A new species of pisang shrimp, *Penaeus symplex* revealed by molecular DNA analyses, as candidate species for aquaculture in Indonesia

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Abstract. In Indonesia there are two species mainly farmed, these are *Penaeus monodon* and *Litopenaeus vannamei*. Due to disease outbreaks which were mainly caused by viruses, shrimp aquaculture is become a high risk, therefore is a need to find new species. Unknown species of shrimp named as *udang pisang* (Pisang shrimp) only found in Indonesian waters. In the present study, six species of shrimp available in Indonesia, those are Pisang shrimp *(Penaeus* sp.*), P. monodon, P. merguensis, P. indicus, P. semisulcatus* and *L. vannamei* were analysed by RAPD with two primers of 2AAM2 and AS15 to confirm their species. Preliminary trials on the culture of Pisang shrimp and *P. monodon* were carried out in earthen ponds to compare their growth. The results revealed that based on the gel banding pattern and molecules weight of DNA, Pisang shrimp genetically is different from other five species analyzed and morphology generally like *P. monodon*. The identity between Pisang shrimp and *P. monodon* was 92%, suggested the two species are different. Pisang shrimp is a new species named *Penaeus symplex* [14, 20] and has been validated. The growth of Pisang shrimp and *P. monodon* for 90 days of culture was comparable (19.89±1.71 g and 20.01±1.08 g). Therefore, Pisang shrimp has high potential for aquaculture diversification.

1 Introduction

Aquaculture supplies over 20% of total protein intake by human and is growing rapidly nearly 10% annually [1]. Aquaculture now makes a significant contribution for food security, human nutrition, improving rural livelihoods and economic growth across the world [1, 2]. Among hundreds of aquaculture species farmed in Asia make a remarkable contribution to seafood production. In Asia Pacific, white shrimp is the pre-eminent farmed species in terms of trade value [3]. Penaeid shrimp are currently the most successful farmed aquaculture commodity around the world. Of the 110 species in 12 genera that are members of the family Penaeidae, the most commercially important farmed shrimps in Asian countries include: *Litopenaeus vannamei, Penaeus monodon, Penaeus indicus, Penaeus merguiensis, Penaeus semisulcatus, Penaeus japonicus* and *Penaeus chinenses* [3, 4]. *P. vannamei* and *P. monodon,* are the most widely farmed species in tropical and sub-tropical areas including Indonesia. These two species' production currently provides 90% of the world's farmed marine shrimp [6, 7]. Penaeid shrimp is a valuable economic resource and has been selected by

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Indonesian Government as a priority culture species for domestic and export commodity for income generation. Unfortunately, most of the shrimp production, research and development efforts have been directed to only two species mainly *Litopenaeus vannamei* and *Penaeus monodon*.

The Ministry of Marine Affairs and Fisheries of Indonesia have been targeted to increase shrimp production for both species up to 2.0 million tonnes by 2024 [8]. The cultured production of *L. vannamei* and *P. monodon* in Indonesia steadily increased during 2005-2013, thereafter a slight drop in production. Production decrease has been due to several factors, but the most prominent cause has been outbreaks of viruses such as white spot syndrome virus (WSSV), infectious myonecroses virus (IMNV) [9] and bacteria, acute hepatopancreatic necrosis disease (AHPND) and parasite, enterocytozoon hepatopenaei (EHP) [10]. Therefore, there is a need to find species that are locally available with market and aquaculture potential as alternatives to *L. vannamei* and *P. monodon* shrimps. One unknown species of shrimp is only found in Aceh waters, mainly along the west coast of Aceh and Simeleu Island. In Aceh named as *udang Pisang* (Pisang shrimp), and the species still needs to be assigned a scientific name [11]. The present study aimed to identify available shrimp species in Indonesian water by RAPD technique and morphology characteristics. The preliminary growth trial between Pisang shrimp and *P. monodon* was also carried out in earthen ponds for 90 days of culture to get an information potential of Pisang shrimp as a candidate species for culture in Indonesia.

