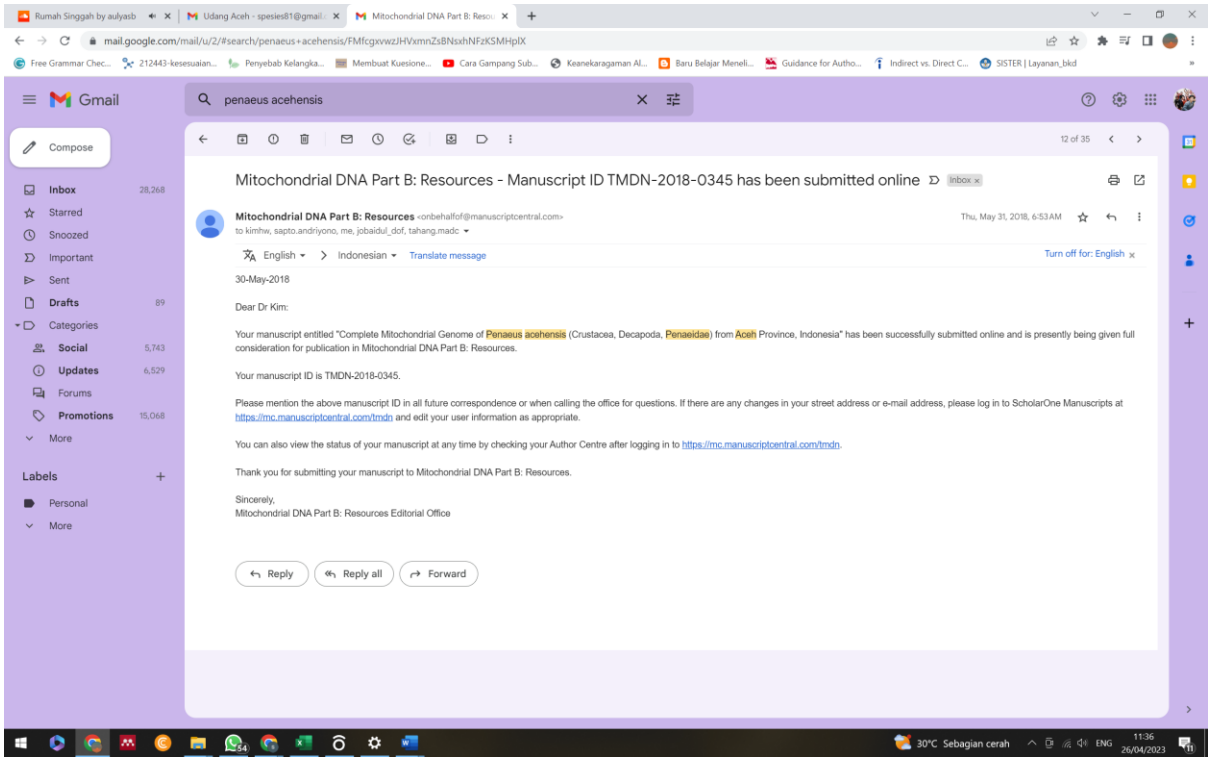
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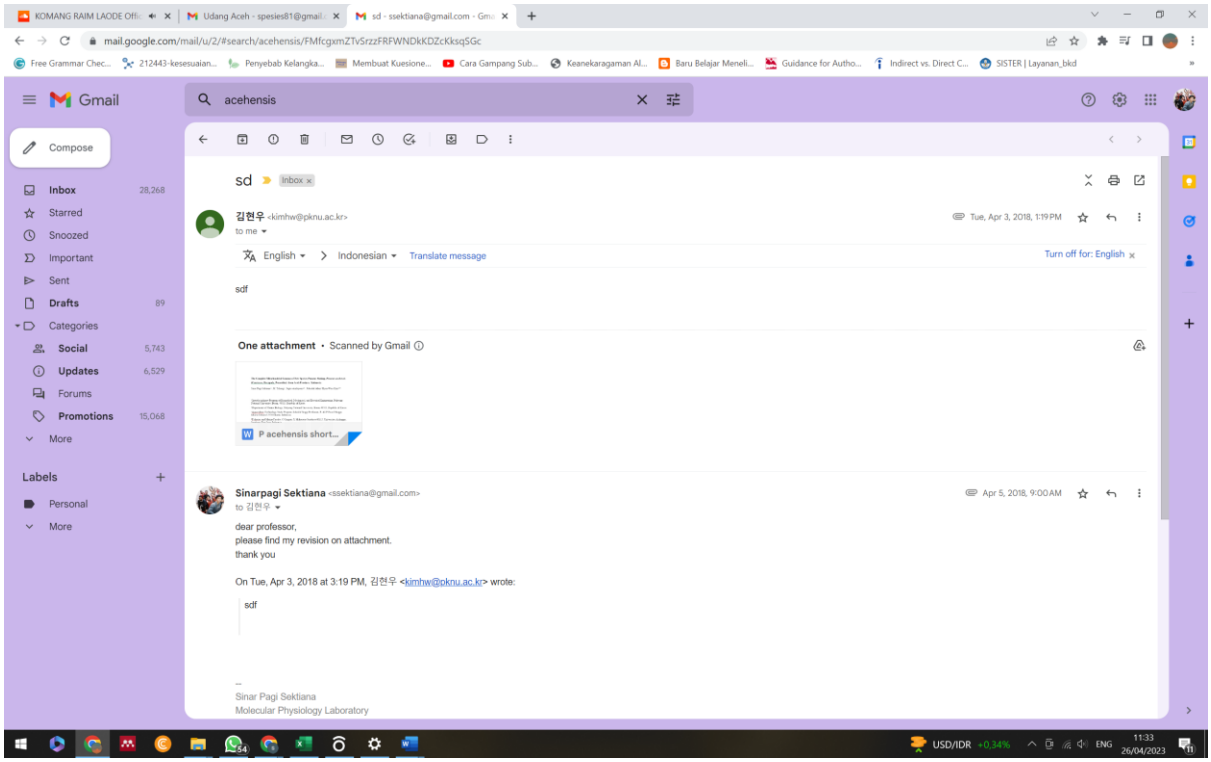
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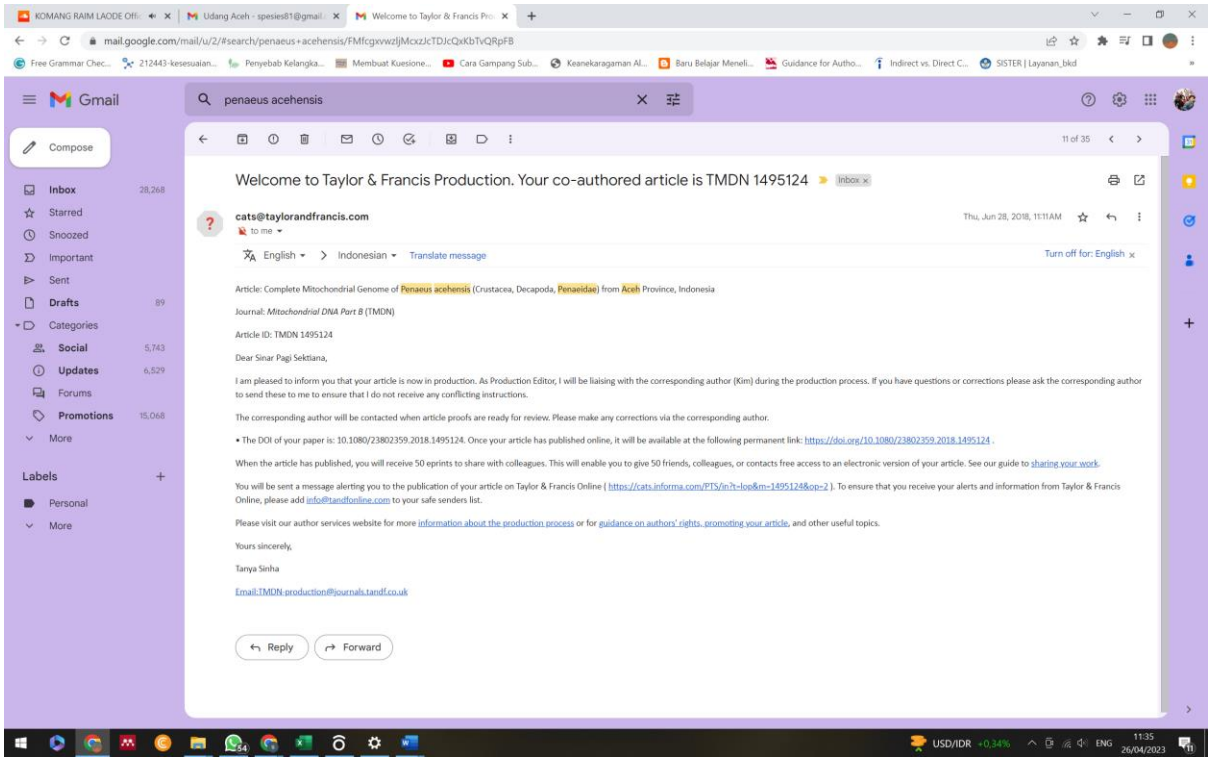
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The Complete Mitochondrial Genome of New Species Penaeus Shrimp, *Penaeus acehensis* (Crustacea, Decapoda, Penaeidae) from Aceh Province, Indonesia

