Evolution of Interacting Proteins in the Mitochondrial Electron Transport System in a Marine Copepod

Christopher S. Willett† and Ronald S. Burton†*

*Department of Biology, University of North Carolina, Chapel Hill; and †Marine Biology Research Division, Scripps Institution of Oceanography, University of California, San Diego, La Jolla

The extensive interaction between mitochondrial-encoded and nuclear-encoded subunits of electron transport system (ETS) enzymes in mitochondria is expected to lead to intergenomic coadaptation. Whether this coadaptation results from adaptation to the environment or from fixation of deleterious mtDNA mutations followed by compensatory nuclear gene evolution is unknown. The intertidal copepod *Tigriopus californicus* shows extreme divergence in mtDNA sequence and provides an excellent model system for study of intergenomic coadaptation. Here, we examine genes encoding subunits of complex III of the ETS, including the mtDNA-encoded cytochrome b (*CYTB*), the nuclear-encoded rieske iron-sulfur protein (*RISP*), and cytochrome c_1 (*CYC1*). We compare levels of polymorphism within populations and divergence between populations in these genes to begin to untangle the selective forces that have shaped evolution in these genes. *CYTB* displays dramatic divergence between populations, but sequence analysis shows no evidence for positive selection driving this divergence. *CYC1* and *RISP* have lower levels of sequence divergence between populations than *CYTB*, but, again, sequence analysis gives no evidence for positive selection acting on them. However, an examination of variation at cytochrome c (CYC), a nuclear-encoded protein that transfers electrons between complex III and complex IV provides evidence for selective divergence. Hence, it appears that rapid evolution in mitochondrial-encoded subunits is not always associated with rapid divergence in interacting subunits (*CYC1* and *RISP*), but can be in some cases (*CYC*). Finally, a comparison of nuclear-encoded and mitochondrial-encoded genes from *T. californicus* suggests that substitution rates in the mitochondrial-encoded genes are dramatically increased relative to nuclear genes.

Introduction

The 13 proteins encoded by mitochondrial DNA (mtDNA) in animals are subunits of the electron transport system (ETS) and, together with some 65 nuclear-encoded subunits, comprise the oligomeric enzyme complexes of the ETS. Given the close association between nuclear-encoded and mitochondrial-encoded proteins in these complexes and the importance of the ETS for energy production, coadaptation between nuclear-encoded and mitochondrial-encoded proteins has been strongly selected during the evolutionary history of these complexes. Two different scenarios of selection/drift could generate intergenomic coadaptation: (1) Selection pressure on ETS enzymes could be generated by the adaptation of a species (or population) to its environment (i.e., thermal environment for ectotherms) leading to changes in both nuclear-encoded and mitochondrial-encoded subunits of ETS complexes. (2) High mutation rates in mtDNA, especially when coupled with fluctuating population sizes, may lead to fixation of deleterious amino acid substitutions and provide selection pressure for subsequent compensatory changes in nuclear genes, a two-step process that could drive evolution of coadaptation in ETS enzymes.

In mammals, several lines of evidence suggest that the interactions involving the nuclear-encoded soluble cytochrome c (CYC) and cytochrome c oxidase (complex IV) have evolved rapidly in higher primates: (1) In vitro studies with heterospecific combinations of CYC and complex IV enzymes across these groups show reduced enzyme activity (Osheroff et al. 1983). Additionally, analyses of human/orangutan cell lines containing portions of both species nuclear genomes and orangutan mtDNA show reduced complex IV enzyme activity consistent with significant mtDNA/nuclear coadaptation (Barrientos et al. 2000). (2) Sequence analyses show that rates of amino acid substitution increase in simian primates in mtDNA-encoded subunits of complex IV (COI and COII) and in seven other nuclear-encoded subunits in concert with increases in substitution in CYC (Adkins, Honeycutt, and Disotell 1996; Wu et al. 2000; Schmidt, Goodman, and Grossman 2002). (3) At the structural level, rates of amino acid evolution are increased at interaction points of proteins for mtDNA-encoded proteins but not nuclear-encoded proteins (Schmidt et al. 2001). (4) Analyses of mtDNA-encoded cytochrome b (CYTB), nuclear-encoded cytochrome c_1 (CYC1), and rieske iron-sulfur protein (RISP) from ubiquinol-cytochrome c reductase (complex III) also show accelerated substitution in humans/simian primates correlated with the changes in complex IV and CYC and may argue for extensive coadaptation of these two ETS complexes and CYC in higher primates (Andrews, Jermiin, and Easteal 1998; Grossman et al. 2001). Given the role of CYC as a carrier of electrons between complex III and complex IV, this coadaptation has an obvious potential functional basis, although the evolutionary forces driving this divergence in higher primates are not known.

Because of high levels of interpopulation genetic divergence, the intertidal copepod *Tigriopus californicus* presents an excellent model system for the study of evolutionary coadaptation between nuclear and mitochondrial genes. Allopatric populations of *T. californicus* along the western coast of North America show extreme divergence in mtDNA genes, sometimes over relatively short physical distances. Two mtDNA-encoded subunits of

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E-mail: willett4@email.unc.edu.

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complex IV, COI, and COII, display divergences above 20% for nucleotide substitutions and up to 15% for amino acid divergence (Burton and Lee 1994; Burton 1998; Burton, Rawson, and Edmands 1999). The nuclear-encoded protein CYC also shows substantial amino acid divergence between populations, particularly given the generally high level of conservation of this protein (Rawson, Brazeau, and Burton 2000). Despite high interpopulation divergence, within-population polymorphism is generally low; the resulting high F_{ST} values suggest highly restricted gene flow. Clearly these populations have evolved independently for long periods of time and could be considered separate species under some species definitions (i.e., phylogenetic species concept); however, based on their reproductive compatibility, we will continue to refer to them as conspecific populations (also see Burton [1998]).

