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Rapid PCR-lateral flow assay for the onsite detection of Atlantic white shrimp

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ABSTRACT

The Atlantic white shrimp (*Litopenaeus setiferus*) is of great economic importance to the United States and risk being substituted with imported species due to a shortage in domestic production. To improve the current methods used for the identification of the Atlantic white shrimp species, we designed and validated a robust multiplex PCR-lateral flow assay for the onsite identification of *L. setiferus*. The standardized assay was validated using a miniaturized, low-cost PCR instrument with 68 shrimp, prawn, and fish samples, spread over fourteen seafood species. *L. setiferus* was simultaneously amplified by the multiplex assay to give three visual bands, which distinguished it from other species having either one or two bands on the dipstick. The standardized assay showed 100% inclusivity for target *L. setiferus* samples, 100% exclusivity for non-target samples and can be completed in less than two hours. The assay standardized in this study can be used for onsite testing of *L. setiferus* samples at processing facilities, restaurants, and wholesalers' facilities.

1. Introduction

Seafood consumption in the United States stands at an average of 19.2 lb per capita, and the most consumed seafood includes shrimp, salmon, tilapia, tuna, crab, Alaska pollock, cod, and clams. As the United States' domestic production is unable to meet this high demand for seafood, it relies on imports from other countries such as Canada, Chile, China, Ecuador, India, Indonesia, and Vietnam, which account for 70%–85% of seafood consumption (NMFS, 2021; NOAA, 2022).

Increasing global demands for seafood and related trading activities have made seafood vulnerable to food fraud (FAO, 2018). Seafood fraud includes species substitution, mislabeling, misrepresentation of the country of origin and/or production method, undercounting, and overtreatment for reasons such as economic gains, avoiding regulations on products and informal supply chains (Fox et al., 2018; Donlan & Luque, 2019; FDA, 2018). These activities are a source of food safety and quality issues that can have adverse effects on consumers' health, trust and result in legal consequences (Silva and Hellberg, 2020).

The Atlantic white shrimp (*Litopenaeus setiferus*) is one of the most economically valuable seafood species in the southern part of the US and is harvested from North Carolina to Florida along the Gulf of Mexico. According to the National Oceanic and Atmospheric Administration (NOAA), the US landed 112 million pounds of Atlantic white shrimp in 2021 with an estimated value of $274 million (NOAA, 2022). To meet the domestic shrimp demands, the US imported 1.64 billion pounds of shrimp valued at $6.4 billion in 2020 (NMFS, 2022), which comprised *Litopenaeus vannamei, Pleoticus muelleri*, and *Penaeus monodon*. Among these, *Litopenaeus vannamei* is one of the cheapest species due to its large production in Southeast Asian countries. These imported shrimps are traded as frozen head-on shell-on, headless shell-on, peeled undeveined, peeled deveined and cooked products (FAO, 2017).

Morphological characteristics are commonly used for the identification of shrimp species. However, with the application of processing steps, these morphological features are lost, and identification becomes challenging, which creates room for species substitution (e.g., the substitution of *L. setiferus* with *L. vannamei*) (Ortea et al., 2012; Sharma et al., 2020). The Seafood Import Monitoring Program (SIMP), a risk-based program, which monitors 13 fish and seafood products has identified shrimps as one species that are vulnerable to seafood fraud (SIMP, 2018). Species substitution is a violation of the Federal Food, Drug, and Cosmetic Act Section 403: Misbranded food, which states that "a food shall be deemed to be misbranded if it is offered for sale under

Abbreviations: SIMP, Seafood Import Monitoring Program; IAC, Internal Amplification Control; DiGN, Digoxigenin; HRM, High-Resolution Melting.

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the name of another food" (FDA, 2020). Even though species substitution is against the law, shrimp species substitution rates as high as 34%, 16%, and 18% have been reported in Northern Carolina (USA), India, and South Africa, respectively (Cawthorn and Hoffman, 2017; Korzik et al., 2020; Wilwet et al., 2021).

Currently, protein and DNA-based methods are used for seafood species identification (Griffiths et al., 2014). Protein-based methods involve the use of high-end laboratory equipment such as electrophoresis units, mass spectrophotometers, and plate readers for antibody-based ELISA assays (Fuller et al., 2006; Ortea et al., 2009). Protein-based methods are less commonly used as they often lack species-level resolution, have a short life of antibodies, and cannot be relied upon to identify shrimp samples that have been processed by heat treatments (Hellberg & Morissey, 2011; Piñeiro et al., 2003).

The DNA-barcoding for fish species identification is a standard method developed by University of Guelph (ON, Canada) for seafood species identification and the method is currently used by the US Food and Drug Administration (FDA) (Handy et al., 2011). The method relies on the sequencing of the cytochrome *c* oxidase subunit I gene (COI) and the comparison of the sequence data with FDA standard barcodes or reference sequences housed in GenBank and/or the Barcode of Life Database (BOLD) (Kress and Erickson, 2012). In addition to DNA barcoding, PCR-based assays targeting the shrimp species-specific regions of COI and 16S ribosomal RNA genes have been published (Ortea et al., 2012; Sharma et al., 2020; Wilwet et al., 2021; Korzik et al., 2020; Pascoal et al., 2011).