2 Method

2.1 Sample and DNA extraction

Twenty individual of each shrimp samples (Pisang shrimp (*Penaeus* sp), *Penaeus monodon, P. merguensis, P. indicus,* and *P. semisulcatus*) were collected from coastal water of Aceh Province (coordinate of 4°10′08″N 96°19′28″E / 4.1689°N 96.3244°E / 4.1689; 96.3244), while *L. vannamei* was collected from cultured pond at Kerawang TIR (*Tambak Inti Rakyat*) shrimp ponds. The Pisang shrimp and *Penaeus monodon* from Aceh Province Indonesia, was collected by staff of The Aceh Coastal Aquaculture Development Center in Ujung Bate. Some samples were sent to The National Taiwan Ocean University, Keelung for further species identification, while another sample both holotype female and male shrimp was stored on deep freezer (-80 °C) at Gondol-Bali Research Center for Mariculture and now belongs to The National Research and Innovation Agency of Indonesia (BRIN).

The samples for molecular analyses were stored in ethanol and transported to the laboratory for further analysis. Genomic DNAs from 20 mg of muscle were put into a microtube containing a particle bed, and added 500 ul of lysis buffer was following the standard procedure of Hans Knoll Institute, Jena University Germany. The microtube was then put in Grinder Paqlab at 5,000 rpm 3 times at 3 seconds each with rest intervals of 10 seconds and incubated for 4 minutes. Subsequently, microtube were incubated for 1 hour at 37 °C and added RNAse A for 20 ul, again microtube were incubated for 1 hour at 37 °C and then added proteinase K at 20 ul, then incubated at 55 °C. The microtubes were then centrifuged at 16,000 rpm for 1 minute and supernatant was moved to new microtube and extracted with Bio and Cell DNA mini kit. Added 500 ul Binding buffer (UCT), and transfer to tube with column filtered then centrifuge for 2 minutes at 16,000 rpm, added 500 ul with wash buffer (IT) and centrifuge for 1 minute at 16,000 rpm, rewashing with 750 ul wash buffer (NT), centrifuge again for 1 minute at 16,000 rpm. They moved the column to the new tube and centrifuge again for 2 minutes at 16,000 rpm. The column was then moved to new microtube added with 100 µl Elution buffer and incubated for 5 minutes, and the end centrifuge again at 10,000 rpm for 3 minutes and DNA genome concentration were measured by Nanodrop. The DNA was amplified by PCR machine.

2.2 Amplification of PCR

Amplification reactions were carried out by master mix with polymerase kit (green) and applied to 6 universal primers presented in Table 1. Master mix for analyze 1 sample consisted of 5 times buffer

(5 µl), 10 nM dNTP (1µl), 100 picomol primer (1 µl, 25 nM $MgCl_2$ (7µl) taq polymerase 0.5 µl and dH_2O 5.5 ul. The total volume master mix becomes 19 ul, and the genome DNA of each sample that is going to be analyzed is 1 ul. Reaction of genome were put into PCR plate with volume of 0.1 ml each ml. There were 96 wells in each plate and covered by PCR film and centrifuge with plate centrifuge for 3 minutes at 1.000 rpm to avoid air bubbles on plate. The plate PCR was placed in PCR machine (ABI Prims) to amplification of DNA, with initial denaturation thermal cycle of 95 °C for 5 minutes, denaturation of 95 °C, annealing at 40 °C for 10 seconds and extension at 68 °C for 30 seconds. This cycle was repeated 40 times, followed by a final extension at 72 °C for 3 minutes and an incubation temperature of 12 °C. Agarose gel 2% were used to express DNA amplification in Bionic buffer of 0.5 times and electrophoresis duration for 30 minutes at 250 voltages. The expression of gel banding patterns was documented.