Several studies suggest that nuclear/mitochondrial coadaptation is evolving between genetically isolated *T. californicus* populations. Edmands and Burton (1999) used repeated backcrossing to study the effect of cytonuclear interactions on cytochrome c oxidase (complex IV) enzyme activity in hybrids. Reductions in complex IV activity in some interpopulation crosses were caused at least in part by nuclear/mitochondrial interactions. Rawson and Burton (2002) found direct evidence for functional coadaptation between CYC and complex IV. CYC derived from a San Diego (SD) population yielded significantly higher activity with SD population–derived complex IV than that derived from a Santa Cruz (SC) population, and reciprocally, SC population–derived CYC functioned best with SC population–derived complex IV. Willett and Burton (2001) found extensive differences in *CYC* genotypic viabilities in F_2 hybrids from controlled crosses between populations (estimated from deviations from Mendelian ratios). *CYC* viability differences were observed in reciprocal crosses between specific populations, suggesting cytoplasmic effects consistent with the inference of nuclear/mitochondrial interactions.

The combination of extensive divergence in mtDNA-encoded genes in *T. californicus* and fitness/functional evidence for mitochondrial/nuclear coadaptation make this system a unique opportunity to examine molecular evolution of these interacting genes. In this paper, we will first examine the evolution of CYTB (the mtDNA-encoded subunit of complex III) and show that it is diverging rapidly between populations. Two nuclear-encoded subunits of complex III have functional domains containing redox centers, the rieske iron-sulfur protein (RISP), and cytochrome c_1 (CYC1). We have obtained nuclear DNA sequences encoding these proteins from *T. californicus* and examine polymorphism within populations and divergence between populations. We then compare these genes, which function in the ETS, with other nuclear genes that have been obtained from *T. californicus*. Two major conclusions can be drawn from these results: (1) Proteins closely interacting with mtDNA-encoded proteins in the ETS can show evidence for selection for high rates of evolution (CYC), but not all appear to be evolving rapidly (CYC1 and RISP). (2) The mtDNA in *T. californicus* appears to have a much higher rate of substitution between populations than has the average nuclear gene for both synonymous and nonsynonymous changes.

Materials and Methods

Cytochrome b Sequencing

To study divergence in mitochondrial and nuclear encoded genes, copepods were collected from high intertidal rock pools at three sites in California, San Diego (SD, 32°45′N, 117°15′W), Abalone Cove in Los Angeles County (AB, 33°44′N, 118°19′W), and Santa Cruz (SC, 36°57′N, 122°03′W). We obtained *CYTB* sequences from SD, SC, and AB copepods to test the extent of population divergence relative to other mitochondrial genes in *T. californicus*. Primers for SD population *CYTB* were designed from the sequence of the complete mtDNA molecule from SD (Rawson and Burton, unpublished data) and amplified the entire gene. PCR products were directly sequenced using Amersham Biosciences MegaBACE ET Terminators chemistry and run on a MegaBACE 500 sequencer following manufacturer's protocols. SD *CYTB* primers did not amplify the entire gene from either AB or SC populations (only smaller fragments were successfully amplified). We designed degenerate primers from comparisons of arthropod mtDNA sequences for both *ND4* and *COXII* (genes flanking *CYTB* in *T. californicus* mtDNA) and within *CYTB* itself. Sequence obtained from this strategy allowed us to design population-specific primers that flanked *CYTB* and permitted us to amplify and sequence this gene from both SC and AB population copepods. Primer sequences and amplification protocols are available upon request from authors. DNA was extracted from single copepods using the proteinase-K cell-lysis method (Hoelzel and Green 1992). Direct sequencing of *CYTB* was performed from PCR products for multiple individuals from each population.

Identification and Sequencing of Nuclear-Encoded Proteins

We sought to characterize two nuclear-encoded genes from complex III of the ETS that contains the mtDNA-encoded protein *CYTB*. The *T. californicus* nuclear genes encoding the proteins *CYC1* and *RISP* were initially identified using a 5′ RACE (rapid amplification of cDNA ends) procedure on mRNA isolated from SD copepods. The kit Generacer (Invitrogen, Carlsbad, Calif.) was used with degenerate primers designed to match conserved regions of these two proteins (from alignments of human, *Drosophila melanogaster*, *Saccharomyces cerevisiae*, and *Caenorhabditis elegans*) to amplify from cDNA. Primer used for *CYC1* was CYC1.rev (5′-GCNCCNCCNGGRAARTANGGRTT-3′) and primer used for *RISP* was RISP.rev (5′-TARTGNSWNCCRTGRCANGGRCA-3′) in conjunction with a Generacer kit primer. Resulting PCR products were gel purified, cloned, and sequenced. The complete mRNA was obtained with 3′ RACE using *T. californicus*–specific primers designed from the new sequence.

Genomic DNA sequences were obtained from the SD population corresponding to the mRNA transcript for *RISP* and *CYC1*. PCR products were generated using species-specific primers and these products were sequenced directly. Polymorphism was inferred for sites when two clear peaks of equal intensity were observed in the

Table 1
Amino Acid Identities for CYTB, CYC1, and RISP Proteins from SD *T. californicus*

	D. melanogaster			*A. gambiae*			Human			*S. cerevisiae*			*T. japonicus*
	CYTB	CYC1	RISP	CYTB	CYC1	RISP	CYTB	CYC1	RISP	CYTB	CYC1	RISP	CYTB
T. californicus	56.1	60.3/51.0	67.0	53.5	61.9	69.2	53.1	64.0	66.8	45.6	54.8	52.6	77.4
D. melanogaster				86.5	87.4/62.8	76.3	56.1	61.9/59.6	69.6	51.3	58.6/51.0	52.1	56.6
A. gambiae							62.1	65.3	69.2	52.4	60.3	54.4	56.6
Human										49.5	57.7	52.6	53.9
S. cerevisiae													47.5

NOTE.—Amino acid identities are calculated for the entire CYTB protein. For both CYC1 and RISP proteins mature proteins, not including mitochondrial signal peptide sequence, were compared (cleavage site for human protein was used to determine approximate N-terminal end of mature proteins). *Drosophila melanogaster* proteins are CYTB-P18935, two homologous gene products for CYC1-AAF50785/AAL90066, and gene product homologous to RISP-AAF51354. *Anonopheles gambiae* proteins are CYTB-P34844, gene product homologous to CYC1-EAA04119, and gene product homologous to RISP-EAA14787. Human proteins are CYTB-P00156, CYC1-P08574, and RISP-P47985. *Saccharomyces cerevisiae* proteins are CYTB-P00163, CYC1-CCBY1H, and RISP-P08067. *Tigriopus japonicus* CYTB protein is BAB97221.

