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Molecular species identification of commercially important penaeid shrimp from the Gulf of Mexico using a multiplex haplotype-specific PCR assay

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ABSTRACT

This study describes a multiplex PCR assay based on the 16S rRNA mitochondrial gene to identify the penaeid shrimp *Farfantepenaeus aztecus*, *Farfantepenaeus duorarum*, *Farfantepenaeus brasiliensis* and *Litopenaeus setiferus*, all native to the Gulf of Mexico, and the exotic *Litopenaeus vannamei*. The assay was validated using positively identified adult shrimp and confirmed by direct sequencing. Samples of postlarvae and early juveniles collected in the eastern and western Gulf of Mexico were tested yielding 119 *F. aztecus*, 78 *F. duorarum* and five *L. setiferus*. Reliable identification of the morphologically similar early life stages of *F. aztecus* and *F. duorarum* has important implications for management and conservation. Similarly, the ability to identify *L. vannamei* is relevant as early detection could help minimize the ecological impact if this species escapes to the wild.

1. Introduction

Multiplex polymerase chain reaction (PCR) is a widely used PCR variant aimed to amplify multiple DNA segments of interest by using more than one pair of primers in a single reaction (Chamberlain et al., 1988). This technique has been typically used in genotyping applications that require the simultaneous analysis of multiple loci. However, single-locus multiplex PCR assays to genotype nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) have also been developed by utilizing taxonomically informative nucleotide differences along the target to position the 3′-end of species-specific primers to generate diagnostic banding profiles (Bottema et al., 1993; Rocha-Olivares, 1998). Accordingly, multiplex PCR assays targeting mtDNA permit the rapid and unambiguous identification (ID) of species belonging to a diverse array of taxonomic groups (Rocha-Olivares, 1998; Hare et al., 2000; Shivji et al., 2002; Hyde et al., 2005). In the present study, a single-locus multiplex PCR was used to ID several species of penaeid shrimp (order Decapoda, suborder Dendobranchiata, family Penaeidae) found in the Gulf of Mexico.

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The penaeid shrimp fishery in the Gulf of Mexico, consisting primarily of brown shrimp (*Farfantepenaeus aztecus*), pink shrimp (*Farfantepenaeus duorarum*) and northern white shrimp (*Litopenaeus setiferus*), is the second most valuable fishery in the U.S. (Anonymous, 2004). Despite their economic and ecological importance, there is substantial uncertainty about the seasonal and geographical patterns of recruitment of these species in the Gulf of Mexico, with much of this controversy associated with discrimination of closely related species during the postlarval (PL) and early juvenile stages (Ditty and Alvarado Bremer, 2011). In addition, the use of the Pacific white shrimp (*Litopenaeus vannamei*) in aquaculture operations in the southeastern U.S. represents a risk to native wild shrimp populations since there is potential for accidental release, and the introduction of disease pathogens and various bacterial, fungal, and viral infections known to be carried by this species (Lightner et al., 1983; Lightner, 1993, 1996; Overstreet et al., 1997; JSA, 1997). Accordingly, the ability to discriminate early stages of shrimp could be used to characterize both spatial and temporal patterns of recruitment, as well as to reveal the presence among larval surveys of exotic species. Such information could be used to improve the management of overfished populations by helping to better delineate both recruitment areas and seasons, as well as to help establish mechanisms to ameliorate the impact of introduced species. In the present study we developed a multiplex haplotype-specific PCR (MHS-PCR; Rocha-Olivares, 1998) assay to discriminate five species of penaeid shrimp. Four of these species, namely *F. aztecus*, *F. duorarum*, the pinkspot shrimp (*Farfantepenaeus brasiliensis*), and *L. setiferus* are native to the Gulf of Mexico and the Florida coast, whereas *L. vannamei* is an introduced species commonly used in aquaculture operations in Florida and Texas (Perry, 2009).

