



New primers for amplification of cytochrome c oxidase subunit I barcode region designed for species of Decapoda (Crustacea)

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ABSTRACT

We designed 14 new primers for amplification of the COI barcode region of decapod crustacean species. We tested, with high level of success, the generation of $\sim 640 \pm 49$ base-pair sequences in selected groups of decapods (hermit crabs, squat lobsters, marine and freshwater crabs and shrimps), encompassing representatives of 27 genera of 15 families, 11 of Pleocyemata (Anomura, Brachyura, and Caridea) and 4 of Dendrobranchiata. Based on the results we expect the applicability of these primers for several studies with different taxa within Decapoda.

KEY WORDS

COI, DNA Barcoding, molecular markers, molecular techniques.

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During the last three decades, molecular techniques have become a large, and in some cases, indispensable ally for advances in our knowledge of biodiversity. There is sufficient available literature providing evidence for the suitability and credibility of DNA-based investigations at different taxonomic levels. Among those taxa for which molecular analyses have proven their efficiency and allowed innumerable advances in different areas is a diverse and species-rich group: decapod crustaceans. The molecular methodological support has helped to advance knowledge about many aspects of this taxon, including systematics, biogeography, ecology, conservation, and taxonomy by the identification of larvae and eggs, cryptic species, and damaged specimens.

Since 2011, the Laboratory of Bioecology and Systematics of Crustaceans (LBSC) has been involved in two long-term projects aiming the characterization of the marine and estuarine decapod crustaceans biodiversity of the Brazilian coast, supported by the Brazilian agencies “Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP)” through Biota-FAPESP Program and “Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES)” through Ciências do Mar II. Both projects made use of combined analysis techniques to elucidate various aspects of the life cycle and evolution in decapod crustaceans, and molecular analyses were one of the main tools to support the assumptions of these studies. To this end, we attempted to generate DNA sequences of two mitochondrial markers, the barcode region of cytochrome c oxidase subunit I (COI) and a fragment of 16S rRNA, of all decapod species sampled along the coast of São Paulo in order to develop a genetic library to serve as base line to all researchers in this field (F. Mantelatto *et al.*, unpubl. data). However, particularly for the COI region, we had considerable difficulties in obtaining successful amplifications using some previous standard universal pairs of primers: LCO1-1490/HCO1-2198 (Folmer *et al.*, 1994) or COL6b/COH6 (Schubart and Huber, 2006), designed for crayfishes based on the primers of Folmer *et al.* (1994).

Thus, we designed 14 new primers for the COI barcode region (Tab. 1, Fig. 1), five of them with degenerate bases. Four nucleotides of the previously designed primer COL6b (5'-ACAAATCATAAAGATATYGG-3') (Schubart

and Huber, 2006) were replaced by variable bases to constitute the primer COL6b2. The primers COIAL2o, COIAH2o, and COIAH2m were designed using the software NetPrimer, available at the PREMIER Biosoft International website <<http://www.premierbiosoft.com/netprimer>>. The others were designed using the Primer-Blast software tool developed at NCBI, which generates target-specific primer pairs (available at <<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>>) (see Ye *et al.*, 2012 for further details).

All decapod specimens used for DNA sequencing were preserved in 70–80% ethanol. For each COI amplification, the polymerase chain reaction (PCR) was performed in reactions containing 0.5 µl of AmpliTaq DNA polymerase Thermo Fisher, 1 µl of each primer (20 mM), 2 µl of bovine serum albumin 1%, 3 µl of 10X Taq Buffer [(NH₄)₂SO₄ or KCl], 3 µl of MgCl₂ (25 mM), 4 µl of dNTP (5 mM), 4.5 µl of ultrapure water, 5 µl of betaine (5 M) and DNA volume according to extraction quality, with the following thermal cycle: initial denaturing for 2 min at 94°C; pairing for 35–40 cycles [45 s at 94°C, 45 s at 38°C–60°C (see Tab. 1 for details), and 1 min at 72°C]; final extension 10 min at 72°C.

