



# Bioinformatic Analysis Strategy in Restriction Enzyme Selection for Indonesian *Panulirus homarus* Identification by PCR-RFLP

Indriatmoko<sup>1,2\*</sup> & Adi Pancoro<sup>3</sup>

<sup>1</sup>Graduate Program in Biology, School of Life Sciences and Technology,  
Institut Teknologi Bandung, Jalan Ganesha 10, Bandung 40132, Indonesia

<sup>2</sup>Research Institute for Fish Enhancement, Agency for Marine and Fisheries Research  
and Human Resources, Ministry of Marine Affairs and Fisheries, Jalan Cilalawi 1,  
Purwakarta 41152, Indonesia

<sup>3</sup>Research Expertise Group of Genetics and Molecular Biotechnology, School of Life  
Science and Technology, Institut Teknologi Bandung, Jalan Ganesha 10, Bandung  
40132, Indonesia

\*Email: indriatmoko.bp2ksi@outlook.com

**Abstract.** The spiny lobster (*Panulirus homarus*) is a valuable fishery commodity in Indonesia, particularly in its juvenile life form. However, identifying the early life forms of the spiny lobster can be challenging, as it exhibits similar morphological features compared to the juveniles of other Panilurid lobsters. Molecular-based identification, specifically DNA sequencing, is the best method for species identification, but it requires advanced instruments and is costly. An alternative method is proposed here, using the PCR-RFLP technique, which is low-cost, rapid, and has standard instrumentation requirements. The challenge with this method is selecting the appropriate restriction enzyme to determine the targeted species' identity. This study proposes using the REfind (<https://github.com/indriatmoko07/REfind>), R package to select the best restriction enzyme for identifying *P. homarus*, applicable to other species. The bioinformatics workflow used in this study successfully identified BseSI or BmgI as the most suitable restriction enzymes among 739 restriction enzymes to differentiate *P. homarus* from other Panilurid species. This result was validated by employing a wet lab test using the BseSI enzyme and successfully validated the bioinformatics result. These findings may be useful for biologists in conducting various studies that require rapid, low-cost, and identification of specific species in the future.

**Keywords:** *bioinformatics; identification; PCR-RFLP; restriction enzymes; spiny lobster.*

## 1 Introduction

Species identity is essential information in ecological studies [1]. This primary information can be used either to build a biodiversity database for specific areas

or to carry out more complex analyses in order to gain a better perspective for the understanding of ecological phenomena [2]. It is well known that the identity of species can be distinguished on the basis of the phenotypic and morphological character of the individual's appearance. Nevertheless, this method is mostly only acceptable for the species in the adult stage or for those with permanent visual parameters [3]. In the past few decades, the use of molecular information has been seen as the gold standard for identifying animals at the species level [4].

Cytochrome Oxidase Subunit I (COI) has been used in several studies to identify various species and is considered a superior marker in species-level identification [5-7]. With the rapid development of sequencing technology, species identification by reading DNA sequences is advancing [8]. However, this advanced technology is associated with high investment costs in the molecular instrument. Another strategy for the development of molecular-based species identification is the novel species-specific primer design [9,10]. This approach is aimed at isolating a pair of oligonucleotides with their ability to specifically amplify certain species. However, this technique is challenging when most species share the conserved DNA region with their closest related taxa [11]. This could result in decreasing primer specificity, possibly causing false-positive results.

DNA fingerprinting techniques, e.g., Restriction Fragment Length Polymorphism (RFLP), offer better alternatives for species-level identification. Compared to other genotyping techniques, i.e., RAPD and microsatellite variation, RFLP is known for its advantages in providing reproducible results and its applicability under standard molecular laboratory facilities [12]. In addition, RFLP does not require sequence reading to determine the identification result. Previous reports have proposed this method as a simple and low-cost utility for several species identification such as shrimp [13], fungi [14], and bacteria [15].

The Indonesian spiny lobster (*P. homarus*) is a valuable commodity either from capture fishery or aquaculture production [16]. This species is becoming a prime commodity for Indonesian lobsters fisheries due to their high abundance in Indonesian waters, especially in the juvenile stages, as well as their high market price [17]. This species is an object of international trade, in the adult stage as well as in the juvenile stage. Unfortunately, the status of the *P. homarus* population has been categorized as overfished [18]. Identifying spiny lobsters in the early life stages is important, but it is a challenging task because in the early life stages they have a similar appearance to other Panilurid lobsters. Thus, a rapid, low-cost method that is applicable in a standard molecular laboratory is desirable.

Several efforts have been conducted by Chow *et al.* [19] and Dharani *et al.* [20] in determining a simple approach for *P. homarus* identification via the RFLP technique. However, information regarding the considerations used in restriction enzyme (RE) selection in PCR-RFLP-based identification remains unelaborated. Today, molecular data acquisition and the development of molecular data in public databases (e.g., the NCBI gene bank) have been rapidly improved. This is correlated with the advance of the sequencer instrument and outstanding improvement in bioinformatics. The huge reported number of sequence data available in public databases allows gaining more knowledge by conducting deeper analyses. This also provides the opportunity to estimate suitable REs for identification of *P. homarus*. This study aimed to provide a step-by-step workflow recommended for exploring suitable REs to identify the spiny lobster (*P. homarus*) from Indonesian waters, which also has potential application in identifying other species.

