estimated the number of genetic populations (K) and assigned individuals to those populations using the program STRUCTURE v.2.2.4 (Pritchard et al. 2000). STRUCTURE uses a MCMC Bayesian method to examine deviations from Hardy–Weinberg equilibrium and linkage disequilibrium to test for population structure. All mallards, mottled ducks, and presumed hybrids were included in this analysis. For each locus, alleles were numbered from 1 to n, where n is the total number of different alleles for that locus. We used an admixture model with allelic frequencies assumed to be independent and estimated $\Pr(X|K)$ for $K = 1$–5 populations. Each analysis was run for a burn-in of 100,000 generations followed by 500,000 generations of sampling and was replicated ten times. We then calculated ΔK (Evanno et al. 2005) to determine the best value of K as implemented in the program STRUCTURE HARVESTER (Earl and vonHoldt 2012). No prior information about sampling localities or species was included in these analyses. We also tested for more subtle population structure by examining mottled ducks and mallards in separate STRUCTURE analyses.

Demographic history

We examined the contributions of paleohistorical demographic changes and gene flow from mallards to extant genetic diversity in mottled ducks using coalescent analyses in the program IM (Hey and Nielsen 2004). The 78 individuals used for hybrid identification were excluded from this analysis (see below). We ran three different models that differed in complexity to test the sensitivity of estimates of effective population size (N_e, measured as θ, where $\theta = 4N_eu$ and u is the geometric mean of the per-locus mutation rate in substitutions/locus/generation) to various interactions between mottled ducks and mallards. Our first model (evolutionary constant N_e) assumed drift-mutation equilibrium and treated mottled ducks as a single, panmictic population with an evolutionary N_e of θ_{modu}. We also ran this model independently for mallards to obtain an estimate of θ_{mall}. Our second model (divergence-isolation) included four parameters, θ_{modu}, θ_{mall}, θ_A (a measure of the effective population size of the ancestral population at the time of population divergence), and t (where $t = Tu$ and T is the time of divergence in years before present). This model, compared to the model of an evolutionary constant N_e, provides information on the contribution of the shared ancestry between mottled ducks and mallards to θ_{modu}, and thus, to genetic diversity in the absence of gene flow. Because this model estimates both ancestral and contemporary population sizes, it also accounts for long-term changes in population sizes. Our final model (isolation-migration) included bidirectional gene flow (m_{modu} and m_{mall}, where $m_i = M_i/u$, and M_i is the rate at which alleles enter species i from the other species) in addition to the parameters included in the divergence-isolation model. Comparing θ_{modu} inferred from the first two models with θ_{modu} from this model provides a measure of the contribution of hybridization with mallards to extant levels of genetic diversity in mottled ducks.

IM analyses included mtDNA and all five nuclear loci. We truncated nuDNA sequences to be consistent with no recombination using IMgc (Woerner et al. 2007) to retain the maximum number of polymorphic sites. We iteratively changed the chromosomal weighting so that a maximum of 5 % of allelic copies were removed from the analysis. We only used IMgc as a guide for truncating sequences, and in contrast to the default method, we retained sites that segregated for more than two nucleotides. These truncated sequences were used for each of the three models described above.

We defined inheritance scalars for mtDNA (0.25), CHD1Z (0.75), and autosomal DNA (1.0) to reflect the different modes of inheritance. We defined an infinite-sites model of evolution for CHD1Z and CD4 and a HKY model for the remaining loci, all of which contained at least one site segregating for more than two nucleotides. Preliminary runs of IM were conducted with wide priors. On the basis of these results, we set new priors that encompassed the full posterior distributions for each parameter, and we assumed

these uniform priors were uninformative. The large sample sizes of sequences caused IM to converge slowly, especially for the more complex models, and therefore, we randomly partitioned the sequences from each locus into two separate datasets of equal sizes, which we treated as replicates of the inferred population histories. We ran IM for 1,000,000 generations of burn-in, followed by ~10,000,000 generations sampling parameters every 20 generations. We assessed convergence based on ESS (effective sample size) values and trace plots to determine whether the algorithm reached stationary distributions for all parameters. Because the partitioned datasets reached the same stationary distributions, we report the posteriors averaged between the two runs.