2. Material and methods

2.1. Collection of reference samples and DNA extraction

Adults of *F. aztecus* ($n = 6$) and *F. duorarum* ($n = 6$) and *L. setiferus* ($n = 5$) were collected in Galveston Bay, Texas, and *F. brasiliensis* ($n = 6$) in Johnson Bay, St. John's, U.S. Virgin Islands. Adults of *L. vannamei* ($n = 6$) came from the Texas A&M shrimp farm in Corpus Christi, Texas (Table 1). All specimens were stored in 95% ethanol, except the *L. vannamei* reference specimens, which were preserved in 70% isopropyl alcohol. Total DNA was extracted from axial muscle by digesting 5–10 mg of tissue with proteinase-K followed by phenol–chloroform extraction and ethanol precipitation (Sambrook et al., 1989). DNA pellets were eluted in 50 μL of TE buffer (10 mM Tris–HCl [pH 8.0], 1 mM EDTA). The quality of the extractions was verified by running 5 μL of isolated DNA through ethidium bromide-stained agarose gels (1%) visualized using a UV transilluminator.

2.2. PCR amplification and sequencing

Two regions of the mtDNA genome were targeted to identify fixed nucleotide differences that could be used in the design of a shrimp species ID assay. Initially, a segment of the cytochrome oxidase I (COI) gene was amplified using the primers CO9 and CO10 (Baldwin et al., 1998) as described in Hunter et al. (2008). Sequence analysis of this fragment revealed the presence of the recognition sites for the restriction endonucleases *Apo*I and *Mfe*I (New England Biolabs) that resulted in restriction fragment length polymorphisms (RFLPs) diagnostic for *F. aztecus*, *F. duorarum* and *L. setiferus* (Fig. 1). However, the ID of other species of penaeid shrimp called for additional restriction endonucleases increasing the cost and complicating the PCR–RFLP assay. Other molecular assays, such as amplified fragment length polymorphisms (AFLP), have been employed to identify penaeid shrimp (Wang et al., 2004), but this technology involves multiple steps, including restriction assays, ligation to adapters, PCR, and polyacrylamide gels. Alternatively, the potential to develop a multiplex PCR assay for species identification was explored because of its inherent simplicity. However, analysis of the COI gene revealed difficulties in identifying stretches of sequence that met the minimum criteria for the design of diagnostic primers (see Section 2.3). Instead, a segment of the 16S rRNA (16S) mitochondrial gene was amplified using primers 16SarL and 16SbrH (Palumbi et al., 1991) for a representative sample of each of the species of interest (Table 1). These 16S sequences were aligned in BioEdit (Hall, 1999), against the orthologous sequences for these species characterized by Maggioni et al. (2001) with GenBank accession numbers: AF279811–AF279812, AF279818, AF192051–AF192056, AF192071, AF192087–AF192089, AF255054–AF255057, AJ297970–AJ297971, AJ132780 and A40446914. PCR cycling parameters for 16S are those in Hunter et al. (2008). The quality of amplified products was verified by gel electrophoresis. Negative controls were included in all reactions to screen for cross-contamination. Amplicons selected for sequencing were cleaned and cycle sequenced as described in Viñas et al. (in press).

Table 1
Data for the adult reference specimens employed to design a multiplex PCR assay for species ID. GenBank accession numbers correspond to the distinct haplotype sequences (*M*) of the mtDNA 16S rRNA gene characterized for each species from *n*-individuals.

Species	*n*	*M*	Locality of capture	Date of capture	GenBank accession numbers
F. aztecus	6	1	Galveston Bay, Texas	Fall 2000	HM014401
F. brasiliensis	6	4	Johnson Bay, St. John, U.S. Virgin Islands	June 11, 2003	HM014402–014405
F. duorarum	6	2	Galveston Bay, Texas	Fall 2000	HM014406–014407
L. setiferus	5	2	Corpus Christi, Texas	June 16, 2003	HM014408–014409
L. vannamei	6	3	Texas A&M Shrimp Farm, Corpus Christi, TX	July 10, 2003	HM014410–014412