## 2 Material and Methods

### 2.1 DNA Sequences Used

The reference sequences used in this study were complete mitochondrial genome sequences of the Panilurid species and partial COI genes from the NCBI gene bank. A set of 10 mtDNA (~16000 bp) was used from different Panilurus genera, e.g., *P. homarus* (NC\_016015, hom), *P. argus* (NC\_039671, arg), *P. cygnus* (NC\_028024, cyg), *P. japonicus* (NC\_004251, jap), *P. longipes* (MN817128, lon), *P. ornatus* (NC\_014854, orn), *P. penicillatus* (MT533488, pen), *P. polyphagus* (MK503959, pol), *P. stimpsoni* (NC\_014339, sti), and *P. versicolor* (NC\_028627, ver). The consistency of the resulting fragments in this study was tested over a large set of partial COI sequences (n = 271) available in the gene bank.

### 2.2 Bioinformatic Strategy

The bioinformatics strategy in exploring and selecting the best REs for the identification of *P. homarus* was carried out in five steps:

a) *Reference sequence acquisition.* An essential type of reference sequence to explore suitable enzymes for species identification is the complete mitochondrial DNA (mtDNA) genome. This type of data can be accessed through the NCBI gene bank via Rentrez [21]. It is recommended to check the number of available reference mtDNA genome sequences before analysis. This is because multiple reported sequences can be found for individual species. If multiple mtDNA sequences are available, we recommend using the longest reference sequence. Prior to the selection of the mtDNA genome sequences, a

multiple sequence alignment (MSA) analysis was performed using ClustalW [22] in MEGA X [23] to determine the best sequence by the absence of nucleotide gaps and/or nucleotide ambiguity detection. It is important to use not only the mtDNA sequence of the target species but also that of the closest related species, as far as an mtDNA sequence is available. In this study, we used all the mtDNA genome sequences available for Panilurid lobsters.

b) *Estimation of the amplicon sequences.* In this step, each of the reference sequences was trimmed to obtain the region of the sequenced amplicon using a particular set of DNA primers. We used a set of DNA primers suitable to amplify the upper-stream region of the COI gene [24]. The lobster's mtDNA sequences were aligned using local alignment and trimmed based on the first aligned nucleotides for the direction of each primer.

c) *Estimation of DNA fragments size.* All the amplicons of the target species (*P. homarus*) and the closest species within the genus (all Panilurid lobsters) from step b were used to estimate the size of the DNA fragments resulting after the digestion of 739 REs. This analysis process was carried out with the seqRFLP package [25]. After the estimation analysis, a data set with 739 series of DNA fragments was created from the analyzed sequence.

d) *Finding potential REs.* The series of DNA fragment sizes (bp) resulting from step b were used in filtering potential REs according to several required conditions (Table 1). After RE filtering, the REs meeting the selection criteria were recorded for the species sequences analyzed. In order to find the best REs for *P. homarus* identification, the DNA fragment size from the filtered REs was compared to the DNA fragment size from Panilurid lobsters. It is inefficient to manually compare DNA fragments from large amounts of REs. Therefore, we performed data conversion by using Formula (1) to convert both DNA fragment sizes ( $F_n$  = size of fragment n, and  $F_t$  = PCR amplicon size) to a single value that is comparable to the data for other species. This value represents a fragment with no DNA fragment produced while the lower value represents a digested DNA fragment.

$$\sum \left( \frac{F_n}{F_t} \right)^2 \quad (1)$$

e) *Final selection.* During step d, the 739 REs from the initial process were filtered down to a lower number of potential REs. These processes minimized the researcher's efforts in selecting the best REs from a large amount of available REs. In making this final selection, some potential single nucleotide polymorphisms (SNPs) should be selected manually based on a few considerations, i.e., the presence of isoschizomer REs and the availability of

commercial REs. To gain more confidence in selecting REs with a consistent result, a trial simulation was conducted using the 271 *P. homarus* sequences. Prior to the analysis, these sequences were aligned using ClustalW and trimmed to remove the gap at both 5' and 3' ends. Furthermore, the trimmed sequences were tested using selected REs to estimate the DNA fragment production consistency across large individual sample sizes.

**Table 1** Required conditions for restriction enzyme selection.

Selection conditions	Considerations
DNA fragment produced = 2	In practice, many DNA fragments cannot be easily observed in an agarose gel because of the low resolution of the gel. Thus, REs that produce two DNA fragments will provide a more observable DNA band when visualized on an agarose gel.
Fragment resolution = 50 bp	Electrophoresis elution with a high DNA concentration can be visualized as a bold DNA fragment. Two DNA fragments with a difference in length of less than 50 bp may be observed as a single large DNA band in bold on the agarose gel.
Shortest DNA fragment <150 bp	The presence of primer-dimers (~100 bp) is sometimes inevitable. This phenomenon may be caused by various conditions, such as primer quality, the Taq polymerase used, or the temperature during reagent preparation. The selection of the RE with the shortest fragment close to 100 bp is very doubtful if a primer dimer is found. In addition, the short-length DNA fragment is occasionally found in very low concentrations. Thus, this fragment may not be observable on the agarose gel under UV light.