To convert estimates of θ and t to demographic parameters, we obtained estimates of μ for ENO1, ODC1, CHD1Z, and mtDNA from Peters et al. (2008). We used the average rate calculated from five nuclear loci in that study (1.2×10^{-9} substitutions/site/year; ssy) as the estimate for CD4 and FGB. These values resulted in the geometric mean of μ for six loci as 2.1×10^{-9} ssy. Because IM scales parameters to the geometric mean of mutation rates per locus (u), we multiplied this value by the geometric mean of fragment lengths (280 bp) used in the analyses, to obtain an estimate of $u = 5.9 \times 10^{-7}$ substitutions/locus/year. Converting θ to N_e also requires an estimate of generation time; we used a value of 3.2 years/generation obtained for a close congener, the gadwall *Anas strepera* (Peters et al. 2008), resulting in a rate of 1.9×10^{-6} substitutions/locus/generation.

Hybrid identification

To test our ability to genetically distinguish between mottled ducks and their hybrids, we sampled wings from 178 "pure" mottled ducks, 52 putative mallard-mottled duck hybrids, and 11 mallards. Each wing was identified to age and sex class (Carney 1992). Following protocols for distinguishing between mallards, American black ducks (*Anas rubripes*), and their hybrids (Kirby et al. 2000), we scored six morphological characters, including (1) total number of wing bars, (2) width of the anterior wing bar, (3) width of the posterior wing bar, (4) number of coverts spanned by the anterior wing bar, (5) number of secondary feathers spanned by the posterior wing bar, and (6) number of dark feathers found on the underside of the wing. When wing bars were absent, the associated character was scored as zero.

We used a principal coordinate analysis (PCA) in XLSTAT to transform correlated plumage variables into uncorrelated variables. Principal coordinate scores were compared between pure mottled ducks, hybrids, and mallards using an ANOVA. Using linear regression, PC1 was then compared to assignment probabilities obtained from the genetic data for the 78 individuals (40 putatively pure mottled ducks and 38 putative hybrids) scored for both morphologic and genetic character sets.

Results

Genetic diversity and population structure

Mottled ducks had approximately half the genetic diversity at mtDNA relative to mallards (Table 1), and only 4 of the 95 observed haplotypes were shared between the two species (Fig. 2a). However, mottled ducks harbored nearly as much nucleotide and gene diversity at nuDNA as mallards (Table 1), despite having a census size that is approximately 1.5 % that of North American mallards. Mottled ducks had lower nucleotide diversity at only two of the five nuDNA loci and mean nucleotide diversity was higher than observed for mallards, although differences were not significant ($t = 1.51$, $P = 0.21$). Likewise, despite lower gene diversity at four nuDNA loci, mottled ducks had 96.1 % of the overall diversity observed in mallards ($t = 1.53$, $P = 0.21$). After accounting for differences in sample sizes, however, mottled ducks had 58.6 % the total number of alleles and 19.7 % the number of private alleles found in mallards (Table 1); both measures of diversity differed significantly between species ($t = 3.21$, $P = 0.033$; $t = 3.20$, $P = 0.033$; respectively). In contrast to the pattern in mtDNA, many nuDNA polymorphisms and alleles were shared between species (Fig. 2b).

Differences between mottled ducks and mallards explained 23.6 % of the total mtDNA variation and between 0.0 and 6.7 % of the total nuDNA variation (mean = 2.4 %; Table 2); genetic diversity was significantly partitioned between species at five of the six loci. Within species, 0.2 % of the total mtDNA variation and 0.0–1.9 % (mean = 0.6 %) of the total nuDNA variation was partitioned among populations, and populations were significantly differentiated at CD4 only (Table 2). Pairwise comparisons among populations also revealed a general lack of genetic differentiation within species for nuDNA (mallards: mean $\Phi_{ST} = 0.0074$, range = 0.0–0.043; mottled ducks: mean $\Phi st = 0.0024$, range = 0.0–0.017; Tables 2 and S1 in Supporting Information available online). Mallard populations also were not significantly differentiated in mtDNA (mean $\Phi st < 0.0$, range ~0.0 to 0.020). However, mottled ducks from Atchafalaya Delta WMA differed significantly from all other sampling localities at mtDNA (mean $\Phi st = 0.18$, range 0.13–0.25; $P \leq 0.034$), whereas there were no significant differences among the remaining populations (mean $\Phi_{ST} < 0.0$, range ~0.0–0.024).