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Keywords; Mitogenome, Dendrobranchiata, Next Generation Sequencing, Shrimp, Indonesia

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Abstract

Penaeid shrimps are widely distributed from Indian to western Pacific Oceans and some which are economically important. In this study, we reported full mitochondrial genome of a new endemic shrimp species, *Penaeus acehensis*, which inhabits exclusively in the coastal water of Aceh, Indonesia. Its body color, numbers of dorsal and ventral teeth, and the absence of transverse band are major characteristics of *P. acehensis* which is distinct from its relatives. Full length of circular mitogenome of *P. acehensis* was 15,991 bp in length, which contained 13 protein coding genes, two rRNA genes, 22 tRNA genes and the control region. Start codons of all coding genes were ATN except for COX1 in which ACG was used. The incomplete stop codon (T--) were found in five coding genes including COX2, COX3, NAD5, NAD4, and NAD4L. *P. acehensis* was most closely related to *Penaeus monodon* in which 92% in COI region and 89 % in its full mitogenome were identical each other. Phylogenetic tree result showed that *P. acehensis* was clustered together with those were distributed in Indo-West Pacific region (clade I), which is distinct from Eastern Pacific region (clade II).

Background

Penaeid shrimp are the most economically important species in Southeast Asian countries including Indonesia (Budhiman et al., 2005), Malaysia (Hanafi et al., 1991), Vietnam (Binh et al., 1997) and Thailand (Tookwinas, 1999). Unfortunately, the native populations of penaeid species are being seriously threatened by the careless development in the coastal area (Páez-Osuna, 2001). In addition, intensive cultivation of a few species resulted in the negative effects their sustainability including massive mortality by the outbreak of disease, lower genetic diversity, and destroying the natural habitats (Primavera, 1993). From the reason, it is urgently required to conserve genetic diversity of native penaeid species for their sustainable management.

Indonesia is one of the world's richest nations in terms of its biodiversity which has been considered as a reservoir of the hidden biodiversity (von Rintelen et al., 2017). Recently, a penaeid species locally called ‘udang lambouh’ or ‘udang kelong’ was discovered as a new endemic species in Aceh coastal area during a local survey between 2005 and 2006. It is exclusively distributed from Lamno beach (Aceh Jaya District) to Meulaboh (West Aceh district), Aceh province, Indonesia (Wedjatmiko, 2009). Although it is morphologically similar to its relatives including a banana shrimp (*Penaeus merguensis*) or a black tiger shrimp (*Penaeus monodon*), this species was

