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High resolution real-time PCR melting curve assay for identification of top five Penaeidae shrimp species

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ABSTRACT

In the United States shrimps is the most popular seafood. *Penaeidae* shrimp species are known to share morphology characteristic and identification based on morphological appearance becomes more challenging when shrimp is processed into ready-to-eat products. Misrepresentation of shrimp is common in commercially available shrimp. The objective of the study was to develop a high-resolution melting (HRM) real time PCR assay targeting the 16S rRNA gene fragment for specific identification of top five penaeid species, *Litopenaeus vannamei*, *Penaeus duorarum*, *Penaeus monodon*, *Litopenaeus setiferus* and *Pleoticus muelleri*. The applicability of assay was evaluated using two DNA extraction kits and two real-time PCR master mixes. The HRM assay was evaluated using 43 shrimp samples and results were validated by sequencing shrimp 16S rRNA gene fragment. Assay standardized in this study formed distinct melt curve profile for each species in the normalized and differential melt curve plots. The assay using Apex qPCR 2 × GREEN master mix showed 100% sensitivity and specificity. Further, species identification results obtained by HRM assay was in complete agreement with identification achieved by 16S rRNA gene sequencing. The HRM assay developed in this study can be used as rapid, low-cost, and reliable method for the identification of abovementioned shrimp species.

1. Introduction

Seafood is consumed worldwide as a source of protein, calcium and iodine (Dayal et al., 2013). In the United States (US), shrimp is the most commonly consumed seafood (NFI, 2018). Due to high demand and low domestic landings, in 2018 United States imported 1.5 billion pounds of shrimp, which was valued at $6.2 billion (NOAA, 2018a). Majority of these shrimp are processed and imported from Asian counties. Currently, the major shrimp exporting countries are India, Indonesia, Ecuador, Vietnam, Thailand, China and Bangladesh (NOAA, 2018a). With the increasing seafood popularity and demand for seafood in the US, mislabeling of shrimp has become a common global problem (Gal-al-Khallaf, Ardura, Borrell, & Garcia-Vazquez, 2016; Rittenschober, Stadlmayr, Nowak, Du, & Charrondiere, 2016). Seafood fraud is usually performed by replacing inferior quality shrimp for economic gain (Ananías Pascoal, Barros-Velázquez, Cepeda, Gallardo, & Calo-Mata, 2008; Woolfe & Primrose, 2004). This can impact consumers health, right, trust and promote illegal fishing (Warner, Timme, Lowell, & Hirschfield, 2013).

In the United States the top five shrimp species that dominates the seafood market are Pacific white shrimp (*Litopenaeus vannamei*), Atlantic white shrimp (*Litopenaeus setiferus*), pink shrimp (*Penaeus duorarum*), Black tiger shrimp (*Penaeus monodon*), and Argentine red shrimp (*Pleoticus muelleri*) (NOAA, 2006; Ananías; Pascoal et al., 2008). These shrimps are marketed in raw, frozen or cooked shrimp products (NOAA, 2018b). In seafood market these shrimp are labeled with a common name and country of origin (FDA, 2012). However, these labelling can be inadequate, and may have deceiving information (Stiles, Kagan, Lahr, Pullekines, & Walsh, 2013). Some shrimp species can be visually identified. However, identification becomes visually challenging after processing, such as pink shrimp and white shrimp cannot be differentiated after the processing. Warner et al. (2014) report 30% (n = 43/143) misrepresentation of commercially available shrimp. Highest misrepresentation was observed in New York city (43%) followed by Washington D.C. (33%). Substitution of Pacific white shrimp, which is mostly consumed in US (Gross, Bartlett, Browdy, Chapman, & Warr, 2001) with other low-quality shrimp, (i.e. banded coral shrimp), was commonly observed, along with the presence of species which are not intendent for

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human consumption (Warner et al., 2014).

Seafood Import Monitoring Program (SIMP) was established by NOAA to address illegal unreported and unregulated (IUU) harvesting and fraudulent marketing of imported seafood. In order to decrease ongoing incidence of misrepresentation, National Oceanic and Atmospheric Administration (NOAA) in 2018 included shrimp in the list of 13 species under SIMP program (NOAA, 2019).

Seafood species identification is performed by testing for protein and DNA based biomarkers. Protein based seafood species identification methods targets the identification of stable protein markers using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and isoelectric focusing (Ortea, Cañas, Calo-Mata, Barros-Velázquez, & Gallardo, 2010). These specific protein markers can denature during processing, making identification difficult for processed seafood samples. Isoelectric focusing based seafood identification methods are not limited by thermal impairment of protein structures, however this technique is not sensitive for shrimp species identification (Ortea et al., 2012). Advancement in the area of proteomics have revolutionized specificity of protein-based species identification methods. However, proteomics-based protein analysis requires advance instrumentation (Ananias Pascoal et al., 2012; Grizzle et al., 2005). DNA based species identification methods overcomes limitations of protein-based methods. Previously, various polymerase chain reaction (PCR) methods have been used for reliable identification of closely related food animal species (Ananias Pascoal et al., 2012; Arroyave & Stiassny, 2014; Druml & Cichna-Markl, 2014; Madesis, Ganopoulos, Sakaridis, Argiriou, & Tsaftaris, 2014; Mafra, Ferreira, & Oliveira, 2008). Currently, DNA barcoding is the gold standard method for seafood species identification (Ward, Hanner, & Hebert, 2009; Yancy et al., 2008) and misrepresentation studies (Warner et al., 2013). This method targets the mitochondrial cytochrome oxidase subunit I (COI) gene for seafood species identification (Galal-Khallaf et al., 2016; Haye, Segovia, Vera, de losÁngeles Gallardo, & Gallardo-Escárate, 2012). Although, DNA barcoding is highly specific, it is still time-cost demanding method. More recently, real-time PCR coupled with high-resolution melting (HRM) analysis has gained popularity for genotyping, pathogen identification and food verification (Druml & Cichna-Markl, 2014). The HRM is a post-PCR analysis that allows discrimination of even a single nucleotide variation (Druml & Cichna-Markl, 2014; Fernandes, Silva, Costa, Oliveira, & Mafra, 2017). It is widely used method for the identification of food adulteration (Druml & Cichna-Markl, 2014), species identification (Fernandes et al., 2017), detection of trace amount of food allergen (Costa, Mafra, & Oliveira, 2012; Madesis, Ganopoulos, Bosmali, & Tsaftaris, 2013) and pathogen identification (Aksoy et al., 2014; Liu, Singh, & Mustapha, 2018a, 2018b). The HRM analysis is a sensitive method for identification of variations in the target region. Therefore, the aim of the study was to standardize a rapid method for identification of top five commercially important penaeid shrimp's species.

