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Source: *Evolution*, Vol. 44, No. 4 (Jul., 1990), pp. 1109-1119

Published by: Society for the Study of Evolution

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MITOCHONDRIAL GENE TREES AND THE EVOLUTIONARY RELATIONSHIP OF MALLARD AND BLACK DUCKS

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Abstract.—We assayed restriction site differences in mitochondrial DNA (mtDNA) within and among allopatric populations of the Mallard (*Anas platyrhynchos*) and the American Black Duck (*A. rubripes*). The observed mtDNA clones grouped into two phylogenetically distinct arrays that we estimate differ by about 0.8% in nucleotide sequence. Genotypes in one clonal array were present in both species, while genotypes in the other array were seen only in Mallards. In terms of the mtDNA “gene tree,” the assayed Mallards exhibit a paraphyletic relationship with respect to Black Ducks, meaning that genealogical separations among some extant haplotypes in the Mallard predate the species separation. Evidence is advanced that this pattern probably resulted from demographically based processes of lineage sorting, rather than recent, secondary introgressive hybridization. However, haplotype frequencies were most similar among conspecific populations, so the Mallard and Black Ducks cluster separately in terms of a population phenogram. The results provide a clear example of the distinction between a gene tree and a population tree, and of the distinction between data analyses that view individuals versus populations as operational taxonomic units (OTUs). Overall, the mtDNA data indicate an extremely close evolutionary relationship between Mallards and Black Ducks, and in conjunction with the geographic distributions suggest that the Black Duck is a recent evolutionary derivative of a more broadly distributed Mallard–Black ancestor.

Received June 5, 1989. Accepted October 1, 1989.

The Mallard (*Anas platyrhynchos*) has a widespread native breeding distribution in the Holarctic, including most of western and central North America. Until recently, the related American Black Duck (*A. rubripes*) predominated in the eastern portion of the continent. In this century, the two species have come into increased contact through an eastward expansion of the Mallard (Cade, 1983; Heusmann, 1974; Johnsgard and Di Silvestro, 1976), possibly enhanced by the growing availability of man-made ponds that are favored Mallard habitat, and to the extensive propagation and release of Mallards by state and private organizations (Hepp et al., 1988; Heusmann, 1974).

Concomitant with the increased range overlap of the species has been a decline in the number of birds recognizable as Black Ducks (Bellrose, 1976). Much debate has focused on possible reasons for the decline, such as competitive exclusion, overhunting, or genetic swamping of the Black Duck's gene pool by introgressive hybridization with the Mallard (Goodwin, 1956; Johnsgard, 1961; Heusmann, 1974; Dennis et al., 1984; Ankney et al., 1987). The species now hybridize frequently in nature (Brodsky and

Weatherhead, 1984; Goodwin, 1956; Johnsgard, 1960, 1967; Heusmann, 1974), and various hybrid and backcross classes appear viable and fertile (though to an unknown degree compared to nonhybrids) (Cade, 1983; Phillips, 1915). The reproductive compatibility of Mallard and Black Ducks, and their similarities in other attributes such as courtship display (Johnsgard, 1960), have generated controversy concerning whether the forms warrant recognition as distinct species (Ankney et al., 1986; Hepp et al. 1988).

Further assessment of evolutionary divergence and degree of introgressive hybridization between Mallards and Blacks will clearly require genetic markers with specifiable inheritance, and preferably should include information from genes distinct from those underlying the plumage color differences conventionally used to characterize specimens as Black Ducks versus Mallards. Two prior multilocus allozyme surveys found extremely small genetic distances between Mallard and Black Ducks (Nei's $D = 0.001$), and no fixed allelic differences at 18–29 nuclear genes (Ankney et al., 1986; Patton and Avise, 1986). Here we

survey a large number of restriction sites in mitochondrial DNA (mtDNA) in allopatric populations of the two species.

There are two major reasons to predict that mtDNA might distinguish Mallard and Black Ducks while allozymes have not. First, mtDNA evolves rapidly (Brown et al., 1979), and has provided numerous diagnostic markers for other avian sibling species and subspecies (Avise and Zink, 1988; Shields and Wilson, 1987a). Second, the life history of these ducks is such that geographically separated breeding populations might exhibit a greater degree of divergence in maternally inherited mtDNA (Avise, 1986) than in biparentally transmitted nuclear genes. Pair formation in both Mallard and Black Ducks occurs during fall migration and on the wintering grounds (Bellrose, 1976), where considerable mixing of birds from different breeding areas can take place. Yet females usually return to the locale where they were raised, accompanied by mates that may have been hatched elsewhere (Gollop, 1965). Thus males could provide an effective evolutionary avenue for the exchange of nuclear genes among breeding populations that is largely closed to mtDNA.