clearly distinguished from its relatives by reddish body color without transverse band and numbers of rostral teeth (7 - 8) and ventral teeth (0 - 5) (Wedjatmiko, 2009, Alafanta, 2014, Idami, 2016). This new penaeid shrimp was named as *Penaeus acehensis* and its artificial seed production is currently made to conserve its populations in the area. However, its genetic information to compare with other relatives is still unknown. Although the partial mitochondrial sequence of cytochrome c oxidase 1 gene, COI region, is most widely used as a standard molecular identification (Hebert et al., 2003), mitochondrial genome sequence information can be used in the various fisheries and aquaculture fields including breeding, evolution, conservation, or management. As the result of new sequencing technique called next-generation sequencing (NGS), information on the complete mitochondrial genome data are exponentially increasing (Smith, 2015). In this research, the complete mitochondrial genome sequence of *P. acehensis* was determined by the combination of NGS and conventional PCR-based cloning methods. Bioinformatic analyses of its mitogenome were also made to know the evolutional relationship with other relative penaeid shrimps.

Methods

Collecting samples and genomic DNA extraction

Five juvenile *P. acehensis* were obtained from a hatchery at Brackishwater Aquaculture Development Center (BADC) in Ujung Batee, Aceh province, Indonesia. Fresh shrimp were directly put into 96% ethanol (SK Chemicals, Korea) and kept at -20 °C until further analysis. Genomic DNA was extracted from the muscle using an Accuprep Genomic DNA Extraction Kit (Bioneer) according to the manufacturer's instruction. Purified genomic DNA eluted in TE buffer, quantified with Nanodrop (Thermofisher Scientific D1000) and stored at -70 °C for further analysis.

PCR amplification and sequencing

Full mitochondrial genome sequence of *P. acehensis* was obtained by assembling five fragmental PCR products. First three PCR fragments (2,898 bp, 2,540 bp, and 3034 bp) were obtained with degenerate primers designed by the multiple alignment of mitogenome sequences

from its relatives including *P. monodon* (AF217843), *Penaeus penicilliatus* (KP637169), *Penaeus californiensis* (EU497054), *Penaeus stylorostris* (EU517503), and *Penaeus vannamei* (EF584003). The other two PCR products (4786 bp and 3527 bp) were generated by two sequence-specific primer set designed with the obtained partial sequences of each region (Table 1). The quality of all the primers in this study was analyzed by the OligoAnalyzer 3.1 (http://s.idtdna.com/calc/analyzer) and commercially synthesized by Bioneer Co. (Korea). Each PCR reaction (30µL) contained 18,7 µL ultrapure water, 1 µL of each primer (0.5µM), 0.3 µL Extaq Hotstart version DNA polymerase (TAKARA, Japan), 3µL 10x Extaq buffer, 3 µL dNTPs (1µM, TAKARA, Japan) and 200ng Genomic DNA as a template. PCR was carried out with two-step PCR protocol under the following condition: initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 98 °C for 10 sec and annealing and extension at 68 °C for 2-4 min (1min/ 1kb), the process was completed with a final extension at 72 °C for 10 min. For the sequencing, all PCR products were pooled together in equal concentration and fragmented into 350 bp in length by covaris M220 (Covaris inc.). Thruseq® sample preparation kit V2 (Illumina, USA) was used for the construction of a library, quality and quantity of the constructed library was measured using 2100 bioanalyzer (Agilent Tech, Santa Clara, CA, USA). Sequencing was performed by Illumina Miseq platform (2x300 bp pair ends) (Illumina, USA).

Assembly of the mitochondrial genome

Ambiguous nucleotides with under Qv 20 raw reads from Miseq sequencer were removed using CLC Genomic Workbench V.7.5 (CLC BIO Aarhus, Denmark). Mothür software v.135.0 (Schloss et al., 2009) was used to pairing forward and reverse sequence with more than 7 bp overlapped and without any mismatch. The paired sequence then assembled using geneious ® 11.0.2 (Kearse et al., 2012) with minimum 20 bp of overlapping sequence and 100% overlap identity. The assembled and identified mitochondrial DNA sequences were further annotated and analyzed. The protein coding genes were identified by ORF finder (http://www.ncbi.nlm.nih.gov/orffinder/) and subsequently annotated by alignment of homologous genes of other published penaeids shrimp mitogenomes (Wilson et al., 2000). The tRNA prediction was accomplished by ARWEN (Laslett and Canbäck, 2008) and the total mitochondrial genome visualized by GenomeVx (Conant and Wolfe, 2008)