2.3 DNA fingerprint by chip

This method uses kit Agilent DNA 1000 and Agilent DNA 7500, application of this method depends on molecular weight as result from RAPD, if molecular weight more than 1500 bp, Agilent DNA 7500 can be used. The analyses with chips are a bit simple because the reagent is designed to determine fragment DNA. Every chip interacts with certain micro channels that are used for fragment expression of nucleic acid based on size and controlled by electrophoretic. DNA analyses using DNA chip (16 microwell) were carried out by fill up of 9.0 µl gel dye mix to one microwell (coded G) and pushing for 1 minute with plunger, then gel dye mix (9 µl) and two microwell above also fill up dye mix and remaining 13 microwell were fill up by marker of 5 µl. One microwell below (code G) fills up 1 ul ladder as a marker while the remaining 12 microwell added by amplicon (PCR product) from RAPD. Microchips were put horizontally at the adapter and vortex for 1 minute at 2,400 rpm to remove air bubbles at microwell. The microchip was placed on Agilent 2.00 Bioanalyzer for further analyses. Analysis DNA of Pisang shrimp and *P. monodon* were carried out using DNA chip Agilent 2100 to check sharing fragments of both species and followed by sequencing which was done with manufacturer protocol. The primers used for amplification PCR were LCO1490 (R) and HCO2198. The sequences were aligned pairwise by using EMBL-ABL Stop codons and removed using BLAST. The similarity between the two species was identified by BLAST.

2.4 Molecular phylogenetic analyses

The molecular phylogenetic relationship amongst the members of the genus *Penaeus* sp. including *P. monodon, P. merguensis, P. indicus, P. semisulcatus, P. stylirostris, L. vannamei* and *Penaeus symplex* (Pisang Shrimp) have been done and reported by [14, 20]. The most comprehensive study to date on the phylogenetic relationships amongst the members of the genus *Penaeus* sp. Fabricius, 1798 has been done by [19].

2.5 Morphology comparisons

Based on the size of adult and mature shrimp, morphology comparisons were made for Pisang shrimp and *P. monodon*, these both shrimps were generally similar. Part of the body were checked including rostrum shape and ratio to carapace, number of teeth upper and lower rostrum. Chan et al. [14] have done detailed morphology comparisons between two species, where the sample of Pisang shrimp was collected from Aceh.

2.6 Growth comparison between Pisang shrimp and *P. monodon*

Two groups of post larvae PL-12 (Pisang shrimp and *P. monodon*) were reared in separate two earthen ponds (2000 m^2) at density of 40 Ind./m^2 under similar conditions (i.e., environment, management processes and feeding diets). The seeds of both shrimp produced by Coastal Aquaculture Development Institute of Ujung Bate-Aceh using modified technology of FAO [18]. Thirty days after stocking, growth was monitored every 10 days and was continued up to 90 days of culture. In each sampling period, 30 individuals from the sub-samples were taken by cast net in each pond. Samples

were weighed by a digital balance to nearest 0.01 g. Water temperature, pH, dissolved oxygen, salinity, and transparency were measured two times a day, morning and noon times.

3 Results and Discussion

3.1. Species identification

Six universal primers (Table 1) were tested for their ability to prime PCR for six species of shrimp genomic DNA. Only 2 RAPD primers (33%) (2 AAM2 and AS15) have yield amplification product, while the rest of 4 primers did not amplify the DNA template and resulted in unclear bands. Two primers 2 AAM2 and AS15 gave reproducible RAPD patterns with resolvable fragments that stained intensely were selected for further analysis (Figure.1). RAPD analysis of 20 individuals in each six species of sample revealed different levels of polymorphisms using two primers. Based on the banding pattern fragment of *P. semisulcatus* almost monomorphic using primer 2AAM2 but high polymorphic using primer AS15, while another shrimp the polymorphism similar either using primer 2AAM2 or AS15. Generally, most of the shrimp species analysed by RAPD showed high level of polymorphism, which may be the fact that wild caught shrimp would have a higher level of genetic variation using the allozyme and RAPD technique [12, 13]. Based on the RAPD banding pattern, it confirmed that all shrimp are different species and that Pisang shrimp and *P. monodon* share some genes (Figure 1).