chromatogram. Sequences for most of the coding region and intervening introns were obtained from multiple individuals from SD, SC, and AB individuals (some 5′ end and 3′ end codons were not sequenced). CYC sequences were published previously based on sequences obtained from clones (Rawson, Brazeau, and Burton 2000), and polymorphisms in these sequences were verified by examining directly sequenced products from same individuals as described above. Sequences were edited with the program Sequencer version 4.1 (Genecodes, Ann Arbor, Mich.), and alignments were made using ClustalX with some adjustments by hand in large indels in introns. DNAsp version 3.53 (Rozas and Rozas 1999) was used to calculate π values, M-K tests, and numbers of fixed silent and replacement differences. PAUP* version 4.0b10 (Swofford 2001) was used to calculate amino acid identities between proteins and for phylogenetic analyses.

We also sought to characterize polymorphism and divergence in nuclear-encoded genes that are not a part of the ETS and not likely to interact closely with mtDNA-encoded proteins. Additional sequences of the gene glutamate dehydrogenase (GDH) were obtained from SD, SC, and AB population copepods. A complete genomic sequence of GDH from SD was recently obtained (Willett and Burton 2003) and a region of 1,320 bp, including a single intron of 71 bp, was sequenced from multiple individuals in each population. Sequences were obtained by direct sequencing from PCR products. Population samples from two other nuclear encoded proteins, Δ^1-pyrroline-5-carboxylase synthase (P5CS) and Δ^1-pyrroline-5-carboxylase reductase (P5CR), were previously published (Willett and Burton 2002). To verify heterozygous sites, sequencing traces were visually inspected for polymorphism as discussed above. Alignments of sequences are available as Supplementary Material online (http://www.mbe.oupjournals.org) and representative sequences from each population have been submitted to GenBank with accession numbers AY344446 to AY344470.

Results

Patterns of CYTB Polymorphism and Divergence

We have examined three levels of *CYTB* variation for *T. californicus*, between species, within species, and within populations. The *T. californicus* CYTB protein is predicted to be 376 amino acids in length. The initiation codon appears to be TAA (based on alignments with other species CYTBs), which is reported to be the initiation codon for *CYTB* and four other mtDNA genes in a congener *T. japonicus* (Machida et al. 2002). Not surprisingly, the most similar CYTB among diverse eukaryotes is that of *T. japonicus* at 77% amino acid identity (table 1). CYTB amino acid sequence is not very informative phylogenetically, because few relationships are robustly supported in phylogenetic trees constructed using parsimony analysis of amino acids of largely arthropod CYTB proteins. For example, the clade Crustacea is not recovered in these trees (fig. 1). The well-supported *Tigriopus* clade has comparatively long branches, suggesting rapid amino acid evolution in this group. This impression is supported by relative rates comparisons with other arthropods: *T. californicus* is 2.03-fold faster when compared with *Daphnia pulex*, 2.7-fold

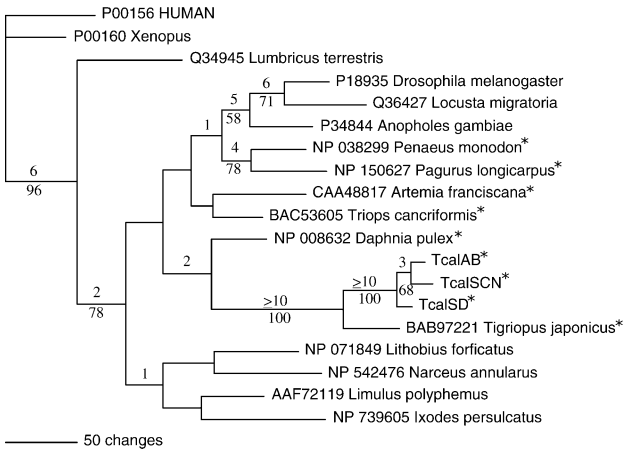

FIG. 1.—Phylogram for CYTB proteins of diverse arthropods, including *T. californicus*. This tree was generated from a parsimony analysis of CYTB complete amino acid sequences and was one of three most-parsimonious trees. This phylogram of a single tree is shown, rather than the consensus, to show branch lengths indicating rapid evolution in the *Tigriopus* clade that is common to all trees. Bremmer support indices (above branch) and bootstrap values from 1,000 replicates (below branch) are given for branches found in the consensus tree. GenBank accession numbers are listed before species names. *T. californicus* is denoted as Tcal with population designation (SD, SCN, or AB). Crustaceans are indicated with an asterisk (*). Outgroups include human, *Xenopus*, and the earthworm (*Lumbricus terrestris*) CYTB.

Table 2
Genetic Divergence in *T. californicus CYTB* in Comparison with *COI* and *COII*

		CYTB			*COI*	*COII*
Pairwise Comparison	Site Type	Pairwise Distance	JC-Corrected Distance	D_n/D_s	JC-Corrected Distance	JC-Corrected Distance
SD/SC	Synonymous	0.701	2.04		0.782[a]	2.23
	Nonsynonymous	0.034	0.035	0.0171	0.029	0.041
AB/SC	Synonymous	0.701	2.05		1.93	1.67
	Nonsynonymous	0.037	0.038	0.0183	0.022	0.092
SD/AB	Synonymous	0.658	1.58		1.31	1.17
	Nonsynonymous	0.039	0.040	0.0253	0.020	0.082

NOTE.—Site type refers to synonymous or nonsynonymous category using Nei/Gojobori method as implemented by the program DNAsp. D_n/D_s value is ratio of nonsynonymous divergence divided by synonymous divergence (using JC-corrected values).

