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A century of hybridization: Decreasing genetic distance between American black ducks and mallards

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Abstract

American black ducks (*Anas rubripes*) and mallards (*A. platyrhynchos*) are morphologically and behaviorally similar species that were primarily allopatric prior to European colonization of North America. Subsequent sympatry has resulted in hybridization, and recent molecular analyses of mallards and black ducks failed to identify two distinct taxa, either due to horizontal gene flow, homoplasy, or shared ancestry. We analyzed microsatellite markers in modern and museum specimens to determine if the inter-relatedness of mallards and black ducks was an ancestral or recent character. Gst, a measure of genetic differentiation, decreased from 0.146 for mallards and black ducks living before 1940, to 0.008 for birds taken in 1998. This is a significant reduction in genetic differentiation, and represents a breakdown in species integrity most likely due to hybridization. Using modern specimens, we observed that despite a lower incidence of sympatry, northern black ducks are now no more distinct from mallards than their southern conspecifics.

Introduction

The taxonomic status of American black ducks (*Anas rubripes*) and mallard ducks (*A. platyrhynchos*) is a matter of much debate. American black ducks are a taxon in the complex North American mallard group (McCracken et al. 2001). Until recently, black ducks were an isolated allopatric offshoot of the much larger mallard population. Habitat alteration that accompanied European settlement and game-farm mallard releases during the 20th century have enabled mallards to colonize territory east of the Appalachian mountains, where they had been only rare wanderers before (Howe and Allen 1901; Knight 1908; Allen 1909; Johnsgard 1959). By 1969, mallards outnumbered black ducks in the Atlantic Flyway (Heusmann 1974). This population trend has continued to the present day and has resulted in hybrid progeny that backcross into the parental populations (Johnsgard 1967).

Recent molecular studies of mallards and black ducks (Morgan et al. 1984; Ankney et al. 1986; Avise et al. 1990) failed to find reciprocal monophyly between these taxa, prompting speculation that black ducks are a color morph of mallards (Hepp et al. 1988) as is common in other taxa in the mallard complex (McCracken et al. 2001). These studies were based on modern specimens and may reflect the consequence of many generations of introgressive hybridization between the two species. It is not possible to determine from studies based on modern specimens alone whether the present-day genetic similarity of mallards and black ducks is the ancestral condition, or is the result of secondary gene flow (Harrison 1990).

Using DNA obtained from modern Atlantic Flyway populations and museum samples taken

prior to 1940, when black ducks were still the most prevalent anatid in eastern North America, we investigate changes in population differentiation between black and mallard ducks that has occurred during the 20th century. We also infer the presence of genetically differentiated clusters in modern populations to test the correlation between genetic identity and plumage characters, which are typically used to assign individuals a taxonomic status (Kirby et al. 2000). Plumage characters can precede other markers in hybrid populations under sexual selection (Parsons et al. 1993; Brumfield et al. 2001). There is some evidence that black duck hens prefer the brighter breeding plumage of mallard drakes in certain conditions (Brodsky et al. 1988), raising questions about the role of sexual selection in mallard–black duck hybridization.

Methods

DNA preparation and amplification of modern specimens

We obtained 135 wings (67 male and 68 female) from the 1998 Atlantic Flyway parts count conducted by the US Fish and Wildlife Service. Wings were hunter-collected from mallards (n = 45), black ducks (n = 42), and their hybrids (n = 48) from Maine to South Carolina (Table 1). The key (Kirby et al. 2000), used to identify these wings, can distinguish black duck and mallard drakes and hens, all F1 hybrids, and some B1 and further backcrossed offspring. Additionally, wings were classed by state of collection, and further classified into northern and southern populations based roughly on percentage of degree of sympatry of mallards and black ducks. Northern states generally had a higher proportion of black ducks to mallards in the 1975–1995 Audubon Christmas Bird Counts, while southern states exhibited the opposite (Mank 2001).

DNA was extracted according to the Leeton et al. (1993) protocol. Following fluorometric DNA quantification on a Packard Fluorocount fluorometer, the samples were diluted to 5 ng DNA/μl in sterile ddH_2O for PCR amplification. PCR was carried out in a total volume of 6.25 μl with 5 ng DNA, 1.5 mM $MgCl_2$, 0.96 μM forward and flourescently-labeled reverse primer, 1 U AmpliTaq Gold DNA polymerase (Applied Biosystems), 2% formamide, and 0.625 μl Applied Biosystems PCR Buffer II in ddH_2O.

