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# Comparison of the complete mitochondrial genome of *Phyllophorus liuwutiensis* (Echinodermata: Holothuroidea: Phyllophoridae) to that of other sea cucumbers

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#### Keywords

mitochondrial genome; *Phyllophorus liuwutiensis*; sea cucumber; sequence analysis; structure characteristic

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Sea cucumber species are abundant (>1400 species) and widely distributed globally. mtDNA sequencing is frequently used to identify the phylogenetic and evolutionary relationships among species. However, there are no reports on the mitochondrial genome of Phyllophorus liuwutiensis. Here, we performed mtDNA sequencing of P. liuwutiensis to examine its phylogenetic relationships with other echinoderms. Its mitochondrial genome (15 969 bp) contains 37 coding genes, including 13 protein-coding genes, 22 tRNA genes and 2 rRNA genes. Except for one protein-coding gene (nad6) and five tRNA genes encoded on the negative strand, all other genes were encoded on the positive strand. The mitochondrial bases of P. liuwutiensis were composed of 29.55% T, 22.16% C, 35.64% A and 12.64% G. The putative control region was 703 bp in length. Seven overlapping regions (1-10 bp) were found. The noncoding region between the genes ranged from 1 to 130 bp in length. One putative control region has been found in the P. liuwutiensis mitogenome. All of the tRNA genes were predicted to fold into a cloverleaf structure. In addition, we compared the gene arrangements of six echinoderms, revealing that the gene order of P. liuwutiensis was a new arrangement.

Sea cucumbers belong to the phylum Echinodermata, which are an important food source for human, particularly in some parts of Asia [1]. Sea cucumbers are nocturnal feeding species, hiding by day in coral reef rocks or sand [2]. The species of sea cucumbers are abundant and widely distributed throughout the world. There are more than 1400 species of sea cucumbers in the world, which are distributed in shallow sea areas, trenches and other marine environments [3]. Despite the variety and wide distribution of sea cucumbers, the phylogenetic and evolutionary relationships of sea cucumbers remain largely unknown. In metazoan animals, the mitochondrial genomes are characterized by exposed circular double-stranded DNA molecules [4]. It has been found that most mitochondrial genomes are self-replicated and inherited in the maternal line. mtDNA sequencing is frequently used to identify the phylogenetic and evolutionary relationships among species [5–7]. Because of its rapid evolution and the lack of genetic recombination, mtDNA can provide important information about rearrangement laws and phylogenetic relationship [8]. The mtDNA contains 37 genes, including 13 proteincoding genes (PCGs), 2 rRNA genes and 22 tRNA

#### Abbreviations

CR, control region; PCG, protein-coding gene; RSCU, relative synonymous codon usage.

FEBS Open Bio **10** (2020) 1587–1600 © 2020 The Authors. Published by FEBS Press and John Wiley & Sons Ltd. This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited. genes [9,10]. At present, many studies have investigated the phylogeny and evolution of echinoderms. Mu *et al.* [3] have explored the adaptation of sea cucumber in a deep-sea environment based on the research of the mtDNA. Fan *et al.* [11] have found a new sequence of genes in the mitochondrial genome of the *Stichopus horrens.* However, no study has been carried out on the mitochondrial genome of *Phyllophorus liuwutiensis.* Therefore, we, in this study, aimed to clarify the structure and composition of the mitochondrial genome of *P. liuwutiensis,* and its phylogeny and evolution were also discussed.

The sea cucumbers, *P. liuwutiensis* (Holothuroidea: Dendrochirotida: Phyllophoridae: Phyllophorus), are popularly distributed in the intertidal sandy bottom. In China, *P. liuwutiensis* is distributed in only the two provinces of Fujian and Guangdong. *P. liuwutiensis* has a thin, cylinder-shaped body with a length of 90–200 mm and a diameter of 10-28 mm [12]. The whole body of *P. liuwutiensis* is covered by tube feet. It has a polian vesicle with a length of about 40 mm. The body wall bone is termed as a table, and the chassis has a round or irregular shape. Moreover, it has a hole in the center, and there are eight (or more than eight) other holes in the peripheral area. The rim of the chassis is an undulating shape, and the diameter of the disc ranges from 50 to 80 µm [12].

# **Materials and methods**

#### Sample collection and identification

All animal handling procedures were reviewed and approved by the ethics committee of the 'Regulations for the Administration of Affairs Concerning Experimental Animals'. The Institutional Animal Care and Use Committee of Fisheries Research Institute of Fujian, China, approved the experiments.

*P. liuwutiensis* were collected in Huandao Road, Xiamen City, Fujian Province, China. The back muscle of sea cucumber was clipped, fixed by anhydrous ethanol and stored at -20 °C [11]. The back wall bone fragment was used to identify the species using a scanning electron microscope [13]. The body wall of the sea cucumber was treated with a 10% NaClO solution for 1–2 min. Then the white precipitate was washed with distilled water four times. The samples were dried and then gold-plated by an ion sputtering device. Finally, the samples were examined using a scanning electron microscope [11].

#### **DNA extraction**

DNA was extracted from 30 mg back muscle tissue using the TIANamp Marine Animals DNA Kit (Tiangen Biochemical Technology, Beijing, Co. Ltd., Beijing, China) according to the manufacturer's instructions. The obtained DNA was stored at -20 °C prior to further analysis.

#### PCR amplification and sequencing

The complete mitochondrial nucleotide sequence of P. liuwutiensis was obtained by general and long-range PCR amplification using the specific primers (Table 1). The primers were designed to match the generally conserved regions of target mtDNA, and the short fragments of *cox*1, cox2, atp6, cox3, nad4, cytb, 12S, nad1 and 16S were amplified. In brief, the amplifications were carried out with 40 cycles at a melting temperature of 94 °C for 30 s, an annealing temperature of 50 °C for 30 s and an extension temperature of 72°C for 1 min per 1 kb. The final MgCl<sub>2</sub> concentration in the reaction was 2.0 mmol· $L^{-1}$ . PCR products were cloned into a pMD18-T vector (Takara, Beijing, China) and then sequenced using the dideoxynucleotide procedure by ABI 3730 automatic sequencer. Sequences were assembled by DNASTAR software [14] and manually adjusted to generate the complete sequence of mtDNA.

