

A genetic polymorphism in the sex-linked ATP5A1 gene is associated with individual fitness in Ovenbirds (*Seiurus aurocapilla*)

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Introduction

Polymorphisms in sex-linked genes have been reported in a wide range of species (Dawson et al. 2001; Jarvi and Farias 2006; Bantock et al. 2008), but were typically thought to represent only neutral genetic variation. Recently, however, polymorphisms in the CHD1 gene have been linked with fitness in Common Moorhens (*Gallinula chloropus*; Lee et al. 2002) and Black-tailed Godwits (*Limosa l. limosa*; Schroeder et al. 2010). This raises the possibility that polymorphisms in sex-linked genes may be under selective pressure, and might thus vary across the range of a species.

Abstract

While testing genetic sexing techniques in Ovenbirds (*Seiurus aurocapilla*), we found a genetic polymorphism in the ATP5A1 gene in 38% of individuals. The Z' allele included changes in both intronic and exonic portions of the sequenced region, but there was no evidence that this changed the resulting ATP synthase product. Males that had one or more copies of this allele had higher relative body mass (mass corrected for size) than other genotypes. This allele was unrelated to stable isotope signatures, and so was not a useful predictor of latitude within the eastern portion of the Ovenbird breeding range. Future studies are needed to determine whether this polymorphism may be a useful geographic marker. This study is the first to link polymorphisms in the sex-linked ATP5A1 gene with fitness effects.

One such application of phylogeographic variability is in understanding migratory connectivity, the range-wide links between breeding and nonbreeding grounds of migrant species. Migratory connectivity has been difficult to establish for small birds because band return rates are very low (less than 1%; Boulet and Norris 2006) and many birds are too small to effectively use transmitters to determine direct flight paths. A variety of techniques have been used to provide information on migratory connectivity: radio and satellite transmitters, geolocators, bands, stable isotopes, trace elements, and genetic variation in individuals or their associated viruses and parasites (Boulet and Norris 2006; Stutchbury



Figure 1. The Ovenbird breeds throughout much of eastern North America and winters in the Caribbean and Central America (photo taken in the Guánica Dry Forest by Mike Morel).

et al. 2009). However, it is important to continue developing new markers because in many species a clear picture of connectivity emerges only when multiple techniques are used (Boulet et al. 2006; Norris et al. 2006).

Although the Ovenbird (*Seiurus aurocapilla*; Fig. 1) is a relatively well-studied species in many respects, little is known about migratory patterns of different regional populations. Some east–west geographic structure may be present in Ovenbirds, as seen in some other species with broad distributions (e.g. Kimura et al. 2002; Milá et al. 2002; Boulet et al. 2006). Band returns suggest that Ovenbirds breeding east of the Appalachian Mountains winter in the West Indies, while individuals breeding west of the Appalachians winter in Middle America (Porneluzi et al. 2011). Moreover, the three recognized subspecies also follow an east–west gradient (Porneluzi et al. 2011). If the Appalachians do act as a geographic barrier, as suggested by band returns, then we would expect the restricted gene flow might result in genetic differences.

While testing two genetic sexing methods in this species (Griffiths et al. 1998; Bantock et al. 2008), we found a genetic polymorphism associated with the ATP5A1 gene that codes for a subunit of mitochondrial ATP synthase. Here, we describe this polymorphism and determine whether it is associated with differences in individual fitness, as found for polymorphisms in another sex-linked gene, CHD1 (Lee et al. 2002; Schroeder et al. 2010). In addition, we determine whether it is associated with variation in stable isotope ratios, one measure of geographic location, to assess its potential for use as a phylogeographic marker.

Methods

Ovenbirds ($n = 113$; 68 female, 45 males) were captured in mist nets, banded, aged, and measured during 2003–2010

in the Guánica Dry Forest, southwest Puerto Rico (Faaborg et al. 2004; Toms 2011). A single central tail feather was collected in the field and stored at room temperature in paper envelopes. We examined two measures of body condition as indices of fitness: pectoral muscle volume scored from 0 to 3 (on a half-point scale) following Latta (2003), and mass corrected for body size. In order to adjust mass for body size, we first used principal components analysis to derive a single combined metric from tarsus length and unflattened wing chord. We then used reduced major axis regression to model mass as a function of the first principal component (Green 2001), and used the residuals as an index of relative body condition. If complete measurement data were available for more than one sampling period, we used only the first complete record because measurements are highly repeatable (Arendt and Faaborg 1989). In addition, hydrogen–deuterium stable isotope ratios were available for a subset of the samples (28 females and 17 males; J. Faaborg, unpubl. data).