[a] Uncorrected distance is given for this comparison because value is too high to use for JC correction.

higher when compared with *Penaeus monodon*, and 2.9-fold higher when compared with *Triops cancriformis* (all comparisons use *D. melanogaster* as an outgroup). In contrast, when *T. californicus* and *T. japonicus* are compared, they show equal rates of evolution with either *D. pulex* or *D. melanogaster* used as outgroups (*T. californicus* 1.02-fold and 1.05-fold higher than *T. japonicus*, respectively).

CYTB is remarkably divergent between the AB, SC, and SD populations, with pairwise amino acid differences averaging 6.9% to 7.4% (AB to SD, and AB and SD to SC, respectively). This translates into 26 and 28 amino acid differences in the *CYTB* protein for these interpopulation comparisons. Divergence was even more extreme at silent sites, with uncorrected pairwise divergences between 65% and 70% (table 2). A Jukes-Cantor correction for multiple hits suggests that the average silent site has undergone at least one change in the evolution of *CYTB* between populations. D_n/D_s values are low for *CYTB*, reflecting the high number of silent substitutions that have occurred. This large amount of genetic divergence between *T. californicus* populations is not unexpected, given previous results for mtDNA genes in this species; levels of nucleotide divergence for nonsynonymous sites in *CYTB* fall between those seen for the mitochondrial-encoded *COI* and *COII* of complex IV for comparisons between the same populations (table 2). Comparisons of these three genes across members of the *Drosophila melanogaster* subgroup of *Drosophila* show the same relative ranks of amino acid conservation with *CYTB* showing an intermediate rate of amino acid evolution (Ballard 2000*a*).

In comparison with the remarkable levels of divergence in *CYTB* between populations, levels of polymorphism within populations are relatively normal. Between 11 and 20 alleles for the complete *CYTB* gene were sequenced from the AB, SC, and SD populations, and levels of polymorphism were calculated within each population (table 3). Average pairwise divergence between alleles within a population was about 0.25% for each of the three populations. These π values for *CYTB* are similar to those reported from a range of animal studies, including armadillos, cod, sardines, and shrews whose π values ranged from 0.3% to 0.53% for a single geographical population (Grant, Clark, and Bowen 1998; Arnason et al. 2000; Frutos and Van Den Bussche 2002; Ratkiewicz et al. 2002). The frequency distribution of alleles differed between *T. californicus* populations as indicated by Fu and Li's *D* test (Fu and Li 1993) and Tajima's *D* statistic (Tajima 1989), which were generally positive in the AB and SC populations and negative in the SD population. A positive value indicates excess high frequency alleles, whereas negative values indicate excess low frequency alleles (in the SD population, there were seven haplotypes, six of which were only represented by one individual). Different population histories or selection on the mtDNA are potential explanations for the differences we observe in haplotype frequency distributions among these populations.

Evidence is accumulating that animal mtDNA polymorphism includes an overrepresentation of slightly deleterious mutations (Rand 2001); this phenomenon is seen in many data sets as excess of amino acid polymorphism and can be detected by McDonald/Kreitman (MK) tests (McDonald and Kreitman 1991; Rand and Kann 1996; Nachman et al. 1996; Weinreich and Rand 2000). Neutrality index (NI) values can be used to measure

Table 3
Polymorphism in *CYTB* Gene in *T. californicus* Populations

	Number of	Segregating Sites		π Values			Fu and Li's	Tajima's
Population	Alleles	Nonsynonymous	Synonymous	Nonsynonymous	Synonymous	Total	*D* Test	*D* Statistic
SD	20	0	13	0	0.00962	0.00224	−2.36	−1.47
SC	12	3	5	0.00148	0.00664	0.00276	1.39*	−0.01
AB	11	1	7	0.00061	0.00973	0.00284	1.38*	0.48

NOTE.—Sites are defined as nonsynonymous, synonymous, and the combination of both (total), and numbers of segregating sites and average pairwise divergences between alleles (π) were calculated using program DNAsp. Significant deviations in Fu and Li's D are indicated with * for $P < 0.05$. No deviations are significant for Tajima's *D* statistic.

Table 4
Neutrality Index Values for CYTB in *T. californicus* Populations

Populations Compared	Neutrality Index (NI)[a]	
	No Correction	JC-Corrected Divergence
SC/SD	1.13	3.17
AB/SD	0.31	0.72
AB/SC	1.27	3.59*[b]

[a] Neutrality index is calculated as the ratio of polymorphic to divergent nonsynonymous difference divided by the ratio of polymorphic to divergent synonymous differences.

[b] This comparison yielded a significant departure in the McDonald/Kreitman test (*$P < 0.05$ by Fisher's exact test) and is consistent with excess replacement polymorphism.

the deviation from expected polymorphism and divergence ratios, with NI values greater than 1 consistent with negative selection and NI values less than 1 consistent with positive selection (Rand and Kann 1996). The levels of divergence at synonymous sites add uncertainty to calculations of NI for comparisons between *T. californicus* populations. The Ni values are greater than 1 for both uncorrected and JC corrected comparisons involving the SC population (table 4). When JC corrected values are used for divergence, the AB/SC comparison is significant in an MK test consistent with elevated replacement polymorphism. Therefore, we find no consistent evidence for positive selection driving amino acid substitution between copepod populations in *CYTB* and some evidence that polymorphism could contain excess slightly deleterious mutations analogous to that seen in the evolution of other animal mtDNAs.

Divergence Between *T. californicus* Populations in Nuclear Genes

Given the extensive amino acid divergence seen in CYTB between populations, it is interesting to ask whether proteins that interact closely with CYTB also show large amino acid divergences between populations. Two nuclear genes that encode protein subunits of complex III and contain the other redox active sites in this complex are CYC1 and RISP. We isolated the genes encoding these proteins from *T. californicus* and found that inferred amino acid sequences show high similarity to CYC1 and RISP proteins of other species (table 1). For most comparisons between the taxa in table 1, the RISP protein appears to be the most highly conserved, with CYC1 being more conserved than CYTB. Similar to the results seen for CYTB, phylogenetic analyses on CYC1 and RISP amino acid alignments do not produce robust and well-resolved phylogenies (data not shown). Rates of evolution between these taxa are more difficult to compare than for CYTB with relative rates comparisons because there are very few sequences of these proteins in arthropods currently available. Comparing the rate for *T. californicus* to *D. melanogaster* CYC1 (AF50785), with human as the outgroup, suggests the rate of amino acid evolution is 0.93-fold slower in *T. californicus* lineage. The same set of comparisons for RISP suggests *T. californicus* lineage is evolving 1.21-fold faster. Finally, a comparison of soluble CYC from

