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Signatures of natural selection in the mitochondrial genomes of *Tachycineta* swallows and their implications for latitudinal patterns of the 'pace of life'

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ABSTRACT

Latitudinal variation in avian life histories can be summarized as a slow–fast continuum, termed the 'pace of life', that encompasses patterns in life span, reproduction, and rates of development among tropical and temperate species. Much of the variation in avian pace of life is tied to differences in rates of long-term metabolic energy expenditure. Given the vital role of the mitochondrion in metabolic processes, studies of variation in the mitochondrial genome may offer opportunities to establish mechanistic links between genetic variation and latitudinal 'pace of life' patterns. Using comparative genomic analyses, we examined complete mitochondrial genome sequences obtained from nine, broadly distributed *Tachycineta* swallow species to test for signatures of natural selection across the mitogenome within a phylogenetic framework. Our results show that although purifying selection is the dominant selective force acting on the mitochondrial genome in *Tachycineta*, three mitochondrial genes (*ND2*, *ND5*, and *CYTB*) contain regions that exhibit signatures of diversifying selection. Two of these genes (*ND2* and *ND5*) encode interacting subunits of NADH dehydrogenase, and amino residues that were inferred to be targets of positive selection were disproportionately concentrated in these genes. Moreover, the positively selected sites exhibited a phylogenetic pattern that could be indicative of adaptive divergence between "fast" and "slow" lineages. These results suggest that functional variation in cytochrome b and NADH dehydrogenase could mechanistically contribute to latitudinal 'pace of life' patterns in *Tachycineta*.

1. Introduction

Over the past century, biologists have accumulated a multitude of comparative data on latitudinal patterns in avian life histories. One of the strongest generalizations emerging from this large body of work is that tropical birds tend to exhibit long life spans and low reproductive and developmental rates compared to their temperate counterparts (James, 1970; Lack, 1947; Ricklefs, 1969, 1976; Skutch, 1976). More recently, these general patterns have been summarized as a "slow–fast" continuum in the 'pace of life' (POL) hypothesis of latitudinal variation in avian life histories (Ricklefs and Wikelski, 2002; Wiersma et al., 2007b). The mechanistic underpinnings of latitudinal pace of life patterns are poorly understood, but recent comparative studies suggest that much of this variation may be linked to the rate of long-term metabolic energy expenditure (Wiersma et al., 2007a). In general, temperate-zone species tend to have higher basal metabolic rates, and higher aerobic capacities than closely related tropical species (Wiersma et al., 2007a,b), both of which may be necessary to support an increased pace of life in the temperate zone. For example, increased energetic investment may be necessary to support faster developmental rates or greater provisioning rates for larger clutches in temperate zone birds. Increased rates of aerobic metabolism can also increase rates of cellular oxidative damage, which may contribute to the reduced life span of temperate zone species (Monaghan et al., 2008; Vleck et al., 2007). Given the central role of the mitochondrion in cellular metabolic processes, mitochondrial variants that are segregating within and among closely related species may affect both cellular and whole-organism metabolic performance (Ballard and Melvin, 2010; Toews et al., 2013), and therefore, studies of mitochondrial variation may offer opportunities to begin to link genetic variation to latitudinal 'pace of life' patterns.

The vertebrate mitochondrial genome contains 13 protein-coding genes, all of which are central to the process of oxidative phosphorylation and aerobic metabolism. Moreover, in animals, the mitochondrial genome exhibits an elevated substitution rate, smaller effective population

Abbreviations: POL, pace of life.

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sizes, and reduced recombination rates compared with the nuclear genome, all of which facilitate rapid evolution and selective pressures that favor compensatory evolutionary changes in interacting nuclear genes (Burton and Barreto, 2012; Osada and Akashi, 2012; Rand et al., 2004). As a result, the mitochondrial genome represents an important potential target of natural selection in taxa that are distributed across environmental gradients. Indeed, recent empirical studies have documented a number of putative examples of local adaptation in mitochondrial variants of species that are distributed along latitudinal and elevational gradients (Cheviron and Brumfield, 2009; Fontanillas et al., 2005; Hassanin et al., 2009; Ribeiro et al., 2011; Scott et al., 2011; Toews et al., 2013). In birds specifically, mitochondrial variants have been linked to differences in kinetic properties of cytochrome c oxidase (Scott et al., 2011), and the degree of coupling efficiency between substrate oxidation and ADP phosphorylation (Toews et al., 2013), both of which can influence overall cellular aerobic capacity. Similarly, a wide variety of mitochondrial variants have been linked to metabolic disorders and the rate of production of reactive oxygen species in humans (Wallace, 2005).

Lineages with broad latitudinal distributions are well suited for examining the mechanistic underpinnings of latitudinal POL patterns. *Tachycineta* is a monophyletic genus of nine swallow species (Dor et al., 2012) that are continuously distributed from Alaska to Cape Horn (Fig. 1a). This exceptionally broad latitudinal distribution makes it possible to make multiple comparisons of temperate and tropical species (Fig. 1b). Multiple aspects of latitudinal variation in the life histories of *Tachycineta* species conform to POL predictions: species that occur nearer to the tropics tend to have smaller clutches, higher investment per offspring, slower developmental rates, and longer nestling periods (Table S1). Additionally, preliminary evidence suggests that tropical *Tachycineta* species also have lower adult provisioning rates (Ardia et al., unpub. data) and generally higher adult survival rates (Winkler et al., unpub. data).