Table 1 Number of chromosomal copies sequenced (*N*) and measures of genetic diversity

	Mallard					Mottled duck				
	N	π	*G*	*A*	*P*	*N*	π	*G*	*A*	*P*
mtDNA	100	0.0138	0.987	70	66 (66.9)	185	0.0065	0.868	29 (13.0)	25 (19.9)
CHD1Z	159	0.0015	0.481	8	3 (3.5)	305	0.0015	0.446	8 (7.0)	3 (2.5)
CD4	198	0.0036	0.735	18	9 (9.5)	378	0.0028	0.637	9 (8.5)	0 (0.0)
ENO1	198	0.0124	0.948	56	26 (29.2)	378	0.0141	0.923	40 (34.0)	10 (7.2))
FGB	198	0.0120	0.822	21	8 (8.8)	378	0.0133	0.847	13 (12.2)	0 (0.0)
OD6	198	0.0190	0.895	30	17 (17.4)	378	0.0230	0.877	17 (16.3)	4 (3.8)
Mean		0.0104	0.811	33.8	21.5 (22.6)		0.0102	0.767	19.3 (15.2)	7 (5.6)

π nucleotide diversity, *G* gene diversity, *A* number of alleles (allelic richness for mottled ducks scaled to mallard sample sizes given *in parentheses*)

On the basis of ΔK, STRUCTURE indicated that the genotypic data best fit a two-population model. Overall, 99.0 % (98 of 99 samples) of mallards were assigned to one genetic cluster (mean $Q = 0.90 \pm 0.130$StDev), whereas 95.2 % (180 of 189 samples) of mottled ducks were assigned to the other cluster (mean $Q = 0.91 \pm 0.162$StDev; Fig. 3). There was no obvious geographic pattern in the distribution of misassigned individuals, and analyzing each species separately did not reveal any additional population structure ($K = 1$ was the best supported model for each).

Demographic history

Similar to levels of genetic diversity, N_e of mottled ducks ($\theta = 2.7$, 90 % HPD = 2.1–3.5) was about 56 % the N_e of mallards ($\theta = 4.8$, 90 % HPD = 3.8–6.2) under models of constant N_e and drift-mutation equilibrium. These values of θ correspond to N_e's of ~360,000 mottled ducks and 640,000 mallards. Thus, the estimated N_e of mottled ducks was approximately 2.7 times larger than the estimated census size ($N = 135{,}000$ individuals), whereas the N_e of mallards was about 0.068 times the census size ($N = 9{,}330{,}000$ individuals). The posterior distributions of θ did not overlap the expected values from census data for either species (Fig. 4a).

Considering a recent shared ancestry in our divergence-isolation model, which accounts for differences between the ancestral and current population sizes, had a modest effect on estimates of N_e in mottled ducks ($\theta = 2.0$, 90 % HPD = 1.3–3.0; $N_e = 270{,}000$ individuals), but a large effect on N_e in mallards ($\theta = 24$, 90 % HPD = 12–81; $N_e = 3{,}100{,}000$ individuals). The estimate of θ for mottled ducks did not overlap the expected value (Fig. 4b). Under this model, we estimated a divergence time of approximately 63,000 years ($t = 0.038$, 90 % HPD = 0.024–0.055) and an ancestral N_e of ~480,000 individuals ($\theta = 3.6$, 90 % HPD = 2.7–4.7).

Incorporating gene flow into our isolation-migration model, we found significant evidence of gene flow from mallards into mottled ducks ($2Nm = 5.0$, 90 % HPD = 0.85–29), but the estimate for the reverse direction had a large confidence interval, and we could not reject the possibility of no gene flow from mottled ducks into mallards ($2Nm = 8.1$, 90 % HPD = 0.0–100; Supplementary Material, Fig. S1). This model had a large effect on estimates of N_e for mottled ducks ($\theta = 0.93$, 90 % HPD = 0.46–1.8; $N_e \approx 120{,}000$ individuals), resulting in values that were close to expectations based on census size (Fig. 4c). The N_e for mallards was similar to that found in the model with no gene flow ($\theta = 18$, 90 % HPD = 9.1–47; $N_e = 2{,}400{,}000$ individuals), although confidence intervals were more narrow. Under this model, we estimated a slightly older divergence time of 100,000 years ($t = 0.066$, 90 % HPD = 0.040–0.10) and a smaller ancestral N_e of ~400,000 individuals ($\theta = 3.0$, 90 % HPD = 2.1–4.0), although the posterior distributions for both parameters broadly overlapped those from the model with no gene flow.

