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**Article:**

Alexander, Michelle orcid.org/0000-0001-8000-3639, Ho, Simon Y W, Molak, Martyna et al. (10 more authors) (2015) Mitogenomic analysis of a 50-generation chicken pedigree reveals a rapid rate of mitochondrial evolution and evidence for paternal mtDNA inheritance. *Biology letters*. pp. 1-4. ISSN: 1744-957X

<https://doi.org/10.1098/rsbl.2015.0561>

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# BIOLOGY

## LETTERS

### Mitogenomic analysis of a 50-generation chicken pedigree reveals a rapid rate of mitochondrial evolution and evidence for paternal mtDNA inheritance

Journal:	<i>Biology Letters</i>
Manuscript ID	RSBL-2015-0561.R2
Article Type:	Research
Date Submitted by the Author:	n/a
Complete List of Authors:	Alexander, Michelle; The University of York, Archaeology, BioArCh Ho, Simon; University of Sydney, School of Biological Sciences; Molak, Martyna; University of Sydney, School of Biological Sciences Barnett, Ross; Durham University, Archaeology Carlborg, Örjan; Swedish University of Agricultural Sciences, Department of Clinical Sciences Dorshorst, Ben; Uppsala University, Science for Life Laboratory, Department of Medical Biochemistry and Microbiology Honaker, Christa; Virginia Tech, Department of Animal and Poultry Sciences Besnier, Francois; Institute of Marine Research, Section of Population Genetics Wahlberg, Per; Uppsala University, Science for Life Laboratory, Department of Medical Biochemistry and Microbiology Dobney, Keith; University of Aberdeen, Department of Archaeology Siegel, Paul; Virginia Tech, Department of Animal and Poultry Sciences Andersson, Leif; Uppsala University, Science for Life Laboratory, Department of Medical Biochemistry and Microbiology; Swedish University of Agricultural Sciences, Department of Animal Breeding and Genetics Larson, Greger; University of Oxford,
Subject:	Molecular Biology < BIOLOGY, Evolution < BIOLOGY
Categories:	Molecular Evolution
Keywords:	mutation rates, paternal leakage, mitochondrial genome, vertebrate pedigree

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Manuscripts

1 **Mitogenomic analysis of a 50-generation chicken pedigree reveals a rapid rate of**  
2 **mitochondrial evolution and evidence for paternal mtDNA inheritance**

3

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27

28 **Abstract**

29 Mitochondrial genomes represent a valuable source of data for evolutionary research, but  
30 studies of their short-term evolution have typically been limited to invertebrates, humans, and  
31 laboratory organisms. Here we present a detailed study of 12 mitochondrial genomes that  
32 span a total of 385 transmissions in a well-documented 50-generation pedigree in which two  
33 lineages of chickens were selected for low and high juvenile body weight. These data allowed  
34 us to test the hypothesis of time-dependent evolutionary rates and the assumption of strict  
35 maternal mitochondrial transmission, and to investigate the role of mitochondrial mutations in  
36 determining phenotype. The identification of a nonsynonymous mutation in *ND4L* and a  
37 synonymous mutation in *CYTB*, both novel mutations in *Gallus*, allowed us to estimate a  
38 molecular rate of  $3.13 \times 10^{-7}$  mutations/site/year (95% confidence interval  $3.75 \times 10^{-8}$  –  
39  $1.12 \times 10^{-6}$ ). This is substantially higher than avian rate estimates based upon fossil calibrations.  
40 Ascertaining which of the two novel mutations were present in an additional 49 individuals  
41 also revealed an instance of paternal inheritance of mtDNA. Lastly, an association analysis  
42 demonstrated that neither of the point mutations was strongly associated with the phenotypic  
43 differences between the two selection lines. Together, these observations reveal the highly  
44 dynamic nature of mitochondrial evolution over short time periods.