2. Materials and methods

2.1. Sample collection

Raw shrimp samples of Pacific white shrimp (*L. vannamei)* (n = 10), Pink shrimp (*P. duorarum)* (n = 3), Black tiger shrimp (*P. monodon)* (n = 3), Atlantic white shrimp (*L. setiferus)* (n = 11) and Argentine red shrimp (*P. muelleri)* (n = 4) and cooked shrimp (ready-to-eat) samples (n = 8) were purchased from seafood supermarkets in Florida and Georgia. All cooked samples lacked species information on the product label. Collected samples were transported to the laboratory on ice and all samples on arrival were immediately stored at −20 °C.

2.2. Primer design

Two pairs of primers based on the 16S ribosomal RNA sequence of *L. vannamei, P. duorarum, P. monodon, L. setiferus and P. muelleri* with respective accession numbers (MK430849.1, FJ943438.1, MK430640.1, JX403862.1, and MK000281.1) were designed using Primer3 software (Untergasser et al., 2012). The amplification potential of the designed primer pairs was tested using the NCBI/Primer-BLAST tool and degenerated bases were added to HRM-1R primer to improve the amplification range of prime pair. The HRM assay was performed using HRM-1F and HRM-1R (Table 1), whereas primer 16S–F and 16S-R (Table 1) were used for sequencing the targeted 16S rRNA gene sequence for each sample.

Table 1
Oligonucleotide used to identify *L. vannamei, P. duorarum, P. monodon, L. setiferus and P. muelleri* in this study.

Name	Sequence	Amplicon Size	Reference
HRM-1F	GGACGATAAGACCCTATAAA	107 - 108 bp	This study
HRM-1R	HDTTATATTCYCGTCGCC		
16S–F	CCGTGCGAAGGTAGCATAAT	236 bp	This study
16S-R	TATATTCTCGTCYCCCCAAC		

2.3. DNA extraction

Collected shrimp samples (cooked and raw) were thawed at 4 °C. In order to compare the applicability of DNA extraction kits for HRM assay, DNA from 100 to 200 mg of each shrimp samples were extracted using Extracta™ DNA prep for PCR (Quanta Biosciences, Beverly, MA, USA) and DNeasy Blood & Tissue Kit (QIAGEN, Valencia, CA, USA). DNA extractions using Extracta™ DNA prep for PCR (Quanta Biosciences, Beverly, MA, USA) was performed according to manufacturer protocols. Extracta DNA Prep for PCR – Tissue is a quick two step method, which uses extraction buffer to dissolve tissue, cell membrane and cell organelles and facilitate release of nucleic acid, followed by addition of stabilizing buffer to maintain stability of extracted DNA. Extractions with DNeasy Blood & Tissue Kit were performed with modification to the manufacturers protocol. The tissue digestion time was increased to 40 min with vortexing every 10 min and DNA was eluted with lower volume (100 μL) of elution buffer. The quality and the quantity of isolated DNA was measured by a Nanodrop One spectrophotometer (Thermo Fisher, Wilmington, DE, USA). Obtained DNA samples were diluted to 8 ng/μL working concentration and stored at −20 °C until further analysis.

2.4. PCR amplification and sequencing

DNA extracted from all the shrimp samples were amplified using 2 × RED Taq Master Mix (Apex, USA) on a T100 Thermal Cycler (Bio-Rad, Hercules, California, USA). A 25 μL of the PCR mixture consisted of 20 ng DNA, 500 nM of 16S–F and 16S-R primer each, 12.5 μL of 2 × Red Master mix (Apex Bioresearch, NC, USA) and nuclease free water was used to adjust reaction volume. The PCR mixture was amplified with pre-initial denaturation at 95 °C for 300 s, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 63 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 300 s. The PCR products after gel electrophoresis were visualized using a UV transilluminator. Thus, obtained PCR products were purified using sodium acetate and ethanol precipitation. Purified amplicons were diluted to 10 ng/μL concentration and samples were sequenced with 3.2 μM of forward primer (16S–F). Sequencing of amplicons were performed at Florida State University sequencing core facility (Tallahassee, FL, USA).

