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

Jaime R. Alvarado Bremer a,b,*, James G. Ditty c, Jennifer S. Turner a, Brandon L. Saxton b

a Department of Marine Biology, Texas A&M University, Galveston, TX 77551, USA
b Department of Wildlife and Fisheries Sciences, Texas A&M University, College Station, TX 77843, USA
c National Oceanographic and Atmospheric Administration, National Marine Fisheries Service, 4700 Avenue U, Galveston, TX 77551, USA

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.

Article info

Article history:
Received 2 October 2009
Accepted 15 May 2010

Keywords:
Multiplex PCR
Penaeid shrimp
Species identification
Farfantepenaeus aztecus
F. brasiliensis
F. duorarum
Litopenaeus vannamei
L. setiferus
Brown shrimp
Pinkspot shrimp
Pink shrimp
Northern white shrimp
Pacific white shrimp

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.

* Corresponding author. Department of Marine Biology, Texas A&M University, 5007 Ave. U, Galveston, TX 77551, USA. Tel.: +1 409 740-4958; fax: +1 409 740-5002.
E-mail address: alvaradj@tamug.edu (J.R. Alvarado Bremer).
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 (MHSPCR; 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 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 Apol and Mfel (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, AF297970–AF297971, 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

<table>
<thead>
<tr>
<th>Species</th>
<th>n</th>
<th>M</th>
<th>Locality of capture</th>
<th>Date of capture</th>
<th>GenBank accession numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>F. aztecus</td>
<td>6</td>
<td>1</td>
<td>Galveston Bay, Texas</td>
<td>Fall 2000</td>
<td>HM014401</td>
</tr>
<tr>
<td>F. brasiliensis</td>
<td>6</td>
<td>4</td>
<td>Johnson Bay, St. John, U.S. Virgin Islands</td>
<td>June 11, 2003</td>
<td>HM014402–014405</td>
</tr>
<tr>
<td>F. duorarum</td>
<td>6</td>
<td>2</td>
<td>Galveston Bay, Texas</td>
<td>Fall 2000</td>
<td>HM014406–014407</td>
</tr>
<tr>
<td>L. setiferus</td>
<td>5</td>
<td>2</td>
<td>Corpus Christi, Texas</td>
<td>June 16, 2003</td>
<td>HM014408–014409</td>
</tr>
<tr>
<td>L. vannamei</td>
<td>6</td>
<td>3</td>
<td>Texas A&amp;M Shrimp Farm, Corpus Christi, TX</td>
<td>July 10, 2003</td>
<td>HM014410–014412</td>
</tr>
</tbody>
</table>
2.3. Design of a multiplex PCR assay for species identification