Although DNA-barcoding and PCR-based methods are highly specific, they generally require overnight shipment of samples to a diagnostic laboratory, expensive high-end equipment (e.g., PCR, real-time PCR, sequencer), pre- and post-processing of samples (e.g., DNA extraction, PCR product clean-up, samples preparation for sequencing). They further require trained personnel to carry out tests and analyze the data. Thus, making these methods expensive, time-consuming, and less desirable for adoption by the seafood industry (Pascoal et al., 2008). Therefore, to fight species substitution, the seafood industry has expressed a need for rapid, low-cost, robust, species-specific methods that can be routinely performed onsite using raw or processed or cooked seafood samples (Ortea et al., 2012).

The lateral flow-coupled PCR approach is an emerging approach and can be used for rapid onsite detection of PCR products. The approach involves PCR amplification of the species-specific target DNA sequence and the detection of the amplicon using a lateral flow strip. The approach has been previously used for the species identification of fish (Taboada et al., 2017), horse, pork, beef, sheep (Magiati et al., 2018; Chen et al., 2020), and pork (Yin et al., 2020) samples.

Therefore, this study aimed to develop a species-specific multiplex PCR-coupled lateral flow assay with internal amplification control (IAC) for the onsite identification of *L. setiferus* that can be performed using low-cost equipment in a resource-limited setting.

2. Materials and methods

2.1. Sample collections

A total of 60 shrimp and prawn samples spread over seven species were collected across commercial retail stores across the states of Florida and Georgia. Collected shrimp samples were saved in Styrofoam boxes containing ice and transported to the laboratory. Fin fish samples were provided by a seafood wholesaler and were shipped to the laboratory under refrigerated conditions. The samples included Argentine red shrimp (*Pleoticus muelleri*) (n = 8), giant freshwater prawn (*Macrobrachium rosenbergii*) (n = 3), pink shrimp (*Farfantepenaeus duorarum*) (n = 4), speckled shrimp (*Metapenaeus monoceros*) (n = 3), black tiger shrimp (*Penaeus monodon*) (n = 8), Pacific white shrimp (*Litopenaeus vannamei*) (raw and cooked) (n = 15), Atlantic white shrimp (*L. setiferus*) (n = 19), black grouper (*Mycteroperca bonaci*) (n = 1), blue catfish (*Ictalurus furcatus*) (n = 1), red snapper (*Lutjanus campechanus*) (n = 1), red grouper (*Epinephelus morio*) (n = 2), rose snapper (*Lutjanus guttatus*) (n = 1), vermilion snapper (*Rhomboplites aurorubens*) (n = 1), and yellowtail snapper (*Ocyurus chrysurus*) (n = 1). About 1 g of collected shrimp tissue samples were placed in 2 ml cryotubes and preserved with 1 ml of DNA/RNA Shield ™ (Zymo Research, Irvine, CA, USA) and stored at −20 °C for the long-term preservation of tissue samples. Thirty shrimp samples used in the study have been barcoded in our previous study (Sharma et al., 2020) and they served as positive and negative controls.

2.2. Rapid tissue lysis and DNA extraction

Stored tissue samples were defrosted at 4 °C. About 0.5–1 mm of tissue was collected using fine forceps and placed into a microcentrifuge tube. We tested the applicability of the DNeasy® Blood & Tissue kit (QIAGEN, Valencia, CA, USA), Extracta™ DNA Prep for PCR (Quanta Biosciences, Beverly, MA, USA), and lysis buffer from Platinum Direct PCR Universal Master Mix (Invitrogen, Waltham, MA, USA) for rapid isolation of shrimp tissue DNA. DNA isolation was performed following the manufacturer's recommendations. The DNA concentration was measured using the NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.3. Primer-design for lateral flow PCR assay

A species-specific primer pair targeting *L. setiferus* was designed using Primer3 software (Table 1) (Untergasser et al., 2012). The forward primer at the 5′ end was labeled with biotin, and the reverse primer at the 5′ end was labeled with 6 FAM and used for PCR amplification. To identify any PCR reaction failures, a conserved primer pair targeting the shrimp 16S rRNA gene (IAC-Shrimp HRM-3F, IAC-Shrimp HRM-1R) was designed; the forward primer was labeled with biotin, and the reverse primer was labeled with Digoxigenin (DiGN) at the 5′ end (Table 1). The primers were synthesized by IDT (Coralville, Iowa, USA).

2.4. Primer optimization

A gradient PCR was used to standardize the annealing temperature of *L. setiferus* and IAC primer pairs. The gradient PCR was performed on a LightCycler® 96 instrument (Roche Diagnostics Corp., Indianapolis, IN, USA), and the reaction mixture consisted of 5 μl of 2 × Apex qPCR GREEN Master Mix (Genesee Scientific, El Cajon, CA, USA), 0.30 μM of forward and reverse primers and 20 ng of DNA. The qPCR amplification profile consisted of an initial denaturation step at 95 °C for 15 min followed by 35 cycles of denaturation at 95 °C for 30 s, annealing between 55 and 62 °C for 30 s and extension at 72 °C for 5 s. A melt curve step was added after the completion of 35 amplification cycles, which consisted of 95 °C for 10 s, 65 °C for 60 s, and 97 °C for 1 s. The real-time PCR results were analyzed using the LightCycler® 96 SW 1.1 software (Roche Diagnostics Corp., Indianapolis, IN, USA). Annealing temperature showing reproducible results and a specific melting peak was used for the PCR assay.