The applicability of these primers was highly satisfactory for the amplification of fragments from 584 to 712 base pairs, given the diversity of species, genera and families used as models (see Tab. 1). These new primers also showed good performance for samples from different populations and geographic regions.

According to this scenario and considering many other projects and publications that are in progress by our team, we are convinced that the new primers presented herein were successful in amplifying the target species and have proven their utility for several studies with different taxa within Decapoda. In addition, these new primers may help in different ways: 1) they have been used and may be useful in future studies to obtain comprehensive phylogenies and/or biogeographical variability of specific target genera and species; 2) to avoid pseudogenes during amplifications, since the occurrence of pseudogenes strongly decreases when using taxon specific (optimized) primers (Schubart, 2009); 3) based on our experience from the data obtained during this research, and pending future tests, we can speculate that some of the present primers can be used for other related genera and species.

Table 1. New primers of cytochrome c oxidase subunit I and the taxonomic groups with successful amplifications showed by pairs of primers. F: forward primer; R: reverse primer. PMT: Primer Melting Temperature; *used with COH6 (Schubart and Huber, 2006). Degenerate bases: Y = C or T, R = A or G, W = A or T. †“Turk” is in reference to the past researcher Dr. Michael Türkay who contributed significantly to studies on crustaceans, in especial on freshwater crabs.

Name	Sequence (5'→3')	Size (bp)	PMT (°C)	Used PMT (°C)	Family	Species
COI – Turk2 (F) [†]	GGAGCITGAGCAGGTATAGTAGG	617	59.7	55.0–59.0	Pseudothelphusidae	<i>Allacanthos</i> spp., <i>Fredius</i> spp., <i>Ptychophallus</i> spp., <i>Potamocarcinus</i> spp.
COI – Turk1 (R) [†]	TAAAATAGGGTCTCCACCCCCAG		61.2			
COILCH 1 (F)	TCGAGCAGAATTAGGTCAACCAG	584	60.4	58.0–60.0	Portunidae	<i>Charybdis hellerii</i> (A. Milne-Edwards, 1867)
COIHCH 1 (R)	GYTAAAGAACGGGTCRCCTC		59.8			
COILCH 2 (F)	CCAGACACTTTATTTTGGAGCTTG	651	59.1	52.0–57.0	Portunidae	<i>Charybdis hellerii</i>
COIHCH 2 (R)	ATGTTGGTAGAGGACGGGGT		60.2			
COL6b2 (F)*	ACWAAYCAYAAAGAYATYGG	680	54.3	48.0–50.0	Alpheidae	<i>Alpheus</i> spp., <i>Synalpheus</i> spp.
					Diogenidae	<i>Clibanarius antillensis</i> Stimpson, 1859
					Pandalidae	<i>Plesionika longicauda</i> (Rathbun, 1901)
COIAL1o (F)	GAGCITGAGCCGGAATAGTAGG	606	59.5	48.0–50.0	Sergestidae	<i>Acetes americanus</i> Ortmann, 1893, <i>Peisos petrunkevitchi</i> Burkenroad, 1945
COIAH1o (R)	CTCCAGCAGGGTCAAAGAAAAGA		57.7			
COIAL1m (F)	GAGCITGAGCYGGRATAGTAGG	606	62.9	48.0–50.0	Hippolytidae	<i>Tozeuma carolinense</i> Kingsley, 1878
					Penaeidae	<i>Litopenaeus schmitti</i> (Burkenroad, 1936)
					Pinnotheridae	<i>Clypeasterophilus stebbingi</i> (Rathbun, 1918)
COIAH1m (R)	CTCCWGRGGGTCAAAGAAAAGA	606	61.3	48.0–50.0	Sergestidae	<i>Acetes americanus</i> , <i>Peisos petrunkevitchi</i>
					Sicyoniidae	<i>Sicyonia</i> spp.
COIAL2o (F)	ACGCAACGATGATTATTCTAC	712	56.4	38.0–50.0	Alpheidae	<i>Alpheus</i> spp., <i>Salmoneus carvachoi</i> Anker, 2007, <i>Synalpheus</i> spp.
					Diogenidae	<i>Clibanarius antillensis</i> , <i>Paguristes tortugae</i> Schmitt, 1933, <i>Pseudopaguristes calliopsis</i> (Forest and de Saint Laurent, 1968)
					Hippolytidae	<i>Hippolyte</i> spp., <i>Latreutes</i> spp.
					Munididae	<i>Munida</i> spp.
					Paguridae	<i>Pagurus exilis</i> (Benedict, 1892)
					Palaemonidae	<i>Leander paulensis</i> Ortmann, 1897, <i>Nematopalaemon schmitti</i> (Holthuis, 1950)
COIAH2m (R)	GACCRAAAAATCARAATAAATGTTG	712	59.8	38.0–50.0	Penaeidae	<i>Xiphopenaeus kroyeri</i> (Heller, 1862)
					Processidae	<i>Processa hemphilli</i> Manning and Chace, 1971
					Sergestidae	<i>Acetes americanus</i> , <i>Peisos petrunkevitchi</i>
					Sicyoniidae	<i>Sicyonia</i> spp.
					Solenoceridae	<i>Pleoticus muelleri</i> (Spence Bate, 1888)
COIAL2o (F) cited above		712	57.7	44.0–46.0	Penaeidae	<i>Xiphopenaeus kroyeri</i>
COIAH2o (R)	GACCAAAAATCAGAATAAATGTTG				Sergestidae	<i>Acetes americanus</i> , <i>Peisos petrunkevitchi</i>
					Solenoceridae	<i>Pleoticus muelleri</i>