Here, we utilized a suite of comparative genomic approaches to analyze complete mitochondrial genome sequences from each of the nine *Tachycineta* species. Using a phylogenetic framework, we specifically tested for evidence of adaptive mitochondrial divergence among lineages along a "slow–fast" POL continuum. We summarized interspecific life-history variation as a multivariate POL score to test the prediction that mitochondrial variation is associated with latitudinal patterns of POL variation in *Tachycineta*. First, we predicted that substitutions at codon positions that are under positive diversifying selection would disproportionately occur along branches that separate taxa that exhibit the greatest differences in POL scores (i.e. adaptive divergence at mitochondrial genes should be associated with divergence between sister taxa that differ strongly in their life histories). Second, we predicted that the "slowest", most derived species would also exhibit the largest accumulation of derived, positively selected substitutions. We show that although purifying selection is the dominant selective force acting on the mitochondrial genome in *Tachycineta*, 3 of 13 mitochondrial genes contain regions that exhibit signatures of diversifying selection. These three genes (*ND2*, *ND5* and *CYTB*) encode two enzymes (NADH dehydrogenase and cytochrome b) that are central to cellular metabolic processes.

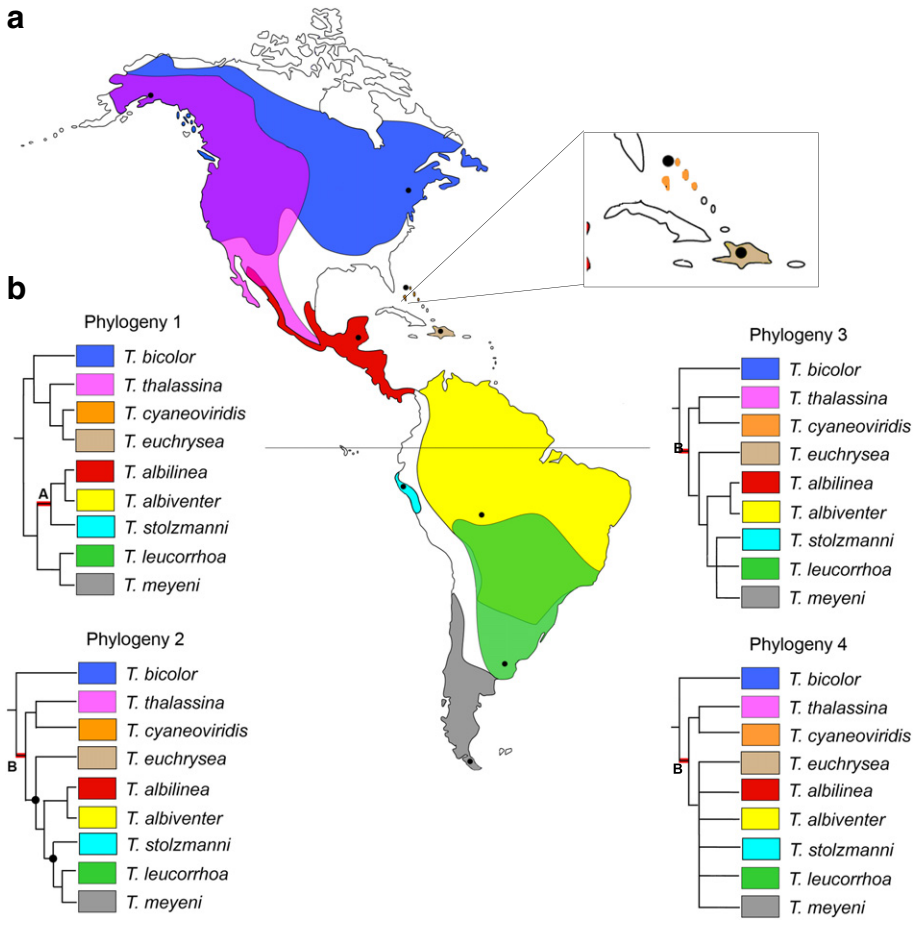

Fig. 1. (a) Color-coded distributions of the nine *Tachycineta* species; colors correspond to those used in phylogenies. Dots indicate approximate localities of specimen origin for each sequence. Gray line indicates the equator. (b) The four phylogenies employed in CODEML analyses are shown: the consensus mitochondrial (Phylogeny 1; Cerasale et al., 2012), consensus nuclear (Phylogeny 2; Dor et al., 2012), and nuclear trees with polytomy at node 17 (Phylogeny 3) and at node 14 (Phylogeny 4). Nodes 14 and 17 indicated by black dots in Phylogeny 2. Branches A and B are highlighted in red. Branch lengths not to scale.

Moreover, the number of positively selected sites was positively correlated with POL score, demonstrating that derived species with slow life histories (high POL scores) are characterized by relatively high levels of adaptive variation at these mitochondrial genes. Taken together, these results suggest that mitochondrial variation could potentially contribute to metabolic differences that are related to latitudinal pace of life patterns in *Tachycineta*.