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Results

The total mitochondrial genome of *P. acehensis* is 15,991 bp in length was determined by the bioinformatic sequence assembly of five fragmental PCR products, which were generated by Illumina MiSeq platform. It comprised 13 protein-coding genes, 22 transfer RNAs (RNAs) two ribosomal RNAs (rRNAs), and the putative control region (Fig 1). A+T content (71 %) was higher than G+C content (29 %) and the highest A+T content was observed in the putative control region (83%). G/C skew and A/T skew measurement of the H-strand were -0.172 and -0.042, respectively (Table 2). Total 14 genes (tRNA-Gln, tRNA Cys, tRNA Tyr, tRNA-Phe, ND5, tRNA His, ND4, ND4L, tRNA Pro, ND1, tRNA Leu, Lr-RNA, tRNA Val, Sr-RNA) were located at L strand whereas the other remaining 23 genes were at H strands (Fig. 1). Overlapping protein-coding genes were detected between ATP8 and ATP6 (7 bp), between ND4 and ND4L (19 bp). Additionally, 2 bp were overlapped between $tRNA^{-Ser}$ and $tRNA^{-Glu}$. 1,140 bp of non-coding nucleotides were spread within *P. acehensis* mitogenome among which putative control region (933 bp) was longest (Table 3).

Protein-coding gene was observed in the *P. acehensis* which 11,150 bp (69.72 %) of total mitochondrial genome sequence encoded 13 protein and the size of each gene ranged from 160 bp (ATP8) to 1723 bp (ND5) (Table 3). Four protein gene was encoded by L-strand and the other protein gene were encoded by H-strand (Fig 1). Seven protein-coding genes appear to start with the ATG codon (COX2, ATP6, COX3, ND3, ND5, ND4L, and CYT B), and the other began with ATT (ND2, ND4, ND6), ATA (ND1) and ACG (COX1). Meanwhile, eight of 13 genes have complete termination codon, either TAA (ND2, COX1, ATP8, ATP6, ND4, ND6, and ND1) or TAG (CYT B), and five coding genes (COX2, COX3, ND5, ND4, and ND4L) is terminated with an incomplete codon T.

Twenty-two tRNA genes are spread throughout the mitochondrial genome and their sizes ranged from 67 bp and 74 bp (Fig 2). Each tRNA genes are predicted to be folded into a typical clover-leaf secondary structure except for $tRNA^{ser(GCT)}$ which was predicted lacked D-arm. Fourteen tRNA genes encoding H strand and 8 genes encoded in L strand (Fig. 1). It genome also contained 1,375 bp in length large subunit of rRNA and 848 bp in length small subunit of rRNA. The large subunit rRNA gene was located between the $tRNA^{leu}$ and $tRNA^{val}$ and small subunit rRNA was located between $tRNA^{val}$ and control region.

The phylogenetic trees were reconstructed based on the two different data set (CO1 sequence and total mitogenome sequence) from *P. acehensis* with minimum evolution algorithm and *Acetes chinensis* was employed as the outgroup (Fig. 3). The CO1 sequence (622 bp in length) was aligned with the other 18 Penaeus shrimp and based on online BLAST from NCBI database showed 84-92% identity, which is *P. monodon* has the highest identity to *P. acehensis.* It'sphylogenetic trees produced two clades (Fig 3a). A longer sequence, the total mitochondrial of *P. acehensis* showed similar topology of the phylogenetic tree (Fig 3b) and shared 81-89% identity to 9 Penaeus shrimp.

