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Optimization and evaluation of a method for the generation of DNA barcodes for the identification of crustaceans☆

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ABSTRACT

Efficient methods for the species identification of seafood are important for ensuring food safety and detecting fraud. DNA barcoding, a technique based on PCR amplification and capillary sequencing of a short, standardized segment of a gene, is a reliable method for species identification of processed and packaged seafood when the species cannot be determined visually. Here we report the optimization and evaluation of a DNA barcoding method for identifying representative commercial decapod crustacean species, including select shrimp, crab, crayfish, and lobster. Two different segments of the mitochondrial cytochrome *c* oxidase 1 (COI) gene were evaluated —a 655 base pair fragment beginning near the 5′ end, representing the "standard" DNA barcode fragment, and a non-overlapping 475 base pair fragment beginning near the 3′ end, representing an alternative DNA barcode marker for shrimp. The standard 5′ fragment was successfully amplified and sufficient to identify most crustacean species tested, but amplification of both the 5′ and 3′ barcodes were often required to efficiently identify shrimp.

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1. Introduction

Seafood is one of the most highly traded commodities in the world. In the interest of public health, it is vital that both domestically processed and imported seafood is safe, wholesome, and properly identified through labeling. To aid in the proper labeling of seafood, the Food and Drug Administration (FDA) maintains a list of acceptable market names for seafood sold in U.S. interstate commerce: The Seafood List (http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/ucm113260.htm). Commercial seafood is often processed in a manner that precludes traditional morphological identification of species, so reliable analytical methods are required. One of the earliest analytical identification methods used for regulatory purposes was protein profiling by isoelectric focusing, or IEF (AOAC, 1980). This analytical technique requires subjective interpretations of gel results and the inclusion of perishable frozen tissue standards in each run. Species identification methods such as DNA barcoding have come into favor because they have improved specificity, and they are more objective and more rugged.

A region near the 5′ end of the cytochrome *c* oxidase subunit 1 gene (COI) has been shown to be highly effective in species identification (Hebert, Cywinska, Ball, & deWaard, 2003a; Hebert, Ratnasingham, & deWaard, 2003b). FDA has demonstrated the utility of DNA barcoding with this COI region for seafood identification (Yancy et al., 2008), has validated a standardized protocol for DNA barcode generation in fish (Handy et al., 2011) and is currently in the process of building a library of reference sequence standards suitable for regulatory use (Deeds, Handy, Fry, Granade, Williams, Powers, et al., 2014). As the fish method has proven to be a valuable regulatory tool for uses ranging from product label confirmations (http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/Seafood/ucm419982.htm) to the identification of meal remnants involved in outbreaks of illness (Cohen et al., 2009; Cole, Heegaard, Deeds, McGrath, & Handy, 2015), there is interest in extending it to other types of seafood, particularly crustaceans.

Numerous researchers have found that DNA barcoding using the COI gene is effective for identification of crustaceans. Thorough investigations have been conducted at the levels of order, family, genus, and species (Costa et al., 2007; da Silva et al., 2011; Meyer, Weis, and Melzer et al., 2013). COI DNA barcoding of crustaceans has also been used successfully in studies of regional variation and on animals at different life stages (Kumar, John, Khan, Lyla, & Jalal,

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2012; Radulovici, Sainte-Marie, & Dufresne, 2009). Crustaceans show greater sequence variation in the COI DNA barcoding region than many animal groups. They have a high ratio of inter/intra-specific sequence divergence, referred to as the "barcode gap," and average sequence divergence has been found to be 17% between different species of the same genus and 0.46% within species (Costa et al., 2007; da Silva et al., 2011). Though COI sequence variation in crustaceans has generally proved favorable to barcoding, successful PCR amplification of the standard 5′ COI DNA barcoding fragment has proven to be a challenge in some cases, particularly in decapods. Investigators have primarily addressed this through minor modification of PCR primer sequences, the use of numerous and/or mixed primers, and variations in reaction composition or thermal cycling (Costa et al., 2007; da Silva et al., 2011; Radulovici et al., 2009). However, most published reports do not clearly indicate which conditions were or were not effective for particular species or groups. DNA barcoding of some shrimp species has been successfully carried out via amplification of a non-overlapping fragment beginning near the 3′ end of the COI gene (Fig. 1). Data obtained from seven metapenaeopsis species indicate that sequence divergence in this 3′ COI fragment ranges from 6 to 20%, and averages 16% (Tong, Chan, & Chu, 2000). However, this region has not been evaluated in decapod crustacean groups outside of shrimp.

