Functional coadaptation between cytochrome *c* and cytochrome *c* oxidase within allopatric populations of a marine copepod

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Geographically isolated populations may accumulate alleles that function well on their own genetic backgrounds but poorly on the genetic backgrounds of other populations. Consequently, interpopulation hybridization may produce offspring of low fitness as a result of incompatibilities arising in allopatry. Genes participating in these epistatic incompatibility systems remain largely unknown. In fact, despite the widely recognized importance of epistatic interactions among gene products, few data directly address the functional consequences of such interactions among natural genetic variants. In the marine copepod, *Tigriopus californicus*, we found that the cytochrome *c* variants isolated from two different populations each had significantly higher activity with the cytochrome *c* oxidase derived from their respective source population. Three amino acid substitutions in the cytochrome *c* protein appear to be sufficient to confer population specificity. These results suggest that electron transport system (ETS) proteins form coadapted sets of alleles within populations and that disruption of the coadapted ETS gene complex leads to functional incompatibilities that may lower hybrid fitness.

In the absence of gene flow, geographically isolated populations diverge genetically and may become reproductively isolated. The potential role of intrinsic genic interactions (epistasis) in the evolution of postzygotic reproductive isolation was originally pointed out by Dobzhansky and Muller (1, 2). The Dobzhansky-Muller model suggests that some alleles that enhance fitness on their normal genetic background may reduce fitness when hybridization places those same alleles on novel genetic backgrounds, regardless of extrinsic (e.g., environmental) selective forces. Sets of alleles within populations are, therefore, intrinsically coadapted; reduction of fitness in hybrids reflects the disruption of coadaptation by recombination. Although this model has recently received renewed interest as the basis for theoretical models of allopatric speciation (3–5), explicit examples of coadapted alleles have not been identified in natural populations, and the functional basis for coadaptation at the biochemical/molecular level remains largely unexplored (5, 6).

As a model system for analysis of genetic divergence in allopatry, geographically separated populations of the intertidal copepod *Tigriopus californicus* show high levels of divergence at several nuclear and mitochondrial gene loci (7). In the laboratory, there is little or no evidence for prezygotic reproductive isolation among even the most divergent *T. californicus* populations (8). First generation progeny (F_1) of interpopulation crosses are vigorous, but second generation hybrids (F_2) frequently show reduced performance in a broad array of fitness-related traits (9–11). Because the consequences of hybridization are manifest in many traits, we hypothesize that some basic cellular function or functions must be affected; these functions, in turn, might have broad physiological consequences. Although we initially focused on mtDNA variation as a marker for population structure (7), the discovery of multiple amino acid differences among mtDNA-encoded proteins led us to question the functional significance of the mtDNA variation specifically at the cytochrome *c* oxidase subunit I and II genes. Cytochrome c oxidase catalyzes the terminal step of the mitochondrial electron transport system (ETS), where electrons from reduced cytochrome *c* are transferred to molecular oxygen. Because of its central role in cellular energy production, even minor disruption of ETS function can result in reduced fitness via numerous physiological mechanisms.

High levels of amino acid substitutions have been found among populations of the copepod *T. californicus* in three ETS proteins, two of the mtDNA genes (*COX1-COXII*) encoding subunits of cytochrome *c* oxidase (COX) and in the nuclear gene encoding cytochrome *c* (*CYC*) (11, 12). This variation has permitted several approaches to testing the hypothesis that ETS proteins are coadapted within populations. First, Edmands and Burton (13) tested the effects of nuclear/mitochondrial interaction on the activity of COX. Via repeated backcrossing, nuclear genes from one population were introgressed onto the cytoplasmic background of a different population. Such introgression resulted in reduced activity of COX, presumably due to suboptimal interactions between COX subunits encoded in nuclear and mtDNA. Second, by analysis of genotypic frequencies among F_2 hybrids, Willett and Burton found that the fitnesses of alternate *CYC* genotypes differed in reciprocal crosses between populations, suggesting that mitochondrial genotype specifically impacts fitness at this nuclear locus (14). Although both approaches provide evidence for nuclear/mitochondrial interactions, neither allows explicit determination of the molecular basis of the incompatibility. Although the nature of the crosses (i.e., backcross or F_2) allows us to calculate the expected genetic composition of the animals under study, we have no direct knowledge of the actual composition of the nuclear-encoded COX subunits for any given individual; hence, we cannot infer which (if any) nuclear subunits from one population are incompatible with those encoded by the mtDNA from the second population.