45

46 **Keywords:** mitochondrial genome, pedigree, mutation rates, paternal leakage, association  
47 analysis

48 **1. Introduction**

49 Mitochondrial genomes have been widely used in biological research, especially when  
50 studying evolutionary and demographic processes that occur over short timeframes [1]. In  
51 vertebrates, mitochondrial evolution is characterized strictly by maternal inheritance and lack  
52 of recombination. Although various studies have suggested a constant rate of mitochondrial  
53 evolution among lineages and through time [2], there is now considerable evidence of a  
54 disparity between short- and long-term estimates of mitochondrial substitution rates [3-5].  
55 Among the possible explanations for this pattern is that mitochondrial DNA (mtDNA)  
56 evolves non-neutrally, such that purifying selection removes negative mutations over time [6].  
57 This naturally produces a pattern in which transient, deleterious mutations cause a short-term  
58 elevation in rates.

59 There have been few studies of short-term mitochondrial evolution, including both  
60 mutation rates and paternal leakage, particularly in non-human vertebrates [7, 8]. Estimates of  
61 mitogenomic mutation rates have been obtained in studies of Adélie penguins [6, 9] and  
62 humans [10] and these rates greatly exceed those inferred from longer phylogenetic  
63 timescales. Evidence for paternal inheritance of mtDNA (and other 'rare' evolutionary  
64 phenomena) is accumulating in multiple species, including humans [11] and sheep [12], but it  
65 is usually only visible in laboratory or controlled conditions [13-15]. As a result, its frequency  
66 may be underappreciated. This is compounded by the assumption that in natural populations,  
67 without direct knowledge of genetic relatedness and transmission, all mtDNA is maternally  
68 inherited. Combined with the low power associated with standard detection methodologies, it  
69 has been difficult to assess rates of paternal leakage in natural populations [13].

70 Domesticated animals present ideal systems for studying mitochondrial evolution in  
71 vertebrates, particularly if they have documented pedigrees. One such pedigree has been  
72 recorded for the Virginia chicken lines, an experimental White Plymouth Rock population

73 spanning >50 generations. This pedigree, initiated in a founder population of seven partially  
74 inbred lines, was subjected to annual divergent selection for high and low body-weights at 56  
75 days of age. This approach established high (HWS) and low (LWS) weight selected lines that  
76 now possess a greater than tenfold difference in body weight at selection age [16-18].

77 Here, we utilized this well-documented chicken pedigree to perform a detailed  
78 investigation of short-term mitochondrial evolution in a vertebrate system. More specifically,  
79 we estimated the mitochondrial mutation rate, tested for instances of non-maternal inheritance,  
80 and examined the degree to which mitochondrial mutations were responsible for the divergent  
81 phenotypes of the two selected lines.

82

## 83 **2. Material and methods**

84 We identified and sequenced the mitogenomes of the 12 most distantly related individuals on  
85 the maternal pedigree, representing 385 mitochondrial transmissions. This sampling scheme  
86 provided an efficient means of capturing a large number of mitochondrial transmissions with  
87 a limited sample of mitogenomes (figure 1a). We used multiple overlapping PCR and Sanger  
88 sequencing primer pairs (supplementary material, table S2) and aligned the resulting genomes  
89 using CodonCode [19].

90 The single nucleotide polymorphisms (SNPs) detected in the *ND4L* and *CYTB* genes  
91 were genotyped using DNA isolated from blood (supplementary material). In order to  
92 establish potential heteroplasmy, we carried out pyrosequencing of the 12 original individuals  
93 and of an additional 66 chickens from generation S41, the most recent generation of the  
94 pedigree, and the F<sub>8</sub> generation of a deep inter-crossed population of the two selection lines  
95 (figure 1a; supplementary material, table S4). The base for the inter-cross line was reciprocal  
96 parent line and F<sub>1</sub> crosses (supplementary material). An association analysis was carried out  
97 using birds from the F<sub>8</sub> generation to explore the possible link between these mitochondrial

98 mutations in the LWS and the marked phenotypic differences between HWS and LWS  
99 chickens.

100 The rate of evolution was calculated by taking into account the number of observed  
101 mutations in the ~16,000 bp mitochondrial genome over 47 years and 385 transmissions.  
102 Uncertainty in the estimate was calculated using the binomial confidence interval.