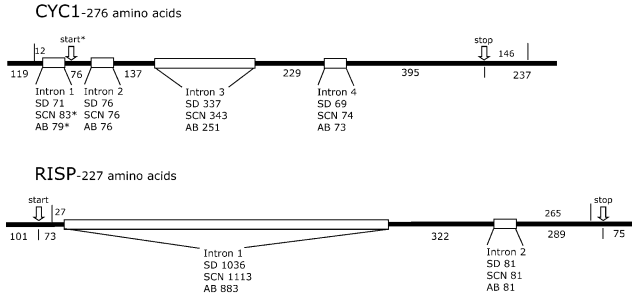

FIG. 2.—Gene structure for *CYC1* and *RISP* from *T. californicus*. Sizes of introns and exons are indicated below figure (introns vary in size between populations as indicated). Region sequenced for all three populations is indicated by lines above alignment and also the distance to the nearest gene feature. Locations of start and stop codons are predicted from sequence and alignments with other homologs. Asterisk (*) indicates intron 1 end appears to have shifted 5 bp closer to start codon in SC and AB (from 6 bp away in SD).

T. californicus and the prawn (*Macrobrachium malcolmsoni*) with a *D. melanogaster* outgroup suggests *T. californicus* CYC is evolving slightly faster (1.2-fold higher).

We obtained sequences of *CYC1* and *RISP* from three *T. californicus* populations, SD, AB, and SC. The intron/exon structure of these genes was determined by comparing mRNA and genomic sequence for SD, and it is largely conserved between populations (fig. 2). A potential exception to this is intron 1 of *CYC1* in the AB and SC populations, where the 3′ intron–ending AG in SD has been changed to GG, and the next closest potential splice AG is 5 bp closer to the predicted start codon (this predicted boundary has not been verified by mRNA sequencing from SC and AB copepods). The four small introns in the two genes vary little in size between populations, whereas the two larger introns vary considerably (fig. 2). Based on the current annotations of the *A. gambiea* and *D. melanogaster CYC1* and *RISP* genes, there appears to be little conservation in intron position among these two Diptera and *T. californicus*. However, unlike the other introns, the first intron in *RISP* does occur in approximately the same position in all three taxa (in the unalignable putative mitochondrial signaling peptide).

The complete coding region for *CYC1* and most of that of *RISP* were sequenced from individual *T. californicus* copepods from each of the SD, SC, and AB populations (sequenced regions indicated in figure 2). Divergence in these genes between populations, although substantial, was considerably lower than that of the mtDNA genes (table 5). The nuclear-encoded CYC has extensive interactions with both complex III and IV and was previously sequenced from these populations (Rawson, Brazeau, and Burton 2000). Divergences between these populations for CYC are also presented in table 5. For these three nuclear-encoded ETS genes, the average rate of nonsynonymous substitution compared with the average for three mtDNA genes was 5.8-fold lower for the SD/SC comparison, sixfold lower for the AB/SC comparison, and 9.7-fold lower for the AB/SD comparison. Insertion and deletion variation was common in noncoding regions of these three genes between these populations but absent within coding regions. Nucleotide divergence between populations was also higher in general

Table 5
Genetic Divergence Between *T. californicus* Populations for Nuclear-Encoded Proteins Interacting Directly with Mitochondrial-Encoded Proteins

		CYC1		*RISP*		*CYC*	
Pairwise Comparison	Site Type	JC-Corrected Distance	D_n/D_s	JC-Corrected Distance	D_n/D_s	JC-Corrected Distance	D_n/D_s
SD/SC	Synonymous	0.0466	0.0683	0.0427	0.0518	0.0289	0.4393
	Total silent	0.0800	0.0398	0.0933	0.0237	0.0564	0.2251
	Nonsynonymous	0.0032		0.0022		0.0127	
AB/SC	Synonymous	0.0361	0.0440	0.0576	0.0387	0.0598	0.3633
	Total silent	0.0792	0.0201	0.0812	0.0274	0.0522	0.4167
	Nonsynonymous	0.0016		0.0022		0.0217	
SD/AB	Synonymous	0.0414	0.0384	0.0428	0.1042	0.0754	0.1145
	Total silent	0.0751	0.0212	0.0627	0.0711	0.0688	0.1255
	Nonsynymous	0.0016		0.0045		0.0086	

NOTE.—Site type refers to synonymous, total silent, which is synonymous plus noncoding, or nonsynonymous category using Nei/Gojobori method as implemented by the program DNAsp. D_n/D_s value is ratio of nonsynonymous divergence divided by synonymous divergence (in synonymous row) or divided by total silent divergence (in total silent row).

for these noncoding regions than for synonymous sites in the coding regions (2.2-fold higher for SC/SD, 1.5-fold higher for AB/SC, and 1.4-fold higher for AB/SD).