PCR was performed on Perkin Elmer 9700 thermal cyclers using seven waterfowl microsatellite primers developed by Fields and Scribner

Table 1. 1998 specimens used for microsatellite analysis

Collection location	State designation based on degree of sympatry[a]	Number *A. rubripes* collected	Number *A. platyrhynchos* collected	Number *A. rubripes x A. platyrhynchos* hybrids collected
Maine	Northern	8	3	6
New Hampshire	Northern	5	1	3
Vermont	Northern	3	4	2
Connecticut	Northern	1	6	5
Rhode Island	Northern	2	0	4
Massachusetts	Southern	5	4	5
New York	Southern	4	9	6
New Jersey	Southern	4	3	9
Pennsylvania	Southern	0	4	2
Delaware	Southern	0	3	0
Maryland	Southern	4	4	4
Virginia	Southern	5	4	1
North Carolina	Southern	1	0	1
South Carolina	Southern	0	0	1

[a] Determined by proportion of black ducks to mallards in the 1975–1995 Audubon Christmas Bird Counts. Northern states generally had a higher proportion of black ducks, while southern states generally had a higher proportion of mallards (Mank 2001).

(1997) and Buchholz et al. (1998) with the following program: *Taq* activation at 94 °C for 10 min, 40 cycles of amplification of 94 °C for 30 s, primer specific (42–55 °C) annealing temperature for 30 s, 72 °C for 30 s, followed by elongation at 72 °C for 7 min.

DNA preparation and amplification of museum specimens

We collected feathers from 74 (Table 2) prepared museum skins in the Carnegie Museum of Natural History Section of Birds collection (Pittsburgh, PA) and The Pennsylvania State University School of Forest Resources skins collection. From all specimens taken before 1940, we collected two minor or marginal coverts from the wing and immediately sealed the feathers in plastic bags. Only feathers with intact calami were used.

DNA extractions and PCR amplifications were conducted in a building with no previous history of avian work. To further reduce contamination risk, feathers and extracts were never handled in the lab on days after PCR products were opened, and PCR amplification and feather DNA extractions were conducted in separate rooms. All DNA extractions and PCR reagents were handled in a laminar flow hood following >1 h UV irradiation and ethanol sterilization.

DNA was extracted with the same protocol reported above for modern samples and final pellets resuspended in 20 μl of sterile ddH_2O. DNA was treated following Pusch et al. (1998), a procedure that repairs single-strand nicks in the DNA prior to denaturation (Handt et al. 1994). In a trial with 10 samples, this protocol resulted in better amplification than untreated museum DNA, without generating false alleles (data not shown). DNA was then

Table 2. Museum specimens used for microsatellite analysis

Species	Collection date	Collection location	Number collected
Anas rubripes			
	1931	Nova Scotia	3
	1932	Nova Scotia	3
	1936	Ontario	1
	1900	Pennsylvania	8
	1908	Pennsylvania	1
	1912	Quebec	7
	1912	Ontario	6
	1914	Quebec	2
	1915	Quebec	1
	1917	Labrador	1
	1917	Quebec	1
	1926	Ontario	1
	1926	Quebec	3
	1931	Manitoba	1
	1931	Ontario	1
	1935	Ontario	1
Anas platyrhynchos			
	1900	Pennsylvania	8
	1903	Pennsylvania	2
	1912	Ontario	5
	1923	Ontario	3
	1931	Manitoba	1
	1931	Ontario	3
	1935	Pennsylvania	2
	1914	Pennsylvania	2
	1926	Pennsylvania	3
	1933	Pennsylvania	4

fluorometrically quantified and, when necessary diluted to 5 ng/μl with TE buffer when necessary.

PCR on museum samples was carried out in a total volume of 12.5 μl at the same concentrations and conditions as the modern specimens (above). Twice the volume was used for PCR amplification of the museum samples, as compared to modern samples, to maximize the likelihood that complete, intact template would be available for amplification. Because there was less available DNA from museum specimens, only three primer pairs were used; *sfi*μ4, *sfi*μ6, and *sfi*μ7 were selected as they were found to be the most variable in modern samples.