Table 1
Primers used for the specific detection of *L. setiferus*.

Primer	Sequence (5′-3′)	Gene	Amplicon size
WS-197F-FAM	6-FAM/TAATATAAGCTTCTGACTTC	CO1	118 bp
WS-313R-biotin	BiosG/GGCGATACTAGCAGATAA		
IAC- Shrimp HRM-3F	6-FAM/GGACGATAAGACCCTATAAA	16S	108 bp
IAC-Shrimp HRM-1R	DiGN/HDTTATATTCYCGTCGCC		

2.5. PCR amplification

DNA samples extracted using the lysis buffer provided in the Platinum Direct PCR Universal Master Mix (Invitrogen, Waltham, MA, USA) were diluted (1:10) using nuclease-free water and used for the PCR reaction. The PCR amplification potential of the DNA extracts obtained using three kits was tested using two PCR master mixes, i.e., 2 × Platinum Direct PCR Universal Master Mix (Invitrogen, Waltham, MA, USA) and Apex 2 × RED Taq Master Mix (Genesee Scientific, CA, USA). The PCR reactions were performed in a 20 µl reaction volume. A 20 µl reaction mixture consisted of 10 µl of PCR master mix, 0.40 µM of forward and reverse primers (WS-197F-FAM and WS-313R-biotin), 0.23 µM of shrimp IAC primers (IAC-Shrimp HRM-3F and IAC-Shrimp HRM-1R), 2 µl of diluted DNA and 7.37 µl of nuclease-free water to reach a final reaction volume of 20 µl. Each PCR reaction was performed in duplicate. The PCR amplification was carried out using a miniaturized Watson PCR instrument (IEH Laboratories & Consulting Group, Seattle, WA, USA). The PCR amplification profile consisted of an initial denaturation step at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 20 s, and a final extension at 72 °C for 5 min.

2.6. Lateral flow detection

HybriDetect 2 T lateral flow kit (Milenia Biotec, GmbH, Germany) was used for the detection of amplified PCR products. Lateral flow assays were performed following the manufacturer's instructions. Briefly, 100 µl of Hybridetect assay buffer was transferred into a microcentrifuge tube. A 5 µl aliquot of PCR products was added into the assay buffer and vortexed for about 10 s to ensure it was mixed in the buffer. The dipsticks were inserted into the solution and incubated for 5 min. Lateral flow sticks were visually observed for band formation and based on the presence or absence of bands, the results were interpreted as positive or negative. A positive species identification was represented by three lines on the lateral flow strip. In comparison, a negative result was represented by either a single control line at the top or two lines at the top of the test strip.

2.7. Assay specificity

The cross-amplification potential of the standardized assay was further tested using eight barcoded fish samples covering seven fish species, which included black grouper (*Mycteroperca bonaci*), blue catfish (*Ictalurus furcatus*), red snapper (*Lutjanus campechanus*), red grouper (*Epinephelus morio*), rose snapper (*Lutjanus guttatus)*, vermilion snapper (*Rhomboplites aurorubens*), and yellowtail snapper (*Ocyurus chrysurus*) (Table 2).

2.8. Assay performance and statistical analysis

The assay sensitivity, specificity, false-positive rate, false-negative rate, positive-predictive value, negative-predictive value, and test accuracy were calculated as previously described (Bosilevac et al., 2019). DNA yield and extraction time were compared using two-way ANOVA (SPSS software version 27) and GraphPad Prism version 9.4 was used to plot the graph.

3. Results

This work was carried out to develop a rapid DNA-based method for the specific and onsite identification of *L. setiferus*. Version one of the assay was standardized using a *L. setiferus*-specific primer pair (Table 1) and the HybriDetect - Universal Lateral Flow Assay Kit (Milenia Biotec), which allows the detection of one FAM/FITC and biotin-labeled analyte. However, as the assay is intended to be performed with crude DNA extracts, it was challenging for version one of the assay to differentiate between a true-negative or a PCR reaction failure result, necessitating the inclusion of an IAC in the PCR assay to detect PCR reaction failures (Fig. 1a). Therefore, version two of the assay consisted of a duplex PCR reaction targeting the shrimp CO1 and 16S rRNA genes. Data from gradient PCR reaction showed annealing at 60 °C for 30 s was best suited for the simultaneous amplification of both targets.

We tested the applicability of three commercially available DNA extraction kits (DNeasy®Blood & Tissue Kit, Extracta® DNA PREP for PCR, and lysis buffer from Platinum Direct PCR Universal Master Mix) for the rapid extraction of shrimp DNA suited for PCR amplification. The average concentration of the crude DNA from the three kits was 104.3, 328.2, and 94.3 ng/µL, respectively. The DNA yield data showed no significant difference ($p < 0.05$) between the platinum direct lysis buffer and the DNeasy® kit (Fig. 1b). However, there was a significant difference in the extraction time ($p < 0.05$) between all the kits used. Out of all kits, the lysis buffer of the Platinum Direct PCR Universal Master Mix was simple to use, took shortest extraction time (3 min) and generated DNA with an average A260/280 ratio of 1.30. Therefore, it was used for assay standardization and validation.