Table 1. Nucleotide sequence of six universal primer tested in the RAPD analysis for six species of shrimp

No	Oligo name	Sequence	Volume for 100 picomol/ul
1	2AAM2	CTGCGACCCAGAGCG	346
2	2AAM2	CTGCGACCCAGAGCG	277
3	AS4	TGTGGGCGCTCGACA	290
4	AS4	TGTGGGCGCTCGACA	322
5	AS15	GGCTAAGCGGTCGTT	318
6	AS15	GGCTAAGCGGTCGTT	334

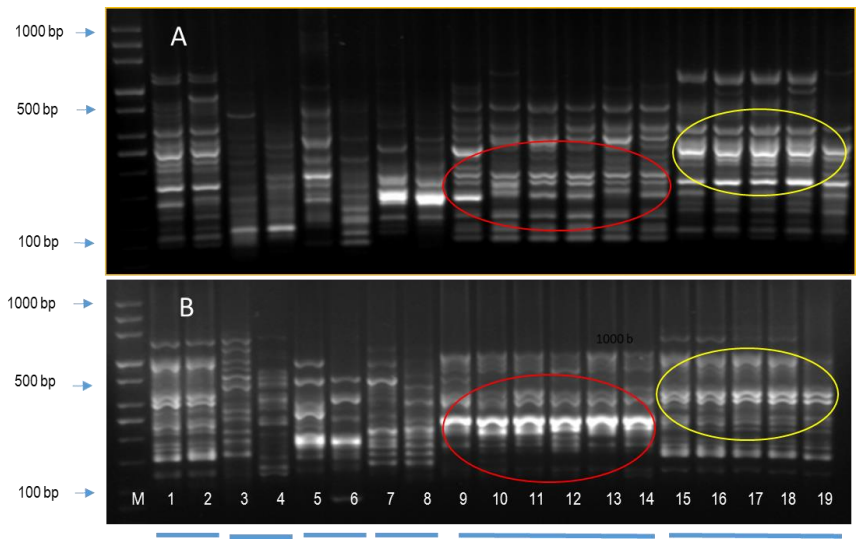

Fig. 1. RAPD pattern using primer 2AAM2 (A) and AS15 (B) of *L. vannamei* (1, 2), *P, semisulcatus* (3, 4), *P. indicus* (5, 6), *P. merguensis* (7, 8), Pisang shrimp (9-14) and *P. monodon* (15-19)

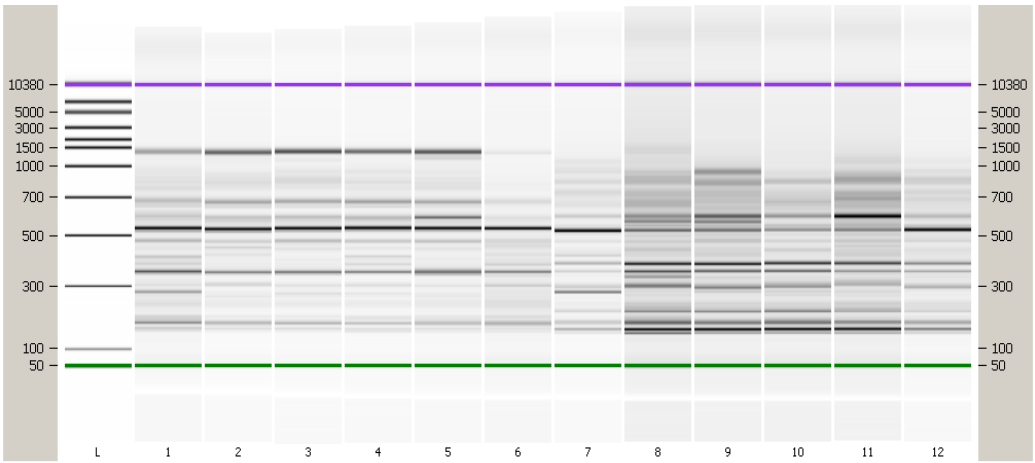

Fig. 2. Fragmentation for DNA, Marker ladder (50 bp~10 380 bp), DNA Pisang shrimp (No.1-6) and *P. monodon* (No.7-12) resulted from of amplification of PCR with AS15 primer