The alignment of 475 bp of the 16S mtDNA gene revealed the presence of 72 polymorphic sites, including several fixed differences or apomorphies that could be employed in the design of an MHS-PCR species ID assay. Initially, the design of diagnostic primers for each species involved identifying stretches of sequence that met the following criteria: 1) Contain at least one apomorphy at the 3’-end of the priming site of each species-specific primer; 2) Have a melting temperature of at least 50 °C; 3) Yield amplification products (amplicons) that differ in size among species by at least 30 bp; and, 4) Free of stable secondary structures including hairpins, self-dimers, and hetero-dimers. Primer parameters were estimated with OligoAnalyzer 3.1 (available at www.idtdna.com). Each species-specific primer was tested individually with the 16S ‘universal’ primer set, and then in combination with the rest of the multiplex primers. Some primers yielded nonspecific amplifications (i.e., false positives) that persisted after increasing the stringency of the PCR reaction to the maximum allowable (i.e., false negatives) with other primer combinations. In all instances, primer dilution eliminated this problem without having to incorporate an additional mismatch at the second nucleotide from the 3’-end as suggested by Cha et al. (1992). Multiplex PCR reactions were optimized as recommended by Henegariu et al. (1997). In total, five multiplex PCR primers (Table 2), one per species surveyed, used in combination with the ‘universal’ 16S primers generated species-specific ‘bands’ differing in size (Fig. 2). The inclusion of the universal primer set was intended as an internal positive control so that amplification failures would not be interpreted as false negatives (Pank et al., 2001; Shivji et al., 2002). Multiplex PCR reactions were initially carried in 12.5 µL volumes containing 0.5 units of Platinum Taq DNA polymerase (Invitrogen), 1 × PCR buffer (Invitrogen), 2.5 mM MgCl₂, 200 µM of each dNTP, 10 pmol of each primer, except for Fbra16S-R, which was diluted to 2.5 pmol, and 1 µl DNA template (= 20 ng). The thermocycling profile was as follows: Initial denaturing step at 94 °C for 4 min, followed by 36 cycles of denaturing at 94 °C for 30 s, annealing at 55 °C for 60 s and extension at 65 °C for 3 min. Amplicons were separated in ethidium bromide-stained 3% agarose gels run at 100 V for 90 min. To simplify the PCR setup when testing larger sample sizes of unknown PL, we used EconoTaq Green Plus (Lucigen Technologies), a DNA polymerase that includes a 2× buffer with all necessary reagents for PCR, inclusive of loading dyes requiring only primers and template. Reaction volumes, concentration of multiplex primers, template amount, and thermocycling parameters were the same as specified above, with the exception of the extension step that was carried at 72 °C.

2.4. Validation of multiplex PCR assay using adult reference samples

After the optimization of the multiplex PCR assay, adult reference specimens (see Table 1) were tested using the multiplex PCR assay. In all instances, the fragment patterns generated from these tests were consistent with the expected banding patterns for the targeted species (see Fig. 2).

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Multiplex PCR primer sequence 5’–3’</th>
<th>Primer direction</th>
<th>Target species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fazt01-R</td>
<td>CGC AAC AAA CAC CAC TTA AT</td>
<td>Reverse</td>
<td>F. aztecus</td>
</tr>
<tr>
<td>Fbra16S-R</td>
<td>CCC CTT AAT CTA TTT AAG CCT TC</td>
<td>Reverse</td>
<td>F. brasiliensis</td>
</tr>
<tr>
<td>Fduo02-R</td>
<td>CAC TCA AAA Gtt AAA TAC TAT TAC AAC</td>
<td>Reverse</td>
<td>F. duorarum</td>
</tr>
<tr>
<td>Lset01-F</td>
<td>AAG AGT TCA TAT CCA CAA GAT CGA</td>
<td>Forward</td>
<td>L. setiferus</td>
</tr>
<tr>
<td>Lvan01-F</td>
<td>TTA GTC TTT TAA TTG GAG GCT C</td>
<td>Forward</td>
<td>L. vannamei</td>
</tr>
</tbody>
</table>
2.5. Species ID of PL and early juvenile penaeid shrimp

We collected a total of 224 PL and early juveniles of penaeid shrimp smaller than 7.0 mm carapace length (CL) by hand-net and benthic sled in the eastern \( n = 32 \) and western \( n = 192 \) Gulf of Mexico from late-February to mid-December. Western Gulf collections were concentrated in Galveston Bay, but extended from the mouth of Calcasieu Lake, Louisiana south-westward to Port Isabel, Texas. Eastern Gulf samples were collected near Panama City, Tampa, and Key West, Florida. Specimens were preserved in chilled 70% ETOH to reduce biological activity and minimize specimen degradation. DNA was isolated from each specimen as described above and then used as the template in the multiplex PCR species ID assay. DNA size markers were included when running gels for fragment size comparison. The accuracy of the multiplex PCR assay was verified by sequencing the 16S gene of four arbitrarily selected individuals of each of the three species identified in the PL and early juvenile collections, namely \( F. aztecus \), \( F. duorarum \), and \( L. setiferus \).