### 2.3 Experimental Validation

In order to confirm the ability of the selected REs to differentiate the identity of *P. homarus* from 9 other Panilurid lobsters, a laboratory experiment was conducted. Forty *P. homarus* samples were acquired from five populations in Indonesian waters (Simeuleu, Mandeh, Pangandaran, Trenggalek, and Makasar) and four samples of panilurid lobsters. DNA extraction was carried out using the gSYNC DNA Extraction kit (Genaid) under the manufacturer's protocol. An aliquot of DNA extract with a lower concentration (95:5) was used after 1x TE dilution. The thermocycler conditions were conducted according to Folmer *et al.* (1994) with the primer pair LCO1490 (5'-GGTCAACAAAT CATAAAGATATTGG-3') and HCO2198 (5'- TAAACTTCAGGGT GACCAAAAATCA-3'). PCR reactions were conducted on a total reaction volume of 50 µL composed by 25 µL MyTaq HS mix 2× (Bioline), 2 µL forward primer, 2 µL reverse primer, 19 µL ddH<sub>2</sub>O, and 2 µL genomic DNA. The touchdown PCR amplification condition was set to 95 °C for 10 min during pre-denaturation, followed by 5 cycles of thermal reaction, 94 °C for 30 s, 45 °C

to 50 °C for 30 s, and 72 °C for 60 s. The next 30 cycles were set to 94 °C for 30 s, 51 °C to 54 °C for 30 s, and 72 °C for 60 s. The reaction ended by a final extension at 72 °C for 10 min. The PCR reaction's success was confirmed through DNA elution under 1% agarose gel in 1× TAE buffer at 100 V for 30 min.

Confirmed PCR products proceeded to DNA digestion from the selected REs by following the RE manufacturer protocols (mixing reagent procedure and specific activation and inactivation heat). The digested PCR products were eluted on 2% agarose gel in 1× TBE buffer at 100 V for 30 min. All agarose gel after electrophoresis were visualized using a gel-doc system.

## 2.4 Data Analysis

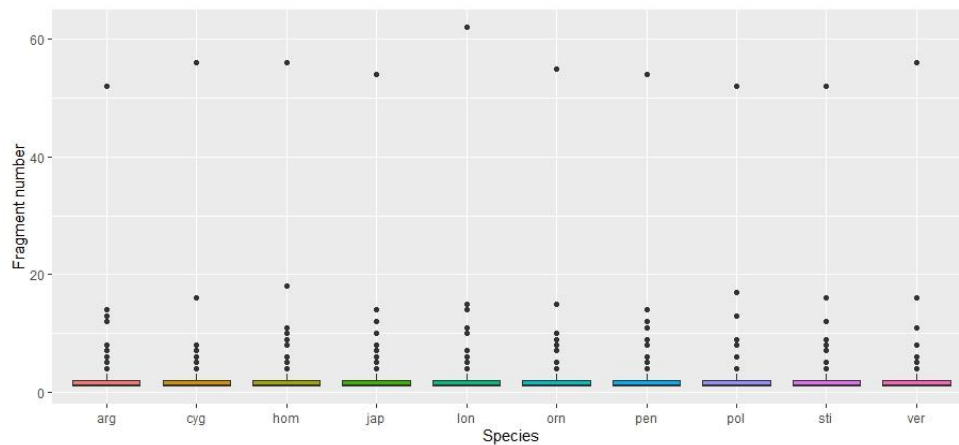
Most of the analyses performed in this study were done using the R software, several packages of which are essential for providing specific functions in conducting bioinformatics analysis. The bioinformatics workflows used in this study have been compiled into an R package accessible via <https://github.com/indriatmoko07/REfind>.

## 3 Result and Discussion

In determining potential REs that have the ability to identify *P. homarus* and distinguish it from other Panilurid species, attention was paid to the availability of a complete mtDNA genome sequence. The mtDNA genome sequence is superior for identification purposes at the species level because of its short sequence (14,000 to 16,000 bp), which can be easily isolated, sequenced, and annotated [26,27].