#### Sequence analysis and gene annotation

After the quality proofing of the obtained fragments, the mtDNA sequences were manually assembled using DNASTAR v7.1 software [14]. First, the raw mtDNA sequences were imported into MITOS web servers to determine the approximate boundaries of genes. Exact positions of PCGs were found by searching open reading frames. All tRNA genes were identified using ARWEN, DOGMA and MITOS [15–17]. MEGAX was used to calculate the DNA base composition and codon preference of the mitochondrial genome of *P. liuwutiensis* [18]. Formula with GC-skew = (G - C)/(G + C) and the AT-skew = (A - T)/(A + T) were calculated for base's preferences [19].

#### **Phylogenetic analysis**

The *P. liuwutiensis* and another 25 echinoderm mitogenomes (obtained from GenBank; https://www.ncbi.nlm. nih.gov/) were used for phylogenetic analysis. *Balanoglossus carnosus* (Enteropneusta) was rooted as the outgroup. Echinoderms were divided into five classes as follows: Holothuroidea, Echinoidea, Asteroidea, Ophiuroidea and Crinoidea [20]. Therefore, the species of these five classes were selected to construct the evolutionary tree, among which more sea cucumber species were selected to study the phylogeny and evolution of the *P. liuwutiensis*. The MEGAX [18] was used to perform the alignment of 13 PCGs. A Bayesian approach using MRBAYES 3.1.2 version [21,22] was employed to analyze the aligned datasets and trees.