The primer pair of HCO2198 and LCO1490 were used to amplify the DNA sequence, while performing BLAST, the sequences of Pisang shrimp and *P. monodon* shrimp species showed degrees of similarity with existing data in the NCBI database. Both Pisang shrimp and *P. monodon* showed 92% identity, meaning that two species are different genetically (Table 2). The photographs of two species and their morphological differences are presented in Figure 3. Generally, Pisang shrimp appearance is like *P. monodon*, the two species are distinguishable by description of rostrum of Pisang shrimp is longer, distinctly sigmoid, slightly curving downward then moderately recurve as long as carapace length, far exceeding antennular peduncle, generally in upper rostrum with 7 teeth and 2-3 teeth in lower rostrum, while *P. monodon* 8 teeth and 2-3 teeth above and below rostrum respectively (Figure 3). Body coloration of Pisang shrimp in large mature adults becomes reddish compared to *P. monodon* (Figure 3). In the wild, Pisang shrimp broodstock is available from August until March, and gravid females are usually found during this period on the Western coast of Aceh Province-Indonesia [11, 17]. According to Chan et al. [14] who identified pisang shrimp with sample collected from Aceh using molecular and morphological technique have been given a scientific name of pisang shrimp as *Penaeus symplex* sp.nov and has been validated by [20]. The name pisang means banana, the color of shrimp during grow out in ponds are like banana leaves although wild broodstock is reddish (Figure 3). The scientific name symplex ("simplicity" in English) refers to the body of the new species being not banded as compared to its close relative, *P. monodon* [11, 14].

Table 2. DNA sequences of Pisang shrimp and *P. monodon* species using primers of HCO2198 and LCO1490

Oligo name	Sequences
HCO2198	TAATAGCTCCTGCTAGGACTGGAAGAGATAATAATAGTAGTAGGGCTGTAATA AATACTGCTCAAACAAAAAGTGGTATTCGATCT ATAGTTATCCCTGTTGATCGTATATTGATAACCGTTGTTATAAAGTTTACAGCT CCTAAAATTGATGAAACTCCTGCTAAATGTAAAG AAAAAATACCTAGATCAACTGAAGCTCCTGCATGAGCAATTCTAGCTGATAGA GGAGGATATACTGTTCATCCGGTTCCTACTCCTC TTTCAACTATACCTCTGGATAAAAGTAAAGTCAGAGAAGGAGGTAAAAGTCA AAAACTTATATTGTTTATTCGGGGAAATGCTATA TCTGGGGCACCTAATATTAAAGGAACAAGTCAATTACCGAAACCTCCAATTAT GATAGGTATAACTATAAAGAAAATTATAACAAAA GCGTGAGCTGTAACCACTACATTATAAATTTGATCATCTCCAATAAGACTTCCT GGTTGACCTAATTCAGCACGGATAATAAGACTAA

Oligo name	Sequences
	GAGCTGTACCCACTATTCCAGCTCAAGCTCCGAAAATAAAATATAAAGTTCCA ATATCTTTATGATTTGTTGAC
LCO1490	GAGATGATCAAATTTATAATGTAGTGGTTACAGCTCACGCTTTTGTTATAATTT TCTTTATAGTTATACCTATCATAATTGGAGGTTTC GGTAATTGACTTGTTCCTTTAATATTAGGTGCCCCAGATATAGCATTTCCCCGA ATAAACAATATAAGTTTTTGACTTTTACCTCCTTC TCTGACTTTACTTTTATCCAGAGGTATAGTTGAAAGAGGAGTAGGAACCGGAT GAACAGTATATCCTCCTCTATCAGCTAGAATTGCT CATGCAGGAGCTTCAGTTGATCTAGGTATTTTTTCTTTACATTTAGCAGGAGTT TCATCAATTTTAGGAGCTGTAAACTTTATAACAAC GGTTATCAATATACGATCAACAGGGATAACTATAGATCGAATACCACTTTTTG TTTGAGCAGTATTTATTACAGCCCTACTACTATTAT TATCTCTTCCAGTCCTAGCAGGAGCTATTACTATATTATTAACAGACCGTAATT TAAACACATCCTTCTTTGACCCTGCAGGGGGTGGT GACCCGGTTTTATATCAACATCTGTTTTGATTTTTTGGTCACCCTGGAAGTTT