We report here on a more direct assessment of genetic coadaptation among *T. californicus* populations in the COX/CYC system. By testing the reaction rates of the different COX variants (from different populations) with each of the CYC variants (again from different populations), we can address two important questions: (*i*) Is the CYC variation itself functionally significant? (*ii*) Are cytochrome *c* variants coadapted to the COX genotype found in the same population (i.e., are the highest reaction rates observed when the CYC/COX combinations are from the same population)?

Materials and Methods

To undertake this work, milligram quantities of each CYC variant are needed for replicated enzyme assays with COX

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Abbreviations: ETS, electron transport system; COX, cytochrome *c* oxidase; CYC, cytochrome *c*; SC, Santa Cruz population; SD, San Diego population.

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obtained from each natural population. Because the small size of *T. californicus* (adult wet weight is approximately 30 μg) makes it difficult to collect more than 1–2 g of tissue from natural populations and because some variation in CYC exists within natural populations (12), direct purification of CYC from pooled, field-collected animals is problematic. Hence, we opted to clone *CYC* cDNA from two natural populations [Santa Cruz (SC) and San Diego (SD)] and express the protein in *Eschericia coli*. We extracted total RNA from ≈100 copepods sampled from each population by using the RNeasy Kit with a Qiashredder column (Qiagen, Valencia, CA) and a DNase digestion step to remove contaminating genomic DNA. First strand cDNA was synthesized by combining 1 μl of RNAsin (40 units/μl), 2 μl of 1 mg/ml oligo(dT) primer, 1 μg of total RNA, and water up to 24 μl. The reaction was incubated at 70°C for 10 min and cooled to 42°C, at which time 8 μl of 5× reverse transcriptase buffer, 4 μl of 0.1 M DTT, and 2 μl of 10 mM dNTPs were added. After incubation at 42°C for 2 min, 1 μl of Superscript II (GIBCO; 200 units/μl) was added, and the reaction was allowed to proceed at 42°C for 90 min, after which it was heated to 70°C for 15 min. Excess oligo(dT) primer, dNTPs, and enzyme were removed from the reaction by using a PCR spin column (Qiagen), and the first strand cDNA was eluted into 40 μl of TE buffer (10 mM Tris/1 mM EDTA, pH 8.0) and stored at −20°C.

PCR reactions were conducted in a total volume of 100 μl containing 1–2 μl of first strand cDNA, 10 μl of 10× PCR buffer, 3.0 μl of 50 mM $MgCl_2$, 2 μl of 10 mM dNTPs, 4 μl each of 30 μM PCR primers (forward, CGCACGCCATGGGTGATATCGACAAGGG; reverse, GAACGCGCTAGCTTACGACGAACTGGCTG), and 0.8 μl of *Taq* polymerase (GIBCO; 5 units/μl). Reactions were heated initially to 94°C for 2 min and then incubated for 30 cycles of 94°C for 30 s, 50°C for 90 s, and 72°C for 150 s, with a final 5-min extension step at 72°C. Because functional cytochrome *c* has a covalently bound heme group, its heterologous expression in *E. coli* requires coexpression of cytochrome *c* heme lyase (15), which is achieved with the cytochrome *c* expression vector pBTR (kindly provided by G. Mauk, University of British Columbia). PCR products were digested with the enzymes *Nco*I and *Nhe*I, gel purified, and ligated into pBTR prepared in the same manner. INVα cells (Invitrogen) were transformed with the recombinant plasmids; isolated colonies bearing recombinant plasmids were used to inoculate 600-ml cultures.