Table	1. Primers	used for	amplification	of	complete	mitochondrial	genome of	Ρ.	liuwutiensis.
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F1 cox1 MIF1 CGAACAGAACTAGCCCAACC 1411   F2 cox1-cox2 MIF2 TCCATCTCCTCCTCAGATGG 708   F3 cox2 MIF3 GACACAATAGAATATTGATAAGAGG 595   F4 cox2-atp6 MIF4 GACGACAAATTGGATTACAAG 584   F5 atp6 MIF4 GACGACAAATGGATGCTCTAGG 607   F6 atp6 MIF5 CGACACAATGGATTCTAGAGG 711   F7 cox3 MIF6 GCCACCTGAGTCCTTAGG 607   F7 cox3 MIF6 GCCACCTGAGTCCTTAGG 711   F7 cox3 MIF7 GTGATCAAGAGCACATGCAACT 714   F8 cox3-nd4 MIF8 CAACCTTCTCAGGCACAGAGC 711   F9 nad4 MIF9 CCAAGGCCTACAGGACTGCACTGAAGC 228   F10 nad4-oytb MIF0 CCTACTCTCACGACATAAGC 228   F11 cytb MIF10 CCTACACCGCTGACAGAAGC 228   F12 cytb-12S MIF13 CACGTTACCAGATATGC 3446   F11 cytb MIF10 CCTACACCGCGCAGAAACC 280   F11 cytb MIF13 CACGTTACCAGATAACC 880   F11 cytb MIF13 CACGTTACCACGCGCGATAAACC 570	Fragment no.	Gene or region	Primer name	Sequence (5'-3')	Length (bp)	
F2MIR1CTTTGAATGTGTGGTGAGATGGF2cox1-cox2MIP2CCAATACCATTGTCAATAGAGCAF3cox2MIP3GAGCACAAATTGGATTACAAGGF4cox2-atp6MIP4GAGCTTAAGGATTCTGAGCAF5atp6MIP4GAGCACTAATGGATTGTGGAGTTCF6atp6MIP5CTGACACAGTGCGAGTCF7cox3MIP6GCACCAGTGGATCACAGATF7cox3MIP6CCACCTGAGTGCATAGAGTF7cox3-nd4MIP6CCACCTGAGTGCTAACGAGTF8cox3-nd4MIP6CCACCTGAGATAGTGCAAGGAF9nad4MIP6CCAACGGCCCACGGAGAGGCF10nad4MIP6CCAACGGCCACGGAGAGGCF11cytbMIP10CCTTACTTATGGAGATAGGF12cytb-12SMIP10CTTACTTCTCAGAGAGATAGTGCF1312SMIP13GAGCTCTTCTCATAAGGATAGTCF1412S-nad1MIP13CCAATAGCATTGTTAAGGAF15nad1MIP14GTTCTCTTATGTAGAAATAGCF16nad1-16SMIP16CTGCGTAACAGTTGTTAAGGAF1716SMIP16CTGCGTAAGGAGATATGCF1816S-cox1MIP16CTGCGTAAGAGAATATGCF1816S-cox1MIP16CTGCGTAAGAGAATAGGF1816S-cox1MIP16CTGCGTAGAGAGCCATTGCTAAGCF1816S-cox1MIP16CTGCGTTAGGAGACCATTGCF1816S-cox1MIP16CTGCGTTAGAGAGCCATCGCAGGF18MIP16CTGCGTTAGGAGAGCCATTGCF18MIP16CTGCGTTAGGAGAGCCATCGCAGGF18MIP16 <td< td=""><td>F1</td><td>cox1</td><td>MIF1</td><td>CGAACAGAACTAGCCCAACC</td><td>1411</td></td<>	F1	cox1	MIF1	CGAACAGAACTAGCCCAACC	1411	
F2 cox1-cox2 MIF2 MIR2 TCCATCTCTCTCATAGGATC 708   F3 cox2 MIR3 GCACATATCATATTGTATAGGAG 595   F4 cox2-atp6 MIR4 GAGCTTAGCAGATTCTGAGCA 594   F5 atp6 MIF5 CGACACAATTGTGGGCTTCTAGG 607   F6 atp6-cox3 MIF5 CGACCTGAGCTTCAGGCACACT 322   F7 cox3 MIF6 CCACCTGAGCTCTAGGC 711   F8 cox3-nd4 MIR6 CATCATCTTATAGCAGTAGCA 711   F8 cox3-nd4 MIF8 CACCTTCCTAGGCCACAGAGC 711   F9 nad4 MIR9 CCAAAGGCCCAGAGAGCACAGAGC 228   F10 nad4 MIR9 CCAAAGGCCCACGAGAGAGC 228   F11 Cytb MIR1 CCTTACCAAGAAGAACAAGACCA 800   F11 cytb MIR9 CCAAAGGCCCTAGGCACAGAAG 714   F11 Cytb-12S MIR1 CACTTACCAAGAAGAACAA 800   F13 12S MIR1 CACGTTACCTACAGCAGATTACCA 800   F14 12S-nad1 MIF13 CACGTAAGCAAGAATTACCA 725   F16 nad1 MIF14 GATCATCAAGACAATTACCA 725   F17 MIR14 GATCATCAAGACAATTACCAAGATTACCA <t< td=""><td></td><td></td><td>MIR1</td><td>CTTTGAATGTGTGGTGAGATGG</td><td></td></t<>			MIR1	CTTTGAATGTGTGGTGAGATGG		
MIR2GCAATAGAATATTGATAAGAGGF3cox2MIF3GAGCACAAATTGGATTACAAG584F4cox2-atp6MIF4GAGCTAAGATGGATGGATGGACTTC584F5atp6MIF4GAACCTTGGGTTCTCAGG607F6atp6-cox3MIF6GCACACAATGGATTGCATGCAAC71F7cox3MIF7GTGATCAAGGAGTGCACT711F8cox3-nd4MIF8CAACCTTCCTACGGACCATGCAC71F8cox3-nd4MIF9GCACACAAGGAGTGCAGT1404F9cox3-nd4MIF9CCAAAGGAGCCAAGGAGCCACGAAGAC71F10nad4-cytbMIF9CCAAAGGAGCCCACGTAGAACC3446F11cytbMIF10CCTACTCCGAAGAAGACCATGACC3446F12cytb-125MIF12CCAATTACGGGCTAACGAGATGCAC70F13125-nad1MIF13CACGTTAACCTATTACGAGAGAC50F14125-nad1MIF15GTACCTCCTTTAGGCATAAC880F15nad1-165MIF16GTACCTCTTATAGAAAAAAAAAAAAAAAAAAAAAAAAAA	F2	cox1-cox2	MIF2	TCCATCTCCTCCATAGGATC	708	
F3 cox2 MIF3 MIR3 GAGCACAAATTGGATTACAAG 595   F4 cox2-stp6 MIR4 GAGCTTAAGATTGCTGAGCA 584   F5 stp6 MIR5 CGACCAAATAGGATTCTCCC 584   F6 stp6-cox3 MIF6 CCACCTGAGGCTCTTAGGAC 711   F7 cox3-nd4 MIF6 CCACCTGAAGTCCTTAGGACC 711   F8 cox3-nd4 MIF6 CACCTCCAAGGCCAGGACCACGAC 228   F9 nad4 MIR9 CGGCCTACAGAGAGATTCCCC 2406   F10 nad4-cytb MIF0 CCTCACCGACGAGAAGC 288   F11 cytb-12S MIF10 CCTACTCCGATCATAACG 880   F12 cytb-12S MIF11 GCACTACTCCTTTAGGATTCCA 846   F14 12S-nad1 MIF13 CGCTCACCAGGACATAACC 860   F15 nad1 MIF13 CACCTTCTTAAGGAATATAGC 570   F16 nad1 MIF13 CGTCCTCTTTAAGGAATATAGC 2206   MIR14 GATCATAAGCGAGACATTGCTAAGGA 570   F16 nad1 MIF13 CACGTCTATAGCGAATAAGC 2206   MIR14 GATCATAAGCAGAGACCATTC 72   F16 nad1 MIF13 CACGTGCTAAGAGACCATTC 72   F17 12S			MIR2	GCAATAGAATATTGATAAGAGG		