2. Methods

2.1. Taxon sampling and mitogenomic data

We downloaded complete mitochondrial genome sequences for each of the nine *Tachycineta* species and one outgroup, *Progne chalybea*, from GenBank (Table 1). From these mitogenomic sequences, we extracted protein-coding regions, and constructed gene-specific alignments using Sequencher (ver. 5.0).

2.2. Summarizing life-history variation

To determine where each *Tachycineta* species fell on the POL continuum, we gathered life-history data for traits that were measured in all nine species from the literature (clutch size, egg size, growth rate, nestling period, adult body mass, and absolute latitude; Table S1). Because the POL hypothesis assumes a continuous axis from "slow" to "fast" life histories, we chose to summarize variation among traits using a principal component analysis, rather than classifying each species as either temperate or tropical. We performed a principal component analysis on the z-normalized values for each trait and retained PC components with eigenvalue >1 (Kaiser, 1960), which proved true of PC1 alone. We used the resulting PC1 scores, which we refer to as the POL score, as a continuous variable with which to perform ancestral state reconstruction. To do this, we reconstructed ancestral states using R (ver. 2.15.0—R Development Core Team, 2012) with ancestral character estimation (*ace*) based on restricted maximum likelihood and a model of Brownian motion (Paradis, 2006). We calculated absolute changes in POL score between the terminal branches and their ancestral nodes for use in determining whether substitutions occur disproportionately along branches with greater POL differences. We then used the POL scores to predict which species are the most derived from the temperate, basal lineage (Fig. S1). Species with the highest POL score are considered "slow" and derived, and thus are likely to exhibit the highest rate of positive selection; while species with the lowest POL scores are considered to be "fast" and are likely to exhibit low rates of positive selection. To test this prediction, we used a generalized linear model with Poisson distribution for count data (i.e. number of substitutions) and verified that overdispersion was not present. We chose not to test for phylogenetic signal among these variables as estimates of phylogenetic signal among datasets of such a limited size (e.g. 9 species) lack power and can therefore be inaccurate (Boettinger et al., 2012). We therefore did not employ phylogenetically independent contrasts (PICs), as use of PICs without proper assessment of phylogenetic signal can be inappropriate or misleading (Revell, 2010).

Table 1
Pace of life variation among the nine *Tachycineta* species. Variation in life history traits was summarized using a principal component analysis on the z-normalized values. The resulting PC1 scores (*POL score*) represent the degree of variation among species; positive scores represent "slow" species and negative scores represent "fast". GenBank accession numbers of mitochondrial sequences for each species and one outgroup used in this study are also indicated (these data were originally reported by Cerasale et al., 2012). Life history data are presented in Table S1.

Species	Accession #	POL score
T. bicolor	JQ071614	−2.81
T. leucorrhoa	JQ071621	−1.95
T. meyeni	JQ071622	−0.76
T. thalassina	JQ071615	−0.69
T. cyaneoviridis	JQ071617	0.38
T. albilinea	JQ071619	0.82
T. euchrysea	JQ071616	0.90
T. albiventer	JQ071620	1.21
T. stolzmanni	JQ071618	2.90
Progne chalybea	JQ071623	

2.3. Detecting selection

We examined each of the thirteen protein-coding genes for evidence of negative selection using the single likelihood ancestor counting (SLAC) method performed with the HyPhy software package (Kosakovosky Pond et al., 2005). We estimated non-synonymous to synonymous substitution (dN/dS) ratios conditioned using neighbor-joining trees, and we identified gene-specific nucleotide substitution models using a model selection procedure performed in HyPhy that tests 203 hierarchical time-reversible substitution models. The most appropriate rate matrix was selected using both likelihood ratio tests (LRTs) and AIC selection. We identified sites under purifying selection at a significance level of $P \leq 0.05$. Although SLAC can also be used to detect signatures of positive selection, it does not allow for variation in dN/dS ratios across branches and individual codons. We therefore employed two additional methods with greater sensitivity for detecting positive selection.

First, we implemented branch-site models, which allowed us to test for positive selection at individual codons using a modified branch-site model A test 2 (Zhang et al., 2005) in the program CODEML (PAML 4.6; Yang, 2007). For each of the thirteen genes, we used LRTs to compare the null neutral model (model = 2, NSsites = 2, fix_omega = 1, omega = 1) against alternate models of branch-specific positive selection (model = 2, NSsites = 2, fix_omega = 0, omega = 1.5). Each branch (terminal and internal) served independently as the foreground lineage and we tested sites within the foreground lineage for selection. To control for phylogenetic uncertainty, we conditioned substitution models using four different trees for each of the models. Input trees were modified from the consensus mitochondrial tree (Cerasale et al., 2012), the consensus nuclear tree comprised of 16 nuclear loci (Dor et al., 2012), and two nuclear trees for which we coded polytomies at each of two nodes with posterior probability less than 0.85 (Fig. 1b). The mitochondrial tree differs from the nuclear tree in that it is composed of two distinct clades—a North American/Caribbean clade and a Central/South American clade—whereas the nuclear tree does not have this distinction, and thus some of the sister relationships, such as that of *Tachycineta euchrysea*, vary between the nuclear and mitochondrial phylogenies. Branch lengths were estimated for each gene in CODEML (Yang, 1996). Positive selection was inferred if the *P*-value derived from the LRT between the neutral and branch-specific models was significant (at $P \leq 0.05$) based on the chi-squared asymptotic distribution. Bayes empirical Bayes (BEB; Yang et al., 2005) procedures were performed for branches exhibiting dN/dS > 1 to identify individual sites subject to diversifying selection.