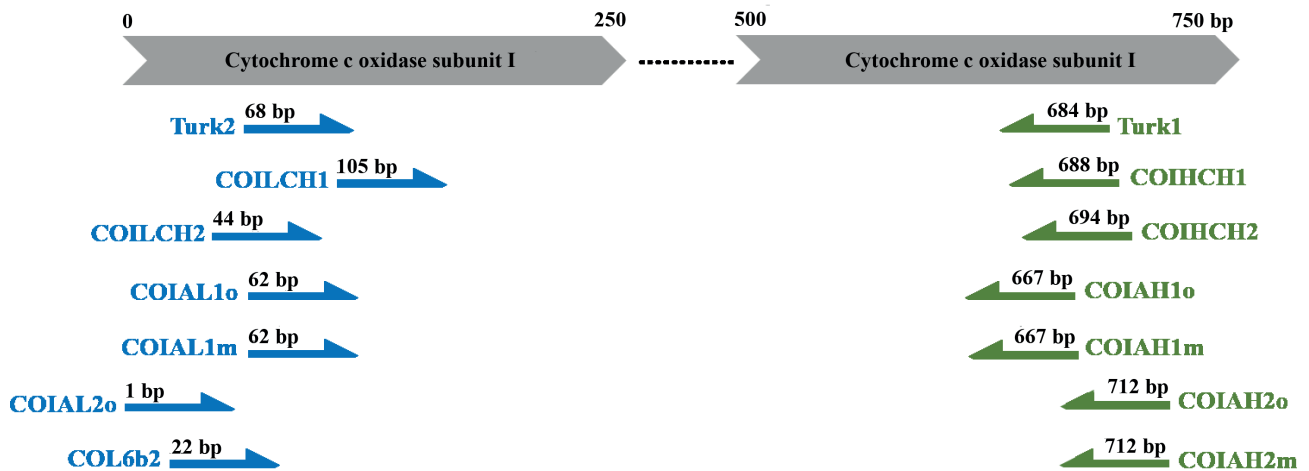


Figure 1. First 750 base pairs of the mitochondrial gene cytochrome c oxidase subunit I showing the primers' alignment region. Blue and green arrows represent the forward and reverse primers, respectively. Numbers above arrows indicate the first nucleotide position where the primers align to the DNA. The dotted line represents 250 base pairs.

Our results evidenced that a successful amplification of the COI region from decapod crustaceans is not always achieved using the universal primers. Therefore, we are happy to share our new findings with the carcinological community. The new primers may contribute to improve the quality and efficiency of molecular markers, aiming to advance the knowledge of evolution of decapod crustaceans and leading to the solution of several systematic issues.

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