MATERIALS AND METHODS

The Mallards were hatched from eggs taken in the wild from two locations: (1) south-central Manitoba, Canada ($N = 10$) and (2) Suisun Marsh, California ($N = 10$). Each egg represented a separate clutch. Because of the nature of earlier, unrelated studies for which these individuals were used, all California birds were males, and all Manitoba birds were females. Breeding Mallards are found throughout much of western North America (Bellrose, 1976). The Manitoba and California samples come from the central and extreme western portions of the species distribution on this continent.

The Black Ducks also came from two sources: (1) a captive breeding population at the Patuxent Wildlife Research Center in Maryland (maintained since the 1970s from eggs taken from Halifax, Nova Scotia, and Fredericton, New Brunswick, Canada) ($N = 10$) and (2) from fall migrating birds collected at Bathurst, New Brunswick, Canada ($N = 10$). From prior experience of Cana-

dian Wildlife Service biologists at the Bathurst site, these latter Black Ducks (all were either juveniles or adult females) almost certainly came from Labrador, Canada. Breeding Black Ducks are found in eastern Canada and the northeastern United States (Bellrose, 1976). The Labrador (and Nova Scotia) populations, which represent northern (and eastern) limits of the species distribution, were chosen for analysis because they are currently far removed from the major Mallard breeding range, and hence should maximize the likelihood of containing "pure" Black Duck genotypes, uncontaminated by introgression from Mallards.

Heart and liver tissues were placed in the MSB-Ca-EDTA buffer of Lansman et al. (1981), and shipped on wet ice to the laboratory. MtDNA was purified (within 7 days of the time of collection) by ultracentrifugation in cesium chloride gradients. This closed-circular mtDNA was then digested by each of the 17 informative endonucleases (those producing at least two cuts in the molecule) listed in Table 1. MtDNA fragments were end-labeled with ^{35}S -radionuclides, and separated through agarose gels (1.0–1.6%). Restriction fragments were revealed by autoradiography of vacuum-dried gels, and compared to molecular weight standards in a 1-kb ladder purchased from Bethesda Research Labs. All of the standard laboratory procedures employed for the DNA isolation, digestion, radioactive labeling, and electrophoretic separation are described in Brown (1980), Lansman et al. (1981), and Maniatis et al. (1982).

Kessler and Avise (1984, 1985) have previously described mtDNA variation in several *Anas* species, including the Mallard (but not the Black Duck). Many of the fragment digestion profiles observed in the present study were identical to those pictured in Kessler (1984), and for consistency are given the same letter designations. Three endonucleases employed here (*Ava*II, *Msp*I, and *Spe*I) were not utilized by Kessler, and the most common profile of each is arbitrarily denoted "C." The multienzyme genotype of each individual (its haplotype or clonal designation) is thus summarized by a 17-letter code, each letter indicating the gel profile for one endonuclease.