Branch-site models of this kind can be limiting due to the necessary specification of foreground lineages and the assumption that dN/dS = 1 for all background lineages (Kosakovosky Pond et al., 2011). We therefore also examined each of the thirteen genes for signatures of episodic diversifying selection using a mixed effects model of evolution (MEME; Murrell et al., 2012) performed with HyPhy. This method allows the distribution of the estimated dN/dS ratio to vary from site to site and from branch to branch and, in so doing, identifies individual episodes of positive selection that affect only a subset of lineages, which are often overlooked using traditional methods that assume that dN/dS is shared by all sites in the alignment or that selective pressures are constant throughout time (Murrell et al., 2012). We identified positively selected sites at a significance level of $P \leq 0.05$.

2.4. Ancestral reconstruction

Following detection of selection, we identified amino acid substitutions for each of the ten species at all sites exhibiting signatures of positive selection and mapped substitutions to the gene tree (the consensus mitochondrial tree; Cerasale et al., 2012). To do this, we reconstructed ancestral states with ancestral character estimation (*ace*) using R (ver. 2.15.0—R Development Core Team, 2012) with three standard models: equal rates, symmetric, and all rates different (Paradis, 2006). We compared alternative models using LRTs at a significance level of $P \leq 0.05$. We calculated the degree of physicochemical similarity of each substitution using Grantham's difference (D), a measure of amino acid exchangeability based on volume, weight, polarity, and carbon composition (Grantham, 1974). Using D, we then classified amino acid substitutions as conservative (0–50), moderately conservative (50–100), moderately radical (100–150), or radical (>150) according to the criteria proposed by Li et al. (1984). Due to our specific interest in sites that were inferred to be under positive selection and the prohibitively large number of negatively selected sites, we did not repeat this procedure for sites exhibiting signatures of negative selection.

2.5. Mapping selected residues

To provide insight regarding the physical location of sites under selection within the protein, we mapped all sites to topologies of the transmembrane segments of electron transport proteins. We predicted topologies based on the consensus of multiple models (Bernsel et al., 2009; Granseth et al., 2006; Hessa et al., 2007) using the software TOPCONS (http://topcons.cbr.su.se/). Topology of these proteins is largely conserved across eukaryotes (Brandt, 2006; Degli Eposti et al., 1993) and so we anticipated that the overall architecture of the proteins would be conserved at the species level. We therefore randomly chose *Tachycineta leucorrhoa* as the query sequence. We then mapped selected sites to predicted topologies for genes exhibiting signatures of both positive and negative selection.

3. Results

Pace of life (POL) scores, representing life-history variation among *Tachycineta* species, show a continuous distribution from "slow" (high POL values) to "fast" (low POL values) with *Tachycineta stolzmanni* and *Tachycineta bicolor* at each end of the spectrum, respectively (Table 1). As predicted, we found a significant correlation between the POL score and the number of amino acid substitutions for a given taxon (Poisson regression, $P = 0.05$), such that "slow", derived species with high POL scores (*Tachycineta albilinea*, *Tachycineta albiventer*, and *T. stolzmanni*) exhibited the highest degree of positive selection (Fig. 2). However, we found equivocal evidence that positively selected substitutions occur disproportionately along branches separating taxa with the greatest differences in POL score (Fig. S1).

Signatures of negative selection were pervasive across the mitogenomes of *Tachycineta* species. We identified 320 negatively selected sites across all 13 protein-coding genes. The percentage of codons under negative purifying selection within a single mitochondrial gene averaged 7.5% with *COX1* exhibiting the highest (11%) and *ATP8* the lowest (3%) rates of purifying selection (Table 2).

Signatures of positive diversifying selection were much less prevalent across the mitogenome than those of purifying selection and were concentrated in three genes: *ND2*, *ND5* and *CYTB*. Using branch-specific models, we detected signatures of positive selection in 2 genes, both of which are subunits of NADH dehydrogenase (Table S2). Two codons in *ND5* were inferred to be under positive selection within the *T. stolzmanni* lineage, site 16 (92.5% probability) and site 545 (55.2%), and this result was robust across tree topologies (Table 3). *ND5* also exhibited signatures of positive selection within branch B at site 27 (86.8–88.7%), and this result was also robust across the three tree topologies to which branch B is common (Phylogenies 2–4). Additionally, two codons in *ND2* exhibited signatures of positive selection along branch A: sites 222 (99.4%) and 327 (79.5%). This model could only be tested with Phylogeny 1 as this branch is not common to the other three trees.