3. Results

Digests of the amplified COI segment with Apol and MfeI resulted in diagnostic RFLPs for \( F. aztecus \), \( F. duorarum \) and \( L. setiferus \) that could be visualized using ethidium bromide-stained agarose gels (Fig. 1). However, since additional penaeid shrimp species potentially present in the Gulf of Mexico could not be diagnosed with these two enzymes, and/or by digesting this COI segment, a multiplex PCR assay that targeted 16S was designed instead. The multiplex PCR assay yielded bands of the expected size for the four native species and for the exotic \( L. vannamei \) (Fig. 3). This included the 563 bp fragment generated by the universal 16S primer set, but only when using the ‘conventional’ PCR setup. By contrast, the amplification of the 563 bp fragment was poor or failed when using EconoTaq Green Plus (Fig. 3; lanes 8–10). The absence of this 16S fragment, included primarily as an internal ‘positive’ control (cf. Pank et al., 2001; Shivji et al., 2002), did not affect the sensitivity of the multiplex PCR assay, as the diagnostic bands were not affected.

Among the 192 PL and juvenile shrimp from the western Gulf of Mexico a total of 118 \( F. aztecus \), 48 \( F. duorarum \) and five \( L. setiferus \) were identified. A total of 21 specimens failed to amplify even after several attempts. We later discovered that these 21 specimens had been preserved in 70% denatured ethanol containing 3.2% methanol (Mallinckrodt Baker, Inc., Phillipsburg NJ), which inhibited PCR. Conversely, the sample of 32 PL and early juvenile shrimp from the eastern Gulf of Mexico consisted of 30 \( F. duorarum \), one \( F. aztecus \) and one \( L. setiferus \). Neither \( F. brasiliensis \) or \( L. vannamei \) were identified among the 224 PL and juveniles examined from the Gulf of Mexico. The species ID obtained with multiplex PCR was corroborated by sequencing the corresponding 16S segment for a representative sample of each of the three species. While in this study we positively identified 197 PL and early juvenile shrimp as \( F. aztecus \) \( (n = 119) \) and \( F. duorarum \) \( (n = 78) \), only 193 specimens were in good physical condition. These well preserved specimens, together with seven more not included here, constituted the sample \( (n = 200) \) used in a separate study to identify a set of morphological characters that would best discriminate early life stages of \( F. aztecus \) and \( F. duorarum \) (Ditty and Alvarado Bremer, 2011).

4. Discussion

Haplotype-specific multiplex PCR is a highly sensitive and rapid method of species identification that has been used to identify the early stages of marine fauna such as rockfishes (Rocha-Olivares, 1998), bivalve larvae (Hare et al., 2000) and billfishes (Hyde et al., 2005). Here, we describe the development of a multiplex PCR assay targeting the 16S mitochondrial gene and demonstrate its utility to identify five species of penaeid shrimp potentially present in the Gulf of Mexico, namely the native \( F. aztecus \), \( F. brasiliensis \), \( F. duorarum \), \( L. setiferus \), and the exotic Pacific white shrimp \( (L. vannamei) \). The multiplex PCR assay generated bands diagnostic for each species (Fig. 3). In addition, the entire 16S rRNA fragment was also amplified, but with substantial variability in yield among specimens. Further, the amplification of this positive control was generally undetectable when employing EconoTaq Green Plus (Fig. 3). The absence of this band did not affect the diagnostic utility of the multiplex PCR assay, since the species-specific bands were not affected. Shivji et al. (2002) encountered a similar problem with their multiplex PCR shark ID assay, with low-yields or undetectable amplification of the positive control when using
silky shark and blue shark as template, possibly due to primer competition. This is not a likely explanation of our results given that the positive control could be amplified with one buffer system, regardless of the species used as template, but not with the other. Henegariu et al. (1997) showed that the ionic strength of the PCR buffer could determine the relative amount of PCR amplification for targets differing in size, which could explain our results.