Essentially all differences among gel pro-

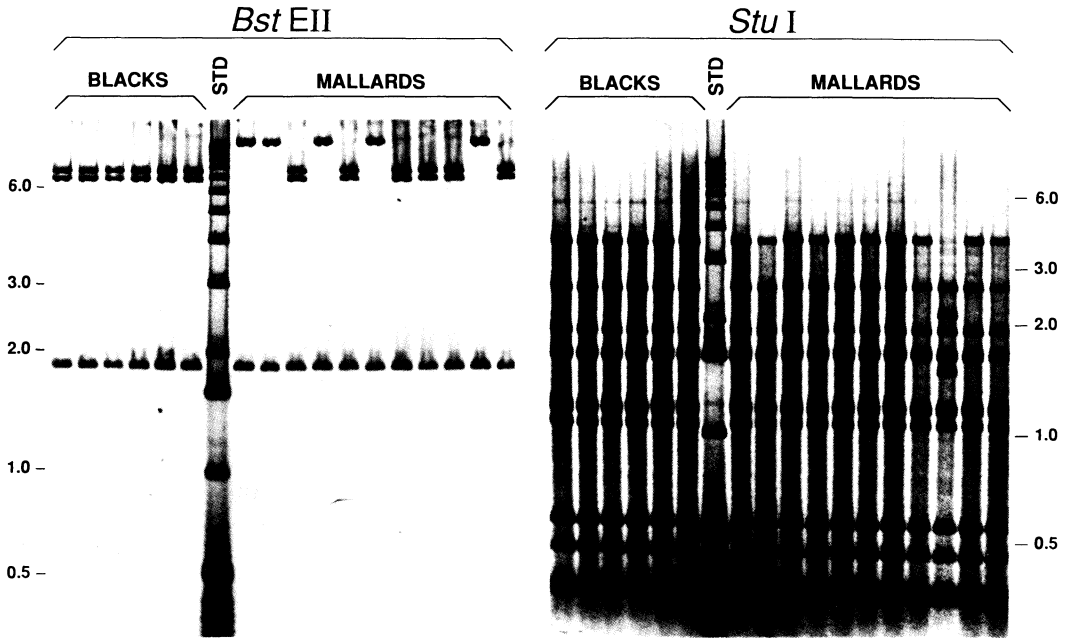


FIG. 1. *BstEII* and *StuI* digests of representative Black and Mallard Ducks. In each gel, the seventh lane from the left is a molecular weight standard, with selected sizes (in kilobase pairs) indicated in the margins. The variant *BstEII* pattern in lanes 8, 9, 11, 13, and 17 results from a site loss from the more common pattern; the *StuI* variant in lane 16 results from a site gain.

size by 0.1 kb; such a small difference in genome size would not likely have been detected in the other gel profiles.] The mtDNA molecule in both the Black Duck and Mallard is about 16.5 kb long, typical of birds and many other vertebrates (Brown, 1983; Shields and Helm-Bychowski, 1988).

mtDNA Gene Trees

Figure 2 shows the UPGMA phenogram for the mtDNA clones observed in the 40 Mallard and Black Duck samples. The genotypes cluster into two distinct groups, differing on average by an estimated 0.86% sequence divergence (the *maximum* genetic distance observed between clones within either cluster was 0.30%). After correction for mean within-cluster divergence (Nei and Li, 1979), the net distinction between the two major clusters remains about 0.69%. Henceforth for simplicity, these two clusters will be referred to as the "A" and "B" groups or lineages.

Groups A and B were also recognized consistently in the qualitative Wagner analyses. Using the PENNY algorithm in PHY-

LIP (which exhaustively searches for minimal-length, unrooted networks), 45 equally parsimonious networks were obtained (each with total branch length of 13 steps). However, all of these networks involved alternative branching arrangements *within* the A or B groups. Furthermore, the distinction between the A and B groups was supported at the 100% level in the bootstrap replicates, while no other assemblage received support at a level greater than 66%.

The Wagner networks are unrooted, but additional information can be obtained by comparison of the mtDNA gel profiles in Mallards and Black Ducks with those previously presented for eight other *Anas* species (Kessler and Avise, 1984; Kessler, 1984). If we view these other species as "outgroups" (with the exception of the Mottled Duck, *A. fulvigula*, which in conventional systematic treatments and in mtDNA composition is very closely related to the Mallard), provisional assignments can be made of the cladistic "status" of various mtDNA gel profiles (see, e.g., Patton and Avise, 1983). For example, because the

TABLE 2. Provisional cladistic assignments (as in Patton and Avise, 1983) of various mtDNA digestion patterns observed in Mallard and Black Ducks, using as outgroups other *Anas* species previously surveyed (Kessler and Avise, 1984).¹

1. Autapomorphies (unique to a single mtDNA clone)		
<i>Bam</i> HI I		
<i>Stu</i> I G		
<i>Stu</i> I H		
<i>Hin</i> dIII E (2 Mottled Ducks only)		
2. Plesiomorphies (to the Mallard/Black/Mottled assemblage)		
A. Patterns shared with at least some outgroups		
<i>Bam</i> HI E	<i>Cl</i> aI A	<i>Hin</i> cII D
<i>Bst</i> EII C	<i>Xba</i> I A	
B. Patterns not shared by outgroups (hence providing unique markers for assayed birds within the Mallard/Black/Mottled lineage)		
<i>Ava</i> I D	<i>Pvu</i> II C	
<i>Bcl</i> I D	<i>Sac</i> I C	
<i>Hin</i> dIII C	<i>Stu</i> I D	
<i>Nde</i> I D		
3. Cladistically uninformative (shared by various clones within Blacks/Mallards, but ancestral condition unclear)		
<i>Bgl</i> I J		
<i>Bgl</i> I F		
<i>Bgl</i> I K		
4. Synapomorphies (identifying clades within Mallards/Blacks)		
<i>Bst</i> EII D	<i>Hin</i> cII J	
<i>Hin</i> cII I	<i>Hin</i> cII E	

¹ Digestion patterns listed in Table 1, but not in this table, involve restriction enzymes not employed in the Kessler and Avise (1984) study.