We also included nine Ovenbirds of known sex collected or captured during the breeding season and fall migration. Five individuals from tower kills in central Missouri were sexed via dissection (three males and two females) and then prepared for the University of Missouri Bird and Mammal Collection (catalogue numbers 2391 and 3295–3298); four males from southern Missouri were captured using mist nets, sexed in the field using cloacal protuberances, and released (Missouri Ozarks Forest Ecosystem Project). We collected 3–4 body feathers from each of these individuals.

DNA was extracted in the lab using InstaGene (BioRad; Eggert et al. 2005), using sterile equipment for each individual feather. We amplified the ATP5A1 gene region using the F2/R1 primer set of Bantock et al. (2008). In all cases, 15 μ L polymerase chain reaction (PCR) reactions were run in a Mastercycle ep gradient S thermocycler (Eppendorf), and consisted of 1X AmpliTaq Gold DNA polymerase buffer (Applied Biosystems, Inc.), 0.2 mM each dNTP, 0.5 μ M forward and reverse primers, 2.5 mM $MgCl_2$, 0.5 mg BSA, 0.5 u AmpliTaq Gold DNA polymerase (Applied Biosystems, Inc.), and 8.0 μ L of genomic DNA extract. After a 10-min preincubation at 95°C, we ran 45 cycles of 95°C denaturing for 1 min, annealing at 52°C for 1 min, and primer extension at 72°C for 1 min. We finished the PCR with a final extension cycle of 10 min at 72°C. Since some band size differences were small, we used 4% agarose gels in TBE buffer to separate PCR products and determine sex.

Sequences of all bands from selected polymorphic individuals (both males and females) were obtained by cloning PCR products using the TOPO[®] TA Cloning[®] Kit for Sequencing (Invitrogen) and high-speed plasmid mini kits (IBI). In addition, we obtained sequences for all bands from a polymorphic male and female by directly excising bands from agarose gels and purifying the DNA using agarase digestion followed by ammonium acetate–cold ethanol precipitation. All purified

products were sequenced using an ABI 3730 DNA Analyzer at the University of Missouri DNA Core facility. Gene sequences were aligned and compared using Sequencher (Gene Codes). Consensus gene sequences are lodged in GenBank (accession numbers JF297598–JF297607).

We tested for heteroduplex molecules using cloned PCR products from a male following Casey *et al.* (2009), except using 10 μ L each of cloned products. We mixed 10 μ L of the Z and Z' cloned alleles into replicate PCR tubes, and then subjected one tube to 10 PCR temperature cycles while leaving the other control tube at room temperature. The resulting products were visualized on a 4% agarose gel.

Statistical analyses were conducted in R (v. 2.9.1; R Development Core Team 2009). Confidence intervals for the expected proportion of Z' among all ATP5A1-Z alleles and the proportion of individuals with one or more Z' alleles within the population were obtained by inverting a standard score test for proportions (the prop. test function). We calculated the expected frequency of all genotypes (WZ, WZ', ZZ, ZZ', and Z'Z'), accounting for both the proportion of Z' alleles and the observed sex ratio, and then used a Chi-squared test to determine whether the ratio of genotypes within the population was consistent with Hardy–Weinberg equilibrium. For the individuals from Guánica only, we used Kruskal–Wallis rank sum tests to determine whether stable isotope values differed among genotypes, and to determine whether body condition differed among genotypes.

We recaptured 26 individuals at least once during the study period, allowing us to compare whether survival differed among genotypes using capture–recapture analyses with R-Mark (Laake and Rexstad 2008). We excluded the three Z'Z' males due to the small sample size. Few individuals genotyped were captured before 2003 (Table 1). Mark-recapture models of a much larger sample of Ovenbirds from Guánica indicate

Table 1. Sample sizes of genotyped individuals available for mark-recapture survival analysis.