F4 ox2-stp6 MIR3 GCTCCACAGATTTCTGAGCA 584   F5 atp6 MIF4 GAAGCTTAGTGGATGCATGCAGCTTCTAGG 607   F5 atp6 MIF5 GTAACCTTGGATACATGCAACC 607   F6 atp6-cox3 MIF6 GCACCATAGGATTCTCC 607   F7 cox3-nd4 MIF7 GTGATCAAAGACCATGGACC 711   F8 cox3-nd4 MIF9 CAACCTTCCTAACAGCAGAGA 716   F9 nad4-cytb MIF9 CCAAAGGCCCACGAGAGAGAC 228   F10 nad4-cytb MIF1 GCCTCCACGACTTGGCACCAGAAGAG 286   F11 GYb GTGGCTACACAGAATATGC 346   F12 nad4-cytb MIF1 GCACTCCTCCTGCACAATAAGC 346   F12 cytb-12S MIF12 CCAATCATCCTCCTTTGGCACATAACC 646   F14 12S-nad1 MIF14 GATCATACTCTCTTTAGCAAATAAGC 2206   F15 nad1-nBS MIF16 GTAGCTACAAGAATATGC 725   F16 nad1-nBS MIF16 CTAGGCGTAACGAATAGCATTGCTAAACCTTTAGGAACCATTC 195   F17 16S MIF16 CTAGCGGCTACAGAAATAGCA 725   F14 GAACCTTCTTTAGGAGACCATTGC 725 725   F15 nad1-nBS MIF16 CT	F3	cox2	MIF3	GAGCACAAATTGGATTACAAG	595	
F4 cox2-atp6 MIF4 GAGTTAAGATGGATGGATGGAGTTC 584   F5 atp6 MIR5 GAAGCTTAGGGATTCTCAGG 607   F6 atp6-cox3 MIF6 GCACCATGAGTCTTAGGATTCTCC 322   F7 cox3 MIF6 GTGATCTTATAGCAGTTGAGC 711   F7 cox3-nd4 MIF8 CAACCATCGAGCACAGAGAAGC 711   F8 cox3-nd4 MIF9 CAAGGCCACGAGAGAGC 228   F9 nad4 MIF9 CCAAAGGCCACGAGAGC 228   F10 nad4-cytb MIF10 CCTCTCTACAGCAGAGACCAAGAGC 228   F11 cytb-12S MIF11 GCACTACCGCGCGACAAAAACCAATAGC 880   F12 cytb-12S MIF13 CACCTTAGCGGGGTATCTAAAGC 880   F14 12S-nad1 MIF13 CACCTCTTAAGCAATAAGC 206   F15 nad1 MIF16 GTAGCCTCTTAAGAATAAGG 206   F16 nad1-16S MIF16 CTAGCGGTAGAAACCAATAGC 725   F16 nad1-16S MIF16 CTAGCCTTTAGGACCACTTC 195   F17 16S-cox1 MIF18 CTAGCCGTTGGACACAAACCATTC 195   F18 18S-cox1 MIF18 CTAGCCGCTTAAAACCATTAACCTTAAGC 568			MIR3	GCTCCACAGATTTCTGAGCA		
F5MIR4GAAGCTTTGTGGCTTCTAGGF5atp6MIF5CGAACAATAGGATTCTCCC607F6atp6-cox3MIF6GCCACCTGGATCCTTAGATC322F7cox3MIF7GTAGACTAGAACGAACGATAGG711F8cox3-nd4MIF8CAACCTTCCTAACGGAAGCATGACC711F9nad4MIF9CCAAGGACCTGCGAGGAAGAAGA228F10nad4MIF9CCAAGGACCTAGGCACAGAAG218F11cytbMIF10CTTGATTATAGAGATCCA3446F11cytbMIF11GACGTCACCGCGAAGAACACCATCC3446F11cytbMIF12CCAATCATCCCGTGACTAAAGAATCC570F12cytb-12SMIF12CCAATCATCCTTAGCGTAAAGAATAAC880MIR13GGTCACCTCTTCTTCTATGTAAGC570111F1412S-nad1MIF14GTACCTCTTATGTAAAGCAATTGCTAAAG226F15nad1-16SMIF16CTAGCTGCAAAGAAGAATTAGC226F16nad1-16SMIF17CCTTGGCTAAAGAAGACCATTCC145F1716SMIF16CTAGCTCCTTAAAGACCATTCC155F1816S-cox1MIF18CTAGCTACCAAGAGCCATTCC683	F4	cox2-atp6	MIF4	GAGTTAAGATGGATGGAGTTC	584	
F5 atp6 MIF5 CGACACAATAGGATTTCTCC 607   MIR5 GTAGCTTGGATACATGCAAC 322   F6 atp6-cox3 MIF6 CATCATCTTATAGCAGTTAGC 322   F7 cox3 MIF7 GTTGATCAAGACCATGACC 711   F8 cox3-nd4 MIF8 CACGTCAACGAGTGTCAGT 1404   F9 nad4 MIF9 CCAAGGCCAACGAAGAAC 228   F10 nad4 MIF9 CCAAGGCCCACGTAGAAGAC 228   F11 cytb MIF10 CTTGATTATGTAGGATCCAC 3446   F11 cytb MIF10 CTTGATTATTATGAGATCACA 880   F11 cytb-12S MIF11 GCACGTCACCACGTAGAAGAC 880   F13 12S MIF12 CTGATCATCTTTATGTAGGATCCA 880   F14 12S-nad1 MIF13 CACGTTACTTATGTAGGC 570   F15 nad1 MIF13 CACGTTACTTTATGTAAGA 725   F16 nad1-16S MIF13 CATCATTTATGTAGGAGCTGTAAC 725   F17 16S MIF16 CTGGCTTTGTTATGTAAGGAGCTGTAAC 725   F16 nad1-16S MIF16 CTGGCTTTGTTATGTAAGGAGCTGTAAC 725   F17 16S MIF16 CTGGCGTAGAGAGCTGTAC 725			MIR4	GAAGCTTTGTGGCTTCTAGG		
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F7MIR6CATCATCTTATAGCAGTTAGGF7cox3MIF7GTTGATCAAGACCATGACC711MIR7GACGTCAACGAAGTGTCAGT711F8cox3-nd4MIF8TAGGAGCCTACGAGAGAG1404MIR8TAGGAGCCTAGGGCACAGAAG1404F9nad4MIF9CCAAAGGCCCACGAGAAGC228F10nad4-cytbMIF10CCTACTCCGACTCTTCCCTC3446MIR10CTTGATTATGTAGGATCCA160164F11GCACTACACGCGCGACATAAC880F12cytbMIF11GCACTACTCTGCTTTCGACAGAC646MIR12GTATCACCGCGTACATAATCC646MIR13GGTACACCTTATGTAGCAGATTAAGC570F1412S-nad1MIF14GATCATAAGCAATTGCTAAGC570F15nad1MIF15GTAGTGGGCATACAAGAATAGC2266F16nad1-16SMIF16CTAGGCGAGAGACCATTC725F1716SMIF17CCTTTGGTTTATGTTATGTTCC1955F1816S-cox1MIF18CAATCATTAGGAGCGAGAGAGCA568	F6	atp6-cox3	MIF6	GCCACCTGAGTCCTTAGATC	322	
F7 cox3 MIF7 GTTGATCAAGACCATGACC 711   F8 cox3-nd4 MIF8 CAACCTCCTAACGAAGTGTCAGT 1404   F9 nad4 MIF9 CCAAAGGCCACGAAGAG 228   F10 nad4-cytb MIF9 CCAAAGGCCACGTAGAAGAC 28   F11 cytb MIF10 CCTACTCCGACTTCCCTC 3446   F11 cytb MIF10 CCTACTCCGACTGTACCA 3466   F11 cytb MIF10 CCTACTCCGACTGTCCCTC 3446   F11 cytb MIF11 GCACTACACGGTGTACAAAGACCA 880   F11 cytb-12S MIF11 GCACTACACCGCTGTGGC 646   F12 cytb-12S MIF13 CACGTTAACCTTATGGTAAAG 570   F14 12S-nad1 MIF13 CACGTAAACCTACTTGTAAGG 725   F15 nad1 MIF15 GTAGTGGGCCATACGGATTAAC 725   F16 nad1-16S MIF16 CTAGGCGTAGAAGACCATTC 1955   F17 16S MIF17 CCTTGTTTTGTTATGTTAAGGACCTAAAGC 863   MIR16 CTTAGTGTGTGCACTAAAGGCAGAGG 1955   F17 16S MIF16 CTAGGCGTAGAAGACCATTC 1955   F18 NIF17 GGTCTTTCGTAAAGGCGTGGAAGAGG 863			MIR6	CATCATCTTATAGCAGTTAGG		