Individual 600-ml cultures in LB broth (with 200 μg/ml ampicillin) were incubated in an orbital shaker at 35°C and 150 rpm for 48 to 72 h until a pinkish-red color developed. Cells from 8 to 10 liters of bacterial culture for each CYC variant were pelleted and then resuspended in 150–200 ml of 100 mM NaH_2PO_4 (pH 7.1) buffer to which 0.5 g lysozyme and several crystals of DNase and RNase had been added. The suspensions were stirred at 4°C until a smooth slurry had formed (at least 24 h). After pelleting the cellular debris, the supernatant was removed to a separate tube and saved, and the pellet was resuspended in digestion buffer; this procedure was repeated until the pellet was colorless. The supernatants from each round of digestion were combined before dialysis against phosphate buffer containing 350 g/liter $(NH_4)_2SO_4$ to concentrate the supernatant and precipitate other proteins. After centrifuging for 2 h at 27,000 × *g*, the supernatant was removed, and the buffer was replaced by several passes through Centricon YM10 columns till the concentration of $(NH_4)_2SO_4$ was <0.5%.

Aliquots (3–5 ml) of each of the preparations of CYC were reduced by addition of 5 mg ascorbic acid; ascorbate was then removed by using a PD-10 desalting column (Amersham Pharmacia Biosciences) equilibrated with 0.05 M potassium phosphate buffer (PB) at pH 7.3. Finally, concentrations of the reduced cytochrome *c* preparations were diluted with PB to 25 μM (based on the millimolar extinction of horse CYC at 550 nm = 27.8). As inferred from the insert sequences, the expressed cytochrome *c* variants represented the common alleles found in the SC and SD populations (12).

As a source of population-specific COX, crude homogenates were prepared from groups of 15 adult female *T. californicus* from each population. Animals were homogenized in 250 μl of PB with 0.05% Tween 80 and centrifuged for 5 min at 1000 × *g*, and the supernatant was diluted by addition of 250 μl of PB. Activity of each of the two COX preparations was measured with each CYC preparation in two experiments: once at 18°C and then, by using new homogenates, at 25°C. Reaction rates were determined by monitoring the oxidation of CYC at 550 nm (16). Initial cytochrome *c* concentration was set at 20 μM (based on the molar extinction of horse CYC) for all reactions. Reactions were initiated by addition of 20 μl of homogenate, monitored for 4 min, and done in quadruplicate. Under these conditions, reaction rates were linear and were calculated by using the best linear fit to the 4-min trajectories. Reaction rates were normalized to protein concentration of the homogenates as determined with bicinchoninic acid reagent (BCA; Pierce) with BSA standards.

Table 1. ANOVA of *in vitro* COX activity as a function of source (population of origin) of CYC, source of COX, and temperature

Effect	df	SS	MS	F	*P*
CYC	1	0.001	0.001	2.21	0.150
COX	1	5.5×10^{-5}	5.5×10^{-5}	0.217	0.645
Temp	1	0.021	0.021	80.7	0.0001
CYC × COX	1	0.036	0.036	141.9	0.0001
CYC × temp	1	0.014	0.014	56.5	0.0001
COX × temp	1	0.024	0.024	94.8	0.0001
CYC × COX × temp	1	9.1×10^{-5}	9.1×10^{-5}	0.359	0.5548
Residual	24	0.006	0.0002	—	—

SS, sum of squares; MS, mean squares.

Results

Our results clearly establish that the functional properties of COX and CYC vary significantly between copepod populations. Three-way analysis of variance indicated that there was a significant effect of temperature on COX activity (Table 1). Although the main effects of source of cytochrome oxidase and source of cytochrome *c* were not significant, the two-way interactions between these effects were highly significant. The bases for these interactions are immediately apparent from the graphs in Fig. 1. The activity of SC COX when incubated with SC CYC was consistently higher than the activity of SC COX with SD CYC, regardless of the temperature at which activity was measured. In contrast, whereas SD COX had similar activities with the two CYC variants at 25°C, the activity with SD CYC was 3-fold higher than with SC CYC at 18°C.

The second major result is that intra-population COX/CYC protein pairs consistently show higher activity than the interpopulation pairs (Fig. 2). The reduction in activity due to interpopulation protein pairings depends on both the specific pairing and the temperature. When intra-population SC CYC/SC COX activity is normalized to 1.0, the relative activity of the SC CYC/SD COX pair was reduced by 50% at 18°C but only by 14% at 25°C. In contrast, when SD CYC/SD COX activity was normalized to 1.0, the activity with the SD CYC/SC COX pair was reduced by 70% at 18°C; the reduction was not statistically significant at 25°C. The consistent reduction of activity between interpopulation pairs is strong evidence for CYC/COX coadaptation within the SC and SD populations.