2.5. HRM real-time PCR assay

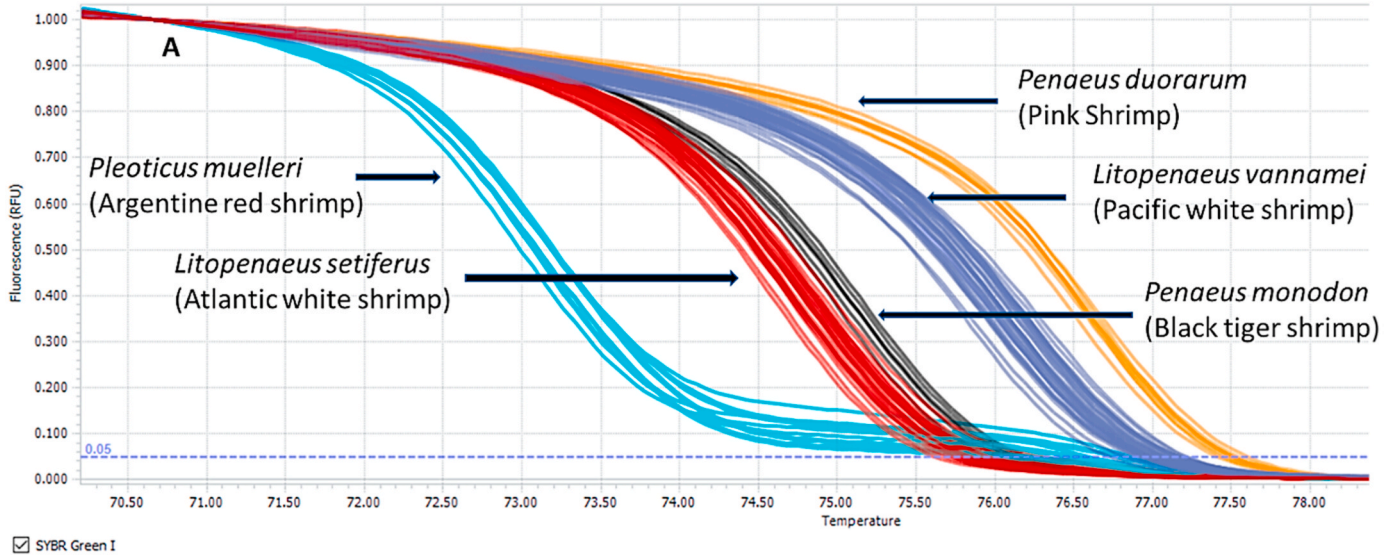
The HRM assays were performed on the LightCycler® 96 instrument (Roche Diagnostics Corp., Indianapolis, USA) with HRM-1F and HRM-1R primer-pair and Apex qPCR 2 × GREEN master mix (without ROX) (Apex Bioresearch, NC, USA) or 2 × LightCycler® 480 High Resolution Melting (Roche, USA) with some modifications. All PCR amplifications

Table 2
Identification of shrimp species by real-time high-resolution melting analysis and its comparison with Sanger sequencing.