PCR amplification potential of crude DNA extract was tested using two master mixes, i.e., 2 × Platinum Direct PCR Universal Master Mix and Apex 2 × RED Taq Master Mix. Out of two master mixes, the Platinum Direct PCR Universal Master Mix Kit generated reproducible results for the crude shrimp DNA extracts and showed no inhibition from any possible PCR inhibitors present in the crude extract. Both gene targets were reliably amplified for all white shrimp; hence, the master mix was selected for assay validation with other shrimp and fish samples. The amplicons from the duplex PCR reaction were tested using the HybriDetect 2 T lateral flow kit (Milenia Biotec). All *L. setiferus* samples tested in this study generated three bands (Fig. 2) showing 100% inclusivity. In contrast, non-target shrimp samples belonging to *L. vannamei*, *P. muelleri*, *F. duorarum*, and *P. monodon* formed only two bands (Fig. 3), i.e., IAC and the lateral flow control band, demonstrating 100% assay exclusivity.

The sensitivity, specificity, false-positive rate, false-negative rate, positive-predictive value, negative-predictive value, and test accuracy of the standardized assay was tested using 52 shrimp samples. The assay showed 100% sensitivity, specificity, positive predictive value, negative predictive value, and test accuracy. The assay showed no false-positive rate or false-negative rate. Hence, it can be inferred from the test results that the samples collected from the market were accurately labeled.

The cross-amplification potential of the assay was further tested using eight barcoded fish samples (Table 2). All eight fish samples tested negative for the white shrimp-specific primer pair, as shown in Supplementary Fig. 1, whereas the blue catfish and yellowtail snapper samples showed amplification for the IAC primer.

4. Discussion

The most crucial aspect of this study was to develop a robust assay suited for the onsite testing of shrimp samples, which can be performed without any major laboratory equipment. DNA extraction is one of the crucial parts of a diagnostic assay, and during assay standardization, it was observed that the performance of DNA extraction and subsequent PCR amplification was dependent on the size of the shrimp tissue used for the DNA isolation. The smallest tissue sample size that can be transferred using a pair of fine forceps generated more reliable results. The use of larger tissue samples resulted in higher DNA concentrations with high protein contamination levels, making DNA samples unsuitable for PCR amplification.

PCR master mixes vary in their performance and resistance to PCR inhibitors. Therefore, master mix selection is another critical component for the development of a diagnostic assay. Out of all master mixes screened and validated in this study, the Platinum Direct PCR Universal Master Mix generated reproducible results for all samples tested in this study. In the past, the Platinum Direct PCR Universal Master Mix kit has

Table 2
Sample used for the assay validation and results obtained using the standardized assay.