103

### 104 **3. Results and Discussion**

105 The reconstruction of the maternal pedigree based on the mitogenome sequences allowed us  
106 to identify two separate point mutations and an instance of paternal leakage, all of which  
107 occurred in the LWS line (figure 1b). The first mutation, a non-synonymous G-A transition in  
108 *ND4L*, occurred between generations S15 and S29 on branch 1. The most likely explanation  
109 for the presence of this mutation in LWS branch 2 (figure 1b) is an instance of paternal  
110 leakage that took place in generation S39 (supplementary material). A second mutation, a  
111 synonymous A-G transition in the *CYTB* gene, occurred between generations S30 and S40 in  
112 an individual that already possessed the *ND4L* mutation. We found evidence for mtDNA  
113 heteroplasmy with subsequent fixation in these lines (figure 1b, supplementary material), a  
114 common observation in maternal lineages after a new mtDNA mutation has occurred [20].

115 The presence of these two novel mutations allowed us to estimate a mutation rate of  
116  $3.13 \times 10^{-7}$  mutations/site/year (95% confidence interval  $3.75 \times 10^{-8} - 1.12 \times 10^{-6}$ ). Our estimate  
117 is consistent with an expectation of a faster rate estimate over shorter timescales as  
118 demonstrated by the trendline resulting from a correlation between previously published avian  
119 rate estimates and the timescale over which they were estimated (figure 2). We observe this  
120 strong relationship despite evidence of substantial rate heterogeneity in birds, with  
121 synonymous substitution rates in mitochondria varying among taxa by more than a factor of  
122 30 [21]. Our pedigree-based estimate of the mutation rate is consistent with consistent with

123 the short-term elevation of rate estimates caused by the presence of transient mutations, a  
124 phenomenon that has been observed in pedigree studies of humans and other mammals [22].  
125 Combined with previous evidence of a time-dependent pattern in rate estimates [5], this has  
126 important consequences for estimating the timescales of recent evolutionary events using  
127 molecular clocks [4].

128 Mapping the mutations onto the pedigree not only allowed us to establish when the  
129 mutations occurred, but also to identify a clear instance of paternal leakage in the LWS line  
130 (figure 1b). A subsequent investigation of the combined maternal and paternal records  
131 allowed us to identify the specific individuals in which the paternal leakage occurred  
132 (supplementary material). This phenomenon is likely to be generally underappreciated given  
133 the difficulty in confidently recognizing the phenomenon in wild populations and the lack of  
134 sensitivity in detection methods. Our observation of an instance of paternal leakage in this  
135 pedigree suggests that this phenomenon might not be as rare as is commonly assumed.

136 The non-synonymous mutation at a first codon position in *ND4L* has, to our  
137 knowledge, not been previously reported in chickens but another galliform, *Polyplectron*  
138 *germaini*, possesses the same nucleotide and amino acid (supplementary material, figure S1).  
139 The second mutation (a synonymous change in *CYTB*) has been previously identified in other  
140 vertebrates (figure S2).

141 Because the observed mutations occurred solely in the LWS line, they may have been  
142 partially responsible for the divergent phenotypes of the two selected lines. To investigate this,  
143 an association analysis was carried out to assess whether the two mitochondrial mutations had  
144 a major effect on body weight at hatch, and at 2, 4, 6, 8, and 10 weeks of age that  
145 differentiated the two lines. A previous QTL analysis of the F<sub>2</sub> generation suggested that  
146 phenotypic differences between reciprocal matings may have been caused by genetic  
147 variation in mtDNA [23]. Here, however, we found no significant effect between the presence

148 of these mutations and growth traits in the F<sub>8</sub> generation for either *CYTB* or *ND4L*  
149 (supplementary material, table S5). As a result, these data suggest that neither of these  
150 mutations is significantly correlated with the extreme difference in early growth between the  
151 two lines.