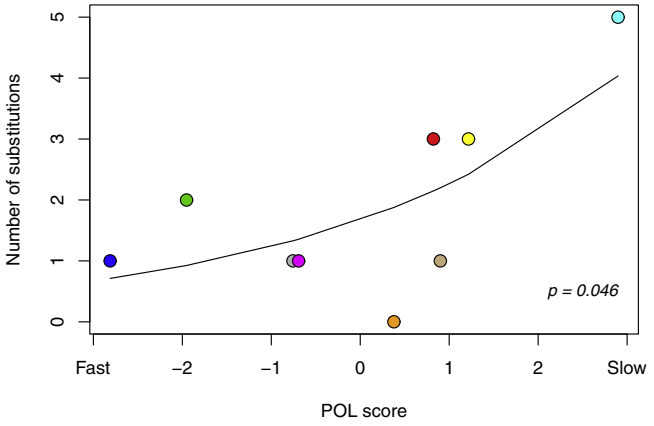

Fig. 2. Number of substitutions exhibited by each species as a function of their respective pace of life (POL) scores. Line fit by Poisson regression, *P*-value shown. Colors correspond to Fig. 1.

Using MEME, we identified episodic positive selection in 2 of 13 genes: *CYTB* (site 161) and *ND5* (sites 27, 67, 190, and 519; Table 2). Thus, 1 of the 10 sites (*ND5* 27) was identified by both methods. Additionally, one site each in *ND2* and *ND4* exhibited signatures of positive selection that were marginally significant (sites *ND2* 222, $P = 0.07$; *ND4* 192, $P = 0.06$).

The equal rates model proved best for all instances of ancestral state reconstruction (Table S3). Positively selected substitutions produce polarity changes in the resulting amino acids for 3 of the 5 sites identified by branch-site models and in 2 additional sites of episodic selection; the remaining 3 substitutions do not result in polarity changes (Fig. 3). The physicochemical dissimilarity of the amino acid change was categorized as conservative for 4 substitutions, moderately conservative for 3 substitutions, and moderately radical for 1 substitution (*ND5* 519, $D = 103$; Fig. 3). When mapped to the phylogeny, the outgroup alone exhibited differences for sites *ND4* 192 and *ND5* 67 (Figs. S3 & S4), and they are therefore not considered for the remainder of this work.

Consistent with other vertebrate studies (e.g., Birrell and Hirst, 2010; Whitehead, 2009), predicted protein topologies contained 8, 11, and 14 transmembrane regions in *CYTB*, *ND2*, and *ND5*, respectively. However, mammalian *ND5* has been shown to contain an additional one to two transmembrane helices (Bridges et al., 2011; da Fonseca et al., 2008; Lemay et al., 2013). All of the sites that exhibited signatures of episodic

Table 2
Proportion of sites undergoing positive episodic (*Pos*) or negative (*Neg*) selection (identified with MEME and SLAC, respectively) for each of thirteen *Tachycineta* mitochondrial genes. Sites identified at $P \leq 0.05$.

Gene	*Pos.*	*Neg.*
ATP6	0	0.044
ATP8	0	0.036
COX1	0	0.112
COX2	0	0.070
COX3	0	0.080
CYTB	0.003	0.097
ND1	0	0.111
ND2	0	0.058
ND3	0	0.034
ND4	0	0.089
ND4L	0	0.071
ND5	0.007	0.091
ND6	0	0.076

Table 3
Genes inferred to be under positive selection using branch-site models with CODEML. Trees and branches correspond to Fig. 1b. Degrees of freedom = 1.

Gene	Branch	Tree	$\ln L_{Neutral}$	$\ln L_{Selection}$	LRT	*P*
ND2	A	1	−3488.26	−3486.06	4.41	0.0357
ND5	B	2	−5791.16	−5787.08	8.15	0.0043
ND5	B	3	−5849.67	−5845.53	8.27	0.0040
ND5	B	4	−6005.78	−6000.92	9.72	0.0018
ND5	*T. stolzmanni*	1	−5767.91	−5770.08	4.33	0.0374
ND5	*T. stolzmanni*	2	−5789.65	−5787.52	4.25	0.0392
ND5	*T. stolzmanni*	3	−5849.29	−5847.01	4.56	0.0327
ND5	*T. stolzmanni*	4	−6004.54	−6003.22	2.64	0.1040

positive selection in the MEME analyses were restricted to the predicted loop regions of their respective proteins (Fig. 4). However, 2 of the sites identified by branch-site models were located wholly within transmembrane regions (*ND2* 327 and *ND5* 16). Sites of negative selection occurred in both loop and transmembrane regions for all three genes that also exhibited positive selection (Fig. 4).

4. Discussion

4.1. Mitochondrial variation and latitudinal patterns in the 'pace of life'

Latitudinal variation in the 'pace of life' (POL) has been well documented in a wide variety of taxa (Ricklefs and Wikelski, 2002; Wiersma et al., 2007b). However, little is known about the mechanistic underpinnings of POL variation within and among species. Several lines of evidence suggest that much of this variation may be functionally linked to differences in rates of metabolic energy expenditure. Given the vital role of the mitochondrion in cellular metabolism, the mitochondrial genome therefore represents a suitable target to begin investigations of the

