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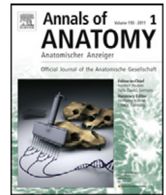

RESEARCH ARTICLE

Nuclear DNA from the extinct Passenger Pigeon (*Ectopistes migratorius*) confirms a single origin of New World pigeons

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SUMMARY

Passenger Pigeons (*Ectopistes migratorius*) were once the most abundant bird in North America, with flock sizes estimated in the billions. However, by the turn of the 20th century, this previously abundant species had been driven to extinction. Morphological analyses linked the Passenger Pigeon with the New World mourning doves of the genus *Zenaida*. However, mitochondrial analyses strongly support its placement within the group of typical pigeons and doves (New and Old World pigeons, cuckoo-doves, turtledoves). Here, the first nuclear DNA sequence obtained for this extinct species confirms the placement of the Passenger Pigeon as sister to the New World pigeons, *Patagioenas*. These findings have implications for the colonization of North America by pigeons and doves.

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1. Introduction

Passenger Pigeons (*Ectopistes migratorius*) were once the most abundant birds in North America, comprising an estimated 20–40% of the total avian population (Schorger, 1973). Initial descriptions referred to "infinite" flocks, while later reports estimated between one and three billion birds flying together (Schorger, 1973). However, these numbers began to fall with European settlement, and the Passenger Pigeon population declined precipitously through the 18th and 19th centuries, culminating in the death of the last known individual in captivity in 1914.

Hunting and deforestation were the primary factors leading to the rapid demise of the Passenger Pigeon during the 18th and 19th centuries. Passenger Pigeons were hunted both in flight or while roosting, and were killed by the thousands; in 1851 nearly two million birds were taken from a single nesting site in New York (Schorger, 1973). Mating pairs of Passenger Pigeons lay a single egg per year; many too few to sustain the population given these heavy losses to hunting. In addition, Passenger Pigeons lived, on average, only five years. As they became increasingly scarce, hunters became less selective in the birds that they captured and even young individuals were taken. By the mid-19th century, the species was nearing extinction, and hunting bans were put into place to try to conserve the remaining birds. New York protected nesting sites in 1862 and similar legislation followed over the course of the next fifteen years in Michigan, Wisconsin, Pennsylvania, Ohio, and Massachusetts (Schorger, 1973). These bans were some of the earliest examples of conservation legislation, paving the way for future conservation action and highlighting the ease with which humans can drive even the most abundant species to extinction. Unfortunately for the Passenger Pigeon, these bans were widely ignored (Schorger, 1973).

Despite widespread attention, very little research has focused on the evolutionary history of the Passenger Pigeon. Originally, the Passenger Pigeon was described as conspecific with the Mourning Dove (*Zenaida macroura*). It was later placed in the monotypic genus, *Ectopistes* (Swainson, 1827) but remained within the radiation of the New World mourning doves (*Zenaida*) (Goodwin, 1967). The Passenger Pigeon is superficially similar to *Zenaida*. It has fewer tail feathers than the Mourning Dove, *Z. macroura*, but the same number (twelve) as *Z. aurita* and *Z. galapagoensis* (Schorger, 1973; Johnson and Clayton, 2000). It is, however, considerably larger than *Zenaida*, lacks a facial stripe, has more pronounced sexual colour dimorphism, and smaller clutch size of a single egg (Goodwin, 1967).

In contrast to the morphological assessment, mitochondrial DNA (mtDNA) obtained from museum specimens using ancient DNA (aDNA) techniques suggested an association with the typical pigeons and doves, rather than with *Zenaida* and the ground doves

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(Shapiro et al., 2002). Specifically, 1448 base-pairs (bp) of mitochondrial 12S and cytochrome *b* (*cytb*) indicated that *Ectopistes* is most closely related to the cuckoo-doves of genus *Macropygia* (Shapiro et al., 2002). In a reanalysis of this *cytb* data plus an additional 169 bp of the mitochondrial gene *ATP8* and a more extensive sample of New World pigeons, Johnson et al. (2010) confirmed that the Passenger Pigeon was not sister to *Zenaida*, suggesting instead a close evolutionary relationship with the New World pigeons, *Patagioenas*. While this association was not well-supported statistically (maximum parsimony bootstrap support of 52%), placement of the Passenger Pigeon within the typical pigeons and doves was well supported. Unfortunately, basal relationships within this group have been difficult to resolve, even when nuclear data is applied (Pereira et al., 2007). However, ancient nuclear DNA has shed light on a wide variety of questions regarding extinct species including moas (Huynen et al., 2003; Allentoft et al., 2010), mammoths (Römpler et al., 2006), and Neanderthals (Green et al., 2010) and can provide key evidence in resolving rapid radiations when gene trees (particularly mtDNA gene trees) may not represent the true species tree. Here, we provide the first nuclear DNA from the extinct Passenger Pigeon and use these data to address its evolutionary history.