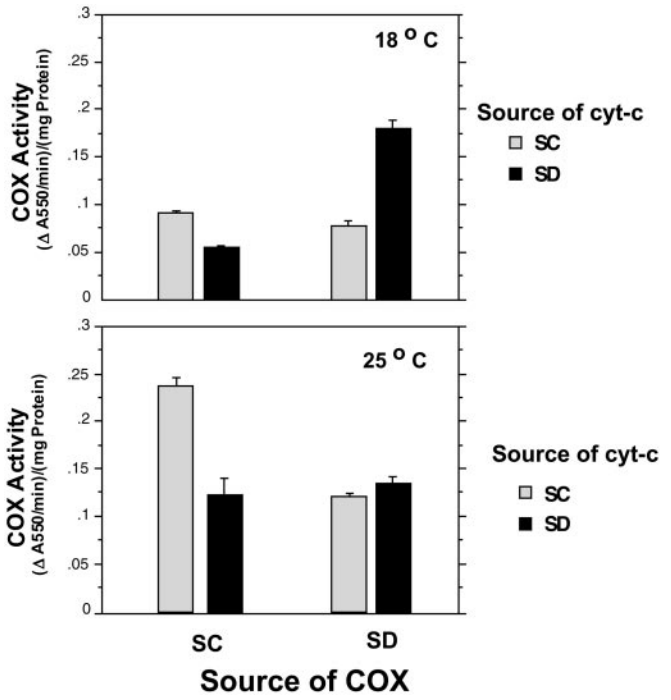

Fig. 1. COX activity (mean ± SE, $n = 4$ measurements per treatment) as determined for pairwise combinations of CYC and COX from SCN and SD populations at two different temperatures.

Discussion

The high levels of population divergence observed in *T. californicus* indicate that geographic populations of this species have long independent evolutionary histories, making it an attractive model system for investigation of coadaptive evolution in allopatry. Although we cannot directly determine whether genetic changes in either CYC or COX were initially favored by selection or fixed by random drift, we can address two questions: (*i*) Do known sequence differences in CYC and COX have functional consequences? (*ii*) Are the CYC/COX pairs found within populations coadapted?

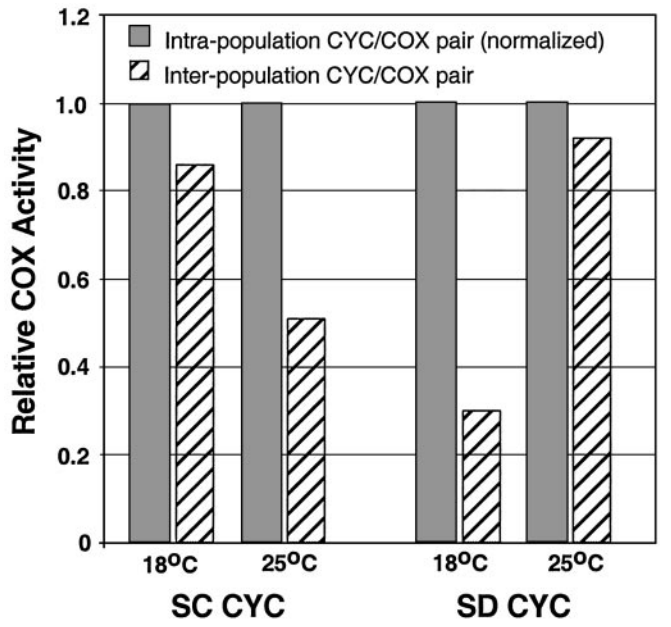

Fig. 2. Relative activities of pairwise combinations of SC and SD CYC and COX. For CYC/COX/temperature combination, mean activity of the intrapopulation pairing was normalized to 1.0. Interpopulation CYC/COX pairs all resulted in reduced activity.

	Residue Number					
	43	44	45	46	47	48
CYC Variant						
SC	Gln	Ala	Ala	Gly	Phe	Asn
SD	Lys	.	.	.	Tyr	Ser

Fig. 3. Amino acid composition of the expressed CYC variants at positions 43–48. All other positions were invariant.