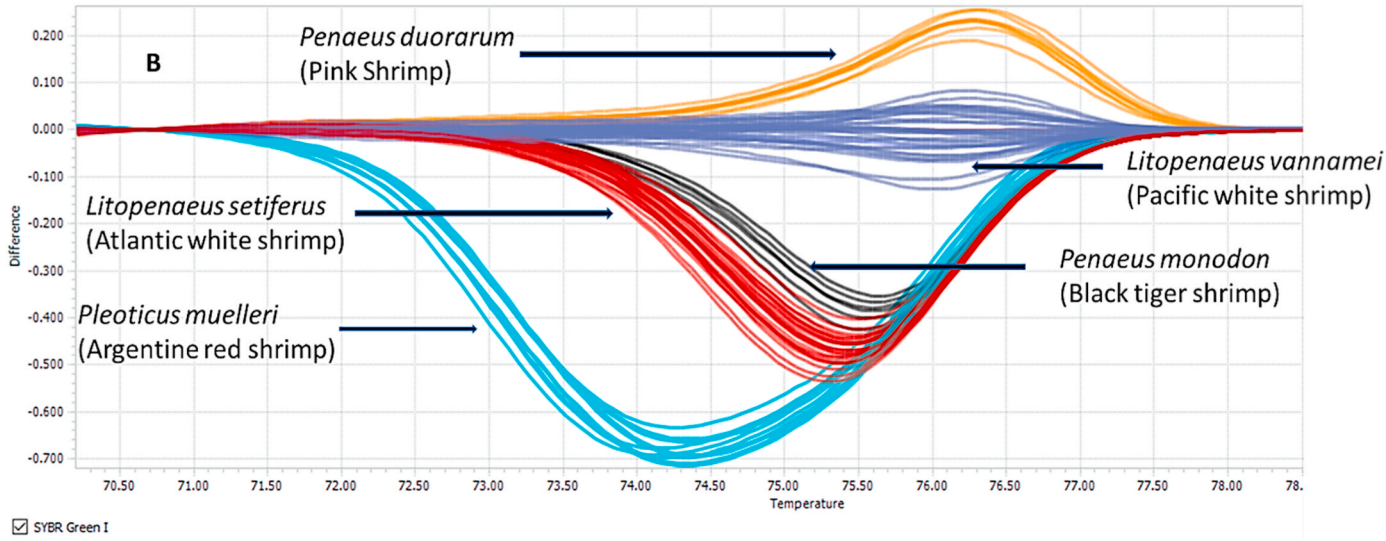
Sample number	Sample details	Shrimp species (Sanger Sequencing)	HRM Group	T_m (Apex)	T_m (ResoLight)
1	Raw	*Litopenaeus setiferus* (Atlantic white shrimp)	5	74.6	74.0
4	Raw	*Litopenaeus setiferus* (Atlantic white shrimp)	5	74.5	73.9
17	Raw	*Litopenaeus setiferus* (Atlantic white shrimp)	5	74.7	74.0
26	Raw	*Litopenaeus setiferus* (Atlantic white shrimp)	5	74.6	73.9
27	Raw	*Litopenaeus setiferus* (Atlantic white shrimp)	5	74.6	74.0
35	Raw	*Litopenaeus setiferus* (Atlantic white shrimp)	5	74.7	74.0
39	Raw	*Litopenaeus setiferus* (Atlantic white shrimp)	5	74.7	74.0
40	Raw	*Litopenaeus setiferus* (Atlantic white shrimp)	5	74.6	74.0
42	Raw	*Litopenaeus setiferus* (Atlantic white shrimp)	5	74.7	74.1
43	Raw	*Litopenaeus setiferus* (Atlantic white shrimp)	5	74.7	74.0
44	Raw	*Litopenaeus setiferus* (Atlantic white shrimp)	5	74.5	74.0
45	Raw	*Litopenaeus setiferus* (Atlantic white shrimp)	5	74.5	74.0
2	Raw	*Litopenaeus vannamei* (Pacific white shrimp)	1	75.8	75.7
3	Raw	*Litopenaeus vannamei* (Pacific white shrimp)	1	75.8	75.7
6	Raw	*Litopenaeus vannamei* (Pacific white shrimp)	1	75.8	75.7
9	Cooked*	*Litopenaeus vannamei* (Pacific white shrimp)	1	75.9	75.8
14	Cooked*	*Litopenaeus vannamei* (Pacific white shrimp)	1	76.0	75.8
15	Cooked*	*Litopenaeus vannamei* (Pacific white shrimp)	1	75.9	75.8
16	Cooked*	*Litopenaeus vannamei* (Pacific white shrimp)	1	75.9	75.7
19	Cooked*	*Litopenaeus vannamei* (Pacific white shrimp)	1	76.0	75.7
21	Cooked*	*Litopenaeus vannamei* (Pacific white shrimp)	1	76.0	75.8
22	Cooked*	*Litopenaeus vannamei* (Pacific white shrimp)	1	75.9	75.8
23	Raw	*Litopenaeus vannamei* (Pacific white shrimp)	1	75.9	75.7
24	Raw	*Litopenaeus vannamei* (Pacific white shrimp)	1	75.8	75.7
30	Cooked*	*Litopenaeus vannamei* (Pacific white shrimp)	1	75.9	75.8
33	Raw	*Litopenaeus vannamei* (Pacific white shrimp)	1	76.0	75.7
41	Raw	*Litopenaeus vannamei* (Pacific white shrimp)	1	76.0	75.6
46	Raw	*Litopenaeus vannamei* (Pacific white shrimp)	1	76.0	75.7
47	Raw	*Litopenaeus vannamei* (Pacific white shrimp)	1	76.1	75.8
48	Raw	*Litopenaeus vannamei* (Pacific white shrimp)	1	76.1	75.8
5	Raw	*Pleoticus muelleri* (Argentine red shrimp)	3	73.0	71.1
32	Raw	*Pleoticus muelleri* (Argentine red shrimp)	3	73.0	71.1
25	Raw	*Pleoticus muelleri* (Argentine red shrimp)	3	73.1	71.1
49	Raw	*Pleoticus muelleri* (Argentine red shrimp)	3	73.1	71.2
50	Raw	*Pleoticus muelleri* (Argentine red shrimp)	3	73.1	71.1
54	Raw	*Pleoticus muelleri* (Argentine red shrimp)	3	73.1	71.1
34	Raw	*Penaeus duorarum* (Pink shrimp)	4	76.5	76.2
36	Raw	*Penaeus duorarum* (Pink shrimp)	4	76.5	76.2
37	Raw	*Penaeus duorarum* (Pink shrimp)	4	76.5	76.2
38	Raw	*Penaeus duorarum* (Pink shrimp)	4	76.6	76.2
51	Raw	*Penaeus monodon* (Black tiger Shrimp)	2	75.0	73.9
52	Raw	*Penaeus monodon* (Black tiger Shrimp)	2	75.0	73.8
53	Raw	*Penaeus monodon* (Black tiger Shrimp)	2	75.0	73.9

* The cooked samples tested in this study lacked species information on the product label.

were performed in 10 μL reaction volume and in duplicates. Reactions with Apex qPCR 2 × GREEN master mix consisted of 16 ng DNA, 0.5 μM of forward and reverse primer each, 1 mM of additional $MgCl_2$ and 5 μL of 2 × master mix. The PCR amplification protocol included an initial denaturation step at 95 °C for 930 s followed by 40 cycles of 95 °C for 15 s and 62 °C for 40 s and 72 °C for 30s. PCR reaction using LightCycler® 480 High Resolution Melting master mix, consisted of 16 ng DNA, 0.5 μM of forward and reverse primer each, 3.75 mM $MgCl_2$ and 5 μL of 2 × master mix. The PCR amplification condition included an initial denaturation step at 95 °C for 615 s followed by 45 cycles of 95 °C for 15 s and 60 °C for 30 s and 72 °C for 10 s. At the end of amplification cycles for both master mixes, HRM step was performed. The HRM step consisted of



A. Normalized melting peaks



B. Differential melting plot

Fig. 1. A high resolution melting curve assay for identification of *L. vannamei, P. duorarum, P. monodon, L. setiferus,* and *P. muelleri* using 2 × Apex qPCR GREEN master mix: 1A: Normalized melting curve; 1B: Differential melting plot. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

gradual temperature increments of 0.07 °C/s (i.e. 15 reading/°C) and 0.04 °C/s (i.e. 25 reading/°C) from 65 °C to 95 °C for the qPCR 2 × GREEN master mix and 2 × LightCycler 480 High Resolution Melting, respectively. Amplification and HRM data were collected in the channel 1 of the real-time PCR instrument. The sensitivity and specificity of the developed assay was calculated as previously descried by Lemmon & Gardner (Lemmon & Gardner, 2008).