Year	Total number of captures	Number of recaptures	%Recaptures
1995	1	0	0
1996	1	0	0
2000	1	0	0
2001	2	0	0
2002	3	1	33
2003	15	6	40
2004	2	1	50
2005	9	2	22
2006	18	6	33
2007	10	1	10
2008	6	1	17
2009	17	5	29
2010	17	4	24
2011	6	5	83

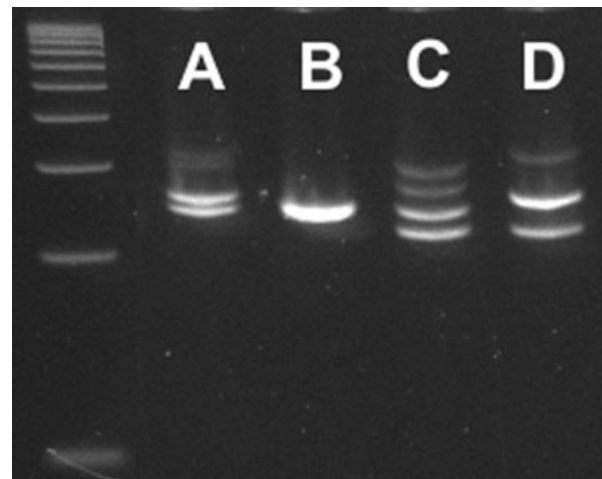


Figure 2. When using the F2/R1 primers to amplify the ATP5A1 genes, some individuals produce three or four bands instead of the expected one or two bands. (A) 100 bp DNA ladder; (B) normal female ZW pattern with PCR products of 242 bp and 257 bp and faint heteroduplex molecules; (C) normal male ZZ pattern with PCR products of 242 bp; (D) polymorphic male with PCR products of 219 bp and 242 bp as well as heteroduplex molecules; (E) polymorphic female with PCR products of 219 bp and 257 bp as well as a heteroduplex molecule. All birds with one or four bands were identified as male and all birds with two or three bands were identified as female using the P2/P8 primers of Griffiths *et al.* (1998).

that survival has remained constant from 1989 through the present (Faaborg *et al.* unpubl. data). Therefore, we determined whether models including genotype were supported over models holding survival constant; capture rates were modelled with a linear trend. Models were compared using AICc.

Results

Polymorphic Z-associated bands were found in 38% of individuals (95% CI 29.2–47.0%). In these individuals, females produced three bands and males typically produced four (Fig. 2); in addition, three males had only a single band corresponding to the smallest of the polymorphic bands. By cloning, we were able to obtain a sequence for the smallest of these bands (219 bp Z'), which is a true polymorphism of the ATP5A1-Z gene (242 bp). The Z' allele comprised 29% (95% CI 23.3–37.4%) of all Z alleles in our test population. The Z' allele resulted from a 23 bp deletion and nine single base pair substitutions within the intron, and a single synonymous transition in the exons (Fig. 3). We were unable to obtain sequences for the three larger additional bands by cloning (the two larger bands seen in polymorphic males and the single larger band seen in polymorphic females), and direct sequencing of DNA obtained from excising these larger bands indicated they had sequences identical to the anomalous 219 bp Z' and the normal 242 bp Z and

1		1		70
2	OV58W	GCCAATTGGTTCGTGGCCAGCGTGAACCTGATAAATGGTGACAGGCAGACCGGGTAAGTTAAATGCCCAAGC		
3	OV64W	GCCAATTGGTTCGTGGCCAGCGTGAACCTGATAAATGGTGACAGGCAGACCGGGTAAGTTAAATGCCCAAGC		
4	OV42Z-241	GCCCATCGGCCGTGGCCAGCGTGAGCTGATCATTGGTGACAGACAGACCGGGTAAGTTAAGTGCTCAAAC		
5	OV64Z'-219	GCCCATCGGCCGTGGCCAGCGTGAGCTGATCATTGGTGACAGACAGACTGGGTAAGTTAGTGCTCAAAC		
6		* * * * * * * # * * *		
7		P I G R G Q R E L I I G D R Q T G		
8				
9		71		140
10	OV58W	CAAAAAGTTTCCTTTAAGAGAGACTTGAGTGCAGTGTGCACCAATGTACAGAACTGAATACAAAATTGT		
11	OV64W	CAAAAAGTTTCCTTTAAGAGAGACTTGAGTGCAGTGTGCACCAATGTACAGAACTGAATACAAAATTGT		
12	OV42Z-241	C:TAAAATGTTTCCTTAAACAGAGACTGAGGTGCAGTG:::GAACTGGACATAGGATTGA		
13	OV64Z'-219	C:TAAAATGTTTCCTTGAACAGAGACTGAGGTGCAGTG:::GAACTGGACATAGGATTGA		
14		** ** # * *** * * * *		
15				
16				
17				
18				
19		141		210
20	OV58W	GTAGAGGTTGGGAGACTGCAGGCAGCTTAACCTACCAACCTCTGTGTTACCCAGGAAAACCTCAATTGC		
21	OV64W	GTAGAGGTTGGGAGAACTGCAGGCAGCTTAACCTACCAACCTCTGTGTTACCCAGGAAAACCTCAATTGC		
22	OV42Z-241	GTAAGGGCTGGGAGAACCTTCGGCAGCTTAACCTACCAACCTCTGTGTTCCACAGGAAAACCTCAATTGC		
23	OV64Z'-219	:::GAGCTGGGAGAACCTTCGGCAGCTTAGCTTACCAGCCTCTGTGTTCCACAGGAAAACCTCAATTGC		
24		# * ***# # # # * # * *		
25				K T S I A
26				
27		211 218		
28	OV58W	AATTGACA		
29	OV64W	AATTGACA		
30	OV42Z-241	AATTGACA		
31	OV64Z'-219	AATTGACA		
32				
33		I D		
34				
35				