Although not as rapidly evolving as mtDNA genes, are the rates of *CYC1*, *RISP*, and *CYC* nonsynonymous substitution elevated above typical nuclear genes that do not interact with mitochondrial-encoded proteins in *T. californicus*? To address this question, nuclear divergences for such ''typical'' nuclear genes are compiled in table 6. In addition to divergences from three previously published genes (P5CS and P5CR [Willett and Burton 2002] and H1 [Burton and Lee 1994; Burton 1998]), sequences were also obtained from glutamate dehydrogenase (*GDH*) of just over 1,300 bp, including parts of exons 1 and 2 and the 71-bp intron 1 for the AB and SC populations (SD sequence obtained previously [Willett and Burton 2003]). The divergence in these genes at synonymous sites is roughly equal to that of *CYC*, *CYC1*, and *RISP* with these three genes evolving 1.14-fold faster for SD/SC, 1.16-fold faster for AB/SC, and 1.83-fold faster for AB/SD. These results contrast sharply with rates of synonymous substitution for the average of these seven nuclear genes compared with the average rate of synonymous substitution in the three mtDNA genes (using the JC-corrected values in table 2): 38.2-fold lower for SD/SC, 34.6-fold lower for AB/SC, and 26.7-fold lower for AB/SD. To compare rates of nonsynonymous substitution normalized to regional mutation rate differences in *CYC1*, *CYC*, and *RISP* with the ''typical'' genes, we can look at D_n/D_s ratios for each set of genes. On average, the four ''typical'' genes are evolving at a 2.7-fold lower rate for the SD/SC comparison, a 3.9-fold lower rate for the AB/SC comparison, and a 1.3-fold lower rate for the AB/SD comparison. On the surface, it appears that the proteins interacting with mitochondrial encoded genes are evolving more rapidly; however, these averages mask differences between the genes. *CYC* and *H1* stand out with high D_n/D_s ratios in their respective sets of proteins and bias the averages. Based upon comparisons with *D. melanogaster* amino acid sequences, *CYC* is a more conserved protein than *CYC1* or *RISP* (75% identity), so its elevated rate of evolution is unexpected and could indicate positive selection in the *T. californicus* lineage. With regard to the elevated rate of evolution observed for histone H1, it is important to note that *H1* is the linker histone and is not as conserved as other core histones. Although *T. californicus* and *D. melanogaster* amino acid identity (63%) appears to

Table 6
Genetic Divergence Between *T. californicus* Population for Non-ETS Nuclear-Encoded Proteins

		GDH		*P5CR*		*P5CS*		*H1*	
Pairwise Comparison	Site Type	JC-Corrected Distance	D_n/D_s	JC-Corrected Distance	D_n/D_s	JC-Corrected Distance	D_n/D_s	JC-Corrected Distance	D_n/D_s
SD/SC	Synonymous	0.0865	0	0.0453	0	0.0724	0.0178	0.0682	0.1680
	Total silent	0.0724	0	0.0440	0	0.0593	0.0217	0.0929	0.1233
	Nonsynonymous	0		0		0.0013		0.0115	
AB/SC	Synonymous	0.0821	0	0.0452	0	0.0716	0.0264	0.0286	0.3029
	Total silent	0.0684	0	0.0478	0	0.0599	0.0316	0.0680	0.1271
	Nonsynonymous	0		0		0.0019		0.0086	
SD/AB	Synonymous	0.0484	0	0.0360	0	0.0614	0.0105	0.0482	0.1794
	Total silent	0.0388	0	0.0219	0	0.0495	0.0130	0.0404	0.2141
	Nonsynonymous	0		0		0.0006		0.0086	

NOTE.—Site type refers to synonymous, total silent, which is synonymous plus noncoding, or nonsynonymous sites calculated using Nei/Gojobori method as implemented by the program DNAsp. D_n/D_s value is ratio of nonsynonymous divergence divided by synonymous divergence (in synonymous row) or divided by total silent divergence (in total silent row).

Table 7
Polymorphism Within Populations for Six *T. californicus* Nuclear Genes

Population	Gene	Numbers[a]				Pairwise Differences[b]		
		Alleles	Nonsynonymous	Synonymous	Total Silent	π_{nonsyn}	π_{syn}	$\pi_{tot\text{-}sil}$
SD	CYC1	8	1	2	5	0.00041	0.00437	0.00322
	RISP	6	0	0	8	0	0	0.00235
	CYC	10	0	2	14	0	0.01255	0.01170
	GDH	6	0	4	4	0	0.00441	0.00357
	P5CR	2	0	0	0	0	0	0
	P5CS	4	0	1	1	0	0.00163	0.00066
						0.00007[c]	0.00383[c]	0.00358[c]
SC	CYC1	4	0	3	18	0	0.00939	0.01352
	RISP	4	0	1	4	0	0.00346	0.00176
	CYC	6	0	3	28	0	0.02090	0.01981
	GDH	4	0	1	2	0	0.00531	0.00679
	P5CR	4	0	6	6	0	0.02865	0.01166
	P5CS	4	0	10	14	0	0.01269	0.00855
						0.00000[d]	0.01340[d]	0.01035[d]
AB[e]	CYC1	4	1	0	1	0.00084	0	0.00079
	RISP	4	0	0	1	0	0	0.00045
	CYC	4	0	1	13	0	0.00713	0.01263
	GDH	4	0	3	3	0	0.00634	0.00509
	P5CR	4	0	3	3	0	0.01355	0.00552
						0.00017[f]	0.00540[f]	0.00490[f]

[a] Number of alleles sequenced for each locus, nonsynonymous segregating sites, synonymous segregating sites, and the total number of noncoding and synonymous segregating sites (total silent).

[b] Average number of pairwise differences for nonsynonymous sites (π_{nonsyn}), synonymous sites (π_{syn}), and total synonymous and noncoding sites ($\pi_{tot\text{-}sil.}$). All values calculated using program DNAsp.

[c] SD average.

[d] SC average.

[e] Only a single allele of *P5CS* was obtained from the AB population.

[f] AB average.

be reasonably high, the H1 proteins align poorly and the value excludes numerous gaps and potentially high homoplasy, as H1 has a large bias toward lysine, proline, and alanine residues (61% of *T. californicus* H1). Hence, *H1* could evolve faster than other nuclear genes because of lower constraint on the H1 gene product, but this is not likely to explain evolution at *CYC*.

Polymorphism Within T. californicus Populations for Nuclear Genes

We examined polymorphism within *T. californicus* populations for these nuclear genes to both contrast levels of polymorphism in nuclear and mtDNA genes and to use in MK tests for evidence of selection. Nonsynonymous polymorphisms are extremely rare in six nuclear proteins, with only two segregating sites of this type observed (table 7), but more common in *H1* (see table 8). In contrast, synonymous sites and noncoding regions show moderate levels of polymorphism in all three populations. The π_{syn} and $\pi_{tot\text{-}sil}$ values vary considerably between genes with a high of 1.98% for *CYC* in SC and a nonzero low of 0.045% for *RISP* in AB. Average values of π_{syn} and $\pi_{tot\text{-}sil}$ for nuclear-encoded genes are higher in the SC population than the AB or SD populations; this pattern did not hold for the mtDNA gene *CYTB* (table 3). With equal mutation rates, the neutral expectation is a fourfold reduction in polymorphism for mtDNA-encoded genes in comparison with nuclear-encoded genes. For the genes we have surveyed, the difference in average levels of polymorphism (less than a fourfold decrease for mtDNA-encoded genes) suggest that synonymous sites in mtDNA have a higher neutral mutation rate (SD is 10.5-fold higher, AB is 7.2-fold higher, and SC is twofold higher).