*Bam*HI “E” pattern observed in most Mallards and Black Ducks (Table 1) is also common in an outgroup species (*A. acuta*), it likely is plesiomorphic to Mallards–Blacks, while the *Bam*HI “I” pattern, observed in one mtDNA clone in Black Ducks but not elsewhere in *Anas*, is probably an autapomorph, derived from *Bam*HI “E” by a single site gain. Table 2 lists such provisional cladistic assignments of the various digestion patterns. By strict Hennigian principles (Hennig, 1966), most of the patterns are uninformative with respect to clade definition within the Black–Mallard complex. These include the autapomorphs (derived, but unique to one mtDNA clone) and plesiomorphs (ancestral to the Mallard–Black–Mottled group). Nonetheless, seven of these gel profiles provide unique genetic markers for the Mallard–Black–Mottled assemblage,

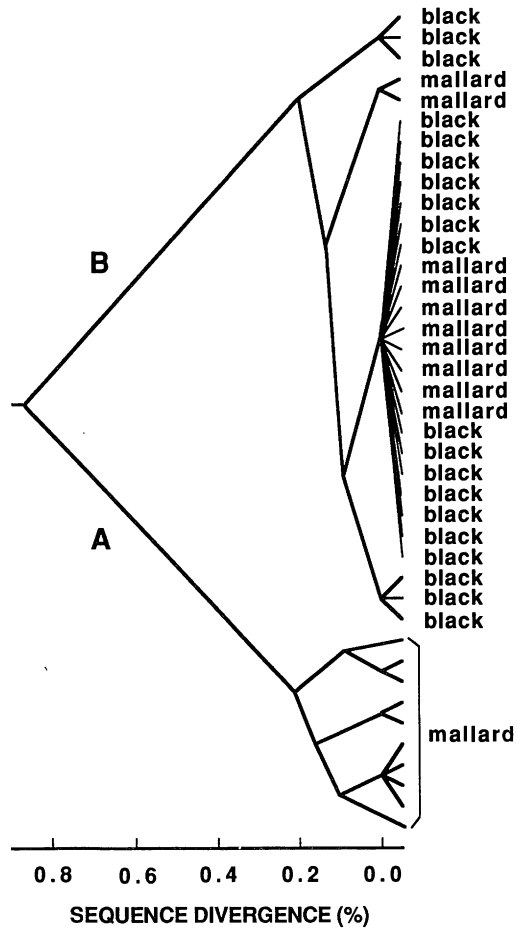


FIG. 2. UPGMA phenogram based on mtDNA haplotype distances, and treating each individual as an OTU.

having been observed in essentially all samples of these species yet nowhere else in *Anas*. Two additional endonucleases (Table 2) produced synapomorphic digestion patterns that tentatively identify clades within the Mallard–Black assemblage: both *Bst*EII and *Hin*cII exhibit derived profiles in members of the A lineage that distinguish them from B (Table 1; Fig. 2).

Overall, in terms of the analyses that treat individual mtDNA genotypes as OTUs, Mallards appear to exhibit a paraphyletic relationship (Neigel and Avise, 1986) with respect to Black Ducks in matriarchal phylogeny. In other words, some mtDNA haplotypes in Mallards appear genetically closer to those in Black Ducks than they do to other mtDNA haplotypes in Mallards.

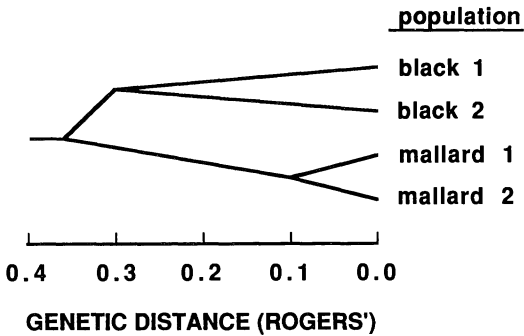


FIG. 3. UPGMA phenogram based on mtDNA haplotype frequency differences, summarized by a distance measure treating each population as an OTU.

mtDNA Population Relationships

A different perspective on these same data comes from analyses that focus on the haplotype *frequencies* in populations (which obviously represent composite information from multiple individuals). A UPGMA phenogram, generated from the Rogers' (1972) distance matrix derived from the mtDNA clone frequencies in Table 1, is shown in Figure 3. In this treatment, the conspecific populations cluster together, due to high similarities in haplotype frequency.