Electrophoresis

PCR products from three loci, each with its own fluorescent label, were pooled with ROX 350^{TM} and separated on an ABI 3700 capillary sequencer under standard electrophoresis conditions.

Data analysis

Electrophoretic mobility data were extracted into Genotyper 3.1 (Applied Biosystems). Peaks from 90 to 1000 base pairs were labeled, and the data were filtered to remove stutter bands. For all data analysis involving comparisons of museum and modern specimens, only data from loci that were amplified in both museum and modern sample sets were used (i.e., from loci *sfi*μ4, *sfi*μ6, and *sfi*μ7). In analyses among only modern specimens, data from all seven loci were used. Data were analyzed for allele frequencies and the results entered into Poptree (Takezaki 1998) to determine Gst values and standard errors. Allele frequency data was also entered into Dispan (Ota 1993) to determine expected population heterozygosity (Hs) and expected total heterozygosity (Ht) (Nei 1973, 1978). Phylip 3.5c (Felsenstein 1989) was used to determine Nei's standard genetic distance (Dst) (Nei 1972) and to draw a UPGMA (Sneath and Sokal 1973), as well as a neighbor-joining tree (Saitou and Nei 1987) based on that distance. To investigate the correlation between plumage characters and genetic identity, we used STRUCTURE (Pritchard et al. 2000) to cluster individual genotypes with a burnin of 20,000 and 100,000 Monte Carlo Markov Chain steps. Bayesian clustering was based on molecular marker data, and taxonomic identity was assigned by plumage characters.

Results

Changes in genetic differences

Gst, a measure of genetic divergence between populations, changed drastically between mallards and black ducks over the course of the 20th century. Gst was estimated from allele frequencies from the three primer sets used on the museum specimens. The mallard–black duck Gst estimate for the pooled sample of specimens taken prior to 1940 was 0.146 (SE = 0.08). In contrast, Gst for the 1998 sample was 0.008 (SE = 0.002). Observed heterozygosity values ranged from 0.000 to 0.538 and expected heterozygosities from 0.180 to 0.839. There were no significant differences in the ranges of observed and expected heterozygosities among black duck versus mallard populations, nor among modern versus museum samples (Table 3).

DNA yields from feathers

Each museum feather yielded an average of 38.0 ng of DNA, roughly one quarter of the yield from each modern feather. DNA yield ranged from 0 to 300 ng for large feathers with a visible tissue plug. PCR amplification was successful in 75% of the museum samples, while over 90% of the modern samples were successfully amplified. A heterozygote deficiency from Hardy–Weinberg expectation was observed in the museum samples, one indication that contamination was not a problem (Table 3) but that null alleles might be present due to DNA degradation. The DNA repair protocol of Pusch et al. (1998) was performed to minimize null alleles, however the protocol cannot repair extensive damage that cleaves both strands of DNA (Handt et al. 1994). Microsatellite alleles from museum specimens fell within the size range of alleles from modern specimens (Table 4), indicating that false allele amplification was not likely.

Modern specimens

The availability of more DNA, and therefore the ability to analyze more loci allowed for a more in-depth scrutiny of the modern samples. Gst estimates for among-population partitioning ranged from 0.017 to 0.045, with an average of 0.031.

Population size was not related to heterozygosity values (Table 4). Population heterozygosity ran-

Table 3. Observed and expected heterozygosity values for microsatellite loci in museum[a] and modern[b] mallard and black duck populations

	Museum				Modern			
	Black duck n = 41		Mallard n = 33		Black duck n = 42		Mallard n = 45	
Locus	He[c]	Ho[d]	He	Ho	He	Ho	He	Ho
*sfiμ*4	0.746	0.289	0.803	**0.538**	0.600	**0.474**	0.594	**0.533**
*sfiμ*6	0.550	**0.217**	0.519	0.000	0.830	0.146	0.839	0.159
*sfiμ*7	0.243	0.000	0.653	0.000	0.180	**0.135**	0.194	**0.205**
Average	0.513	**0.169**	0.658	0.179	0.537	**0.252**	0.542	**0.299**

Significant departures from Hardy–Weinberg expectation are shown in bold ($P < 0.01$).
[a] Museum samples from the Carnegie Museum of Natural History, collected between 1900–1939.
[b] Modern samples, obtained from the USFWS Eastern Flyway Parts Count.
[c] Expected heterozygosity based on Nei (1978).
[d] Observed heterozygosity.