F8MIR7GACGTCAACGAAGTGTCAGTF8cox3-nd4MIF8CAACCTTCCTAACAGTATGTC1404MIR8TAGGGACCTAGGGCACAGAAG228F9nad4MIF9CCAAAGGCCCACGAGAAGC228F10nad4-cytbMIF10CCTACTCCGACTCTTCCCTC3446MIR10CTTGATTTATGTAGGATCCAMIR10CTTGATTTATGTAGGATCCA880F11cytbMIF11GCACTACACCGCTGACATAAC880F12cytb-12SMIF12CCAATCCTCTCTCTTCGAC646F1312SMIF13CACGTTAACCTTTAGCTAAAG570F1412S-nad1MIF14GATCATCATCCTCTTAAGGATTAAC206F15nad1-16SMIF15CGTGGGTAGGAGGCTGTAAC226F16nad1-16SMIF16CTAGCTGGTAGAGACCATTC1955F1716SMIF17CCTTTAGTAGACCTAAAGCA863F18MIF13MIF16CTAGCGGTAGAAGACCATTC1955F18MIF14MIF16CTAGCTGGTAGAAGACCATTC1955MIR16CTAGCTGGTAGAAGACCATTC1955MIR16CTAGCTGGTAGAAGACCATTCCF1716SMIF17CCTTTAGTAGACCTAAAAGCA863MIR17GGTCCTTTCGTACTAAAAGCAAAGGMIR17GGTCCTTTCGTACTAAAGAAGGF18MIF18CAATCATTAGGGCACAGAG568	F7	cox3	MIF7	GTTGATCAAAGACCATGACC	711	
F8 cox3-nd4 MIF8 CAACCTTCCTAACAGTATGTC 1404   MIR8 TAGGGAGCCTAGGGCACAGAAG 228   F9 nad4 MIF9 CCAAAGGCCCACGTAGAAGAC 228   F10 nad4-cytb MIF10 CTTGATTATGTAGGATCCA 3446   F11 cytb MIF11 GCACTACCGACGTAGAAGAC 880   F12 cytb-12S MIF12 CCAATCATCCTCTTTGGC 646   MIR13 GGTACACCTATATGTAGGATCCA 70   F13 12S MIF12 CCAGTTAACCTTTAGCAAAAATAAG 570   F14 12S-nad1 MIF14 GTACACCTACTTGTAAGGATTACC 70   F15 nad1 MIF15 GTAGTGGGGGATGCTAAAG 206   F16 nad1-16S MIF16 CTTGGTTTAGGAGAGCCGAATAGC 75   F17 16S MIF17 CCTTGGTTAGGAGCACCTATCC 1955   F18 MIF18 GTAACCAAAGGCGCAAAGACCAATTCC 1955			MIR7	GACGTCAACGAAGTGTCAGT		
F9nad4MIR8TAGGGAGCCTAGGGCACAGAAG228F10nad4-cytbMIF9CCAAAGGCCCACGTAGAAGAC228F11nad4-cytbMIF10CCTACTCCGACTCTCCCCTC3446F11cytbMIF10CTTGATTTATGTAGGATCCA80F12cytbMIF11GCACTACACCGCTGACATAAC860F1312SMIF12CCAATCATCCTCTTTCGAC646MIR13GGTACACCTACTTTATGTAGGATTACCMIR1367TACACCTTAGCTAAAGCF1412S-nad1MIF14GTACACCTACTTCTAAGGAATAAG2206F15nad1MIF15GTAGGGCGATGAAATAAG725F16nad1-16SMIF16CTAGGCGGTAGAAGACCATTC1955F1716SMIF17CCTTTAGTAGACCTAAAGC863MIR14GTACTTTTTTGTTTATGTTTCC1955F1716S-cox1MIF18GTAACCAAAGGGTGGCAGCAG568	F8	cox3-nd4	MIF8	CAACCTTCCTAACAGTATGTC	1404	
F9 nad4 MIF9 CCAAAGGCCCACGTAGAAGC 228   MIR9 GTGGCCTACAGAAGAATATGC 3446   F10 nad4-cytb MIF10 CCTACTCCGACTCTTCCCTC 3446   MIR10 CTTGATTTATGTAGGATCCA 880   F11 cytb MIF11 GCACTACACCGCTGACATAAC 880   F12 cytb-12S MIF12 CCAATCATCCTCTTTTGGTGGC 646   F13 12S MIF12 CCAATCATCTTCTAATGCTAAAGC 570   F14 12S-nad1 MIF14 GTACACCTTAAAGCAATTACC 2206   F15 nad1 MIF15 GTAGGTGGGCATACGAATAAC 2206   F16 nad1-16S MIF16 CTAGGCGGTAGGAGGCTCGTAC 725   F17 16S MIF17 CCTTAGGTAGAAGACCATTC 1955   F18 16S-cox1 MIF17 CCTTTAGTAGACCTAAAGGAAGACGAGG 663			MIR8	TAGGGAGCCTAGGGCACAGAAG		
$ \begin{array}{ccccc} & \mbox{MIR9} & \mbox{GTGGCTACAGAAGAATATGC} & & & & & & & & & & & & & & & & & & &$	F9	nad4	MIF9	CCAAAGGCCCACGTAGAAGC	228	
F10 nad4-cytb MIF10 CCTACTCCGACTCTTCCCTC 3446   MIR10 CTTGATTTATGTAGGATCCA 880   F11 cytb MIF11 GCACTACACCGCTGACATAAC 880   MIR11 AGGTTCTTCTACTGGTTGGC MIR1 AGGTTCTTCTACTGGTTGGC 646   F12 cytb-12S MIF12 CCAATCATCCTCTTTCGAC 646   MIR12 GTATAGCGGGGTATCTAATCC F13 12S MIF13 CACGTTAACCTTTGTTACG 570   F14 12S-nad1 MIF14 GTACACCTCTTAAGCAATAAG 2206   MIR15 CGTGGGTAGGAGGCTCGTAC 725   F16 nad1-16S MIF16 CTTGGTTTTGTTATGTTCTCC 1955   F17 16S MIF17 CCTTTGGTACTAAAGCAATGCTAAAGG 863   F18 16S-cox1 MIF18 GTAACCAAAGGGTGCAGAG 663			MIR9	GTGGCCTACAGAAGAATATGC		
MIR10CTTGATTTATGTAGGATCCAF11 $cytb$ MIF11GCACTACACCGCTGACATAAC880MIR11AGGTTCTTCTACTGGTTGGCMIR11AGGTTCTTCTACTGGTTGGC646F12 $cytb-12S$ MIF12CCAATCATCCTCCTTTCGAC646MIR12GTATAGCGGGGTATCTAATCCMIR13GGTACACCTACTTTGGTACG570F1312SMIF13CACGTTAACCTTTGGTACG570F1412S-nad1MIF14GTACCTCCTTAAAGAAATAAG2206F15nad1MIF15GTAGTTGGGCCATACGGATTAC725F16nad1-16SMIF16CTAGGCGGTAGAAGACCATTC1955F1716SMIF17CCTTTAGTAGACCTAAAAGC863F1816S-cox1MIF18GTAACCCAAAGGGTGCAGCAG568	F10	nad4-cytb	MIF10	CCTACTCCGACTCTTCCCTC	3446	
F11 cytb MIF11 GCACTACACCGCTGACATAAC 880   MIR11 AGGTTCTTCTACTGGTTGGC MIR12 AGGTTCTTCTACTGGTTGGC 646   F12 cytb-12S MIF12 CCAATCATCCTCTTTCGAC 646   MIR12 GTATAGCGGGGTATCTAATCC MIR13 CACGTTAACCTTTAGCTAAAG 570   F13 12S MIF13 CACGTTAACCTACTTTGTTACG 570   F14 12S-nad1 MIF14 GTACACCTACTTTGTTACG 2206   MIR14 GATCATAAAGCAATTGCTAAAG 2206   F15 nad1 MIF15 GTAGGGTAGGAGGCTCGTAC 725   F16 nad1-16S MIF16 CTAGGTGGTAGAAGACCATTC 1955   F17 16S MIF17 CCTTTAGTAGACCTAAAGGC 863   F18 16S-cox1 MIF18 GTAACCAAAGGGTGCAGCAGCAG 568			MIR10	CTTGATTTATGTAGGATCCA		