Particularly striking is the very close similarity in mtDNA genotype frequencies at the two Mallard locales (Table 1), separated by more than 2,000 km. Clearly, these populations must have been in extensive contact in recent evolutionary times. Two possibilities may account for this finding. First, Manitoba may have been colonized recently by Mallards from California (or other related southern) populations. Since Manitoba was unsuitable for nesting during the Wisconsin glacial period, an upper time limit on colonization of this region is about 15,000 years ago. Of course, such a colonization may have occurred much more recently than this. Second, the similarities in haplotype frequency between our California and Manitoba samples could be due to ongoing northward dispersal by young female California Mallards. There are data to suggest that this occurs, although the frequency is unknown (R. M. McLandress, pers. comm.). Clearly, if these females nested in prairie Canada, then the likelihood of genetic exchange between California and Manitoba

Mallards is increased. Whether the haplotype similarities reflect a population interconnection through recent colonization, and/or contemporary interchange of birds, the mtDNA data predict that the California and Manitoba Mallard populations should be very closely related in nuclear genes as well.

DISCUSSION

The most salient features of the mtDNA data in Mallards and Black Ducks are (1) the presence of two genetically distinct arrays of haplotypes (groups A and B) that do not coincide with the species designations of the individuals in which they are housed, and (2) the close similarities (particularly in the Mallard) in haplotype frequency among conspecific populations. The first conclusion derives from analyses that view the haplotypes of individuals as OTUs, a treatment that is justified because mtDNA in vertebrates is maternally inherited, nonrecombining, and effectively haploid. Thus the evolutionary history of mtDNA represents one particular "gene genealogy" (the matrilineal phylogeny) within an organismal pedigree (Avise, 1986, 1989; Wilson et al., 1985). The second conclusion derives from analyses that focus on the *frequencies* of haplotypes (irrespective of their genetic relationships) within and among populations. Together, these two perspectives are more informative than either is alone.

mtDNA Gene Trees

Several prior studies with other vertebrate and invertebrate taxa have also reported discordancies between mtDNA genotypes and species designation (Ferris et al., 1983; Powell, 1983; Spolsky and Uzzell, 1984; Tegelstrom, 1986). In each case, the authors reasonably attributed the findings to introgressive hybridization mediated by backcrossing of hybrid females in secondary contact zones. In principle, even a low level of such introgressive hybridization can be sufficient to establish a neutral mtDNA genotype in a foreign population or species (Takahata and Slatkin, 1984). Could introgressive hybridization between Mallards and Black Ducks similarly account for the results summarized in Figure 2? We doubt it, for at least three reasons.

First, both of our Mallard samples came from locales well outside the current breeding range of the Black Duck (and, our Labrador sample of Black Ducks falls outside the breeding range of Mallards). Second, if hybridization is responsible, introgression of Mallard mtDNA into Black Ducks must already be complete or nearly so, because we observed no Black Ducks with mtDNA genotypes highly distinct from those of Mallards. Third, if mtDNA types in the B lineage (Fig. 2) were originally characteristic of some Mallards, but now appear in Black Ducks because of introgression, then the hybridization events must have involved female Mallards with male Black Ducks or hybrids (rather than the reverse). But this direction of cross is inconsistent with what is known about present-day hybridization between these species, which normally involves male Mallards with female Black Ducks. In these waterfowl, male dominance exerts an important influence on intra- and intersexual social interactions, including mating. Male Mallards are typically dominant to Black Duck males, and commonly displace them when competing for females of either species (Brodsky and Weatherhead, 1984; Brodsky et al., 1988).