Discussion

In this study, the total mitochondrial genome of *P. acehensis* was determined and this information supported the previous morphological description, which was distinct from other penaeid shrimps (Wedjatmiko, 2009, Alafanta, 2014, Idami, 2016). Three pairs of Penaeus specific primers were succeeded to distinguished with 86-90% shared identity to *P. monodon*, whereas the Folmer universal primer fails (Folmer et al., 1994). Failures of the Folmer primers also found in other penaeids shrimp (Rajkumar et al., 2015). These are at least in part due to mismatches with the target annealing position because of a few sequences database available in 1994 (Geller et al., 2013). Therefore sequences database were needed to increase detection. Even though mitochondrial genome database reached more than 10.000 (https://www.ncbi.nlm.nih.gov), biodiversity is remaining unrevealed because of higher species density in the tropical region (Dowle et al., 2013) and lack of a thorough survey of all taxa (Lo et al., 2017). For this reason, total mitochondrial DNA (mtDNA) became attractive for molecular species identification (Raupach and Radulovici, 2015)

Both morphological identification and molecular detection showed the similar result which is *P. monodon* as a closest related species. Its also bear out shrimp collected from Aceh as a new species. Although its sequence identity was distinct from other penaeid shrimp which is sharing from 81 % to 89 % sequence identity, the mitogenomic organization of *P. acehensis* was identical to other of penaeid shrimps (Wilson et al., 2000, Shen et al., 2007, Peregrino-Uriarte et al., 2009). Its A+T content value is similar to *P. monodon* (Wilson et al., 2000) and higher than another penaeid shrimp (Peregrino-Uriarte et al., 2009). In contrast, the control region A+T value higher than *P. monodon* and similar to *P. vannamei* which is 82.5 % (Shen et al., 2007). The negative of

G+C and A+T skew indicating a compositional bias associated with an excess of C over G nucleotides and excess of T over A nucleotides (Perna and Kocher, 1995).

The initiation codon of protein-coding genes start with ATN and mostly ended with a complete stop codon (TAA, TAG) (Table 3). Even though the initiation codon of COX1 is unclear, it appears to start with ACG based on alignment with other species (Wilson et al., 2000). These atypical ACG as a start codon is converted to ATG in the RNA editing (Kadowaki et al., 1995, Quiñones et al., 1995). During the maturation process of mRNA, the incomplete stop codon (T--/TA-) were found in six protein-coding genes may be completed by polyadenylated (Anderson et al., 1981, Ojala et al., 1981).

Twenty-one tRNAs secondary structure was classified as normal clover-leaf structure (Watanabe, 2010) and one tRNA $^{Ser(GCT)}$ were absent of D arm structure. Although the D-armless tRNA structure is unusual however it occurs in mammals (Watanabe et al., 1994), insect (Kim et al., 2005), echinoderm (Hyouta et al., 1987) and crustacean (Shen et al., 2007). The most closely related species, *P. monodon* also showed similar structure for tRNA $^{Ser(GCT)}$ (Wilson et al., 2000).

As shown in the phylogenetic tree analysis, sequence of *P. acehensis* is the most closely related to *P. monodon* with 92% shared identity for CO1 sequence and 89% shared identity for the longer sequence of total mitogenome. The other two closely related species, *P. indicus* and *P. merguensis* were located in the same clade which is attested similar distribution with *P. acehensis* and *P. monodon* (Wedjatmiko, 2009). The penaeid shrimp distribution limited to a single zoogeographical region, it's suggesting the clade that constructing the phylogenetic tree were Indo west Pacific and Eastern Pacific geographical distribution (Voloch et al., 2009). CO1 gene known as a powerful tool to resolved species-level phylogenetic. It widely used to assess molecular phylogenetic and identification due to slowly envolving compare to another protein-coding gene (Patwardhan et al., 2014). However, the total mitochondrial DNA resulted in a better resolution and supported phylogeny (Yu et al., 2007). This result clearly separated *P. acehensis* with the other species from Indo-West Pacific in a different branch.