Table 4. Heterozygosity and distance values for the microsatellite loci in mallards, black ducks, and hybrids collected from the 1998 USFWS Eastern Flyway Parts Count

Locus	N[a]	Number of alleles	Range of alleles	Ho[b]	Hs[c]	Ht[d]	Gst[e]
*Bcaμ*6	124	8	147–166	0.250	0.614	0.643	0.045
*Bcaμ*10	126	6	93–110	0.102	0.108	0.111	0.027
*Bcaμ*11	124	8	105–157	0.125	0.188	0.192	0.023
*sfiμ*4	130	17	135–159	0.462	0.596	0.609	0.022
*sfiμ*5	117	10	145–166	0.134	0.179	0.183	0.024
*sfiμ*6	134	11	123–203	0.169	0.805	0.832	0.032
*sfiμ*7	127	9	100–160	0.176	0.177	0.180	0.017
Average	126	9.9		0.208	0.381	0.393	0.031

[a] Number of individuals.
[b] Observed heterozygousity from direct count.
[c] Expected population heterozygosity, from Nei (1978).
[d] Expected total heterozygosity, from Nei (1978).
[e] Proportion among-population differentiation, from Nei (1978).

ged from 0.362 to 0.416 with standard errors of approximately 0.1 in all populations. There were no statistically significant differences in the heterozygosity among any populations. Despite the vastly larger mallard population, this species did not have significantly higher heterozygosity values.

A UPGMA tree (Sneath and Sokal 1973) as well as a neighbor-joining tree (Saitou and Nei 1987) was generated based on Dst distances computed with Phylip (Figure 1) from data from the three loci amplified from both museum and modern specimens. Bootstrap values are based on 1000 replicates. The UPGMA phenogram shows strong support for genetic separation of the museum Mallards from all other samples. Within the second general clade, museum black duck samples were genetically distinct from all modern samples. Modern samples formed one intercalated cluster.

We chose to concentrate on the UPGMA tree because of the assumptions implicit in the method. The neighbor-joining method of phylogenetic tree reconstruction assumes that two neighbors (two nearest taxa on an unrooted tree) are connected by a single node (Nei and Kumar 2000). This does not appear to be a fair assumption among taxa that are known to hybridize, and therefore create multiple nodes due to repeated gene flow events in various directions. The UPGMA algorithm makes

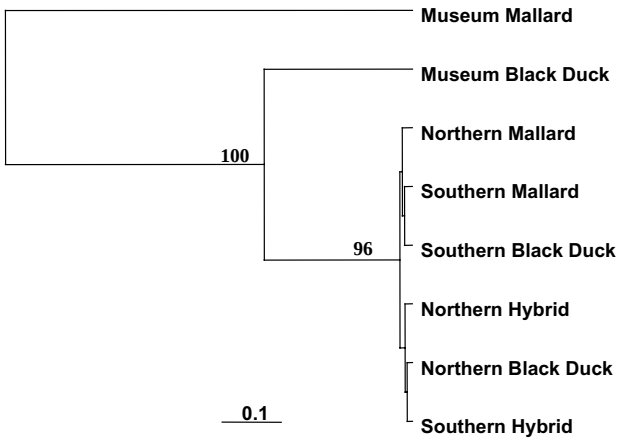

Figure 1. Phenogram of historic mallard, black duck, and hybrid genetic relationships. Museum populations came from samples collected prior to 1940, and all other populations are from wing samples from the 1998 USFWS Eastern Flyway Parts Count. Phylogenetic tree is calculated based on the UPGMA algorithm from Nei's standard genetic distance (Dst). Dst = 0.1 is shown at the scale bar. Significant bootstrap values are shown at the nodes. Northern and southern populations were delimited by the proportion of mallards to black ducks in the Audubon Christmas Bird Count, averaged from 1975 to 1995. Northern states generally had a higher proportion of black ducks, while southern states generally had a higher proportion of mallards (Mank 2001).

no such assumptions as to linear descent, and simply measures amount of divergence among populations (Grauer and Li 2000). This is more appropriate for hybridizing taxa. Despite this, and to ensure thorough data evaluation, we also reconstructed the phylogenetic relationships among these taxa with the neighbor-joining algorithm. The resulting tree was identical in topology to the UPGMA tree, with expected differences in branch length (data not shown). This adds further support to the recovered topology.