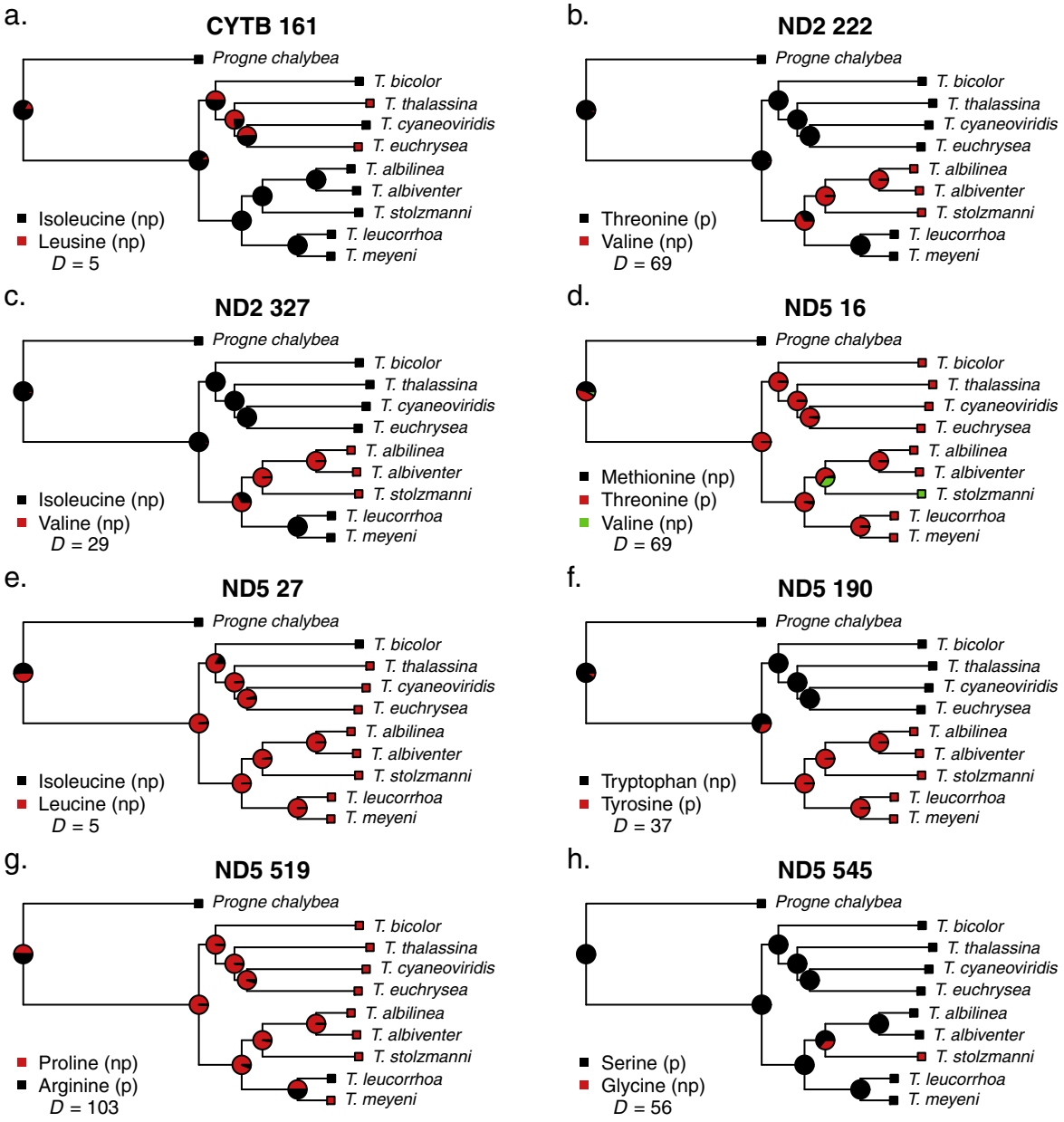

Fig. 3. Ancestral state reconstruction of polarity changes resulting from amino acid substitutions for 8 residues exhibiting positive selection: sites (a) *CYTB* 161; (b) *ND2* 222; (c) *ND2* 327; (d) *ND5* 16; (e) *ND5* 27; (f) *ND5* 190; (g) *ND5* 519; (h) and *ND5* 545. Amino acids are indicated by color and their polarity specified: nonpolar (np) or polar (p). Pie charts represent the proportional likelihood of particular state changes at each of the ancestral nodes. Grantham's difference (*D*) shown for each amino acid pair within *Tachycineta* (Grantham, 1974).