MIR11AGGTTCTTCTACTGGTTGGCF12cytb-12SMIF12CCAATCATCCTCCTTTCGAC646MIR12GTATAGCGGGGTATCTAATCCMIR12GTATAGCGGGGTATCTAATCCF1312SMIF13CACGTTAACCTTTGTTACG570MIR13GGTACACCTACTTTGTTACGMIR13GGTACACCTACTTTGTTACG2206F1412S-nad1MIF14GATCATAAAGCAATTGCTAAAG2206MIR14GATCATAAAGCAATTGCTAAAG2206F15nad1MIF15GTAGTTGGGCCATACGGATTAC725F16nad1-16SMIF16CTAGGCGGTAGAAGACCATTC1955F1716SMIF17CCTTTAGTAGACCTAAAAGC863MIR17GGTCCTTTCGTACTAAAGAAGGMIF18GTAACCAAAGGGTGCAGCAG568F1816S-cox1MIF18GTAACCAAAGGGTGCAGCAGCAG568	F11	cytb	MIF11	GCACTACACCGCTGACATAAC	880	
F12   cytb-12S   MIF12   CCAATCATCCTCCTTTCGAC   646     MIR12   GTATAGCGGGGTATCTAATCC   646     F13   12S   MIF13   CACGTTAACCTTTAGCTAAAG   570     F14   12S-nad1   MIF14   GTACACCTCTTAAGAAATAAG   2206     MIR14   GATCATAAAGCAATTGCTAAAG   2206     F15   nad1   MIF15   GTAGTTGGGCCATACGGATTAC   725     F16   nad1-16S   MIF16   CTAGGCGGTAGAAGACCATTC   1955     F17   16S   MIF17   CCTTTAGTAGAAGAAGC   863     F18   16S-cox1   MIF18   GTAACCAAAGGGTGCAGCAG   568			MIR11	AGGTTCTTCTACTGGTTGGC		
MIR12GTATAGCGGGGTATCTAATCCF1312SMIF13CACGTTAACCTTTAGCTAAAG570MIR13GGTACACCTACTTTGTTACGMIR13GGTACACCTACTTTGTTACG2206F1412S-nad1MIF14GTACTCCTCTAAAGAAATAAG2206MIR14GATCATAAAGCAATTGCTAAAG2206F15nad1MIF15GTAGTTGGGCCATACGGATTAC725F16nad1-16SMIF16CTAGGCGGTAGAAGACCATTC1955F1716SMIF17CCTTTAGTAGACCTAAAAGC863F1816S-cox1MIF18GTAACCAAAGGGTGCAGCAG568	F12	cytb-12S	MIF12	CCAATCATCCTCCTTTCGAC	646	
F13   12S   MIF13   CACGTTAACCTTTAGCTAAAG   570     MIR13   GGTACACCTACTTTGTTACG   MIR13   GGTACACCTACTTTGTTACG   2206     F14   12S-nad1   MIF14   GTACTCTTAAAGAAATAAG   2206     MIR14   GATCATAAAGCAATTGCTAAAG   725     F15   nad1   MIF15   GTGGGGTAGGAGGCTCGTAC   725     F16   nad1-16S   MIF16   CTAGGTGGTAGAAGACCATTC   1955     F17   16S   MIF17   CCTTTAGTAGACCTAAAGC   863     MIR17   GGTCCTTTCGTACTAAAGAAGG   568     MIB18   CAATCAATAGCGAATTACTACTACTACTACTACTACTACAAGAGCAAGACCAGACCAGAGAGACCAGAGACCAGAGACCAGAGAGAGAGACCAG			MIR12	GTATAGCGGGGTATCTAATCC		
MIR13GGTACACCTACTTTGTTACGF1412S-nad1MIF14GTACCTCCTTAAAGAAATAAG2206MIR14GATCATAAAGCAATTGCTAAAG725F15nad1MIF15GTAGTTGGGCCATACGGATTAC725F16nad1-16SMIF16CTAGGCGGTAGAAGACCATTC1955F1716SMIF17CCTTTAGTAGACCTAAAGC863F1816S-cox1MIF18GTAACCAAAGGGTGCAGCAG568	F13	12S	MIF13	CACGTTAACCTTTAGCTAAAG	570	
F14 12S-nad1 MIF14 GTACCTCCTTAAAGAAATAAG 2206   MIR14 GATCATAAAGCAATTGCTAAAG 725   F15 nad1 MIF15 GTAGTTGGGCCATACGGATTAC 725   MIR15 CGTGGGTAGGAGGCTCGTAC 725   F16 nad1-16S MIF16 CTAGGCGGTAGAAGACCATTC 1955   F17 16S MIF17 CCTTTAGTAGACCTAAAGC 863   MIR17 GGTCCTTTCGTACTAAAGAAGG 768   F18 16S-cox1 MIF18 GTAACCAAAGGGTGCAGCAG 568			MIR13	GGTACACCTACTTTGTTACG		
MIR14GATCATAAGCAATTGCTAAAGF15nad1MIF15GTAGTTGGGCCATACGGATTAC725MIR15CGTGGGTAGGAGGCTCGTACMIF15CGTGGGTAGAAGACCATTC1955F16nad1-16SMIF16CTTGGTTTTGTTTATGTTTCC1955F1716SMIF17CCTTTAGTAGACCTAAAGC863MIR17GGTCCTTTCGTACTAAAGGAGGMIF18GTAACCAAAGGGTGCAGCAGCAG568F1816S-cox1MIF18CAATCAATAGTGCGAATTAGTTCC568	F14	12S-nad1	MIF14	GTACCTCCTTAAAGAAATAAG	2206	
F15 nad1 MIF15 GTAGTTGGGCCATACGGATTAC 725   MIR15 CGTGGGTAGGAGGCTCGTAC MIR15 CGTGGGTAGAAGACCATTC 1955   F16 nad1-16S MIF16 CTAGGCGGTAGAAGACCATTC 1955   F17 16S MIF17 CCTTTAGTAGACCTAAAAGC 863   MIR17 GGTCCTTTCGTACTAAAGAAGG 165 568   MIB18 CAATCAATAGCGGAATTAGTTCC 568			MIR14	GATCATAAAGCAATTGCTAAAG		
F16 nad1-16S MIR15 CGTGGGTAGGAGGCTCGTAC 1955   F17 16S MIF16 CTTGGTTTTGTTTATGTTTCC 863   F18 16S-cox1 MIF18 GTAACCAAAGGGTGCAGCAGC 568	F15	nad1	MIF15	GTAGTTGGGCCATACGGATTAC	725	
F16 nad1-16S MIF16 CTAGGCGGTAGAAGACCATTC 1955   MIR16 CTTGGTTTTGTTTATGTTTCC 1957   F17 16S MIF17 CCTTTAGTAGACCTAAAAGC 863   MIR17 GGTCCTTTCGTACTAAAGAAGG 1000000000000000000000000000000000000			MIR15	CGTGGGTAGGAGGCTCGTAC		
MIR16 CTTGGTTTTGTTTATGTTTCC   F17 16S MIF17 CCTTTAGTAGACCTAAAAGC 863   MIR17 GGTCCTTTCGTACTAAAGAAGG   F18 16S-cox1 MIF18 GTAACCAAAGGGTGCAGCAG 568   MIB18 CAATCATTAGTGGAATTAGTCC	F16	nad1-16S	MIF16	CTAGGCGGTAGAAGACCATTC	1955	
F17 16S MIF17 CCTTTAGTAGACCTAAAAGC 863   MIR17 GGTCCTTTCGTACTAAAGAAGG   F18 16S-cox1 MIF18 GTAACCAAAGGGTGCAGCAG 568   MIB18 CAATCATTAGTGGAATTAGTC			MIR16	CTTGGTTTTTGTTTATGTTTCC		
MIR17 GGTCCTTTCGTACTAAAGAAGG   F18 16S-cox1 MIF18 GTAACCAAAGGGTGCAGCAG 568   MIB18 CAATCATTAGTGGAATTAGTC	F17	16S	MIF17	CCTTTAGTAGACCTAAAAGC	863	
F18 16S-cox1 MIF18 GTAACCAAAGGGTGCAGCAG 568 MIB18 CAATCATTAGTGGAATTAGTC			MIR17	GGTCCTTTCGTACTAAAGAAGG		
	F18	16S-cox1	MIF18	GTAACCAAAGGGTGCAGCAG	568	
			MIR18	CAATCATTAGTGGAATTAGTC		