Figure 3. ATP5A1 sequences for Ovenbirds (OV). Positions that differ consistently between the W and Z chromosomes are indicated by an asterisk (*); other differences are indicated by a hash mark (#). Amino acids are shown for exons (positions 1–52, 197–218).

257 bp W sequences. Tests confirmed that the larger bands were heteroduplex molecules; cloned products subjected to PCR temperature cycles produced identical banding patterns to those previously seen in polymorphic males (band pattern D in Fig. 2), while cloned products left at room temperature showed only a double-banded 219 bp Z'/242 bp Z pattern. The 219 bp polymorphic Z' allele appears to readily form heteroduplex molecules with both the 242 bp Z and 257 bp W alleles. Fainter extra bands were sometimes observed in normal females (band pattern A in Fig. 2), indicating that the 242 bp Z and 257 bp W alleles can also form heteroduplex molecules under some circumstances.

There was no evidence that genotypes containing the 219 bp Z' allele deviated from the frequencies expected under Hardy–Weinberg equilibrium ($WZ = 44$, $WZ' = 26$, $ZZ = 29$, $ZZ' = 20$, $Z'Z' = 3$; $\chi^2_4 = 2.951$, $P = 0.566$). Stable isotope ratios of individuals captured at Guánica did not differ among genotypes ($\chi^2_3 = 1.33$, $P = 0.721$). Three of the tower-killed individuals (two females and one male) had the Z' allele. However, it was not seen in the four ZZ males breeding at the Missouri Ozarks Forest Ecosystem Project site; in a population with 29% (95% CI 23.3–37.4%) Z' alleles, there is only a 0.058 (0.024–0.120) chance of this observation, so the

allele may not be present in that breeding population at the rates observed in this study. All males homozygous for the Z' allele were captured at Guánica.

For the subset of individuals captured at Guánica, pectoral muscle volume did not differ significantly among genotypes ($\chi^2_4 = 7.93$, $P = 0.094$). However, mass corrected for size did differ ($\chi^2_4 = 11.23$, $P = 0.024$), with males carrying one or more Z' alleles being heavier than other genotypes (Fig. 4). Constant survival rates were best supported by the data (74% model support). However, models allowing survival rate to vary across genotype estimated that heterozygous males had higher survival than other genotypes (Table 2).

Discussion

Polymorphisms have been found in a wide range of birds with the more commonly used CHD1 sex-linked gene (Dawson et al. 2001; Jarvi and Farias 2006; also in one of eight Red-eyed Vireo *Vireo olivaceus* tested by JDT and LSE), but polymorphisms in the ATP5A1 gene have previously been reported only in Corn Buntings (*Emberiza miliaria*; Bantock et al. 2008). Male chromosomes have been found to evolve faster (Ellegren and Fridolfsson 1997; Carmichael et al. 2000),

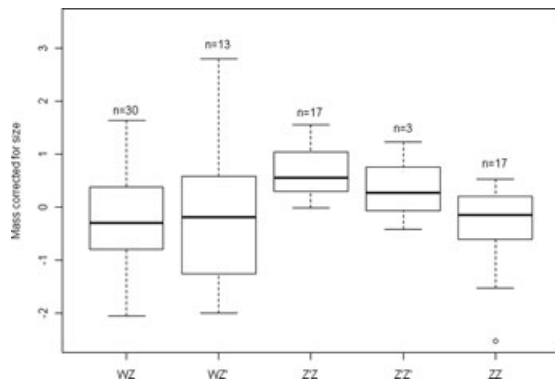


Figure 4. Mass corrected for size (unflattened wing chord and tarsus) was higher in heterozygous males than in other genotypes. Body condition was assessed only in individuals captured at Guánica.