Conclusion

This study determined the complete mitochondrial genome sequence of *P. acehensis*, which is endemic penaeid species in Aceh, Indonesia. Circular mitogenome of *P. ace*hensis (15.991 bp) contained the canonical 2 rRNAs, 22 tRNA genes, 13 protein-coding genes and the

putative control region. Although its overall mitogenome organization and the putative secondary tRNA structures were highly conserved as those of other penaeid species, its nucleotide sequence clearly demonstrated that *P. acehensis* is the endemic species in Aceh, Indonesia. Phylogenetic analysis showed that *P. acehensis* is most closely related to *P. monodon* as shown by the morphological analysis.

Declarations

List of Abbreviations

NGS : Next-Generation Sequencing
CO1 : Cytochrome oxidase 1
COX1 ; Cytochrome c oxidase subunit 1
COX2 : Cytochrome c oxidase subunit 2
COX3 : Cytochrome c oxidase subunit 3
ND1 : NADH dehydrogenase subunit 1
ND2 :NDH dehydrogenase subunit 2
ND3 : NADH dehydrogenase subunit 3
ND4 : NADH dehydrogenase subunit 4
ND4L : NADH dehydrogenase subunit 4L
ND5 : NADH dehydrogenase subunit 5
ND6 : NADH dehydrogenase subunit 6
CYT B : Cytochrome B
tRNA : Transfer Ribonucleic Acid
rRNA : ribosomal Ribonucleic Acid

- Ethics approval and consent to participate

Not applicable

- Consent for publication

Not applicable

Availability of data and materials

The dataset(s) supporting the conclusions of this article is (are) included in the article.

Competing interest

The authors declare that they have no competing interests.

Author contributions

S-PS carried out the whole process, participated in the whole experiment and drafted the manuscript. M-T supported morphological data. S-A added drafted the manuscript and final phylogenetic analysis. J-A finished final drafted. H-WK participated in the design of the study and editing the manuscript. All author read and approved the final manuscript.

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373 Figure Legends

374 Figure 1. Mitochondrial genome structure of *Penaeus acehensis.* The mitogenome map was
375 constructed by GenomeVx (http://wolfe.ucd.ie/GenomeVx/)

376 Figure 2. Putative secondary structure of tRNA genes in the mitochondrial genome. The
377 proposed structure of 22 tRNA genes encoded in the mitochondrial of *P. acehensis* was
378 predicted by ARWEN (Laslett and Canbäck, 2008)

379 Figure 3. Phylogenetic trees of penaeid shrimp constructed by COI regions (A) and full
380 mitochondrial genomes (B). The phylogenetic tree was constructed by molecular
381 evolutionary genetic analyses (MEGA 6, ver 6.0) with the minimum evolutionary
382 algorithm. The evolutionary distance was calculated by Kimura 2 parameter method.
383 Bootstrap replications were 1000. Penaeid shrimps were clustered into two clades (I,
384 II) reflecting the geographical difference. GenBank Accession number for each species
385 was shown in bracket.

386

387 Tabel 1. PCR Primers Set Used for the Mitochondrial Genome of P. *acehensis*

Name	Sequence (5' to 3")	Product size
Penaeus-^{225}-F	GATAAGCTAAGYAAGCTCGTGGG	2898 bp
Penaeus-^{3123}-R	ACCTAAGTGACCTCATGTTGGC	
Penaeus-^{3066}-F	GCATAGGATTTAAGCTCCTACCAGG	2540 bp
Penaeus-^{5606}-R	CTACACCYTCTGGTTGGAAGCC	
Penaeus-^{9921}-F	AWTCTTCTTAAAGCATCAGAG	3034 bp
Penaeus-^{12955}-R	ACCTCGATGTTGAATTAAGG	
P.acehensis-^{5433}-F	CCACTTTGGGTTTGAAGCTGC	4786 bp
P.acehensis-^{10219}-R	CATCAGGAATCTTACAGAAGCCG	
P.acehensis-^{12782}-F	ATAGAAACCGACCTGGCTCAC	3527 bp
P.acehensis-^{318}-R	CAATTCAGGCTCCGAACCAAG	