Analyses had two parallel runs with four chains of each (three hot chains and one cold chain), which were carried out for 1 000 000 generations (sampling every 100 generations). After the first 1000 'burn in' trees were discarded, the remaining 9000 sampled trees were used to estimate the 50% majority rule consensus tree and the Bayesian posterior probabilities.

# Results

# Identification of P. liuwutiensis

We found four types of table body in the body wall of the sea cucumber (Fig. 1), which was in accordance with a previous study [12]. Based on the morphological characteristics, the sea cucumber was identified to be *P. liuwutiensis*.

# **Genome organization**

The complete mtDNA sequence of *P. liuwutiensis* was obtained by general and long-range PCR. Similar to other deuterostomes, the mtDNA of *P. liuwutiensis* was a closed double-stranded loop and composed of 15 969 bp (Fig. 2). The genome encoded 37 genes, including 13 PCGs, 2 rRNA genes and 22 tRNA genes. Among these genes, 31 genes were encoded on the positive strand, whereas others (such as *nad6*,  $tRNA^{ser(tga)}$ ,  $tRNA^{Gln}$ ,  $tRNA^{Ala}$ ,  $tRNA^{Val}$  and  $tRNA^{Asp}$ ) were encoded on the negative strand. The



Fig. 1. The dorsal wall of the *P. liuwutiensis*. Tabular form (A), plates of sea cucumber (B), type rosettes (C) and type buttons (D). Scale bars: 10  $\mu$ m (A, C, D); 50  $\mu$ m (B).

base composition of the mtDNA of *P. liuwutiensis* is 29.55% T, 22.16% C, 35.64% A and 12.64% G.

#### Base composition and AT/GC-skew of mtDNA

Table 2 shows the base composition of P. liuwutiensis and 25 species of echinoderms. We also found that the contents of the bases varied among species, with a T content of 31.63% ranging from 25.24% (Acanthaster planci) to 46.46% (Phanogenia gracilis), a C content of 21.00% ranging from 11.44% (P. gracilis) to 27.95% (A. planci), an A content of 32.25% ranging from 25.82% (P. gracilis) to 35.82% (Echinaster brasiliensis) and a G content of 15.12% ranging from 10.65% (Freyastera benthophila) to 18.22% (Strongylocentrotus droebachiensis; Table 2). The length of the P. liuwutiensis mtDNA was shorter compared with other echinoderms. The higher content of base A (than T) and the lower content of base G (than C) were found in most echinoderms. The data (Table 2) showed that all GC skewness of the P. liuwutiensis mtDNA was negative, and all AT skewness was positive, indicating that the mtDNA of P. liuwutiensis had a preference for A and C.