3.2. Phylogenetic of *Penaeus* sp. Shrimp

Phylogenetic tree of the *Penaeus* sp. Including *P. monodon, P. merguensis, P. indicus, P. semisulcatus, Penaeus stylirostris, L. vannamei* and *Penaeus symplex* (Pisang Shrimp) from Aceh Province have been reported by [14, 20]. Genetic distance between *Penaeus symplex* vs *P. monodon* is close and belonged to one group of *Penaeus monodon* complex in which sample of *P. monodon* were collected from several Asian Countries (Indonesia, Singapore, Thailand and Philippines). This finding agrees with the present study where similarity of *P. monodon* and *P. symplex* is 92 %, almost similar but belonging to different species. The most comprehensive study to date on the phylogenetic relationships among the members of the genus *Penaeus* sp. was by Yang et. al. [19] suggested that a single genus should be reinstated for these commercially important shrimps. Their study proposes that if those molecular clades are revealed in the phylogenetic tree of *Penaeus* sp. [19] are recognized as taxonomic groups, the use of subgenera is preferable ; using this rank would also reduce confusion and maintain stability for non-taxonomists who use the name. In their phylogenetic study, Yang et al. [19] showed that up to 11 subgeneric-level clades can be recognized. While many of these clades have been named in the past, five of them, however, remain un-named. In the interest of nomenclatural stability and consistency in discussing their systematics, they propose to apply formal names for them.

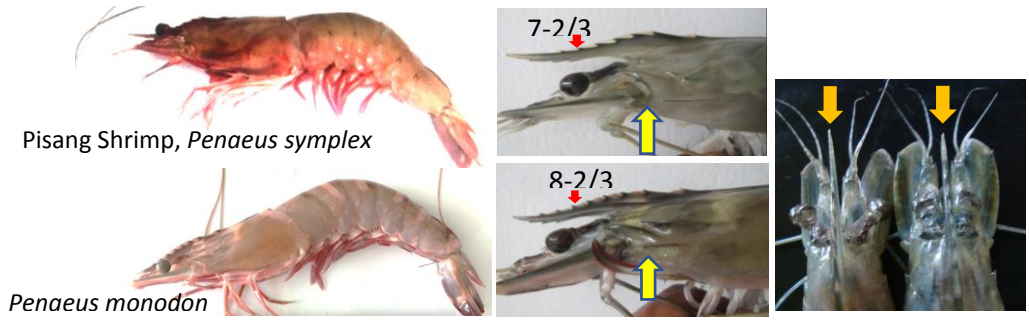

Fig. 3. Pisang shrimp (*Penaeus symplex* sp. nov.) and *Penaeus monodon* samples from Aceh Province. Adult color of shrimp, rostrum, and number of teeth above and below rostrum. (Holotype specimens of both shrimp's male and female were stored at deep freezer -80 °C in The Gondol Research Centre for Mariculture)

Penaeus symplex is most like *P. monodon* but differs in having a relatively longer rostrum (overreaching vs. not exceeding scaphocerite) and lacking distinct dark or light transverse bands on the body in life (body distinctly banded in *P. monodon*) (Figure 3). Detailed photographs, coloration, distribution, and additional distinguishing characters of *Penaeus symplex* are in [14]. Chan et al. [14,

20, 22] described in detail a new species of penaeid shrimp, *Penaeus symplex*, which is closely related to the tiger prawn *P. monodon* Fabricius, 1798, from Aceh Province, Sumatra, Indonesia. The name *Penaeus symplex* [14, 19, 22] is unavailable under the current Zoological Code (International Commission on Zoological Nomenclature /ICZN) [21]. The description of *P. symplex* by Chan et al. [14] did not include a ZooBank registration number (LSID). Article 8.5.3 of the International Code of Zoological Nomenclature /ICZN [21] requires electronic-only publications to be registered in the Official Register of Zoological Nomenclature (ZooBank) and contain evidence in the work itself that such registration has occurred. This note serves to establish the name *Penaeus symplex* as available by fulfilling the requirements of the Code.