Sample no.	Common Name	Scientific Name	Condition	Amplicon A (white shrimp primer)	Amplicon B (IAC)	Interpretation (+positive. '-' negative)
DNA barcoded samples						
1	White shrimp	*Litopenaeus setiferus*	frozen raw	yes	yes	+
2	White shrimp	*Litopenaeus setiferus*	frozen raw	yes	yes	+
3	White shrimp	*Litopenaeus setiferus*	frozen raw	yes	yes	+
4	White shrimp	*Litopenaeus setiferus*	frozen raw	yes	yes	+
5	White shrimp	*Litopenaeus setiferus*	frozen raw	yes	yes	+
6	White shrimp	*Litopenaeus setiferus*	frozen raw	yes	yes	+
7	White shrimp	*Litopenaeus setiferus*	frozen raw	yes	yes	+
8	White shrimp	*Litopenaeus setiferus*	frozen raw	yes	yes	+
9	White shrimp	*Litopenaeus setiferus*	frozen raw	yes	yes	+
10	White shrimp	*Litopenaeus setiferus*	frozen raw	yes	yes	+
11	White shrimp	*Litopenaeus setiferus*	frozen raw	yes	yes	+
12	White shrimp	*Litopenaeus setiferus*	frozen raw	yes	yes	+
13	Whiteleg shrimp	*Litopenaeus vannamei*	frozen raw	no	yes	–
14	Whiteleg shrimp	*Litopenaeus vannamei*	frozen raw	no	yes	–
15	Whiteleg shrimp	*Litopenaeus vannamei*	frozen raw	no	yes	–
16	Whiteleg shrimp	*Litopenaeus vannamei*	cooked and frozen	no	yes	–
17	Whiteleg shrimp	*Litopenaeus vannamei*	cooked and frozen	no	yes	–
18	Whiteleg shrimp	*Litopenaeus vannamei*	frozen raw	no	yes	–
19	Argentine red shrimp	*Pleoticus muelleri*	frozen raw	no	yes	–
20	Argentine red shrimp	*Pleoticus muelleri*	frozen raw	no	yes	–
21	Argentine red shrimp	*Pleoticus muelleri*	frozen raw	no	yes	–
22	Argentine red shrimp	*Pleoticus muelleri*	frozen raw	no	yes	–
23	Pink shrimp	*Farfantepenaeus duorarum*	frozen raw	no	yes	–
24	Pink shrimp	*Farfantepenaeus duorarum*	frozen raw	no	yes	–
25	Pink shrimp	*Farfantepenaeus duorarum*	frozen raw	no	yes	–
26	Pink shrimp	*Farfantepenaeus duorarum*	frozen raw	no	yes	–
27	Tiger shrimp	*Penaeus monodon*	frozen raw	no	yes	–
28	Tiger shrimp	*Penaeus monodon*	frozen raw	no	yes	–
29	Tiger shrimp	*Penaeus monodon*	frozen raw	no	yes	–
Commercially available samples						
30	Giant freshwater prawn	*Macrobrachium rosenbergii*	frozen raw	no	no	–
31	Giant freshwater prawn	*Macrobrachium rosenbergii*	frozen raw	no	no	–
32	Giant freshwater prawn	*Macrobrachium rosenbergii*	frozen raw	no	no	–
33	Speckled shrimp	*Metapenaeus monoceros*	frozen raw	no	no	–
34	Whiteleg shrimp	*Litopenaeus vannamei*	cooked and frozen	no	yes	–
35	Speckled shrimp	*Metapenaeus monoceros*	frozen raw	no	no	–
36	White shrimp	*Litopenaeus setiferus*	frozen raw	yes	no	+
37	White shrimp	*Litopenaeus setiferus*	frozen raw	yes	no	+
38	Whiteleg shrimp	*Litopenaeus vannamei*	frozen raw	no	yes	–
39	Tiger shrimp	*Penaeus monodon*	frozen raw	no	yes	–
40	Tiger shrimp	*Penaeus monodon*	frozen raw	no	yes	–
41	Tiger shrimp	*Penaeus monodon*	frozen raw	no	yes	–
42	Whiteleg shrimp	*Litopenaeus vannamei*	frozen raw	no	yes	–
43	Whiteleg shrimp	*Litopenaeus vannamei*	frozen raw	no	yes	–
44	Tiger shrimp	*Penaeus monodon*	frozen raw	no	yes	–
45	Whiteleg shrimp	*Litopenaeus vannamei*	cooked and frozen	no	yes	–
46	Argentine red shrimp	*Pleoticus muelleri*	frozen raw	no	yes	–
47	Argentine red shrimp	*Pleoticus muelleri*	frozen raw	no	yes	–
48	Argentine red shrimp	*Pleoticus muelleri*	frozen raw	no	yes	–
49	White shrimp	*Litopenaeus setiferus*	frozen raw	yes	yes	+
50	White shrimp	*Litopenaeus setiferus*	frozen raw	yes	yes	+
51	Speckled shrimp	*Metapenaeus monoceros*	frozen raw	no	no	–
52	Argentine red shrimp	*Pleoticus muelleri*	frozen raw	no	yes	–
53	Whiteleg shrimp	*Litopenaeus vannamei*	frozen raw	no	yes	–
54	Whiteleg shrimp	*Litopenaeus vannamei*	frozen raw	no	yes	–
55	Tiger shrimp	*Penaeus monodon*	frozen raw	no	yes	–
56	Whiteleg shrimp	*Litopenaeus vannamei*	frozen raw	no	yes	–
57	Whiteleg shrimp	*Litopenaeus vannamei*	frozen raw	no	yes	–
58	White shrimp	*Litopenaeus setiferus*	frozen raw	yes	yes	+
59	White shrimp	*Litopenaeus setiferus*	frozen raw	yes	yes	+
60	White shrimp	*Litopenaeus setiferus*	frozen raw	yes	yes	+

(*continued on next page*)

Table 2 (*continued*)

Sample no.	Common Name	Scientific Name	Condition	Amplicon A (white shrimp primer)	Amplicon B (IAC)	Interpretation (+positive. '-' negative)
Specificity test using barcoded fish samples						
61	Red Snapper	*Lutjanus campechanus*	frozen raw	no	no	–
62	Red Grouper	*Epinephelus morio*	frozen raw	no	no	–
63	Blue Catfish	*Ictalurus furcatus*	frozen raw	no	yes	–
64	Yellowtail snapper	*Ocyurus chrysurus*	frozen raw	no	yes	–
65	B-line snapper	*Rhomboplites aurorubens*	frozen raw	no	no	–
66	Red Grouper	*Epinephelus morio*	frozen raw	no	no	–
67	Black Grouper	*Mycetoperca bonaci*	frozen raw	no	no	–
68	Rose/Pacific Lane snapper	*Lutjanus guttatus*	frozen raw	no	no	–