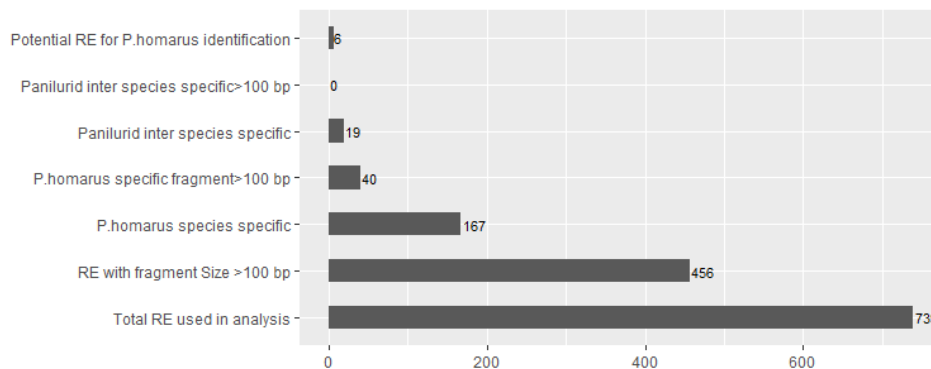
The complete mtDNA genomes have been very well studied and the reported sequences are available in gene banks [26]. This is beneficial for molecular biologists to be able to further explore these genome sequences. MtDNA has been widely used as an object in studying the molecular level of organisms, including identification [28], genetic differentiation [29], genetic diversity [30], and gene-disease association [31].

In studying the animal kingdom, the Cytochrome Oxidase Subunit I (COI) gene is commonly used as the target gene in the mtDNA genome as a DNA barcode [24]. This gene is regarded as a DNA barcode due to the conserved sequence at the intra-species level. Numerous studies have shown that COI is superior in identifying species levels from a wide variety of taxa.



**Figure 1** Distribution of the resulting DNA fragment size among Panilurid lobsters estimated from 739 restriction enzymes.

Our simulation using 10 mtDNA genome sequences from ten Panilurid lobsters, successfully identified the amplification region with a size of 709 bp. This finding was confident, as it was confirmed by a previous report using a similar primer pair amplified at an approximately similar size [24,32]. Prior to further analysis, we also estimated the size of the digested fragments using 739 REs. The fragment size (Figure 1) was distributed from 0 (no DNA digestion occurred) to a number of 62 fragments.



**Figure 2** The number of restriction enzymes acquired from the respective RE exploration steps.

The bioinformatics analyses in this study initially proceed with 739 REs to simulate the digestion (Figure 2). In the first filter step, 456 REs with fragment

sizes of more than 100 bp were obtained. These 456 REs contained different numbers of DNA fragments, ranging from 1 to 5 fragment products.

As the next step in the selection process, 167 REs were successfully identified that have the potential to distinguish *P. homarus* from other species. This large number of potential REs were filtered again to identify REs that have the ability to provide a specific *P. homarus* identity that contains a single cleavage site and produce fragments greater than 150 bp. Eventually, a set of 19 potential REs that met this requirement were acquired. In addition, we also tried to find specific REs that have the ability to specifically differentiate between each of 10 Panilurid lobster sequences, but no RE was found that met this criterion.

The analysis successfully identified 19 REs that have the ability to produce different fragment sizes and numbers to identify Panilurid lobsters. Nevertheless, no REs met the requirement of providing a single cut site. Thus, the potential application of determining Panilurid lobsters using a single RE was only possible by the use of capillary electrophoresis as the DNA fragment separation instrument due to its ability of separating DNA fragments at a higher resolution (2-5 bp). Nevertheless, the use of standard agarose gel electrophoresis was still applicable to distinguish *P. homarus* compared to other Panilurid lobster using the 19 selected REs.

The set of short-listed potential REs (Table 2) provides an opportunity to be used in *P. homarus* identification under the agarose gel technique. This is because of their ability to produce DNA fragment sizes that are specific to *P. homarus* and their large DNA fragment size. Nevertheless, REs with long nucleotide recognition sites were eliminated from the selection (AclI, CjeNII, and Eco57I).

The REs with long nucleotide recognition sites are potentially inconsistent in large sample sizes due to the presence of SNPs. To be more stringent in selecting the best among the potential REs, selection through isoschizomer REs could be used to eliminate REs with similar characteristics of the recognition site [33]. The REs selected as representatives of their isoschizomers were Sau96I as isoschizomer of AspS9I, AsuI, PspPI, Cfr13I, and BseSI as isoschizomers of BaeGI, and BstSLI and PctI as isoschizomers of BsaMI, BsmI, and Mva1269I. Thus, from 19 sets of REs, six RE were selected for the final test, i.e., BmgI, BseSI, FmuI, PctI, Sau96I, and UnbI.



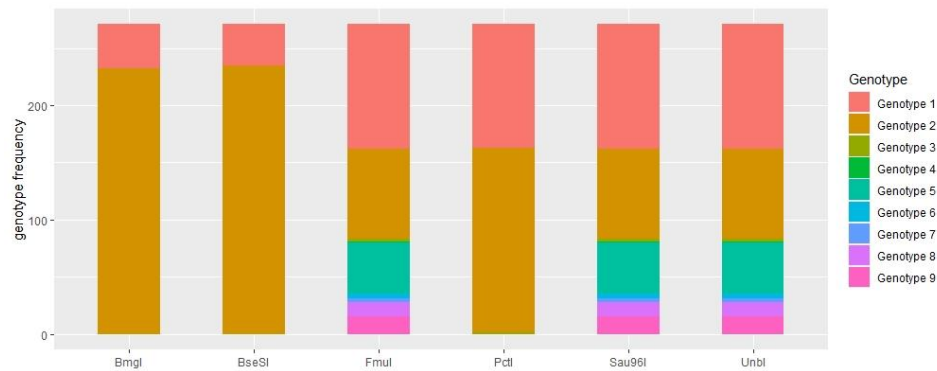
**Table 2** Selected restriction enzymes after eliminating REs with smaller than 150 bp digestion product.