Hybrid identification

The first principal component (PC1) accounted for 55.7 % of the variance in the six wing-plumage characters (eigenvalue = 3.341). Factor loadings were uniformly high and positive (0.557–0.876) for all characters except the number of dark feathers on the underwing (−0.307). PC1 differed significantly among mottled ducks, putative hybrids identified a priori, and mallards (ANOVA: $F_{2,237} = 459.5$, $P < 0.0001$), with mottled ducks receiving the lowest scores (mean $= -0.885 \pm 0.822$StDev), mallards receiving the highest scores (mean $= 5.350 \pm 0.744$StDev), and hybrids receiving intermediate scores (mean $= 1.881 \pm 0.884$StDev).

Among the 38 individuals classified a priori as hybrids, genetic assignment probabilities (*Q*) classified 32 (84.2 %)

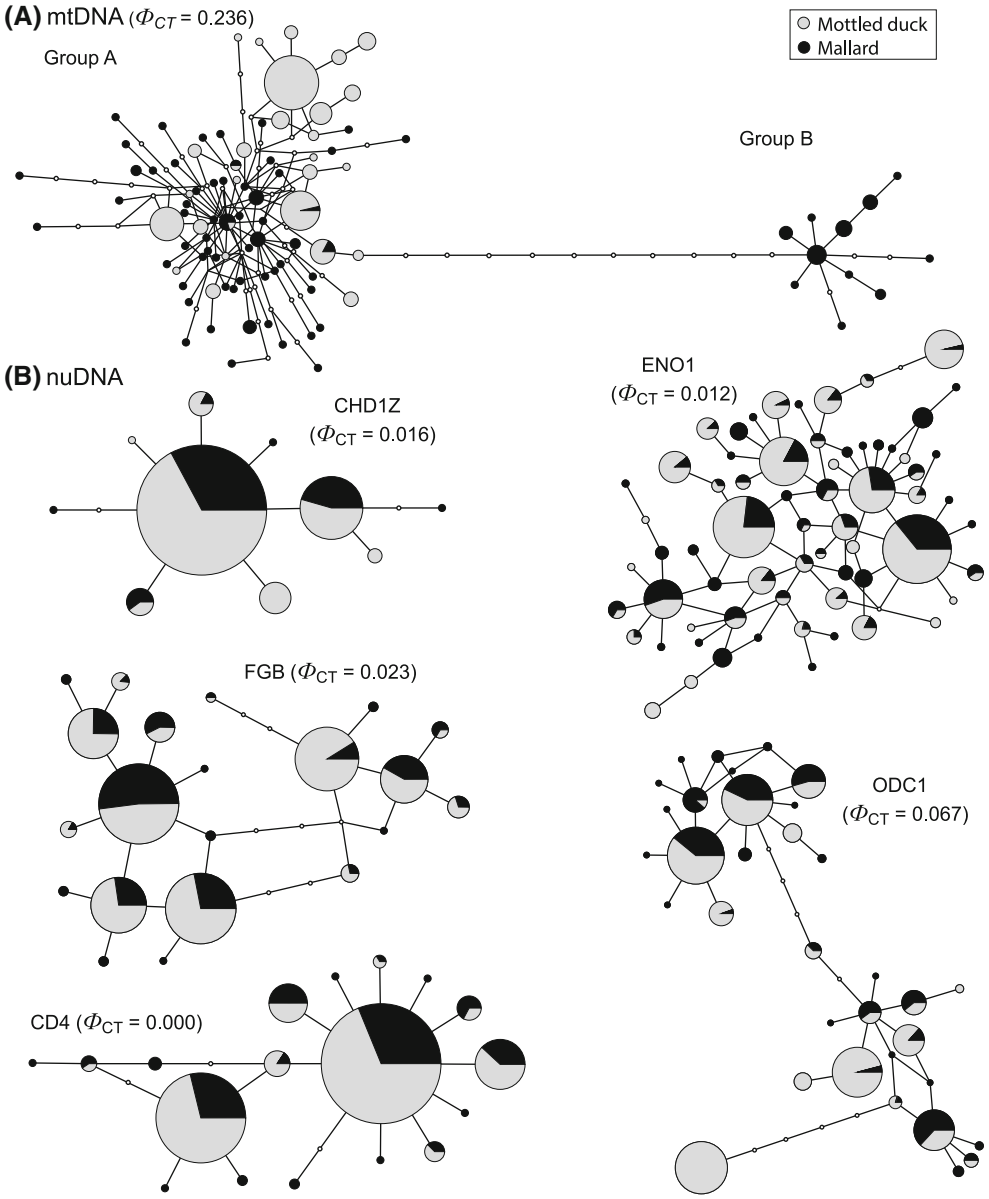

Fig. 2 Haplotype networks for (**a**) mtDNA and (**b**) the 5 nuclear loci sequenced for mottled ducks (*grey shading*) and mallards (*black shading*). The area of each *circle* and each *pie within circles* is proportional to the sample size for that allele. Branches between alleles, separated by *small open circles*, indicate the number of mutations. Φ_{CT} indicates the proportion of the total genetic diversity at each locus that is partitioned among species in a three-hierarchical AMOVA

as mottled ducks ($Q \geq 0.8$), 5 (13.2 %) as hybrids ($0.8 > Q > 0.2$), and 1 (2.6 %) as a mallard ($Q \leq 0.2$). Among the 40 individuals classified as pure mottled ducks, genetic assignments classified 38 (95.0 %) as mottled ducks and 2 (5.0 %) as hybrids; the proportions of individuals assigned to each group was similar to those obtained for the reference population (see above). Q differed significantly between individuals classified as hybrids ($N = 38$; $Q = 0.872 \pm 0.229$StDev) and pure mottled ducks ($N = 40$; $Q = 0.950 \pm 0.072$StDev; t test, $t = 2.06$, df $= 76$, $P = 0.043$). Furthermore, we found a significant regression between Q and PC1 ($R^2 = 0.076$, $F_{1,76} = 7.31$, $P = 0.0085$; Fig. 5). The width of both anterior and posterior wing bars had the strongest correlations with Q ($P < 0.005$).

Discussion

Genetic diversity, N_e, and gene flow

Under drift-mutation equilibrium, genetic diversity is expected to be positively correlated with population size. However, we found that mottled ducks and mallards had