Structure analysis (Pritchard et al. 2000) showed three distinct, separate clusters for black ducks, mallards, and the intermediate hybrids (Figure 2). The typing of individuals by plumage appears to be consistent with clustering based on the microsatellite data.

Discussion

The drastic change in Gst values between pre-1940 and modern black ducks and mallards indicates that a great deal of introgression has occurred. It is known that too much hybridization between closely related species can reduce fitness by diluting specially adapted gene complexes, thereby destroying genetic integrity (Lande 1999). The high degree of gene flow indicated by the lower Gst value for the 1998 sample shows that this breakdown in genetic integrity is well under way between the formerly distinct taxa.

This breakdown does not appear to be due to sexual selection for mallard-type breeding plumage (Figure 2). There is no evidence for invasion of phenotypic mallards within the black duck genetic cluster. This indicates both that captive-derived wing keys (Kirby et al. 2000) are accurate for wild populations, and that black duck hens do not prefer mallard drakes.

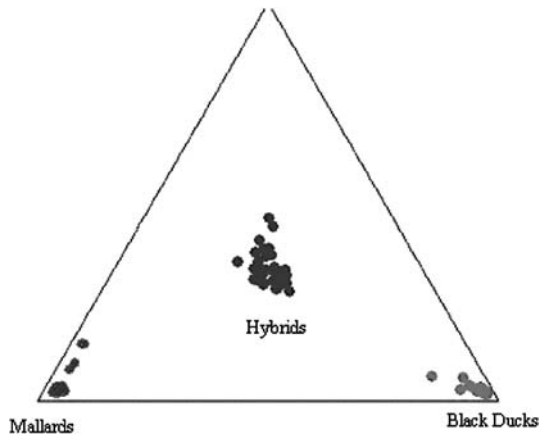

Figure 2. Triangle plot of clustered mallards, black ducks, and hybrids. Clustering was performed using STRUCTURE (Pritchard et al. 2000) with molecular marker data only, taxonomic status was determined by plumage characters.

The Bayesian clustering techniques used to construct Figure 2 do not rely on averages of allele frequencies that are the basis of traditional population genetics. Treating each individual as an operational taxonomic unit, rather than relying on population averages, can offer alternative insights into phylogenetic relationships since the intricacy of the pattern is not lost due to subsumation. This methodology has been used to recover phylogenies of closely related and otherwise cryptic populations (Bowcock et al. 1994), and when combined with Bayesian reconstruction, can determine patterns lost to traditional frequentist population genetics (Rosenberg et al. 2002). This may be why the Bayesian analysis was able to distinguish clear clusters while the genetic distance methods, which are based on averaged allele frequencies, were not.

The genetic similarity shared by black ducks and mallards is a phenomenon that arose in the mid-20th century, resulting from several decades of introgressive hybridization. Our pre-1940 Gst estimate is conservative for two reasons. The sample encompasses birds from 1900 to 1939. This period covers both before and after the cutting of the eastern forests and the concomitant mallard eastward expansion, though it is prior to the large increases in mallard population in the 1960s and 1970s. Additionally, due to limitations in the number of available museum specimens, it was necessary to use museum black duck and mallard samples collected all over eastern North America. If samples from introgressive zones had been excluded, the Gst values for pre-1940 populations might well be higher.

Most of the loci tested in mallards and black ducks did not exhibit the high variability typical of microsatellites. This could be due to the fact that the loci were originally isolated from other waterfowl species, and not selected for variability in anatids. Deka et al. (1994) found ascertainment bias in microsatellites isolated from humans and subsequently amplified in chimpanzees. This may be the case with the loci used in this study, which were originally isolated from spectacled eider, *Somateria fischeri* (Fields and Scribner 1997) and Canada goose, *Branta canadensis* (Buchholtz et al. 1998).