Enzymes*	Recognition Site	Cutting Site	Fragment Length	Iso
AcuI	CTGAAGnnnnnnnnnnnnnnnn_nn'	482	481,228	A
AspS9I	G'GnC_C	387	386,323	B
AsuI	G'GnC_C	387	386,323	B
BaeGI	G_kGCm'C	246	245,464	C
<b>BmgI</b>	<b>GkGCCC</b>	<b>240</b>	<b>239,470</b>	-
BmgT120I	GG'n_CC	388	387,322	-
BsaMI	GAATG_Cn'	524	523,186	D
<b>BseSI</b>	<b>G_kGCm'C</b>	<b>246</b>	<b>245,464</b>	<b>C</b>
BsmI	GAATG_Cn'	524	523,186	D
BstSLI	G_kGCm'C	246	245,464	C
Cfr13I	G'GnC_C	387	386,323	B
CjeNII	GAGnnnnnnGT	396	395,314	-
Eco57I	CTGAAGnnnnnnnnnnnnnnnn_nn'	482	481,228	A
<b>FmuI</b>	<b>G_GnC'C</b>	<b>390</b>	<b>389,320</b>	-
Mva1269I	GAATG_Cn'	524	523,186	D
<b>PctI</b>	<b>GAATG_Cn'</b>	<b>524</b>	<b>523,186</b>	<b>D</b>
PspPI	G'GnC_C	387	386,323	B
<b>Sau96I</b>	<b>G'GnC_C</b>	<b>387</b>	<b>386,323</b>	<b>B</b>
<b>UnbI</b>	<b>'GGnCC_</b>	<b>386</b>	<b>385,324</b>	-

\*Enzymes in bold indicate potential enzymes to analyze in the next step of enzyme selection (i.e., BmgI, BseSI, FmuI, PctI, Sau96I, UnbI).

COI sequences are considered to be a more conservative compared to other genes in mtDNA. Nevertheless, nucleotide polymorphisms can still be found in this gene. Senevirathna & Munasinghe [34] have reported the presence of sixty nucleotide polymorphism sites in *P. homarus* from southern Srilanka and South India, while Permana *et al.* [35] have reported 49 haplotypes from Indonesian *P. homarus* COI sequence data.

Nucleotide polymorphism is the main factor that affects the RE's recognition site to be activated; otherwise it loses its ability to cleave the DNA sequence. In the initial filtering step, the use of a single mtDNA sequence to estimate the PCR amplicon sequence is produced using a single mtDNA sequence for a single species. Thus, the presence of nucleotide polymorphisms at the population level is neglected. Finding six potential sequences, a test to find the most consistent DNA fragment size resulting using multiple individuals was applicable.



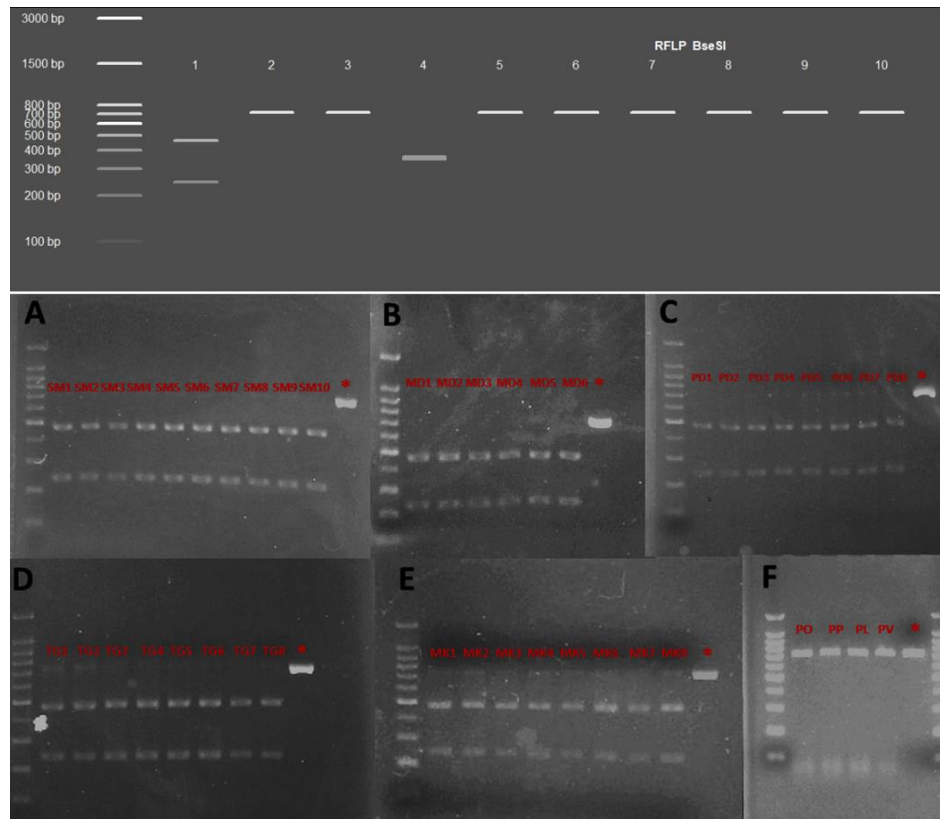
**Figure 3** Cut site consistency of the 6 best restriction enzymes tested using 271 reference sequences.