Table 2 Results of AMOVA showing genetic differentiation between mallards and mottled ducks and among sampling locations within each species ($N = 6$ for mallards & 8 for mottled ducks)

	Mottled ducks vs. mallard (%)	Among populations within species (%)
mtDNA	23.6*	0.2
CHD1Z	1.6*	0.0
CD4	0.0	1.9*
ENO1	1.2*	0.3
FGB	2.3*	1.0
ODC1	6.7*	0.0

*$P < 0.05$ after a correction for the false discovery rate

similar levels of genetic diversity despite nearly two orders-of-magnitude difference in census population sizes. Mottled ducks had higher diversity than expected, and mallards had lower diversity than expected given their respective population sizes. This observation is consistent with extensive evidence that the range of genetic diversity among species is much narrower than the range in population sizes (Frankham 1996; Leffler et al. 2012).

One possible explanation of the observed discrepancy between population size and genetic diversity is that estimates of census sizes are inaccurate. Higher estimates of population size have been obtained from mark-recovery (banding) data for WGC mottled ducks suggesting about 630,000 individuals (North American Waterfowl Management Plan, Plan Committee 2004), which is on par with estimates of N_e from our equilibrium model. This estimate largely pertains to the Fall and Winter population (post-breeding). In contrast, the Delany and Scott (2006) estimate of 135,000 individuals that we used was obtained from Mid-winter Waterfowl Surveys, which were conducted in early January (pre-breeding period for WGC mottled ducks—nesting commences in February and March; Grand 1992; Johnson et al. 2002) and conducted after the fall and early winter population has incurred density-dependent mortality (Newton 2006). Census size estimates obtained from recent aerial surveys of the breeding population are closer to the Delany and Scott (2006) estimate, suggesting population sizes fluctuating between 110,000 and 165,000 breeding individuals (USFWS 2013). Because N_e is influenced by the number of breeding individuals rather than the total population size, the lower estimates are likely to be more appropriate for comparisons between census sizes and genetic effective sizes.