3. Results

The performance of two commercial DNA extraction kits were evaluated based on purity of extracted DNA and their applicability for HRM assay. DNA extracted using DNeasy Blood & Tissue Kit resulted in a A260/A280 ratio range of 1.7–2.0. Whereas A260/A280 using Extracta™ DNA prep for PCR kit were below 1.7. There was no difference in DNA yield (ng/μL) from both kits. However, DNA extracted using the Extracta™ DNA prep for PCR degraded after one month of storage at −20 °C. The 16s rRNA gene sequencing results for all samples were obtained and BLAST results of 236 bp sequenced region showed above 98% sequence identity with respective penaeid species and accurately identified all the shrimp tested in the study (Table 2).

Applicability of real-time PCR assays using SYBR Green I and Reso-Light dye was evaluated for the differentiation of five genetically related penaeid species. SYBR Green I dye based HRM assay showed lower quantitation cycle (Cq) values range of 19- 26, with melting temperature (T_m) of *L. vannamei* (75.8 °C), *P. duorarum* (76.7 °C), *P. monodon* (75 °C),

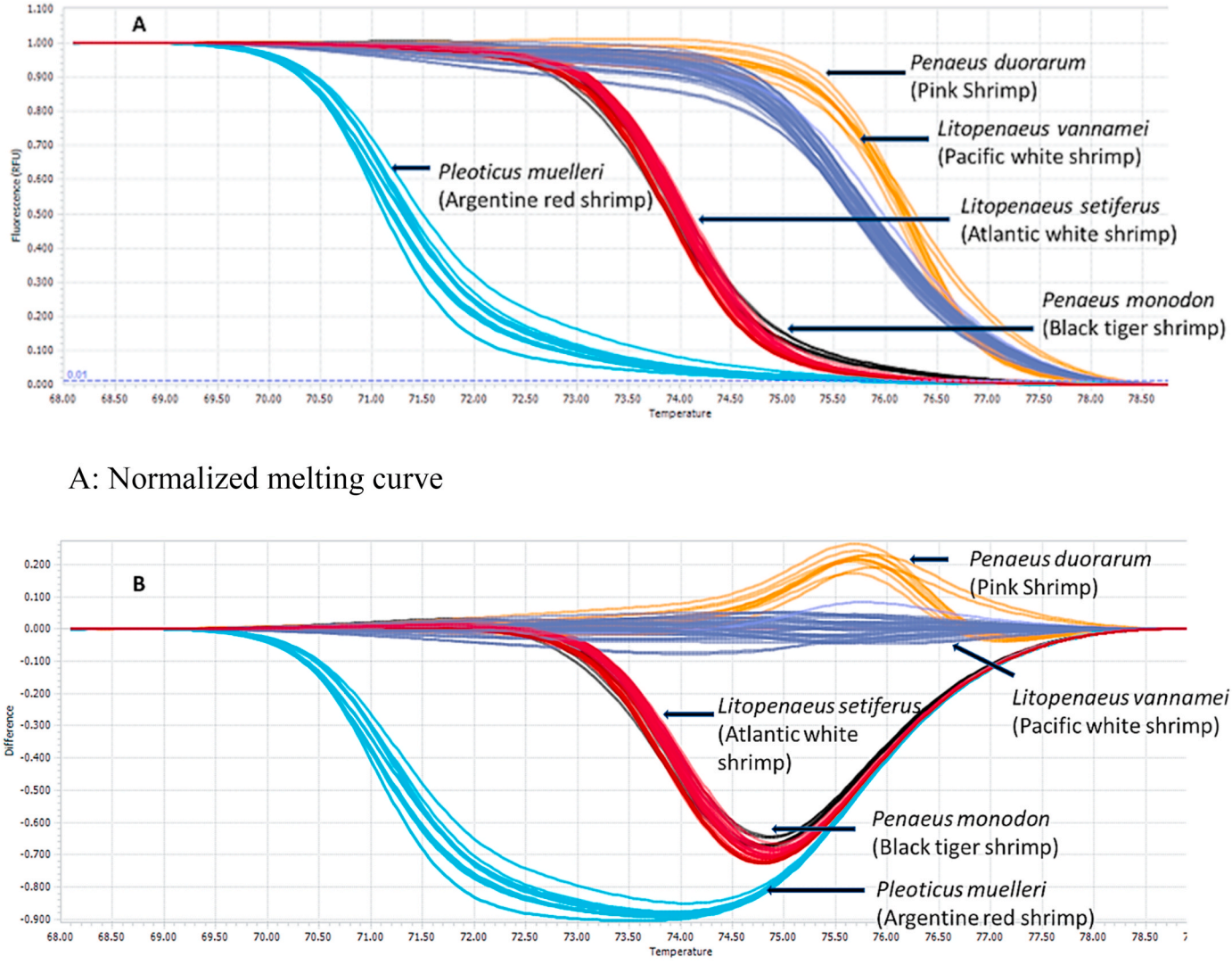


Fig. 2. A high resolution melting curve assay for identification of *L. vannamei, P. duorarum, P. monodon, L. setiferus,* and *P. muelleri* using 2 × LightCycler® 480 High Resolution Melting: 2A: Normalized melting curve; 2B: Differential melting plot.