Figure 3 shows BmgI and BseSI were the two best applicable REs for identification of *P. homarus*. Both REs had more than 85% consistency in producing cleavage in the DNA sequence compared to the other REs when digesting 271 DNA sequences.

BmgI and BseSI produced DNA fragment cleavage in 232 and 235 individual COI sequences, respectively. Nevertheless, 36 and 39 COI sequences failed to be digested using those REs by in silico restriction. This was because *P. homarus* has at least four subspecies that have a distinct molecular characteristic, i.e., *P.h. homarus*, *P.h. megasculpta*, *P.h. rubellus*, and *P.h. 'Brown'* [36]. Our study found that the uncut sequences for BseSI and BmgI were confirmed from the reference sequences of non-*P.h. homarus* subspecies.

According to Lavery *et al.* [36], it is known that Indonesian waters are becoming a distribution area of a single subspecies type, *P.h. homarus*. Thus, BseSI and BmgI can be considered as the two REs with the best practical use for identification of *P. homarus* from Indonesia. This finding was also confirmed by using forty samples of Indonesian *P. homarus* in a wet lab trial (Figure 4).

Because the availability of commercially accessible enzymes in Indonesia is limited, the wet lab trial was conducted using BseSI enzymes. After the trial, all *P. homarus* showed fragmented products with size ~245 bp and ~464 bp (Figure 4A-E) after BseSI digestion, while other species, i.e., *P. ornatus*, *P. penicillatus*, *P. longipes*, and *P. versicolor* showed undigested products (Figure 4F).



**Figure 4** Simulated (upper figure, 1. *P. homarus*, 2. *P. argus*, 3. *P. cygnus*, 4. *P. japonicus*, 5. *P. longipes*, 6. *P. ornatus*, 7. *P. penicillatus*, 8. *P. polyphagus*, 9. *P. stimpsoni*, 10. *P. versicolor*) and laboratory tested (bottom figure, 40 *P. homarus* from five populations; A. Simeleu, B. Mandeh, C. Pangandaran, D. Trenggalek, and E. Makasar, and F. 4 non-*P. homarus* digestion product; PO. *Panulirus ornatus*, PP. *Panulirus penicillatus*, PL. *Panulirus longipes*, PV. *Panulirus versicolor*) electropherogram of BseSI digestion product from 709 bp COI sequence. \* indicates *P. homarus* PCR product.

#### 4 Conclusion

PCR-RFLP techniques using a single RE offer simplicity and low cost in providing species identity information. Large sequence information available in gene banks and the assistance of the REfind package make it easy to consider potential REs for the identification of species. Here, among 739 REs, we successfully identified the two best REs, i.e., BseSI and BmgI, the potential for *P. homarus* in silico restriction, and confirmed the application of BseSI for *P. homarus* identification in a laboratory experiment.

## Acknowledgements

The first author would like to thank the Ministry of Marine Affairs and Fisheries for its support through a PUSDIK-KP post-graduate scholarship programs as well as for funding the research. We would like to thank the Head of the Research Institute of Fish Enhancement (RIFE) and Danu Wijaya (Coordinator of the Lobster Bio-ecology Project) for their permission to use the laboratory facilities and specimens. The authors also thank Aswar Rudi, Dece E Sahertian, and Ingrid N Kailola for their efforts in providing information and specimens of local lobsters.