Our results clearly suggest that there are functional consequences of the observed variation. Although some caution is required in interpreting *in vitro* results based on crude homogenates and partially purified proteins, many potential problems are circumvented by our experimental design. For example, one could question whether crude COX preparations from different populations vary in concentrations of inhibitory substances; although this scenario would produce a significant main effect (for COX), a significant COX/CYC interaction would not be expected. Similarly, differences in purity among the CYC preps would not produce a significant COX/CYC interaction. The consistently significant two-way interactions and the consistently higher activities observed in the within-population protein pairs (relative to between population pairs) strongly support our interpretation of coadaptive evolution within SC and SD populations.

How much genetic change underlies CYC/COX coadaptation? The populations of *T. californicus* studied here are known to be highly divergent at a number of gene loci (7, 11, 17), although nuclear ribosomal genes, 18S and 28S, differ at only one base of over 1,500 surveyed (E. Metz and R.S.B., unpublished results). COX is a large protein composed of 13 subunits (18). We have no information on the interpopulation variability of the 10 COX subunits encoded in the nuclear genome. However, full-length DNA sequences of the *COXII* subunit gene (mtDNA-encoded, 226 codons) indicate that the SC and SD proteins differ by 18 aa substitutions (11). *COXI*, also encoded in the mtDNA, differs at 12 of 518 aa residues (unpublished results). Hence, a minimum of 30 aa differ between SC and SD COX enzymes. At present, we have no system for examining the effects of specific amino acid substitutions in COX, either singly or in combination. In contrast, CYC is a small protein (105 aa including the initial f-met), and differences between populations vary from 1 to 5 aa substitutions (12). The inferred amino acid sequence differences among the expressed CYC proteins are shown in Fig. 3; the reciprocal coadaptation observed between SC and SD appears to rest on one or more of the 3 aa substitutions between the respective CYC proteins: glutamine to lysine (polar to basic) at position 43, phenylalanine to tyrosine (hydrophobic to polar) at position 47, and asparagine to serine (polar to polar) at position 48. Models of the interaction between CYC and COX have not identified a specific functional role for these residues; however, the observed changes (especially the charge change at position 43) may effect the electrostatic forces important to transient protein/protein interactions associated with electron transfer (19).

Do the differences in ETS protein function result in reduced fitness in interpopulation hybrids? Recent studies indicate that *CYC* genotypic viabilities differ in reciprocal interpopulation crosses (14); however, the activities of CYC/COX pairs determined here do not accurately predict the viability differences. A variety of factors probably contribute to the discrepancy, but among the most important are the following: (*i*) in the viability experiments, each CYC is reacting with a hybrid COX enzyme

comprised of known mtDNA encoded subunits but a mix of nuclear subunits from the parent populations, and (*ii*) viability of *CYC* genotypes will also depend on interactions of CYC with proteins other than COX, most notably the ETS cytochrome *c* reductase complex. Although the CYC/COX interaction gives strong evidence for coadaptation, we have not yet directly demonstrated that the changes in COX reaction rates result in differences in ETS flux or organismal energy balance.

Natural populations may undergo coadaptation in response to differentiation of their genetic backgrounds, regardless of whether those differences arise in response to genetic drift or in response to selection for adaptation to local environments (e.g., refs. 5 and 11). Coadaptation in metabolic pathways may be widespread, and interpopulation hybridization will sometimes lead to suboptimal function. When genetic differences lead to reduced performance of central energy-producing pathways such as the mitochondrial ETS, we can expect reduced hybrid fitness. Numerous previous studies have considered the coevolution of ETS proteins; most of this work has focused on sequence analyses and correlations in rates of change among interacting proteins across evolutionary lineages (e.g., refs. 20–23). Functional analyses like those presented here have been limited to comparisons of distant taxa, such as primate vs. non-primate COX/CYC interactions (24) or to analyses of manipulated human/ape cell lines (e.g., ref. 25). Our results indicate that functional coadaptation of ETS proteins can be an important property of evolutionary lineages within nominal species, especially where strong population genetic divergence occurs. To the extent that fitness is related to the function of central metabolic pathways (26), disruption of such pathways in hybrids may represent a significant class of incompatibilities expressed during hybrid breakdown.

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