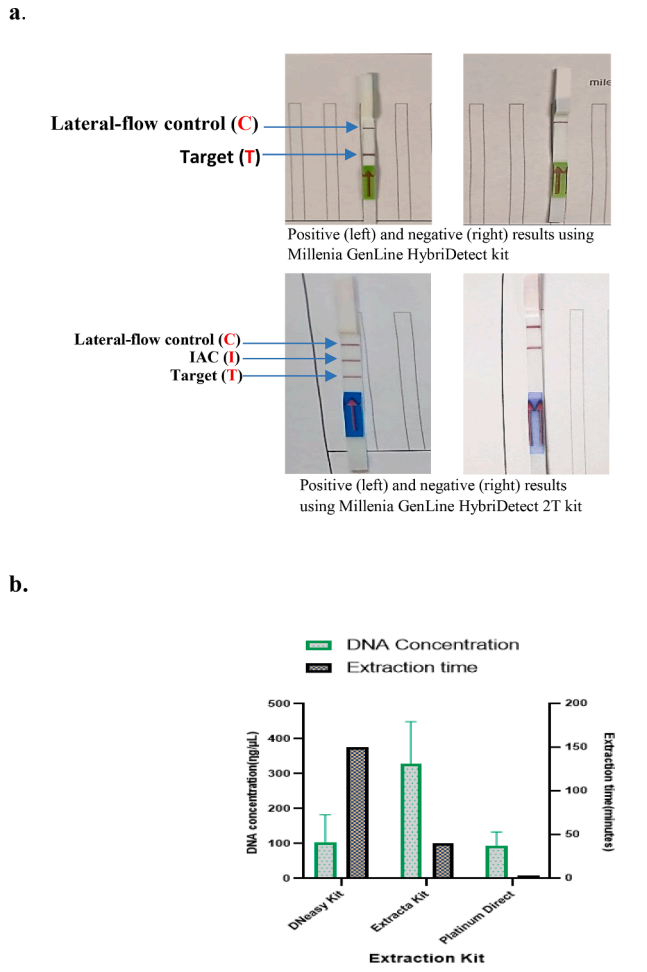

Fig. 1. A. white shrimp assay comparison using the hybridetect kit for the identification of one target without iac (version 1) and using the hybridetect 2 T kit for the identification of white shrimp specific CO1 gene and IAC amplicons (Version 2). Fig. 1b. Comparison of different DNA extraction kits on yield and extraction times for shrimp samples. Error bars represent standard deviation.

been used by researchers for the isolation of DNA from seafood specimens (i.e., ostracode) and subsequent PCR amplification (Echeverría-Galindo et al., 2021). This master mix is designed to facilitate PCR amplification directly from tissue samples. However, direct PCR attempts using shrimp tissue resulted in reaction failure. The presence of shrimp tissue in the PCR reaction mixture may have resulted in high DNA concentrations in the PCR reactions and may have inhibited the amplification. High DNA concentrations are known to cause PCR reaction failure due to the production of PCR amplification byproducts (Kainz, 2000, Singh et al., 2019). Therefore, for subsequent experiments, the crude DNA extracts were diluted by 1/10 and used for the PCR reaction. This approach further facilitated the dilution of PCR inhibitors present in crude DNA extracts and generated reproducible PCR results. Even though this approach improved the assay's reliability, the PCR reaction could still fail due to other factors. Thus, necessitating the inclusion of an IAC in the PCR assay. IAC is another target present in every PCR reaction that co-amplifies with the target, facilitates the identification of false-negative results due to reaction failure, and is considered an essential component of a diagnostic assay (Hoorfar et al., 2004). Therefore, version two of the assay was standardized with a *L. setiferus*-specific primer and a conserved primer targeting the shrimp 16S rRNA gene as an IAC (Table 1). The two amplicons (i.e., *L. setiferus* and IAC) of the duplex PCR reaction generated visually distinct bands on the agarose gel as well as on the HybriDetect 2 T lateral flow dipstick (Fig. 2). Interestingly, samples belonging to the prawn species (i.e., *Macrobrachium rosenbergii, Metapenaeus monoceros*) formed only the lateral flow control band (Fig. 3) and failed to generate the IAC band. The IAC primers used in this study were initially designed to amplify the top five commercially available shrimp species (Sharma et al., 2020). Therefore, they showed amplification for all the shrimp species but failed to amplify the two prawn species tested in this study. Sequence differences at the primer-binding sites of the IAC primer pair could be a probable reason for the lack of IAC amplification for the *M. rosenbergii* and *M. monoceros* samples.

One of the interesting aspects of this study was the usefulness of the assay for testing cooked and frozen shrimp samples. The test results for the two cooked and frozen *L. vannamei* samples showed PCR application and band formation on the lateral flow stick, indicating that the crude DNA isolated from processed shrimp samples can be used for testing with the PCR assay developed in this study (Fig. 4). This is interesting when compared to some protein-based detection assays, which are used for seafood allergen testing, where the application of heat treatment can denature the target protein and interfere with the protein-based assay results (Hellberg & Morissey, 2011; Piñeiro et al., 2003).

These results are comparable to our previous finding, where we developed a real-time PCR high-resolution melting (HRM) assay for the identification of the top five shrimp species (Sharma et al., 2020). Even though our previous assay could identify all five shrimp species, it requires DNA isolated using a high-purity DNA isolation kit (i.e., DNeasy® Blood & Tissue kit), which takes hours to complete, a real-time PCR instrument, software for the HRM data analysis, and can only be performed in a research laboratory. Compared to our HRM assay, the assay developed in this study was performed using a crude DNA extract obtained in 2–3 min and using a miniaturized and low-cost, Watson® PCR instrument, making it best suited for onsite testing. The Watson® PCR instrument took about 80 min to complete the PCR reaction, which was slightly longer than a conventional PCR instrument. Irrespective of the longer run time, all targets were reliably detected for all samples tested in this study. The use of a miniaturized PCR can facilitate the deployment of the standardized assay in low-resource settings (e.g., seafood processing facilities or restaurants) and enable the US shrimp industry to perform onsite testing of shrimp samples.