## References

- [1] Austen, G.E., Bindemann, M., Griffiths, R.A. & Roberts, D.L., *Species Identification by Experts and Non-Experts: Comparing Images from Field Guides*, Scientific Reports, **6**(1), pp. 1-7, 2016.
- [2] Slade, E.M., Kirwan, L., Bell, T., Philipson, C.D., Lewis, O.T. & Roslin, T., *The Importance of Species Identity and Interactions for Multifunctionality Depends on How Ecosystem Functions are Valued*, Ecology, **98**(10), pp. 2626-2639, 2017.
- [3] Wibowo, A., Panggabean, A.S., Zamroni, A., Priatna, A. & Yusuf, H.N., *Using DNA Barcode to Improve the Identification of Marine Fish Larvae, Case Study Coastal Water Near Jakarta and Banda Sea, Indonesia*, Indonesian Fisheries Research Journal, **24**(1), pp. 23-30, 2018.
- [4] Barrett, R.D. & Hebert, P.D., *Identifying Spiders Through DNA Barcodes*, Canadian Journal of Zoology, **83**(3), pp. 481-491, 2005.
- [5] Bingpeng, X., Heshan, L., Zhilan, Z., Chunguang, W., Yanguo, W. & Jianjun, W., *DNA Barcoding for Identification of Fish Species in the Taiwan Strait*, PLoS One, **13**(6), e0198109, 2018.
- [6] Jamaluddin, J.A.F., Mohammed Akib, N.A., Ahmad, S.Z., Abdul Halim, S.A.A., Abdul Hamid, N.K. & Mohd Nor, S.A., *DNA Barcoding of Shrimps from a Mangrove Biodiversity Hotspot, Mitochondrial DNA Part A*, **30**(4), pp. 618-625, 2019.
- [7] Nagy, Z.T., Sonet, G., Glaw, F. & Vences, M., *First Large-Scale DNA Barcoding Assessment of Reptiles in the Biodiversity Hotspot of Madagascar, based on Newly Designed COI Primers*, PLoS One, **7**(3), e34506, 2012.
- [8] Taberlet, P., Coissac, E., Pompanon, F., Brochmann, C. & Willerslev, E., *Towards Next-Generation Biodiversity Assessment using DNA Metabarcoding*, Molecular Ecology, **21**(8), pp. 2045-2050, 2012.
- [9] Blackman, R., Bennucci, M., Donnelly, R., Hänfling, B., Harper, L.R., Sellers, G. & Lawson-Handley, L., *Simple, Sensitive and Species-Specific*

- Assays for Detecting Quagga and Zebra Mussels (Dreissena rostriformis bugensis and D. polymorpha) using Environmental DNA*, Management of Biological Invasions, **11**(2), pp. 218-236, 2020.
- [10] Paoletti, M., Mattiucci, S., Colantoni, A., Levsen, A., Gay, M. & Nascetti, G., *Species-Specific Real Time-PCR Primers/Probe Systems to Identify Fish Parasites of the Genera Anisakis, Pseudoterranova and Hysterothylacium (Nematoda: Ascaridoidea)*, Fisheries Research, **202**, pp. 38-48, 2018.
- [11] Wilcox, T.M., Carim, K.J., McKelvey, K.S., Young, M.K. & Schwartz, M.K., *The Dual Challenges of Generality and Specificity when Developing Environmental DNA Markers for Species and Subspecies of Oncorhynchus*, PLoS One, **10**(11), e0142008, 2015.
- [12] Guan, F., Jin, Y.T., Zhao, J., Xu, A.C. & Luo, Y.Y., *A PCR Method that Can be Further Developed into PCR-RFLP Assay for Eight Animal Species Identification*, Journal of analytical methods in chemistry, 5890140, 2018.
- [13] Wilwet, L., Jeyasekaran, G., Shakila, R.J., Sivaraman, B. & Padmavathy, P., *A Single Enzyme PCR-RFLP Protocol Targeting 16S rRNA/tRNA<sup>Val</sup> Region to Authenticate Four Commercially Important Shrimp Species in India*, Food Chemistry, **239**, pp. 369-376, 2018.
- [14] Diguta, C.F., Vincent, B., Guilloux-Benatier, M., Alexandre, H. & Rousseaux, S., *PCR ITS-RFLP: A Useful Method For Identifying Filamentous Fungi Isolates on Grapes*, Food Microbiology, **28**(6), pp. 1145-1154, 2011.
- [15] Marty, E., Buchs, J., Eugster-Meier, E., Lacroix, C. & Meile, L., *Identification of Staphylococci and Dominant Lactic Acid Bacteria in Spontaneously Fermented Swiss Meat Products using PCR-RFLP*, Food Microbiology, **29**(2), pp. 157-166, 2012.
- [16] Priyambodo, B., Jones, C.M. & Sammut, J., *Assessment of the Lobster Puerulus (Panulirus homarus and Panulirus ornatus, Decapoda: Palinuridae) Resource of Indonesia and its Potential for Sustainable Harvest for Aquaculture*, Aquaculture, **528**, 735563, 2020.
- [17] Jones, C.M., Le Anh, T. & Priyambodo, B., *Lobster Aquaculture Development in Vietnam and Indonesia. In Lobsters: Biology, Fisheries and Aquaculture*, Springer, pp. 541-570, 2019.
- [18] Suman, A., Pane, A.R.P. & Panggabean, A.S., *Fishing, Size Structure and Exploitation Rate for Scalopped Spiny Lobster (Panulirus homarus) and Pronghorn Spiny Lobster (Panulirus penicillatus) in Gunung Kidul and Adjacent Waters*, JPPI, **25**(3), pp. 147-160, 2019.
- [19] Chow, S., Suzuki, N., Imai, H. & Yoshimura, T., *Molecular Species Identification of Spiny Lobster Phyllosoma Larvae of the Genus Panulirus from the Northwestern Pacific*, Marine Biotechnology, **8**(3), pp. 260-267, 2006.