3.3. Growth performance

During the preliminary trial on rearing of Pisang shrimp or *Penaeus symplex* sp.nov compared to *P. monodon* in earthen ponds, the environmental parameters between two ponds are almost uniform. The water temperature ranged from 28.1 to 30.2°C, salinity ranged from 28 to 34 ppt, average dissolved oxygen concentration at 7 am was 4.05±0.44 (mg/L) and at 4 pm was 4.83±0.21 (mg/L). Mean weight of shrimp in every sampling time and at 90 days of culture for each species were summarized in Table 3. No significant difference was observed in average weight at every sampling time. Based on field observation Pisang shrimp prefer to stay on pond bottom for the first 30 days of culture, then the shrimp actively feed in the water column and the shrimp are very active and sometimes eat while upside down. Challenge trials for WSSV infection have been done in laboratory and the result revealed that Pisang shrimp resistant to WSSV but depend on the isolate that is used for challenged [15, 16]. Based on the preliminary trial, growth performance between Pisang shrimp and *P. monodon* was comparable, means that Pisang shrimp is potential for aquaculture to diversify shrimp product of Indonesia. Therefore, an effort to produce year-round broodstock through domestication is urgently needed also improve the broodstock genetically.

Table 3. Growth performance of Pisang shrimp and *P. monodon* cultured in earthen ponds for 90 days

Days of culture	*P. symplex*/Pisang shrimp (g)	*P. monodon* (g)
30	2. 98±0.25	3.03±0.32
40	4.44±0.33	4.52±0.38
50	6.26±0.29	6.10±0.21
60	10.12±0.42	10.28±0.56
70	13.66±0.85	13.68±1.11
80	15.64±1.41	15.88±1.22
90	19.89±1.71[a]	20.01±1.08[a]

4 Conclusion

Udang pisang is genetically different from five shrimp's species available in Indonesia and confirmed as à new species named *Penaeus symplex* sp. nov. The growth performance between Pisang shrimp and *P. monodon* after 90 days of culture is similar, hence Pisang shrimp is potentially as candidate species for aquaculture development in Indonesia.

References

1. Food and Agriculture Organization, The state of world fisheries and aquaculture (FAO, Rome, 2016)
2. Food and Agriculture Organization, Sustainable intensification of aquaculture in the Asia-Pacific Region (FAO, Rome, 2016)
3. G. Kumar, C. R. Engle, Reviews in Fisheries Science & Aquaculture **24**, 2 (2016)
4. F. Andriantahina, X. Liu, H. Huang, J. Xiang, C. Yang, Aquaculture **324** (2012)
5. J. A. Benzie, Reviews in Aquaculture **1** (2009)

6. Food and Agriculture Organization, The state of world fisheries and aquaculture (FAO, Rome, 2018)
7. S. Moss, D. Moss, Woodhead Publishing (2009)
8. Ministry of Marine Affairs and Fisheries, Kebijakan produksi udang (KKP, 2021) (in Indonesian)
9. I. Koesharyani, L. Gardenia, N. L. A. Lasmika, Indonesian Aquaculture Journal **11**, 2 (2016)
10. World Organization for Animal Health, Aquatic animal disease cards (WOAH, Paris, 2008)
11. A. Nur, T. Ridwan, K. Sugama, Journal of The World Aquaculture Society **48**, 1 (2017)
12. K. Sugama, J. A. H. Benzie, E. Ballment, Aquaculture **205**, 1-2 (2002)
13. J. Benzie, E. Ballment, A. Forbes, N. Demetriades, K. Sugama, Moria, Molecular Ecology **11** (2002)
14. T. Y. Chan, Z. A. Muchlisin, A. Hurzaid, Journal of Crustacean Biology **41**, 1 (2021)
15. Nurbariyah, Z. Zurrah, D. Royani I. Sahidhir, Journal of The World Aquaculture Society (2015)
16. D. Novita, T. R. Ferasyi, Z. A. Muchlisin, Jurnal Ilmiah Mahasiswa Kelautan dan Perikanan Unsyiah **1**, 3 (2016)
17. J. Hutabarat, S. W. Saputra, RJOAS: Russian Journal of Agricultural and Socio-Economic Science **9**, 81(2018)
18. Food and Agriculture of the United Nations, FAO Fisheries Technical Paper No. 446 (FAO, Rome, 2007)
19. C. H. Yang, K. Y. Ma, K. H. Chu, T. Y. Chan TY. Aquaculture **563** (2023)
20. T. Y. Chan, Z. A. Muchlisin, A. Hurzaid, Journal of Crustacean Biology **43** (2023)
21. International Commission on Zoological Nomenclature. Zootaxa, **3450** (2012)
22. T. Y. Chan, ZooKeys **1141** (2023)