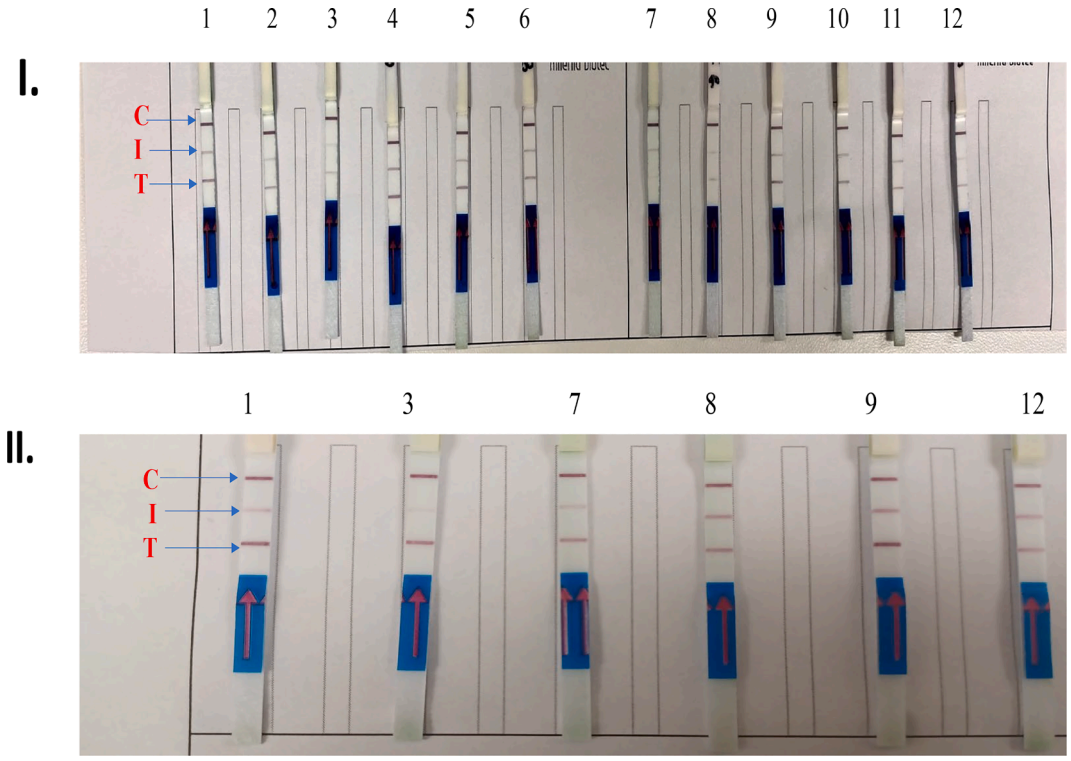

Fig. 2. Lateral flow detection of white shrimp after PCR using barcoded shrimp samples. Panel I: Test results for 12 *L. setiferus* samples. Panel II: Retesting of selected *L. setiferus* samples, which initially showed weak bands.