Discrepancies between population size and genetic diversity also can be explained by a number of neutral and non-neutral factors (Leffler et al. 2012; Cutter and Payseur 2013). Under selective neutrality, N_e will deviate from census sizes if population sizes fluctuate, causing the population to be out of drift-mutation equilibrium (Wright 1938; Vucetich et al. 1997). In this study, we found evidence that long-term changes in population sizes contributed to standing genetic diversity, especially for mallards. Incorporating a shared ancestry between mottled ducks and mallards (i.e., an ancestral population size that accounted for changes in N_e) into our coalescent analyses, we found that N_e was 4.9 times larger for mallards compared to the analysis that assumed drift-mutation equilibrium and 6.5 times larger than the ancestral N_e at the time of population divergence. Thus, the comparatively low genetic diversity observed in mallards can be explained, at least in part, by a population expansion that followed divergence. In contrast, incorporating a shared ancestry only had a modest effect on N_e in mottled ducks; nonequilibrium N_e was 75 % the N_e estimated under equilibrium conditions and 57 % the ancestral N_e.

Gene flow can introduce novel alleles into a population, offset genetic drift, and maintain higher genetic diversity than expected for a given N_e (Lacy 1987). We found evidence that gene flow from mallards, estimated at ca. 1–30 migrants per generation, has contributed prominently to standing genetic diversity in mottled ducks, and therefore, incomplete lineage sorting alone is insufficient for explaining the genetic similarity between these species. Furthermore, incorporating migration into our coalescent analyses resulted in an N_e that was about 34 % the size of

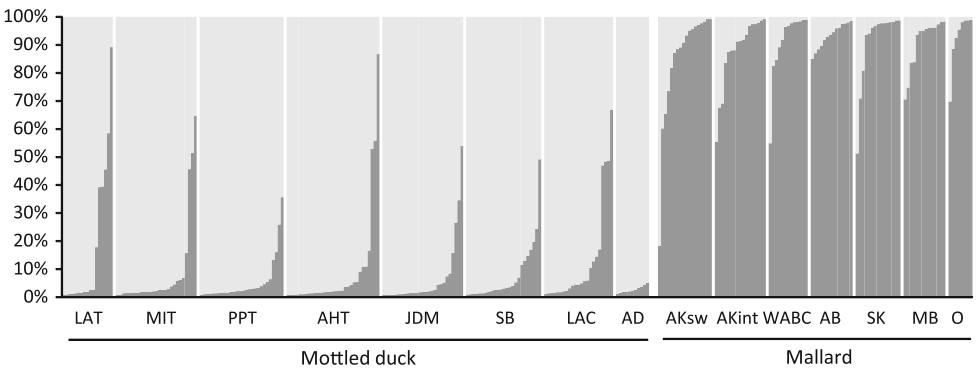

Fig. 3 Population assignment probabilities of 189 mottled ducks and 99 mallards based on genotypes at mtDNA control region and five nuclear introns. Sites arranged from west to east, and the labels are as defined in Fig. 1, except O (other), which indicates widespread mallard samples that were not grouped into sites