A related possibility is that mtDNA types in the B lineage (Fig. 2) were originally characteristic of Black Ducks, and some Mallards secondarily obtained them via introgression. This too seems unlikely for our particular samples, given their geographic origin, although we cannot eliminate the possibility altogether. In particular, the hypothesis that the California population of Mallards exhibits mtDNA introgressed from Black Ducks requires not only past hybridization with female Black Ducks, but also extensive gene flow of such maternal lineages to the extreme west coast. [However, in sympatric areas such as southern Ontario, ducks that appear phenotypically to be Mallards may possibly carry Black Duck mtDNA derived from past hybridizations between male Mallards and female Black Ducks. In general appearance, such later-generation hybrids or backcrosses often closely resemble Mallards in plumage coloration (Johnsgard, 1967).]

An alternative class of hypotheses to account for the apparent discordancy between

species affiliation and mtDNA genotype in Figure 2 involves stochastic matriarchal lineage sorting from a polymorphic ancestral gene pool (Neigel and Avise, 1986; Tajima, 1983; Nei, 1987). Particularly for species or populations that have separated recently (relative to their effective population sizes), the probabilities are high that a state of reciprocal monophyly has not yet been achieved with respect to the true genealogical ancestry of haplotypes at particular loci. Some previous empirical examples of such discordancies between mtDNA genotype and conventional taxon boundaries are probably best attributed to such a lineage sorting process (Avise et al., 1983; Cann et al., 1987).

In a random mating population (or a species with historically high levels of gene flow), the expected mean time to common ancestry (measured in generations) for randomly drawn pairs of mtDNA haplotypes is approximately $N_{(e)}$, the evolutionary effective population size of females (Avise et al., 1988). Thus under a neutral model, large populations are expected to retain large numbers of haplotypes, many of which will have been separated for long periods of time. Suppose such a population is divided into two (e.g., by a geographic or behavioral isolating barrier). Initially, each of the two daughter populations will likely retain polymorphisms of the ancestor, and hence will appear "polyphyletic" with respect to mtDNA (or nuclear) haplotypes. Random extinction of lineages (or directed extinction, if selection is also at work) will occur inevitably, as some females fail to leave daughters (Avise et al., 1984). New mutations will also arise in the surviving lineages. First, one daughter population (likely the smaller one) will lose all ancestral lineages except one, such that all haplotype diversity within it will postdate the original population separation. Eventually, the other population will reach a similar state of affairs, such that the two daughter populations become reciprocally monophyletic in matriarchal ancestry. The time course of these changes in mtDNA phylogenetic status (polyphyly \rightarrow paraphyly \rightarrow monophyly) depends on the demographic conditions specified, but the stage of reciprocal monophyly is generally expected with high probability

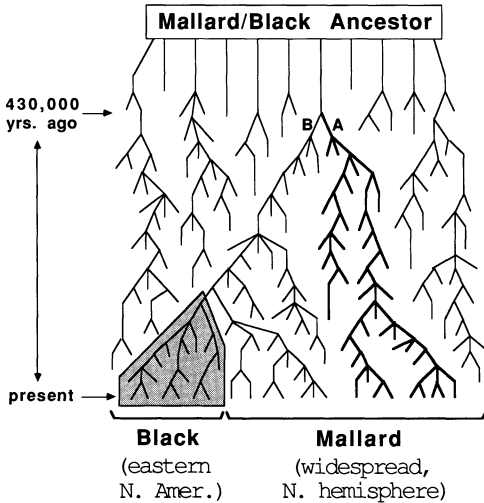


FIG. 4. Simplified diagrammatic representation of one phylogenetic interpretation for the matriarchal ancestry of Mallard and Black Ducks. mtDNA lineage A is shown in dark lines, and the Black Duck portion of the phylogeny is shaded. The time scale is provisional, based on the conventional mtDNA "clock calibration" of 2% sequence divergence per million years. See text for explanation and qualifications.

within at most $4N_{f(e)}$ generations (Neigel and Avise, 1986).

Figure 4 presents one possible schematic interpretation of the mtDNA results in Mallards and Black Ducks, assuming that phylogenetic sorting of lineages is indeed responsible for the paraphyletic relationship in the observed mtDNA gene tree. The assayed Black Ducks are thus a recently derived subset within the B mtDNA lineage. (Relationships of various mtDNA haplotypes within the B group are not well resolved with current data, so it remains unclear whether Black Ducks are mono-, para-, or polypyletic within B; Figure 4 arbitrarily depicts them as a unified clade) If mtDNA in these ducks has evolved at the "conventional" rate reported for birds and some other vertebrates (about 1% sequence divergence per lineage per million years—Brown et al., 1979; Shields and Wilson, 1987b), then the A and B lineages diverged about 400,000 years ago. This time well predates the "separation" of Black Ducks from the B lineage in Mallards (which in fact with current data we cannot distinguish from zero time before present). Heusmann (1974) previously suggested that the Black Duck

"arose" very recently from the Mallard, perhaps during the last Pleistocene glacial episode.