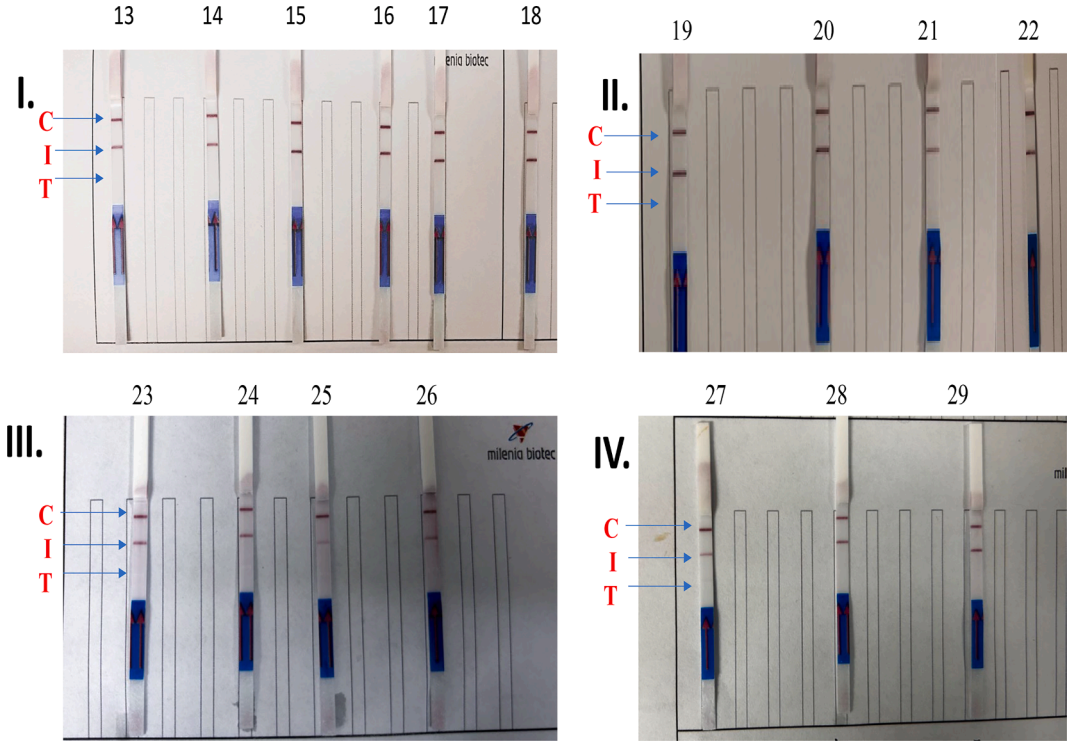

Fig. 3. Lateral flow detection after PCR with the developed assay using samples from four different barcoded shrimp species. Panel I: 13, 14, 15, and 16 were raw *L. vannamei* samples, whereas 17 and 18 were cooked *L. vannamei samples*. Panel II: *Pleoticus muelleri* samples. Panel III: *Fartanpenaus duorarum* samples. Panel IV: *Penaeus monodon* samples.

5. Conclusion

The assay standardized in this study showed 100% sensitivity and specificity. The assay offers a simple and accurate DNA-based solution for the onsite identification of *L. setiferus* samples in a low-resource setting. The assay can be used for testing processed or cooked shrimps

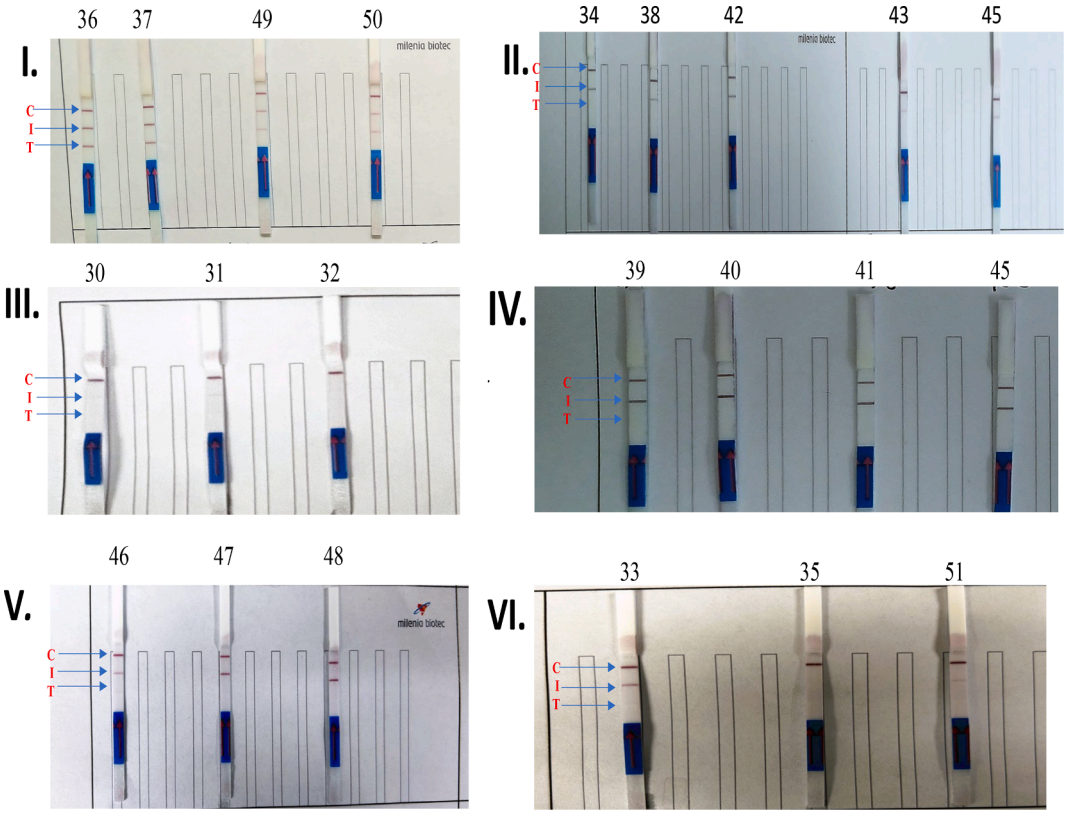

Fig. 4. Assay validation using shrimp samples collected from markets in Florida and Georgia. Panel I: *L. setiferus* samples. Panel II: 38, 42, 43—raw *L. vannamei*, 34, 45—cooked *L. vannamei* samples. Panel III: *Macrobrachium rosenbergii* samples. Panel IV: *Penaeus monodon* samples. Panel V: *Penaeus muelleri* samples. Panel VII: *Metapenaeus monoceros* samples.

lacking morphological identification features. The standardized PCR-coupled lateral flow assay, combined with a miniaturized PCR instrument, is a cost-effective and reliable solution for the food industry's onsite DNA-based testing needs.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [Prashant Singh has patent Methods and compositions useful in discriminating between fish species pending to Florida State University.].

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochms.2023.100164.

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