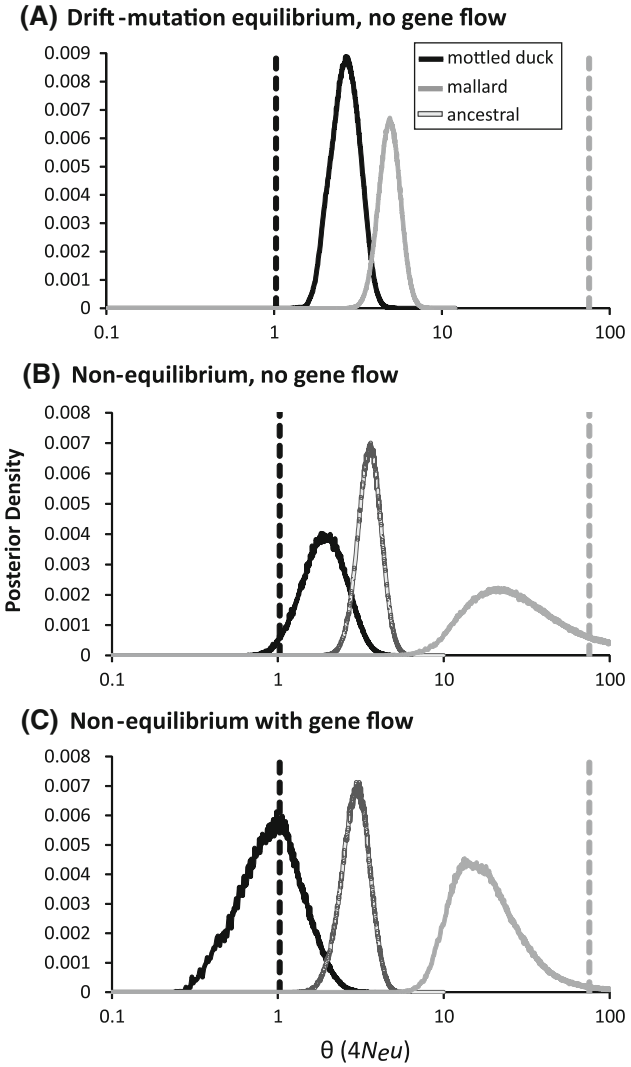

Fig. 4 Coalescent estimates of effective population sizes (where $\theta = 4N_e u$) for mottled ducks (*black curves*), mallards (*grey curves*), and the ancestral population (*outlined curves*) under models of **a** drift-mutation equilibrium (constant N_e, no gene flow), **b** non-equilibrium with no gene flow, and **c** non-equilibrium with gene flow. The *dashed*, *vertical lines* indicate the expected θ for mottled ducks (*black line*) and mallards (*grey lines*) given current estimates of census populations sizes

the equilibrium N_e. Of our three models examined, only the model including gene flow resulted in a posterior distribution of θ that overlapped our expected value for mottled ducks. Thus, gene flow might be maintaining four-times more genetic diversity than would be expected if these species were completely isolated. Although determining the timing of gene flow is difficult (Sousa et al. 2011; Strasburg and Rieseberg 2011), we hypothesize that mallards and mottled ducks have been hybridizing and exchanging genes over the long-term rather than this being a case of secondary contact. IM assumes constant rates of gene flow since divergence (Hey and Nielsen 2004), and therefore, our estimated number of migrants would suggest

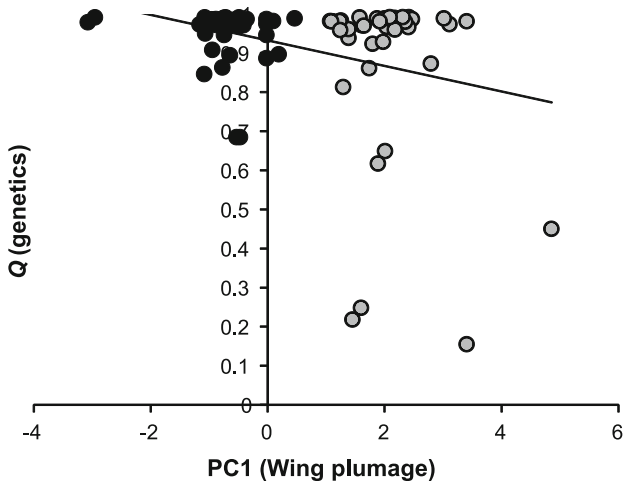

Fig. 5 Population assignment probabilities (Q) from genotypic data were significantly correlated with principle component 1 (PC1) from wing morphology. *Black circles* indicate phenotypically pure mottled ducks; *grey circles* indicate putative hybrids

more than 100,000 effective hybridization events over the course of their divergence. Given a current census size of 135,000, this magnitude of gene flow resulting solely from secondary contact seems unlikely. Thus, this pair of duck species might be another example of parapatric speciation (Peters et al. 2012), perhaps initiated by peripheral isolation of the ancestral mottled duck (Omland 1997).

Because genetic diversity is important to the adaptability and survival of a population (Lande 1995; Frankham 1996; Frankham 2012), limited hybridization with mallards might be beneficial to mottled ducks. In addition to maintaining relatively high levels of genetic diversity, hybridization can potentially introduce novel, beneficial mutations into the population, increasing the efficiency of selection and rate of adaptation (Alleaume-Benharira et al. 2006; Garant et al. 2007). However, at the opposite extreme, excessive hybridization could lead to genetic swamping and 'extinction by hybridization', especially for species represented by small population sizes (Rhymer and Simberloff 1996; Wolf et al. 2001; Lenormand 2002). The level of hybridization that could be tolerated is likely dependent on the strength of selection acting on those genes that are important to the phenotypic distinctiveness of mottled ducks. Given growing evidence of genomic islands of divergence that are important in speciation in the face of gene flow (Ellegren et al. 2012; Nadeau et al. 2012; Renaut et al. 2012), there may be only a few regions within the genome that are responsible for maintaining the genetic distinction between these species. Some of these regions might be under sexual selection, whereas others might be adapted to local environments. Such regions are important for the genetic integrity of mottled ducks, whereas alleles from other regions can freely flow between the species (Rieseberg and Burke 2001; Wu 2001; Wu and Ting 2004) without

compromising what we recognize as a mottled duck. Genome scans offer the power to identify those genetic regions responsible for the distinction between species and populations (Hohenlohe et al. 2010; Bradbury et al. 2013; Hemmer-Hansen et al. 2013). Such information could help in determining the relative roles of genetic drift versus selection in maintaining species integrity between mottled ducks and mallards, and thus the importance of hybridization and gene flow to the conservation of mottled ducks.