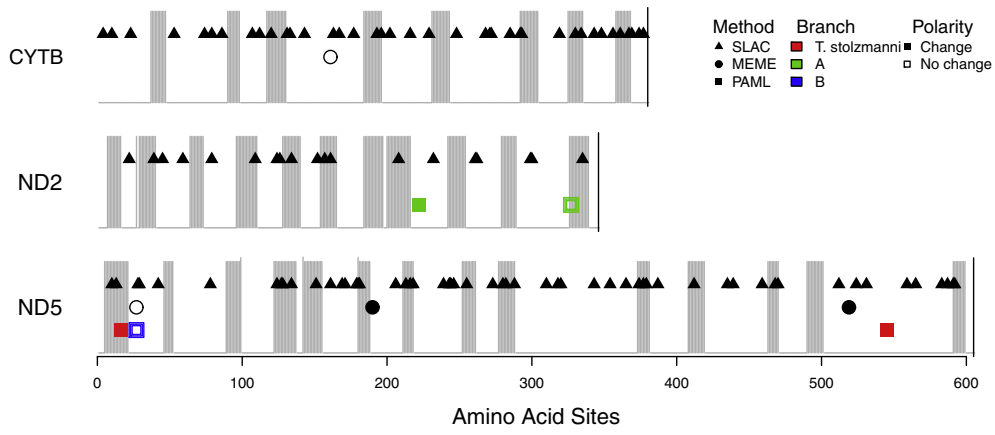

Fig. 4. Predicted topologies of transmembrane domains for genes exhibiting signatures of selection. The x-axis represents amino acid sites for each protein, and transmembrane regions are shown in gray while loop regions are shown in white. Sites under negative selection identified with SLAC are indicated with triangles. Sites that are inferred to be targets of positive selection identified with MEME are indicated with circles, and closed circles represent amino acid substitutions resulting in a change in polarity, whereas open circles represent substitutions that do not change polarity. Sites inferred to be under positive selection in the CODEML analysis are indicated with squares (closed = polarity change; open = no change in polarity). Colors correspond to the different branches on which they were identified: red = *T. stolzmanni*; green = branch A; blue = branch B (branches correspond to Fig. 1).

genetic basis underlying life-history variation. Using a genus of broadly distributed passerines, we identified mitogenomic regions that may contribute to a mechanistic understanding of this macroecological pattern. Although purifying selection is the dominant selective force acting on the mitochondrial genome in *Tachycineta*, we found evidence of diversifying selection in three mitochondrial genes.

The positively selected sites exhibited a phylogenetic pattern that is consistent with adaptive divergence between "fast" temperate and "slow" tropical lineages. Derived substitutions at both positively selected sites in *ND2* were shared by the three equatorial species whose life-history variation is at the "slow" end of the spectrum (*T. albilinea*, *T. albiventer*, and *T. stolzmanni*; Fig. 3). Moreover, substitutions at two positively selected sites within *ND5* were unique to *T. stolzmanni* alone, which is likely to be the "slowest" of the *Tachycineta* species (Table 1), exhibiting both the lowest fecundity and lowest growth rate of the genus (Stager et al., 2012). The remaining sites under positive selection were distributed across the phylogeny resulting in an overall pattern consistent with the POL hypothesis (i.e., "slow" lineages were characterized by more positively selected substitutions than "fast" lineages; Fig. 2).

The high rate of positively selected substitutions within *T. stolzmanni* could alternatively be explained by the narrow distribution and relatively small effective population size of this lineage. However, this pattern does not hold across the genus: the two island species, *T. euchrysea* and *Tachycineta cyaneoviridis*, are characterized by even more restricted range sizes and smaller populations — listed as vulnerable and endangered, respectively (IUCN, 2013) — yet they do not show the same elevation in positively selected sites. In fact, *T. cyaneoviridis* does not exhibit a single positively selected substitution, yielding this an unlikely mechanism for the interspecific patterns we have shown here. Likewise, interspecific divergence levels across the mitochondrial genome are similar among *Tachycineta* species with both small and large distributions (Cerasale et al., 2012). This finding is supported by the work of Bazin et al. (2006) who have demonstrated across ~3000 animal species that mitochondrial DNA diversity cannot be predicted by effective population size, but rather is better explained by adaptive evolution.

The three positively selected genes encode subunits of two separate complexes of the electron transport chain. Seven of the eight positively selected sites occurred within genes (*ND2* and *ND5*) that encode subunits of NADH dehydrogenase, a large, multimeric enzyme comprising respiratory chain complex I. This complex has been previously implicated in the adaptive evolution of the mitogenome in other taxa (41 placental mammals, da Fonseca et al., 2008; salmon, Garvin et al., 2011; monkeys, Yu et al., 2011; wild donkeys, Luo et al., 2012; herring, Teacher et al., 2012; hares, Melo-Ferreira et al., 2014). Located in the arm of L-shaped complex I, subunits *ND2*, *ND4*, and *ND5* collectively serve as a proton pump for H^+ ions across the inner membrane (Brandt, 2006; Radermacher et al., 2006). These three subunits are linked by the arm of *ND5*, allowing for coordinated shifts in proton pumping that in turn drive ATP production (Efremov et al., 2010; Hunte et al., 2010).

Within NADH subunits, sites exhibiting positive selection largely occurred in the predicted loop regions of the protein. Regions lacking sites of positive selection may reflect the presence of conserved functional domains that are subject to purifying selection (Whitehead, 2009), and we do indeed find evidence of purifying selection in many of the regions that are devoid of codons under positive selection (Fig. 4). The low incidence of positively selected sites in these particular regions of complex I is consistent with previous studies of fish (Whitehead, 2009) and mammals (da Fonseca et al., 2008). Although one positively selected site in each of *ND2* (site 327) and *ND5* (site 27) occurred within the transmembrane region, these substitutions are physiochemically conservative. The most radical substitutions were restricted to loop regions (Figs. 3 & 4).