Table 2. Estimated survival rates for each genotype in the second best supported mark-recapture model (26% model support). This model allowed survival to differ among genotypes, while capture rate declined nonsignificantly over time.

Genotype	<i>n</i>	Survival rate	95% Confidence interval
WZ	30	0.632	(0.385–0.825)
WZ'	13	0.772	(0.417–0.941)
ZZ	17	0.785	(0.519–0.925)
ZZ'	17	0.917	(0.463–0.993)

and most polymorphisms have been found on the male versions of sex-linked genes, as we found here.

The interpretation of the band-size polymorphism we found in Ovenbirds was more complicated than expected because the Z' allele readily formed heteroduplex molecules with the normal ATP5A1-W and -Z alleles. Heteroduplex molecules have now been found with both the CHD-1 and ATP5A1 loci (Casey *et al.* 2009, this study), and further investigation may reveal their presence in other species.

We found that male Ovenbirds with the Z' allele had higher mass relative to body size than all other individuals. In the closely related American Redstart (*Setophaga ruticilla*), body condition is closely associated with individual fitness: body condition closely predicts annual survival (Johnson *et al.* 2006), and individuals wintering in lower quality habitat have reduced reproductive success (Reudink *et al.* 2009; Smith *et al.* 2009). Thus, the higher relative body mass of male Ovenbirds carrying this allele may result in higher individual fitness. Although survival rates did not differ significantly among genotypes, the statistical power was likely low due to our small sample size. However, heterozygous individuals had somewhat higher survival, suggesting these differences in body condition may have important consequences at a population level.

In the Common Moorhen, male chicks homozygous for a CHD1-Z' allele had very low survival (Lee *et al.* 2002). In

contrast, in the Black-tailed Godwit, a similar CHD1-Z' allele did not affect survival, but did increase fitness of females with the allele (Schroeder *et al.* 2010). In both studies, the authors speculated that the fitness effects of the polymorphism were the result of a genetic linkage between the CHD1-Z gene and the causative allele, because the polymorphism was located within a noncoding portion of the CHD1 gene. In this study, the differences between the ATP5A1-Z and Z' alleles were largely confined to an intron, and the single base-pair transition within the exon did not result in an amino acid coding change. Therefore, the effects on fitness correlates (body condition) found in this study are also most likely to result from genetic linkage with a different causative gene. Alternatively, it could be due to linkage with another part of the ATP5A1 gene, which codes for part of ATP synthase. ATP synthase is a likely candidate gene for fitness effects: mutations in ATP synthase can result in a variety of negative processes including oxidative stress, reductions in body mass and survival, and a suite of diseases (Johannsen and Ravussin 2009); birds may be particularly susceptible to these effects due to their high metabolic requirements.

Wintering populations of Ovenbirds at Guánica are comprised of a mixture of individuals from a large portion of the eastern U.S. breeding populations (Dugger *et al.* 2004), but we found the presence of the Z' allele to be unrelated to stable isotope signatures. In eastern North America, these isotope signatures differ only along latitudinal bands, suggesting that the Z' allele is not confined to a small latitudinal region. However, none of the individuals breeding in southern Missouri were found to carry the Z' allele, which would be unexpected if it was present in this region at the observed frequency. Further studies on the breeding grounds are needed to determine how useful this allele may be for studies of migratory connectivity (Kimura *et al.* 2002; Webster *et al.* 2002).

This study is the third to link polymorphisms in sex-linked genes with fitness effects, but the first to demonstrate that this can occur with genes linked to the ATP5A1 gene, rather than the CHD1 gene (as in Lee *et al.* 2002; Schroeder *et al.* 2010). Although these associations could be spurious, the fact that they have been identified in three disparate avian lineages makes it more likely that at least some of these effects are real. This raises the question as to whether the underlying causative genes are the same across these three studies. At an evolutionary scale, the CHD1 and ATP5A1 genes ceased to diverge at very different time scales (García-Moreno and Mindell 2000; Ellegren and Carmichael 2001; Nam and Ellegren 2008; Suh *et al.* 2011). Indeed, the male (Z) chromosome has undergone major reorganization, suggesting that numerous rearrangements have occurred across avian lineages (Backström *et al.* 2006; Itoh *et al.* 2006). As a result, these different avian lineages are likely to show different placement of the two genes, and their location relative to other potential causative

genes may be useful in determining the underlying causative genes (The International HapMap Consortium 2003). We encourage other researchers to look for similar fitness effects associated with polymorphisms in other avian lineages, to improve the chances of determining the causative genes.

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