- [20] Dharani, G., Maitrayee, G.A., Karthikayalu, S., Kumar, T.S., Anbarasu, M. & Vijayakumaran, M., *Identification of Panulirus homarus Puerulus Larvae by Restriction Fragment Length Polymorphism of Mitochondrial Cytochrome Oxidase I Gene*, Pakistan Journal of Biological Sciences, **12**(3), 281, 2009.
- [21] Winter, D.J., *Rentrez: An R Package for the NCBI eUtils API*, PeerJ, e3179v2, 2017.
- [22] Thompson, J.D., Gibson, T.J. & Higgins, D.G., *Multiple Sequence Alignment using ClustalW and ClustalX*, Current protocols in bioinformatics, (1), pp. 2-3, 2003.
- [23] Kumar, S., Stecher, G., Li, M., Knyaz, C. & Tamura, K., *MEGA X: Molecular Evolutionary Genetics Analysis Across Computing Platforms*, Molecular Biology and Evolution, **35**(6), 1547, 2018.
- [24] Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R., *DNA Primers for Amplification of Mitochondrial Cytochrome C Oxidase Subunit I from Diverse Metazoan Invertebrates*, Mol Mar Biol Biotechnol, **3**, 294-299, 1994.
- [25] Ding, Q. & Zhang J., *seqRFLP: Simulation and Visualization of Restriction Enzyme Cutting Pattern from DNA Sequences*, R package version 1.0.1, 2012. <http://cran.r-project.org/web/packages/seqRFLP/index.html> accessed November 4<sup>th</sup>, 2021.
- [26] Françoso, E., Gomes, F. & Arias, M.C., *A Protocol for Isolating Insect Mitochondrial Genomes: A Case Study of NUMT in Melipona Flavolineata (Hymenoptera: Apidae)*, Mitochondrial DNA Part A, **27**(4), pp. 2401-2404, 2016.
- [27] Xiao, B.H., Zhang, W., Yao, W., Liu, C.W. & Liu, L., *Analysis of the Complete Mitochondrial Genome Sequence of Palinura homarus*, Mitochondrial DNA Part B, **2**(1), pp. 60-61, 2017.
- [28] DeSalle, R., Schierwater, B. & Hadrys, H. *MtDNA: The Small Workhorse of Evolutionary Studies*, Frontiers in Bioscience, **22**, pp. 873-887, 2017.
- [29] Colihueque, N., Estay, F.J., Crespo, J.E., Arriagada, A., Baessolo, L., Canales-Aguirre, C.B. & Carrasco, R., *Genetic Differentiation and Origin of Naturalized Rainbow Trout Populations from Southern Chile, Revealed by the mtDNA Control Region Marker*, Frontiers in Genetics, **10**, 1212, 2019.
- [30] Yang, T., Yang, S., Meng, W., Gao, T. & Zhang, X., *Genetic Evaluation of Chinese Shrimp (Fenneropenaeus Chinensis) Stock Enhancement in the Yellow Sea and Bohai Sea based on Mitochondrial DNA Control Region*, Aquaculture International, **28**(2), pp. 603-614, 2020.
- [31] Gammage, P.A., Viscomi, C., Simard, M.L., Costa, A.S., Gaude, E., Powell, C.A. & Minczuk, M., *Genome Editing in Mitochondria Corrects a Pathogenic mtDNA Mutation in Vivo*, Nature Medicine, **24**(11), pp. 1691-1695, 2018.

- [32] Pranata, B., Fadjar, M., Iranawati, F. & Toha, A.H., *Phylogeny of the Spiny Lobster Panulirus Versicolor in Cenderawasih Bay, Papua, Indonesia*, Aquaculture, Aquarium, Conservation & Legislation, **11**(4), pp. 1015-1024, 2018.
- [33] David Clark, P. & Nanette Pazdernik, J., *Manipulation of Nucleic Acids*, in Molecular Biology (Second Edition), Academic Press, pp. 125-161, 2013.
- [34] Senevirathna, J.D.M. & Munasinghe, D.H.N., *Genetic Diversity and Population Structure of Panulirus homarus Populations of Southern Sri Lanka and South India Revealed by the Mitochondrial COI Gene Region*, Biological Medical Scienceews (FBMS-2014), Bangkok, 2014.
- [35] Permana, G.N., Slamet, B., Permana, B.A., Dewi, A.K. & Mahardika, G.N., *Population Genetic Structure of Spiny Lobsters, Panulirus homarus and Panulirus ornatus, in the Indian Ocean, Coral Triangle, and South China Sea*, Indonesian Aquaculture Journal, **14**(1), pp. 7-14, 2019.
- [36] Lavery, S.D., Farhadi, A., Farahmand, H., Chan, T.Y. & Azhdehakoshpour, A., *Evolutionary Divergence of Geographic Subspecies within the Scalloped Spiny Lobster Panulirus homarus (Linnaeus 1758)*, PLoS One, **9**(6), e97247, 2014.