Polymorphism within *T. californicus* populations and divergence between populations were compared for the six nuclear genes in table 7. We tested for differences from neutral expectations using the MK test. For five of the six genes (excluding *CYC*), no significant deviations were found from neutral expectations (data not shown). In contrast, for *CYC* comparisons involving the SC population, there are deviations consistent with selection for amino acid replacement (statistically significant for the comparison of AB and SC) (table 8). Histone *H1* data also showed some evidence for rapid evolution based upon D_n/D_s ratios for the SD/SC comparison. In an MK test, however, the excess is in the opposite direction, excess nonsynonymous polymorphism within populations rather than divergence between populations. These results suggest that positive selection has acted on *CYC*, leading to rapid amino acid evolution, whereas lower functional constraint could explain the pattern of evolution at *H1*.

Discussion

Protein-coding mtDNA genes appear to be evolving at a more rapid rate than protein-coding nuclear genes in *T. californicus*. Synonymous sites are evolving approximately 25-fold to 40-fold faster for sequence comparisons between populations for mtDNA genes (*COI*, *COII*, and

Table 8
McDonald/Kreitman Tests for *CYC* and *H1* Genes from *T. californicus* Populations

	SD/SC		AB/SC		AB/SD	
	Nonsynonymous	Total Silent	Nonsynonymous	Total Silent	Nonsynonymous	Total Silent
CYC						
Polymorphism[a]	0	37	0	39	0	26
Divergence	3	28	5	29	2	35
		$P = 0.090$		$P = 0.019$		$P = 0.51$
H1						
Polymorphism[b]	4	8	2	15	4	22
Divergence	4	37	3	27	3	19
		$P = 0.067$		$P = 1.0$		$P = 1.0$

NOTE.—McDonald/Kreitman tests were performed using polymorphism in both species and fixed divergence between species for nonsynonymous and noncoding and synonymous sites (total silent.). Fisher's exact test was used to calculate the probability that observed changes differ from the neutral expectation.

[a] Numbers do not match those in table 7 because some polymorphic sites were found in noncoding regions with alignment gaps between populations and were excluded.

[b] Polymorphism in *H1* is taken from sequences from AB, SD, and SC populations previously obtained (Burton and Lee 1994; Burton 1998).

CYTB) than for the set of seven nuclear-encoded genes. Synonymous site comparisons are likely to be less precise given that multiple hits may have occurred for each mtDNA synonymous site (but probably not for nuclear-encoded synonymous sites). The synonymous AB/SD comparison shows the smallest amount of genetic divergence and the lowest estimated increase in mtDNA synonymous substitution rate (26-fold); hence the JC-correction might not drastically underestimate the number of multiple hits. Synonymous polymorphism within populations suggests a smaller difference between silent substitution rates for mtDNA and nuclear genes (about 10-fold or less); however, this calculation assumes neutrality, which is violated by either selective sweeps or background selection (selection against deleterious polymorphisms). All genes are completely linked in animal mtDNA, so either of these processes would reduce polymorphism for the entire molecule at all sites. Nonsynonymous rates are also higher in mtDNA than in the seven nuclear genes (7.9-fold faster for SD/SC, 9.7-fold faster for AB/SC, and 13.8-fold faster for AB/SD). Accelerated evolution of mtDNA relative to nuclear DNA is of course not unique to *T. californicus*; this pattern has also been seen in mammals and birds (Brown, George, and Wilson 1979; Arctander et al. 1995). However, such increases in mtDNA rates of substitution are not universal. Both *Drosophila* and sea urchins (genus *Strongylocentrotus*) appear to have roughly equal rates of evolution of mtDNA and single-copy nuclear DNA (Powell et al. 1986; Vawter and Brown 1986). The rate of evolution in plant mtDNA is lower than that of nuclear DNA (Palmer and Herbon 1988).

Few other studies have explicitly examined the average level of divergence at synonymous and nonsynonymous sites for comparisons of mtDNA and nuclear genes. Pesole et al. (1999) found 22-fold higher synonymous substitution in complete mtDNA from humans and chimps in comparison with divergence in 20 nuclear genes from both species. Nonsynonymous sites had a threefold higher rate of substitution in mtDNA for comparisons of the same proteins. In an examination of 13 complete animal mtDNA sequences, Lynch and Jarrell (1993) reported that rates of amino acid substitution may be somewhat lower in mitochondrial-encoded proteins than in nuclear-encoded proteins. Comparisons of *COI*, *COII*, and *CYTB* with nuclear genes for *D. melanogaster* and *D. simulans* show less than a two-fold increase in synonymous rates but greater than a three-fold decrease in nonsynonymous rates for the mtDNA-encoded genes (Ballard 2000*b*; Betancourt, Presgraves, and Swanson 2002; C. S. Willett, unpublished results). Both synonymous and nonsynonymous rates of substitution appear to be substantially higher in *T. californicus*, suggesting a much higher mutation rate for mtDNA than nuclear. Noncoding sequence in large introns is likely to be the least constrained sequence we have analyzed, and comparisons of divergences of noncoding and synonymous sites for the three genes with large introns analyzed (*CYC1*, *RISP*, and *CYC*) show a 1.5-fold to twofold higher rate for noncoding sites, suggesting some constraint on synonymous sites in coding regions of nuclear genes. For comparison of mutation rates between nuclear-encoded and mitochondrial-encoded genes, this constraint could inflate the difference if mitochondrial-encoded genes have less constrained synonymous sites (but only by about twofold).