Cytochrome b is an integral component of respiratory chain complex III (cytochrome bc_1 complex), which converts ubiquinol to cytochrome c, thereby generating an electrochemical gradient across the inner mitochondrial membrane that is utilized for ATP production (Hunte et al., 2003; Mitchell, 1976; Trumpower, 1990). In this process, ubiquinol is first converted to ubiquinone via oxidation at the Q_o binding site and ubiquinone is then reduced to cytochrome c at the Q_i binding site (Crofts, 2004; Gao et al., 2003; Kolling et al., 2003). Both of these sites are located within the cytochrome b subunit. This process ultimately relies on the capacity of the Q_i site to bind water molecules for the reduction of ubiquinone to ubiquinol (Crofts, 2004). Because amino acid replacements can result in regional changes to hydrophobicity and structure within the protein, amino acid replacements within *CYTB* have the potential to alter the efficiency of this biding site. Replacements at cytbI7T characteristic of human mitochondrial haplogroup H likely enhance the binding of water at Q_i, and have been linked to patterns of increased longevity for this group (Beckstead et al., 2009). Similarly, mutations to key residues at Q_o in yeast alter this binding site and have resulted in decreased catalysis efficiency, as well as increased damaging, oxygen radical-producing bypass reactions (Wenz et al., 2007). However, the isoleucine to leucine substitution at *CYTB* 161 is among the most conservative amino replacements that occur at positively

selected residues ($D = 5$), making the functional significance of this substitution in *Tachycineta* unclear.

4.2. Model assumptions and consistency of inferences across methods

Our results differ from a previous investigation of mitogenomic variation in *Tachycineta* in which positive selection was not detected (Cerasale et al., 2012). This disparity is likely due to our use of methods that are more sensitive to detecting lineage-specific selection. Cerasale et al. used a two-rate fixed effects likelihood (FEL) approach, whereas we have employed a mixed effects model of evolution (MEME). The two methods differ in that MEME allows dN/dS to vary across both sites and branches (such that some branches may exhibit positive selection at the same time that others exhibit negative selection). Conversely, FEL assumes constant selection across time (prohibiting variation across branches), and thereby limiting its power to detect selection occurring in only a single or few lineages (Murrell et al., 2012). We also implemented a branch-specific model of positive selection in CODEML to control for phylogenetic uncertainty. Cerasale et al. did not employ branch-site models.

CODEML inferences were largely robust to tree topology but only a subset of the sites inferred to be under selection by CODEML were also identified by MEME: one site was detected by both methods (*ND5* 27) and another (*ND2* 222) was found to be marginally significant. The differences can likely be attributed to differences in the underlying assumptions of the two methods (namely the use of one vs. multiple dN/dS ratios across branches), and also to the constraint of testing one focal branch at a time using branch-site tests. The latter issue prevented detection of sites under positive selection at *CYTB* 161, which exhibits a parallel amino acid change in two non-sister taxa (Fig. 3).

5. Conclusions and future directions

Taken together, the results presented here suggest that the mitochondrial genome may be a promising candidate locus to begin to tie molecular genetic variation to latitudinal patterns in the pace of life. We have found evidence of positive selection acting on DNA sequence variation in the mitogenome of a widespread avian genus that exhibits latitudinal patterns of life-history variation that are consistent with POL expectations. While we found weak support for our prediction that branches separating taxa with greater POL variation also exhibit higher rates of positive diversifying selection, "slow" species did harbor the largest accumulation of derived, positively selected substitutions. We speculate that these substitutions are associated with functional metabolic differences among species and hope that our work will motivate future studies to characterize whole-organism metabolic rates, energy expenditure, and cellular metabolic processes within *Tachycineta*. Because the metabolic machinery is also encoded by the nuclear genome, similar investigations should be performed as sequences become available for the adjacent nuclear subunits: these could likely be targets of intergenomic coevolution (Burton and Barreto, 2012) and be responsible for some of the remaining POL variation among species.

At the biochemical level, variation in metabolic rates is manifest through differences in the expression of genes that encode the machinery of cellular metabolism and the kinetic properties of these metabolic enzymes. Thus, although the positively selected amino acid substitutions we have identified here may contribute to functional variation in NADH dehydrogenase and cytochrome b, variation in whole-organism metabolic rate can also be driven through regulatory mechanisms. Indeed, recent studies of metabolic performance in deer mice (*Peromyscus maniculatus*) have demonstrated that elevated thermogenic performance is associated with large-scale upregulation of genes that participate in the beta-oxidation of lipids and oxidative phosphorylation, and these transcriptomic changes paralleled differences in the activities of metabolic enzymes that influence flux through these same pathways (Cheviron et al., 2012, 2014). Therefore, studies of regulatory variation in relation to pace of life expectations are likely to be particularly fruitful, and would complement our analyses of DNA sequence variation. Similarly, detailed studies of mitochondrial respiration rates coupled with measurements of whole-organism metabolic rates could provide insights into the functional consequences of putatively adaptive amino acid substitutions (Storz and Wheat, 2010; Toews et al., 2013). Collectively, these studies may begin to elucidate the genetic and physiological mechanisms that underlie an important broad-scale macrophysiological pattern — latitudinal variation in the 'pace of life' (Gaston et al., 2009).

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2014.05.019.

Conflict of interest

The authors declare that there is no conflict of interest.

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