152 Overall, our analysis of a long-term chicken pedigree has revealed the complex nature  
153 and dynamism of mitochondrial evolution when observed over evolutionarily short time  
154 periods. The observations of a rapid rate of evolution and an incidence of paternal leakage  
155 have several ramifications. Firstly, molecular clock analyses often uncritically import  
156 evolutionary rates calculated using fossil calibrations. Our study provides further evidence  
157 that short-term rates can be much higher and that a failure to take this into account will lead to  
158 overestimation of the timeframe of recent evolutionary events. In addition, understanding the  
159 frequency of paternal inheritance of mtDNA is key to determining how and why different taxa  
160 maintain uniparental inheritance of mitochondria. Lastly, our study provides a demonstration  
161 of the evolutionary insights that can be gleaned from detailed studies of well-documented  
162 animal pedigrees.

163  
164 **Data Accessibility.** The 12 mitochondrial genomes sequenced as part of this project are  
165 available on GenBank, accessions KT626847-KT626858.

166  
167 **Competing interests.** We have no competing interests.

168  
169 **Authors' contributions.** MA carried out lab work, data analysis, sequence alignment, and  
170 drafted the manuscript. GL conceived of, designed, and coordinated the study, carried out lab  
171 work, and drafted the manuscript. SYWH carried out the statistical analyses and drafted the  
172 manuscript; BD carried out lab work (pyrosequencing); MM collected and analysed data; and

173 LA and ÖC carried out the association analysis. PS designed and, with CH, conducted the  
174 long-term selection experiment. All authors contributed to the manuscript and gave final  
175 approval.

176

177 **Acknowledgements.** We would like to thank Gabi Márquez Betz for assistance with  
178 additional information regarding the chicken pedigree.

179

180 **Funding.** GL was supported by the European Molecular Biology Organisation, the European  
181 Research Council (ERC-2013-StG 337574-UNDEAD) and the Arts and Humanities Research  
182 Council (AH/L006979/1).

183

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- 245

246 **FIGURE LEGENDS**

247

248 **Figure 1.** Chicken pedigree from which mitochondrial genomes were sequenced. (a)  
249 Overview of the maternal lineages of the chicken pedigree, comprising high weight selected  
250 (HWS) and low weight selected (LWS) lines. Pink circles indicate individuals from which we  
251 sequenced complete mitochondrial genomes and grey circles represent those that were typed  
252 for the mutations in *CYTB* and *ND4L*. Black circles indicate individuals that were either not

253 sampled or not successfully sampled. Codes on the left hand side refer to generations before  
254 (P) and after (S) the selection experiment began, and following the initiation of the intercross  
255 experiments (F). (b) Subset of the pedigree from S13 to F<sub>8</sub> and additional detail of the LWS  
256 line. Blue and yellow shading indicates the timing and lineage on which the *ND4L* and *CYTB*  
257 mutations occurred on the pedigree. Genotyped individuals that possessed the *ND4L* mutation  
258 are shown in blue and those that were heteroplasmic for *ND4L* are shown in white and blue.  
259 Those that possessed both mutations but were heteroplasmic for the *CYTB* mutation are  
260 shown in green and blue, the individual that was homoplasmic for both mutations is shown in  
261 green. Those that were tested but possessed neither mutation are shown in white. The blue  
262 arrow represents the instance of paternal leakage. It starts on the lineage from which the male  
263 involved in the paternal leakage was derived, and points to the female whose offspring  
264 inherited the male's mitochondrial genome. Further details are in the supplementary material.  
265

266 **Figure 2** Relationship between published estimates of substitution rates and calibration age  
267 from 65 bird datasets (empty circles) using only coding mtDNA (data from [5]). The filled  
268 circle represents the pedigree estimate from this study and was not used to derive the  
269 regression line. Our calculation may be an underestimate given the potential for back  
270 mutations between the founding line and the sampled birds in generation S41, though this is  
271 unlikely. The dashed line is a regression trendline estimated solely from the 65 published rate  
272 estimates. Grey shading represents the 95% confidence interval of the trendline.