Given the central importance of the mitochondrial-encoded subunits in the ETS, a high mutation rate of mtDNA in comparison with nuclear DNA in *T. californicus* can only explain a rapid rate of amino acid replacement if the fixed substitutions behave neutrally. An alternate explanation for increased amino acid divergence in mtDNA-encoded proteins could be environmental selection on the ETS. Is there evidence for selection for amino acid replacement acting on *CYTB*? A negative Tajima's *D* statistic or Fu and Li's *D* test could indicate recovery from a recent selective sweep. Polymorphism for *CYTB* has nonsignificant negative values for both of these measures in the SD population. Both SC and AB populations have positive Fu and Li's *D* test values and nonsignificant Tajima's *D* statistic values. These tests then provide no strong evidence for recent selective sweeps on mtDNA. MK tests can detect repeated fixations driven by selection, but comparisons of polymorphism and divergence across these three populations for *CYTB* do not show a pattern consistent with positive selection. In fact, for comparisons involving SC, an opposite deviation consistent with excess replacement polymorphism is seen. Therefore, examination

of polymorphism and divergence in *T. californicus* populations lends little support for selection acting on *CYTB* to fix amino acid changes; instead, there is some evidence for excess slightly deleterious polymorphism, a pattern common in animal mtDNA.

If amino acid substitution in CYTB has not been driven by positive selection, could fixed differences be deleterious? Because of its ephemeral high intertidal habitat, *T. californicus* experiences dramatic fluctuations in population size (Burton 1997), which could facilitate the stochastic fixation of deleterious mutations. Extinction and recolonization in subdivided populations has been shown to increase the probability of fixation of deleterious mutations (Cherry 2003). Given the close physical and functional contacts of the mitochondrial-encoded and nuclear-encoded subunits of the ETS, compensatory amino acid change (via selection on nuclear-encoded subunits) is a potential consequence of accumulated deleterious substitutions. For two key subunits of complex III, RISP and CYC1, we find little evidence for selection for increased amino acid replacement in MK tests on these two proteins. This contrasts with apparently pervasive coadaptation occurring in primate complex IV proteins where amino acid substitution appears to be accelerated in many subunits of the complex (Adkins, Honeycutt, and Disotell 1996; Andrews, Jermiin, and Easteal 1998; Wu et al. 2000; Schmidt, Goodman, and Grossman 2002). Our finding shows that substantial change in CYTB can occur without numerous compensatory changes in RISP or CYC1. This could imply that the majority of the amino acid changes observed in CYTB between *T. californicus* populations are functionally neutral, or alternatively, most compensatory change occurs within the same subunit (for example within CYTB).

Mapping the positions of the amino acid changes from *T. californicus* populations in RISP and CYC1 on vertebrate complex III structures could also provide functional insights. Both differences from the SD sequence for CYC1 are found in the $\alpha 1'$ helix, which is thought to be near the interaction site with soluble CYC (Iwata et al. 1998; Zhang et al. 1998) although they are not the specific interacting residues in yeast (Lange and Hunte 2002). They include adjacent sites 68 and 69 (based on the vertebrate CYC1 mature protein positions) and are Val to Ile in SC/AB, and Met to Ile in SC. In RISP, residue 24 (based on vertebrate RISP mature protein position) is in the matrix portion of the protein and is a Lys to Asn change in AB. At position 50 in the transmembrane helix, there is a change from Gly to Ala in SC/AB. None of these residues have been implicated in a specific intermolecular interaction in yeast or vertebrate complex III.

Sequence comparisons provide no evidence for selection acting on RISP or CYC1 in the evolutionary divergence of these two proteins between *T. californicus* populations; however, there is evidence for selection acting on the soluble CYC protein that transports electrons between complex III and complex IV. This evidence comes from MK tests that suggest an excess of amino acid fixation for comparisons involving the SC population (table 8). Divergences between *T. californicus* populations for CYC appear high based on the high level of conservation of CYC in animals, but a relative rate comparison with the CYC of prawn does not suggest a dramatic increase in the rate of CYC in the *Tigriopus* lineage in general (1.2-fold higher). CYTB in contrast shows a twofold to threefold increase along the *Tigriopus* lineage, suggesting that rates of amino acid substitution in CYC and in CYTB may not be evolving in concert over long periods of time. A more relevant comparison for CYC evolution may be with COII of complex IV rather than CYTB with which CYC has no direct interactions. Like CYTB, COII is highly divergent between *T. californicus* populations, and hydrophobic portions of this protein (involved in CYC binding) are unexpectedly divergent (Burton, Rawson, and Edmands 1999; Rawson and Burton unpublished results).

Functional and fitness data also suggest selective divergence of CYC between genetically isolated *T. californicus* populations. Rawson and Burton (2002) showed that the enzymatic activity of complex IV differed when using SD population–derived CYC versus SC population–derived CYC as substrate. Enzymatic activity was consistently higher when complex IV and CYC were derived from the same natural population. Large temperature/enzyme interaction effects were also detected. Other studies show that fitnesses (as determined by deviations from expected Mendelian ratios) of different *CYC* genotypes vary dramatically in F_2 hybrids of AB, SC, and SD populations (Willett and Burton 2001). For AB/SC hybrids, dramatic influences of the rearing environment (including a different temperature regime) were observed; selection on *CYC* genotype was totally eliminated by a change from one environmental regime to another (Willett and Burton, 2003). The prevalence of environmental influences on *CYC* fitness and function suggest that local adaptation to temperature environment could be a selective force on CYC and influence its evolution. A model for molecular evolution in this system could involve a high mutation rate on mtDNA, leading to frequent fixation of neutral or slightly deleterious mtDNA mutations perhaps compensated by intramolecular changes. Coadaptation between mtDNA-encoded and nuclear-encoded proteins may then be driven by occasional bouts of adaptation to environmental conditions, leading to the evolution of specific components of the ETS complexes.

Supplementary Material

Amino acid alignments for RISP, CYC1, and CYTB for comparisons of the *T. californicus* proteins with homologs in other species are available online at the journal's website (http://www.mbe.oupjournals.org). Sequences for genes are available in GenBank with accession numbers AY344446 to AY344470. DNA sequence alignments in nexus format for all genes are also available online.

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David Rand, Associate Editor

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