We observed an overall heterozygote deficiency across all loci in the modern sample set, indicating some form of assortative mating. Heterozygote deficiency signifies that the mallard, black duck, and hybrid gene pool is not completely panmictic, indicating that there may be some remaining barriers to hybridization.

Locus *sfi*μ6 showed a severe heterozygote deficiency, which may indicate the presence of one or more null alleles (Neumann and Wetton 1996; Dawson et al. 1997). It is unlikely that null alleles would affect the trees reconstructed with the UPGMA algorithm more so than trees reconstructed with alternative methods. Amplifiable alleles were not confined by taxonomic designation, thus it is therefore unlikely that null alleles were restricted to specific taxa. Additionally, estimates of null allele frequency (Chakraborty et al. 1992) did not differ statistically among identified taxa, and deviations from Hardy–Weinberg equilibrium, an indicator of null alleles, was not statistically different among taxa. This indicates that any effects of null alleles would be distributed roughly evenly across the tree, and would be negligible.

The small genetic distance values we observed were not surprising given the small amount of genetic distance between avian taxa in general, as well as the recent speciation and century of introgressive hybridization. Earlier molecular studies have found similarly low distance values (Ankney et al. 1986; Avise et al. 1990), as we obtained in this study with microsatellite loci.

The 20th century thus brought a volte-face in the course of evolution of mallards and black ducks. The divergent path of evolution was reversed after the thousands of years that the species were geographically isolated. The primitive ability to hybridize was retained and gene flow recommenced after humans removed the geographic barriers to reproduction. The UPGMA phylogeny places the museum mallards as an outgroup to all other populations on the tree. This suggests that the black ducks speciated from an isolated group of mallards, as suggested by Heusmann (1974), but does not rule out the more complicated speciation scenarios recently suggested (Omland 1997; Johnson and Sorenson 1999; McCracken et al. 2001). The museum mallard and black duck branches are deep, and well supported by bootstrap values, indicating unambiguous genetic distance between them. In sharp contrast, the clustering of modern taxa, separated by short branches and lacking significant bootstrap values, demonstrates how much these groups have converged after a century

of hybridization, how their lineages are now intertwined through gene flow, and how genetic drift has changes the allelic composition of these taxa over time (Goldstein and Schlotterer 1999). Despite this, the genomes of these groups have not intermingled enough to dissociate plumage characters from the remainder of the genome. There is some evidence (Brodsky et al. 1988) that plumage characters can proceed other markers in hybrid populations under sexual selection (Parsons et al. 1993; Brumfield et al. 2001). Microsatellite analysis and genotypic clustering thus offers a molecular method to type individuals for which plumage characters are ambiguous.

The black duck–mallard hybridization phenomenon represents a classical gene flow event in a natural laboratory. This study shows the utility of using museum specimens to follow population-level genetic changes over time, and in determining whether genetic similarity is due to ancestral or secondary gene flow. This approach allows the utility of theoretical and laboratory methods to be tested in real world situations over time. This direct view is rarely possible in population genetic analysis, a field that has spent considerable time in developing indirect inference methods for the same purpose. Further research to illuminate the phenomenon of mallard–black duck hybridization should focus on cyto-nuclear disequilibrium studies contrasting maternally inherited mitochondrial markers with bi-parental nuclear loci to determine the direction of introgression. The inclusion of more and even older specimens could give a better estimate of the amount of hybridization and could be used to determine the primary geographic locations of hybridization.

The implications of our findings for the conservation of the black duck are grim. Without preventing hybridization, conservation of pristine black duck habitat will be ineffective in preserving the species. Hybrids, regardless of where they originate, are capable of migrating to prime black duck breeding grounds and introgressing into the black duck population, bringing foreign mallard genetic material with them and with it, the threat of subsumation into the larger mallard gene pool (Nichols et al. 1987; Rhodes et al. 1995; Schaeffer and Malecki 1996). Though there is a possibility that isolated black duck populations can persist in the extreme northern limits of the black duck range of eastern North America, if hybridization continues, the genetic and phenotypic integrity of the black duck will continue to diminish.

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