The phylogenetic pattern exhibited by mtDNA also suggests that $N_{f(e)}$ in Black Ducks has been considerably smaller than that in Mallards. The level of mtDNA diversity in Mallards, and the retention of at least two mtDNA lineages separated for 400,000 years (Fig. 4), is a likely outcome under a neutral model with an $N_{f(e)}$ in that species of a few hundred thousand individuals (Neigel and Avise, 1986; Avise et al., 1988); the limited sequence diversity observed within Black Ducks is compatible with an $N_{f(e)}$ of only perhaps a few tens of thousands of females. Currently, the total North American breeding population of female Mallards fluctuates between about 5 and 7.5 million, while that of the Black Duck is currently about 0.5 million. Thus the mtDNA-derived estimates of $N_{f(e)}$ for Mallards and Black Ducks are roughly proportional to the current relative abundances of these species. They are also about one order-of-magnitude lower than the present-day absolute population sizes, a finding that is consistent with previous observations for other vertebrate species with high gene flow (Avise et al., 1988).

Population History and Taxonomy

Of what relevance are these findings to the taxonomic status and management of Mallard and Black Ducks? Unfortunately, the mtDNA data cannot decide unambiguously the "species status" of *A. platyrhynchos* and *A. rubripes*, other than to demonstrate that the two forms are extremely closely related. In terms of the mtDNA phylogeny, although the Black Duck appears to be a poorly defined subset of lineages within the larger mtDNA tree of Mallards, it nonetheless appears to be a coherent biological entity in terms of plumage characteristics and original geographic range (and perhaps mtDNA clonal frequencies). As interpreted against the mtDNA phylogenetic backdrop, this raises the likelihood that the morphological features that make a Black Duck recognizable as such may have evolved very rapidly and recently within an eastern North American isolate of the more broadly distributed Mallard-Black ancestor. Many

workers consider the Black (and Mottled) Ducks to be merely sexually nondimorphic forms of the Mallard (Bellrose, 1976; Graham, 1979; Ankney et al., 1986), and otherwise to exhibit little evolutionary separation from the latter.

The original intent of this preliminary project was to identify diagnostic genetic markers for Mallard and Black Duck populations in allopatry that might subsequently be of use in examining the extent of hybridization and introgression where the species come into contact. While we have found some distinctive mtDNA genotypes in Mallards not yet seen in Black Ducks (the A lineage), all mtDNA genotypes in Black Ducks were essentially indistinguishable in our assays from those also present in Mallards, presumably due to the phylogenetic retention of matriarchal lineages from a polymorphic ancestral population. The mtDNA tree is the first "gene genealogy" (Avice, 1989) monitored in the Mallard-Black complex, but the haplotypes clearly have not yet evolved to a condition of reciprocal monophyly in the two "species." This raises the likely spectre that comparable assays of haplotypes at many randomly chosen nuclear genes would also reveal either poly- or paraphyletic gene trees (under a 1:1 sex ratio, the effective population size for nuclear haplotypes is roughly four times greater than that for mtDNA, and hence the expected times to reciprocal monophyly correspondingly greater—Ball et al., 1990).

The data and theory in this report provide an important message: proper interpretations of any observed discordance between a gene tree and a presumptive organismal tree should recognize and assess the competing possibilities of ancestral lineage sorting versus contemporary introgression. For Mallards and Black Ducks in particular, and for recently separated populations or species in general, it is theoretically likely and empirically plausible that haplotypes retained in extant forms may date to gene-lineage separations predating the partitioning of the populations in which they are found.

ACKNOWLEDGMENTS

We would like to thank Myrtle Bateman, Dick Jackowski, Dennis Jorde, Pat Kehoe,

Ken Risi, and the Delta Waterfowl and Wetlands Research Station for their assistance in obtaining the specimens for this study, Marty Ball for assisting with the data analysis, David Scott and Shiva Singh for comments on the paper, and Darrell Dennis for providing the idea from which this research arose. Work was supported by NSF Grants BSR-8603775 and BSR-8805360 to JCA, and a National Science and Engineering Research Council grant to CDA.

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Corresponding Editor: M. G. Kidwell