2. Materials and methods

2.1. DNA isolation, amplification, and sequencing

A toe pad sample of *E. migratorius* was received from National Museums Liverpool (museum ID: Canon H.B. Tristram Collection LIV T17065). DNA extraction and all pre-PCR work was performed in a dedicated aDNA facility at The Pennsylvania State University that is housed in a separate building from any laboratories that perform genetic analysis. Ancient DNA protocols were strictly adhered to at all stages including, but not limited to, the use of full body suits and face masks, "one-way" movement of reagents, supplies, and workers prohibiting any PCR-contaminated material from entering the facility, sterile reagents and plasticware, and sterilization of surfaces and equipment with bleach and ethanol (Cooper and Poinar, 2000; Gilbert et al., 2005). Toe pad tissue was cut into small pieces and DNA was isolated using the Qiagen DNeasy Tissue Kit (Qiagen) with an extended initial lysis step of four days and additional proteinase K to ensure complete tissue lysis. An extraction negative control (no tissue sample) was carried out simultaneously.

In order to compare sequences for *Ectopistes* to the greatest number of species within Columbidae, intron 7 of the nuclear-encoded fibrinogen beta chain (*FGB*) gene was selected for sequencing. Due to the level of fragmentation incurred by aDNA, a series of primers were designed to amplify a series of short overlapping fragments (Table 1). PCR amplifications were performed in 25 μl reactions comprising 50 μg rabbit serum albumin, 0.25 mM dNTPs, 1× High Fidelity buffer, 1.25 units Platinum *Taq* High Fidelity (Invitrogen), 3mM $MgSO_4$, 1 μM of each primer, and 1 μl DNA extract. Cycling conditions were 94 °C for 90 s, 50 cycles of 94 °C for 45 s, 45 s at 48–56 °C (Table 1), 68 °C for 90 s followed by 10 min of 68 °C. Negative PCR reactions (containing no DNA extract) were included for each amplification reaction. PCR products were cleaned using Millipore Multiscreen PCRμ96 filter plates and sequenced with BigDye v3.1 chemistry (Applied Biosystems). Following ethanol/EDTA precipitation, sequences were resolved on an ABI3730xl DNA Analyzer (Applied Biosystems) at the Penn State Genomics Core Facility (University Park). To assess DNA damage and screen for contamination, each amplification was also cloned using the TOPO TA cloning kit (Invitrogen) according to manufacturers' instructions. Several clones from each sample were sequenced using the M13F primer (TOPO manual). All sequences were visualized and aligned using Lasergene 8 (DNASTAR).

2.2. Phylogenetic analysis

DNA sequence is available from *E. migratorius* for three mitochondrial genes: *ATP8*, 12S rRNA, and *cytb* (Shapiro et al., 2002; Johnson et al., 2010). The sequence for these genes and *FGB* for a wide selection of Columbiformes was retrieved from GenBank and each locus was aligned using MAFFT v.6 online under the 'moderately accurate' automatic strategy (Katoh et al., 2002). The final aligned data set includes 122 taxa (116 Columbiformes, 6 outgroups), 415 bp for 12S rRNA, 1045 bp for *cytb*, 169 bp for *ATP8*, and 529 bp for *FGB*, as alignments were trimmed to maximize overlap with *Ectopistes* sequence. Forty-seven taxa were missing data for 12S, one was missing *cytb*, 29 were missing for *ATP8*, and 30 were missing *FGB*. GenBank accession numbers and references for those sequences that were included in our analysis are provided as Supplementary Table S1. An additional nine taxa were removed from analyses including only *FGB* because they were either identical to or differed by a single bp from another taxon from the same genus and fell within a well-represented, well-supported clade (analyses not shown). This is indicated in Supplementary Table S1.

The best fitting model of evolution was selected using jModelTest (Posada, 2008). The general time reversible model, a proportion of invariant sites and gamma distributed rate heterogeneity (GTR + I + G) was selected for each gene. Maximum likelihood (ML) and ML bootstrapping (MLBP; 100 pseudoreplicates) were performed in RAxML 7.0.4 (Stamatakis, 2006) using a partitioned analysis and rapid bootstrapping (option -f a) with the GTRGAMMA model for both the combined data set and *FGB* alone. Bayesian inference was conducted in MrBayes v. 3.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) for both the combined data set and *FGB* alone. Two runs of 5 million generations each were performed simultaneously, sampling every 200 generations. Each gene was given its own partition with unlinked model parameters. Convergence was assessed using the sump command to visualize the trace and calculate a potential scale reduction factor (PSRF) of ∼1.00. A burnin of 10% of samples was removed. All trees were visualized in FigTree v.1.3.1 (http://tree.bio.ed.ac.uk/software/figtree).

Table 1
Overlapping primer sets to amplify *FGB* intron 7 from ancient pigeons.

Primer set	Forward primer	Reverse primer	Annealing temp. (°C)	Fragment length[a] (bp)
F2R3	AGCACTGTTTTCTTGGATCTGAAGT	GTTTATTATGGTTTGAAAATTCCAGT	52	133
F3R4	TAGATCAACAGAGTACCTAGACCTGC	TTCACCTTCCAAGTGCCCTGTGT	56	136
F4R5	ACCATAATAAACATTTAAAATCCTCTC	AATTATCAATTGATAAACTAAAATGACA	48	164
F5R6	AGCAGCTAAGAAAAACAAGTAAAA	GGGAAGACATACATTTCTCATTGTT	48	157
F6R7	CATAATGATGATTGCAATATCAA	GTGTGCTGTGCCTTTACCTTA	48	130
F7R8	TTCCTTTATTCATGAATGTGTGA	AAAGTTCTGCCTACTTAGAAGACA	48	114

[a] Fragment length includes primers.

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