L. setiferus (74.5 °C) and *P. muelleri* (73 °C). Whereas, HRM assay with ResoLight dye, had a Cq value of 17–30 with T_m *L. vannamei* (75.7 °C), *P. duorarum* (76.2 °C), *P. monodon* (73.9 °C), *L. setiferus* (74 °C) and *P. muelleri* (71.1 °C) (Table 2).

In this study the HRM assay using the SYBR Green I dye was able to accurately differentiate all five target species (Fig. 1). Whereas HRM assay using ResoLight dye resulted in only four HRM groups in which melt curves of *P. monodon* and *L. setiferus* were in one group and failed to resolve (Fig. 2). The standardize HRM assay using SYBR Green I dye when tested on cooked shrimp samples lacking shrimp species description correctly identified all cooked samples tested in the study. Interestingly, one raw shrimp sample (sample No. 41), which was sold as *L. setiferus* was identified as *L. vannamei* by the HRM assay. These results were further confirmed by sequencing.

4. Discussion

Real-time PCR HRM analysis is an rapid and reliable method for the species identification (Fernandes et al., 2017). The sensitivity of HRM assay relies on the purity of the template DNA. Presence of any impurities or PCR inhibitors in the isolated DNA sample can interferes with the melting profiles of samples. Therefore, selection of appropriate DNA isolation kit or method is of utmost importance for a real-time PCR HRM assay.

In this study, we compared applicability of two DNA extraction kits for HRM analysis assay. The instability of DNA isolated using Extracta DNA Prep for PCR – Tissue can be due to presence of double stranded DNase in the isolated DNA samples. This quick DNA isolation method excludes proteinase K treatment, which can be useful for digesting of DNase and other DNA degrading enzymes (Rossmanith, Röder, Frühwirth, Vogl, & Wagner, 2011). Compared to DNA isolated by above-mentioned method, DNA isolated from the DNeasy Blood and Tissue kit were of higher purity, with A_{260}/A_{280} ratio between 1.7 and 2.0, remained stable during storage and were more suitable for the real-time PCR HRM assay.

Magnesium chloride concentration is another critical component for HRM analysis. The Apex qPCR 2 × GREEN master mix has $MgCl_2$ pre-added to the mix. Whereas, the 2 × LightCycler® 480 High Resolution Melting master mix requires optimization of $MgCl_2$ for each assay. In order to facilitate identification of all target species the $MgCl_2$

concentrations for both master mixes were individually optimized. Optimization of $MgCl_2$ to the reaction mixture resulted in improved PCR amplification efficiency, increased amplicons T_m and the most importantly improved separation of melt curves, facilitating identification of penaeid species by the HRM analysis. Among five target species the *L. setiferus* and *P. monodon* showed very similar melt profiles and were discernable using only Apex qPCR 2 × GREEN master mix with supplementation of 1 mM $MgCl_2$.

Mitochondrial genomic region (1.38 kb) (i.e.16S rRNA, 12S rRNA and $tRNA^{val)}$ of penaeid shrimp species, are significant for phylogenetic analysis (Bremer, Ditty, Turner, & Saxton, 2010). Further several DNA-based techniques like PCR (Bremer et al., 2010), PCR coupled with restriction fragment length polymorphism (RFLP) (Hisar, Aksakal, Hisar, Yanik, & Mol, 2008; Khamnamtong, Klinbunga, & Menasveta, 2005; Ananías; Pascoal et al., 2008) and DNA barcoding (Bilgin, Utkan, Kalkan, Karhan, & Bekbölet, 2015) targeting mitochondrial DNA fragments has been previously used to study and identify economically important shrimp species. In this study we targeted the 16S rRNA region of shrimp for identification of penaeid species. Alvarado Bremer et al. (2010) standardized a conventional multiplex PCR assay targeting the shrimp 16S rRNA gene sequence for the identification of Farfantepenaeus aztecus, Farfantepenaeus duorarum, Farfantepenaeus brasiliensis and L. setiferus, and L. vannamei (Bremer et al., 2010). Similarly, Pascoal et al. (2011) developed another PCR assay using two sets of primer pairs targeting the mitochondrial 16S rRNA/$tRNA^{Val}$ region for the identification of *L. vannamei, F. indicus and P. monodon* (Ananías Pascoal et al., 2011). Currently, DNA barcoding is the gold standard method for the identification of fish/crustacean's species (Arroyave & Stiassny, 2014; Günther, Raupach, & Knebelsberger, 2017; Yancy et al., 2008) and this method is extensively used for shrimp species identification (Bilgin et al., 2015; Cutarelli et al., 2014). Although these DNA-based methods (e.g. RFLP, multiplex PCR, DNA barcoding) have been developed for the identification of shrimp species, these methods require post-PCR analysis such as gel electrophoresis, digestion by multiple restriction enzymes, or sequencing of PCR amplicons. Compared to these methods the real time PCR HRM analysis is a single tube method that does not require any additional steps and can be completed in the same reaction tube within 3 h, which includes DNA isolation and real time PCR and high-resolution melting.

Fernandes et al. (2017) developed an HRM assay for the identification of five shrimp species (i.e. *L. vannamei, P. indicus, P. monodon, Metapenaeus affinis* and *Melicertus kerathurus*) using universal primer pair targeting COI genes. The assay was evaluated using commercially available food product containing crustaceans (Fernandes et al., 2017). Whereas, in our study we targeted the shrimp 16S rRNA gene for the specific identification of top five shrimp species (*i.e. L. vannamei, P. duorarum, P. monodon, L. setiferus,* and *P. muelleri*), which are commonly available in the United States seafood market, making the assay more suitable for United States.