While DNA barcoding of either the 5′ or 3′ end of the COI gene, depending on the taxonomic group, shows promise for the identification of a wide range of commercial crustacean species, no single set of conditions has been shown to work consistently across the cited studies, or even within a given study. Lack of a standardized procedure makes it difficult to compare results between studies and between species, complicates the study of crustaceans using the valuable technique of DNA barcoding, and precludes the use of barcoding in crustaceans for regulatory purposes. The purpose of the work described here was to establish and evaluate such a standardized procedure, and this is the first study we know of to report a method that works consistently with different crustaceans. Here we have: 1) established a single, standardized procedure which can be used to successfully DNA barcode a wide variety of crustacean species, and 2) demonstrated that the standardized procedure can be used to effectively differentiate crustaceans by evaluating variation both between and within species. In addition, we report on a more novel 3′ COI barcode fragment which is especially beneficial for shrimp identification but has received little attention in previous work on crustacean DNA barcoding. With respect to this 3′ COI fragment, we have: 1) generated a previously unreported reference for sequence alignments and demonstrated its utility in 3′ COI barcoding of shrimp, 2) demonstrated that the 3′ COI fragment can be used to differentiate shrimp by evaluation of variation both within and between species, and 3) provided the first direct comparison in crustaceans using results from the 3′ COI fragment and the 5′ COI fragment.

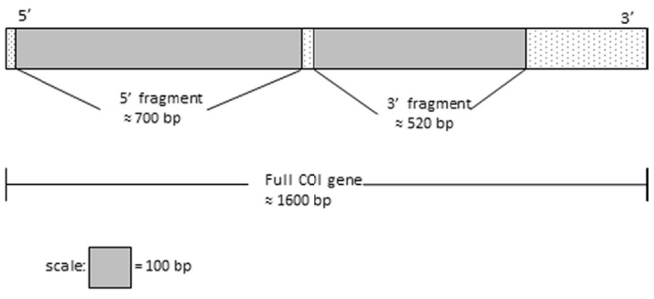

Fig. 1. Schematic showing the entire cytochrome *c* oxidase subunit 1 (COI) gene and locations of the 5′ and 3′ barcoding fragments used in this study.

2. Materials and methods

2.1. Primers

Primers used for amplification of the 5′ COI DNA barcode, the 3′ COI DNA barcode, and the sequencing reaction are given in Table 1.

2.2. Reference sequences

For the 5′COI standard barcoding fragment, the reference trimming sequence previously used for fish was found to be applicable for this work; for the 3′COI shrimp barcoding fragment, a new reference trimming sequence was generated (Table 1). The new 3′ reference sequence was created by generating a consensus sequence from all shrimp sequences obtained as part of this study; forward and reverse primers were aligned to the consensus and trimmed off. The new 3′ shrimp reference sequence was tested using all available overlapping shrimp and shrimp-like crustacean sequences from GenBank (http://www.ncbi.nlm.nih.gov/genbank/), including 216 sequences from 10 genera of shrimp (*Crangon*, *Farfantepenaeus*, *Fenneropenaeus*, *Litopenaeus*, *Metapenaeopsis*, *Penaeus*, *Pleoticus*, *Stenopus*, *Solencera* and, *Upogebia*) and 2 genera of krill (*Nyctiphanes* and *Thysanoessa*); all produced successful alignments.

2.3. Samples

Tests were conducted on a variety of commercial decapod crustacean species (Table 2). All samples were acquired from either commercial sources or from research cruises in the Gulf of Mexico, USA (provided as a generous donation from the National Marine Fisheries Service-National Seafood Inspection Laboratory, Pascagoula, MS). To confirm the identity of crustacean samples used in this study, sequences from each sample were compared to publically available COI sequences found in Genbank, the Barcode of Life Datasystems (BOLD) database (www.boldsystems.org; Ratnasingham & Hebert, 2007), or to sequences from the FDA Reference Standard Sequence Library (RSSL) for Seafood found at http://www.fda.gov/Food/FoodScienceResearch/DNASeafoodIdentification/ucm238880.htm.

2.4. Optimization

Optimization experiments were carried out prior to the formal evaluation because published papers on crustacean DNA barcoding included such a wide variety of experimental conditions (Costa et al., 2007; da Silva et al., 2011; Radulovici et al., 2009), and those conditions found to be effective in FDA's method for generating DNA barcodes for fish (Handy et al., 2011) did not consistently yield high quality DNA barcodes with all crustacean samples (data not shown). Optimization included DNA extraction, COI PCR, and cycle sequencing reaction conditions (Table 3).

2.5. Method evaluation

To assess method precision (measured as sequence variability) for the same sample between multiple sequencing runs, tissue from a single individual was sampled on four different days, then sequenced and analyzed in four independent experiments (Table 2; 1 individual, 4 replicates). This set of experiments established: a) day-to-day repeatability, b) the ability of the method to reliably differentiate a variety of crustacean species, and c) differences in performance between the 5′ and 3′ COI barcoding fragments. To