The ability to reliably differentiate between the morphologically similar early stages of *F. aztecus* and *F. duorarum* has important implications for management and conservation. Differences in seasonal occurrence can assist with species identification when recruitment periods are distinct, like in the Atlantic Ocean (Williams, 1959), but spawning seasons for *F. aztecus* and *F. duorarum* overlap in the Gulf and young can co-occur in estuarine nurseries (Cook and Murphy, 1971). Management decisions based on erroneous species ID could compromise inferences about shrimp population dynamics. Consequently, of particular relevance is the ability to discriminate molecularly the morphologically similar young of *F. aztecus* from those of *F. duorarum* during the period of estuarine residency. In addition, the data set of molecularly identified PL and early juveniles was employed in a discriminant analysis to help identify the best set of morphological characters to distinguish *F. aztecus* from *F. duorarum*. This approach was analogous to that used by Pendrey et al. (1999) to separate small juveniles of the banana prawns *Penaeus indicus* and *Penaeus merguiensis*. Using laboratory-reared *F. aztecus* and *F. duorarum* from the Gulf the early development was described for these two species (i.e., Dobkin, 1961; Cook and Murphy, 1971; Kitani, 1985). However, our use of the molecular techniques described here to positively ID penaeid shrimp revealed that several conventional characters that have been used to discriminate PL and early juveniles were unreliable and resulted in misclassification of 35% of the *F. duorarum* and 28% of the *F. aztecus* (for details see Ditty and Alvarado Bremer, 2011). In sharp contrast, using the set of positively identified individuals from a wide geographical range to select the ‘best’ subset of anatomical characters for species ID resulted in correct classification of 90% or higher. Altogether, the combined analysis of anatomical and molecular approaches for penaeid shrimp species ID in the Gulf of Mexico resulted in: 1) A multiplex PCR assay that can unambiguously ID all five species of penaeids that could potentially occur in the Gulf of Mexico (this study), and 2) A dramatic improvement of the selection of anatomical characters that can be used to discriminate PL and early juveniles of *F. aztecus* and *F. duorarum* (Ditty and Alvarado Bremer, submitted for publication). Furthermore, the identity of those individuals that fall outside of confidence intervals that encompass 90% of the mean for a character important in species discrimination would be classified as *Farfantepenaeus* spp., but their species identity could be ascertained using the multiplex haplotype-specific PCR assay described here.

Finally, the ability to identify early stages of exotic shrimp, like the Pacific white shrimp, is relevant because mass escapes of *L. vannamei* have occurred in the United States (Balboa et al., 1991; Wenner and Knott, 1992; Howells, 2001). The ability to monitor their presence is particularly important as the introduction of non-native species could have important ecological...
consequences by promoting change in ecosystem functioning, disease and pathogen transfer, and other factors that may affect native species and potentially result in loss of species biodiversity (Cook et al., 2008).

Acknowledgements

We thank Dr. Jim Tolan of the Texas Parks and Wildlife Department for providing L. vannamei specimens. Thanks to Dr. Ronnie Baker, Juan Salas, Shawn Hillen, Jennifer Doerr, Seth King, Kirk Kilfoyle, Enji Guy from NOAA, Galveston Laboratory for collecting shrimp in Galveston Bay. We thank Dr. Ron Hill for providing shrimp from the U.S. Virgin Islands, and to Dr. Ed Matheson of Florida Freshwater Fish Commission and Dr. Maria Criales of NOAA, Miami Laboratory, for shrimp from Florida waters. Thanks also to Dr. Darryl Felder and Dr. Heather Bracken of the University of Louisiana, Lafayette for their technical suggestions. Expert technical assistance in the laboratory at the Molecular Ecology and Fisheries Genetics lab (MEFGEN), Texas A&M University at Galveston (TAMUG) was provided by Dr. Jordi Viñas, Jennifer Atchinson, Kristine Hiltunen, Heather Walker, Danielle Higgins, Chris Courtney, Carlos Ruiz and Alex Chapman. This study was supported with funds provided by the Texas Institute of Oceanography (TIO) and by Texas Sea Grant (Project 424013).

References


Perry H., 2009. Litopenaeus vannamei. USGS Nonindigenous Aquatic Species Database, Gainesville, FL.