5. Conclusion

To the best of our knowledge, this is the first real-time PCR assay for specific identification and differentiated Atlantic white shrimp, Pink Shrimp, Pacific white shrimp, Black tiger shrimp, and Argentine red shrimp. This low-cost assay can be useful for identification of shrimp species, prevention of misrepresentation of commercially available shrimp and suitable for implementation by NOAA SIMP program.

CRediT authorship contribution statement

Laxmi Sharma: Investigation, Writing - original draft. **Evelyn Watts:** Writing - review & editing. **Prashant Singh:** Conceptualization, Methodology, Validation, Data curation, Supervision, Project administration.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2020.109983.

References

Aksoy, U., Marangi, M., Papini, R., Ozkoc, S., Delibas, S. B., & Giangaspero, A. (2014). Detection of Toxoplasma gondii and Cyclospora cayetanensis in Mytilus galloprovincialis from Izmir Province coast (Turkey) by real time PCR/high-resolution melting analysis (HRM). *Food Microbiology, 44*, 128–135.

Arroyave, J., & Stiassny, M. L. (2014). DNA barcoding reveals novel insights into pterygophagy and prey selection in distichodontid fishes (Characiformes: Distichodontidae). *Ecology and Evolution, 4*(23), 4534–4542.

Bilgin, R., Utkan, M., Kalkan, E., Karhan, S., & Bekbölet, M. (2015). DNA barcoding of twelve shrimp species (Crustacea: Decapoda) from Turkish seas reveals cryptic diversity. *Mediterranean Marine Science, 16*(1), 36–45.

Bremer, J. R. A., Ditty, J. G., Turner, J. S., & Saxton, B. L. (2010). Molecular species identification of commercially important penaeid shrimp from the Gulf of Mexico using a multiplex haplotype-specific PCR assay. *Biochemical Systematics and Ecology, 38*(4), 715–721.

Costa, J., Mafra, I., & Oliveira, M. B. P. (2012). High resolution melting analysis as a new approach to detect almond DNA encoding for Pru du 5 allergen in foods. *Food Chemistry, 133*(3), 1062–1069.

Cutarelli, A., Amoroso, M. G., De Roma, A., Girardi, S., Galiero, G., Guarino, A., et al. (2014). Italian market fish species identification and commercial frauds revealing by DNA sequencing. *Food Control, 37*, 46–50.

Dayal, J. S., Ponniah, A., Khan, H. I., Babu, E. M., Ambasankar, K., & Vasagam, K. K. (2013). Shrimps–a nutritional perspective. *Current Science*, 1487–1491.

Druml, B., & Cichna-Markl, M. (2014). High resolution melting (HRM) analysis of DNA–Its role and potential in food analysis. *Food Chemistry, 158*, 245–254.

Fernandes, T. J., Silva, C. R., Costa, J., Oliveira, M. B. P., & Mafra, I. (2017). High resolution melting analysis of a COI mini-barcode as a new approach for Penaeidae shrimp species discrimination. *Food Control, 82*, 8–17.

Food and Drug Administration (FDA). (2012). FDA's guide to acceptable market names for seafood sold in interstate commerce. Retrieved March 3, 2020, from https://www.fda.gov/regulatory-information/search-fda-guidance-documents/guidance-industry-seafood-list#thelist.

Galal-Khallaf, A., Ardura, A., Borrell, Y. J., & Garcia-Vazquez, E. (2016). PCR-based assessment of shellfish traceability and sustainability in international Mediterranean seafood markets. *Food Chemistry, 202*, 302–308.

Grizzle, W. E., Semmes, O. J., Bigbee, W., Zhu, L., Malik, G., Oelschlager, D. K., & Manne, U. (2005). The need for review and understanding of SELDI/MALDI mass spectroscopy data prior to analysis. *Cancer Informatics, 1*, 117693510500100106.

Gross, P., Bartlett, T., Browdy, C., Chapman, R., & Warr, G. (2001). Immune gene discovery by expressed sequence tag analysis of hemocytes and hepatopancreas in the Pacific White Shrimp, Litopenaeus vannamei, and the Atlantic White Shrimp, L. setiferus. *Developmental & Comparative Immunology, 25*(7), 565–577.

Günther, B., Raupach, M. J., & Knebelsberger, T. (2017). Full-length and mini-length DNA barcoding for the identification of seafood commercially traded in Germany. *Food Control, 73*, 922–929.

Haye, P. A., Segovia, N. I., Vera, R., de los Ángeles Gallardo, M., & Gallardo-Escárate, C. (2012). Authentication of commercialized crab-meat in Chile using DNA barcoding. *Food Control, 25*(1), 239–244.

Hisar, S. A., Aksakal, E., Hisar, O., Yanik, T., & Mol, S. (2008). Discrimination of penaeid shrimps with PCR-RFLP analysis. *Journal of Shellfish Research, 27*(4), 917–920.

Khamnamtong, B., Klinbunga, S., & Menasveta, P. (2005). Species identification of five penaeid shrimps using PCR-RFLP and SSCP analyses of 16S ribosomal DNA. *BMB Reports, 38*(4), 491–499.

Lemmon, G. H., & Gardner, S. N. (2008). Predicting the sensitivity and specificity of published real-time PCR assays. *Annals of Clinical Microbiology and Antimicrobials, 7*(1), 18.