388

389 Table 2. Percentage of Base Composition from Complete Mitochondrial Genome *P. acehensis*

Gene	Base composition (%)						GC skew	AT skew
	A	T	G	C	A+T	G+C		
COX1	28	39	16	17	67	33	- 0.030	-0.164
COX2	31	40	15	14	71	29	0.034	-0.127
ATP8	36	38	8	18	74	26	-0.385	-0.027
ATP6	29	41	12	18	70	30	-0.200	-0.171
COX3	25	39	16	20	64	36	-0.111	-0.219
NAD3	25	43	15	17	68	32	-0.063	-0.265
NAD5	42	30	12	16	72	28	-0.143	0.167
NAD4	42	30	10	18	72	28	-0.286	0.167
NAD4L	43	31	9	17	74	26	-0.308	0.162
NAD6	27	47	9	17	74	26	-0.308	-0.270
COB	27	41	14	18	68	32	-0.125	-0.206
NAD1	43	27	12	18	70	30	-0.200	0.229
NAD2	28	41	11	20	69	31	-0.290	-0.188
tRNA	35	34	15	16	69	31	-0.032	0.014
rRNA	35	38	10	17	73	27	-0.259	-0.041
Control region	38	45	9	8	83	17	0.059	0.084
Total	34	37	12	17	71	29		

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393

394 Table 3. Organization of the full length mitochondrial genome of *P. acehensis*

Name	Position		Strand	Length	ovl/nc	Codons	Anti-codon
	Start	Stop					
tRNA-Ile(gat)	1	68	+	68	12	-	GAT
tRNA-Gln(ttg)	81	150	-	70	29	-	TTG
tRNA-Met(cat)	180	248	+	69	0	-	CAT
ND2	249	1250	+	1002	-3	ATT/TAA	
tRNA-Trp(tca)	1249	1317	+	69	8	-	TCA
tRNA-Cys(gca)	1326	1392	-	67	1	-	GCA
tRNA-Tyr(gta)	1394	1460	-	67	-1	-	GTA
COX1	1463	3001	+	1539	-6	ACG/TAA	
tRNA-Leu(taa)	2996	3063	+	68	4	-	TAA
COX2	3068	3755	+	688	0	ATG/T--	
tRNA-Lys(ttt)	3756	3824	+	69	5	-	TTT
tRNA-Asp(gtc)	3830	3897	+	68	0	-	GTC
ATP8	3898	4056	+	159	-7	ATC/TAA	
ATP6	4050	4724	+	675	10	ATG/TAA	
COX3	4735	5524	+	790	-1	ATG/T--	
tRNA-Gln(tcc)	5524	5592	+	69	-1	-	TCC
ND3	5592	5943	+	352	-1	ATG/TAA	
tRNA-ala(tgc)	5943	6009	+	67	4	-	TGC
tRNA-ARG(tcg)	6014	6080	+	67	0	-	TCG
tRNA-Asn(gtt)	6081	6147	+	67	2	-	GTT
tRNA-Ser(gct)	6150	6218	+	69	-2	-	GCT
tRNA-Glu(ttc)	6217	6288	+	72	18	-	TTC
tRNA-Phe(gaa)	6307	6376	-	70	-1	-	GAA
ND5	6376	8098	-	1723	9	ATG/T--	
tRNA-His(gtg)	8108	8174	-	67	0	-	GTG
ND4	8175	9530	-	1356	-19	ATT/T--	
ND4L	9511	9808	-	297	2	ATG/T--	
tRNA-Thr(tgt)	9811	9877	+	67	0	-	TGT
tRNA-Pro(tgg)	9878	9943	-	66	13	-	TGG
ND6	9945	10463	+	519	3	ATT/T--	
Cytb	10467	11603	+	1137	-2	ATG/TAG	
tRNA-Ser(tga)	11602	11671	+	70	17	-	TGA
ND1	11689	12627	-	939	4	ATA/TAA	
tRNA Leu(tag)	12632	12701	-	70	0	-	TAG
l-rRNA	12702	14076	-	1375	0	-	
trnV(tac)	14077	14150	-	74	0	-	TAC
s-rRNA	14151	14998	-	848	0	-	
control region	14999	15991		993	0	-	

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