Numerous additional life-history characteristics, such as population genetic structure, mating system, generational overlap, and variance in family sizes, might also contribute to N_e being smaller than the actual census size. However, these life-history characteristics are unlikely to vary sufficiently between mottled ducks and mallards to explain the strong disparity between census size and genetic diversity observed in this study. In addition, molecular evidence suggests that substitution rates can be higher for small population sizes as a result of selection being less effective at removing slightly deleterious mutations (Eyre-Walker et al. 2002; Hughes 2005; Smith and Klicka 2013), and a higher substitution rate could cause mottled ducks to have higher genetic diversity than expected relative to mallards. However, given the differences in census sizes, the substitution rate would have to be nearly two-orders of magnitude faster in mottled ducks to explain the observed genetic diversity. Overall, gene flow into mottled ducks from mallards and a population expansion in mallards are the better explanations for the similar levels of genetic diversity observed in these species.

Genetic differentiation and contemporary hybridization

Coalescent analyses revealed significant evidence of gene flow between mottled ducks and mallards, and our combined analysis of plumage and DNA demonstrate that hybridization with mallards is potentially a concern for mottled ducks in the WGC. Although some level of introgression can be beneficial to the adaptability of populations, excessive gene flow can be detrimental to the invaded population (i.e. extinction by hybridization). Therefore, understanding the frequency and spatial extent of hybridization in contemporary mottled duck populations is important and requires long-term monitoring. Of the 38 suspected hybrids examined, only six had genotypes suggestive of a hybrid ancestry. Thus, whereas the six loci examined in this study seem sufficient for distinguishing between mottled ducks and mallards, they seem to offer low power for reliably identifying hybrids.

An alternative explanation for the inability of our genetic markers to detect what appear to be hybrids is that some individuals might be progeny of F1 backcrosses to parental mottled ducks. In the closely related American black duck, for example, plumage characters of such progeny are typically recognizable as having had a hybrid ancestry (Kirby et al. 2000), and the same is likely true for mottled ducks. Because our methods rely on statistical differences in allelic frequencies for identification, hybrids will be increasingly difficult to identify with more generations of backcrossing. Without an understanding of the genetic basis of the plumage characters (e.g., dominance vs. recessiveness, quantitative traits, etc.), we cannot know how backcrosses will express these traits. Indeed, these characters could persist for generations after hybridization and be expressed by what is otherwise a pure mottled duck. Examining genes associated with plumage will be necessary to understand the morphological-genetic discord observed in putative hybrids and increase our confidence of hybrid identification with both morphological and genetic techniques.

Conservation implications

DNA sequences from six independent loci sampled from WGC mottled ducks and North American mallards revealed two primary findings relevant to future conservation and management of mottled ducks. First, despite large differences in North American census sizes (135,000 mottled ducks versus 9,330,000 mallards), these two species had similar levels of genetic diversity, especially at nuDNA. Coalescent analyses suggested that gene flow from mallards into mottled ducks (and to a lesser extent, shared ancestry) explains the higher-than-expected genetic diversity in mottled ducks. Second, mottled ducks and mallards share many genetic polymorphisms but are sufficiently differentiated in allele frequencies to allow species identification. However, the six loci examined here seem to offer low power for detecting hybrids. Given generally weak allelic frequency differences across nuclear loci, monitoring hybridization using molecular methods and evaluating the utility of plumage characteristics for hybrid identification will require many markers. New techniques in next-generation sequencing will likely offer high power for detecting those loci important in the species integrity of mottled ducks and for use as a monitoring tool for conservation. Although some level of hybridization might be beneficial, our results demonstrate that hybridization with mallards is a phenomenon that should be studied more closely in WGC mottled ducks, and that continued monitoring of phenotypic and genotypic characters is important to determine the frequency and spatial extent of hybridization.

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