Liu, Y., Singh, P., & Mustapha, A. (2018a). High-resolution melt curve PCR assay for specific detection of E. coli O157: H7 in beef. *Food Control, 86*, 275–282.

Liu, Y., Singh, P., & Mustapha, A. (2018b). Multiplex high resolution melt-curve real-time PCR assay for reliable detection of Salmonella. *Food Control, 91*, 225–230.

Madesis, P., Ganopoulos, I., Bosmali, I., & Tsaftaris, A. (2013). Barcode high resolution melting analysis for forensic uses in nuts: A case study on allergenic hazelnuts (Corylus avellana). *Food Research International, 50*(1), 351–360.

Madesis, P., Ganopoulos, I., Sakaridis, I., Argiriou, A., & Tsaftaris, A. (2014). Advances of DNA-based methods for tracing the botanical origin of food products. *Food Research International, 60*, 163–172.

Mafra, I., Ferreira, I. M., & Oliveira, M. B. P. (2008). Food authentication by PCR-based methods. *European Food Research and Technology, 227*(3), 649–665.

Natinal Fisheries Institute (NFI). (2018). Top 10 list for seafood consumption. Retrieved February 29, 2020, from https://www.aboutseafood.com/about/top-ten-list-for-seafood-consumption/ .

National Oceanic and Atmospheric Administration (NOAA). (2006). *Noaa fisheries glossary* (Vol. 69). NOAA Technical Memorandum NMFS-F/SPO.

National Oceanic and Atmospheric Administration (NOAA). (2018a). *Fisheries of the United States*. Retrieved from file:///C:/Users/Laxmi/Downloads/FUS_2018_report.pdf.

National Oceanic and Atmospheric Administration (NOAA). (2018b). Fisheries of the United States, 2017 report. Retrieved February 29, 2020 https://www.fisheries.noaa.gov/resource/document/fisheries-united-states-2017-report.

National Oceanic and Atmospheric Administration (NOAA). (2019). Seafood Import monitoring program. Retrieved March 3, 2020 https://www.fisheries.noaa.gov/international/seafood-import-monitoring-program.

Ortea, I., Cañas, B., Calo-Mata, P., Barros-Velázquez, J., & Gallardo, J. M. (2010). Identification of commercial prawn and shrimp species of food interest by native isoelectric focusing. *Food Chemistry, 121*(2), 569–574.

Ortea, I., Pascoal, A., Cañas, B., Gallardo, J. M., Barros-Velázquez, J., & Calo-Mata, P. (2012). Food authentication of commercially-relevant shrimp and prawn species: From classical methods to foodomics. *Electrophoresis, 33*(15), 2201–2211.

Pascoal, A., Barros-Velázquez, J., Cepeda, A., Gallardo, J. M., & Calo-Mata, P. (2008). Survey of the authenticity of prawn and shrimp species in commercial food products by PCR-RFLP analysis of a 16S rRNA/tRNAVal mitochondrial region. *Food Chemistry, 109*(3), 638–646.

Pascoal, A., Barros-Velázquez, J., Ortea, I., Cepeda, A., Gallardo, J. M., & Calo-Mata, P. (2011). Molecular identification of the black tiger shrimp (Penaeus monodon), the white leg shrimp (Litopenaeus vannamei) and the Indian white shrimp (Fenneropenaeus indicus) by PCR targeted to the 16S rRNA mtDNA. *Food Chemistry, 125*(4), 1457–1461.

Pascoal, A., Ortea, I., Gallardo, J. M., Cañas, B., Barros-Velázquez, J., & Calo-Mata, P. (2012). Species identification of the Northern shrimp (Pandalus borealis) by polymerase chain reaction–restriction fragment length polymorphism and proteomic analysis. *Analytical Biochemistry, 421*(1), 56–67.

Rittenschober, D., Stadlmayr, B., Nowak, V., Du, J., & Charrondiere, U. R. (2016). Report on the development of the FAO/INFOODS user database for fish and shellfish (uFiSh)–Challenges and possible solutions. *Food Chemistry, 193*, 112–120.

Rossmanith, P., Röder, B., Frühwirth, K., Vogl, C., & Wagner, M. (2011). Mechanisms of degradation of DNA standards for calibration function during storage. *Applied Microbiology and Biotechnology, 89*(2), 407–417.

Stiles, M. L., Kagan, A., Lahr, H. J., Pullekines, E., & Walsh, A. (2013). Seafood Sticker Shock Why you may be paying too much for your fish. *Oceana, 30*.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M., et al. (2012). Primer3—new capabilities and interfaces. *Nucleic Acids Research, 40*(15). e115-e115.

Ward, R. D., Hanner, R., & Hebert, P. D. (2009). The campaign to DNA barcode all fishes, FISH-BOL. *Journal of Fish Biology, 74*(2), 329–356.

Warner, K., Golden, R., Lowell, B., Disla, C., Savitz, J., & Hirshfield, M. (2014). *Shrimp: Oceana reveals misrepresentation of America's favorite seafood*. Oceana.

Warner, K., Timme, W., Lowell, B., & Hirschfield, M. (2013). *Oceana study reveals seafood fraud nationwide*. Washington, DC: Oceana.

Woolfe, M., & Primrose, S. (2004). Food forensics: Using DNA technology to combat misdescription and fraud. *Trends in Biotechnology, 22*(5), 222–226.

Yancy, H. F., Fry, S., Randolph, S. C., Deeds, J., Ivanova, N. V., Grainger, C. M., & Hunt, J. (2008). *A protocol for validation of DNA-barcoding for the species identification of fish for FDA regulatory compliance*. FDA Laboratory Information Bulletin.