# Overlapping and noncoding regions

In this study, we identified seven overlapping regions of genes (*trnP/trnQ*, *trnL1/trnA*, *trnY/trnG*, *atp8/atp6*,

cox3/trnS2, nad4/trnH and trnS1/nad5) in the complete genome of *P. liuwutiensis* (Table 3). The length of these overlaps varied from 1 to 10 bp, with the longest length between nad4 and trnH (Table 3). Among the 20 noncoding regions, the length was 1–703 bp (Table 3). The longest noncoding sequence (703 bp; AT% = 69.30%) was found between tRNA<sup>thr</sup> and tRNA<sup>pro</sup>. Due to its location and AT richness, the noncoding sequence in *P. liuwutiensis* was identified as the putative control regions (CRs; Table 3), which were similar to that in the genomic studies of Stichopus sp. [23].

#### PCGs and codon usage

The length of PCGs in the mtDNA of *P. liuwutiensis* was 11 352 bp. The longest (1830 bp) and shortest (165 bp) lengths were *nad5* and *atp8*, respectively (Table 3). The bases C and A were found to be predominant in most genes, while the base T was predominant in some genes (Table 4). Except for *nad6*, which was encoded on the negative strand, all PCGs were encoded on the positive strand (Table 3 and Fig. 2), which was similar to the results found in other sea cucumber species [3]. ATG was found to be the start codon in most PCGs, except for *nad1* (GTG as the start codon), *nad5* (TTG) and *nad6* (CTA), while TAA was the termination codon of most PCGs, excepting



Fig. 2. Gene map of the complete mitochondrial genome of *P. liuwutiensis*. Genes encoded on the positive and negative strands are shown outside and inside the circular gene map, respectively.

*nad4* (TAG as the start codon) and *nad6* (CAT; Table 3).

Table 5 and Fig. 3 summarize the relative synonymous codon usage (RSCU) values for the 13 PCGs. The mtDNA of *P. liuwutiensis* contained 5323 codons. Among the 13 PCGs, the most frequently used amino acid was Ser (12.91%), followed by Leu (10.82%), Lys (8.34%), Pro (7.06%) and Thr (6.01%). A common feature in most metazoan mtDNA is a bias toward a higher representation of nucleotides A and T, leading to a subsequent bias in the corresponding encoded amino acids [8,24]. The content of A + T in the 13 PCGs was 64.3% and the AT-skew was positive, indicating a higher occurrence of A than T (Table 4).

#### rRNA and tRNA genes

The results revealed that the two rRNA of *P. liuwutiensis* were encoded on the positive strand, and the 16S and 12S genes were 1348 bp (G + C% = 33.16%)

Table 2. List of species used for species identification based on the whole mitochondrial genome and the analysis of the base composition of each species.

Species	T (%)	C (%)	A (%)	G (%)	A + T (%)	GC-skew	AT-skew
P. liuwutiensis	29.55	22.16	35.64	12.64	65.19	-0.273	0.093
Amphiura digitula	30.89	22.98	32.74	13.39	63.63	-0.263	0.029
Apostichopus japonicus	30.14	20.21	31.75	17.89	61.89	-0.061	0.026
Parastichopus nigripunctatus	30.14	20.15	31.68	18.03	61.82	-0.055	0.025
Parastichopus parvimensis	29.91	20.38	31.78	17.93	61.69	-0.064	0.030
Parastichopus californicus	29.87	20.57	31.53	18.04	61.40	-0.066	0.027
Stichopus sp. SF-2010	29.29	23.76	30.95	16.01	60.24	-0.195	0.028
Stichopus horrens	29.31	23.72	30.80	16.17	60.11	-0.189	0.025
Holothuria scabra	27.07	24.34	32.67	15.91	59.74	-0.209	0.094
Holothuria forskali	30.82	21.41	31.40	16.37	62.22	-0.134	0.009
Cucumaria miniata	28.09	22.95	35.74	13.22	63.83	-0.269	0.120
Benthodytes marianensis	36.91	17.83	32.33	12.93	69.24	-0.160	-0.066
Freyastera benthophila	33.53	21.13	34.70	10.65	68.23	-0.330	0.017
Astropecten polyacanthus	31.51	22.44	32.50	13.55	64.01	-0.247	0.015
Acanthaster planci	25.24	27.95	31.10	15.71	56.34	-0.280	0.104
Echinaster brasiliensis	25.83	26.43	35.82	11.91	61.65	-0.379	0.162
Heliocidaris crassispina	27.86	24.37	31.03	16.74	58.89	-0.186	0.054
Heterocentrotus mammillatus	29.02	23.55	29.89	17.54	58.91	-0.146	0.015
Strongylocentrotus droebachiensis	30.18	22.80	28.80	18.22	58.98	-0.112	-0.023
Hemicentrotus pulcherrimus	30.58	22.43	29.19	17.79	59.77	-0.115	-0.023
Florometra serratissima	46.37	11.57	26.45	15.61	72.82	0.149	-0.274
Phanogenia gracilis	46.46	11.44	25.82	16.27	72.28	0.174	-0.286
Ophiura lutkeni	33.11	19.23	32.77	14.90	65.88	-0.127	-0.005

and 801 bp (G + C% = 37.2%) in length, respectively (Tables 3 and 4). The *16S* gene was between *nad2* and *cox1*, and *12S* was between *trnF* and *trnE*. The results showed that 5/22 tRNA genes, including *trna-gln*, *trna-ala*, *trna-ser* (*tga*), *trna-asp* and *trna-val*, were encoded on the negative strand, while the remaining genes were encoded on the positive strand. The length of the tRNA genes ranged from 64 to 73 bp, and the shortest (64 bp) and longest (73 bp) lengths were found from *trna-lys* and *trna-leu*<sup>(*tag*)</sup>, respectively (Table 3). In addition, Table 4 shows the base composition of tRNA A + T bias. All 22 tRNA genes were predicted to be capable of folding into a cloverleaf secondary structure using the MITOS web server (Fig. 4).

#### **Phylogenetic analysis**

Phylogenetic analysis revealed that the complete mtDNA of *P. liuwutiensis* and the other 25 echinoderm species were separated into five major clades as follows: Holothuroidea, Echinoidea, Asteroidea, Crinoidea and Ophiuroidea (Fig. 5). Almost all of the clades were strongly supported. The two species, *P. liuwutiensis* and *Cucumaria miniata*, formed a cluster, which were distinguished from other species, indicating that these two species together belonged to the same order (Dendrochirotida).

#### Gene order

Figure 6 illustrates the gene arrangement of P. liuwutiensis and seven other echinoderms. Similar gene arrangements exist in the species used to construct evolutionary trees or that had a few gene rearrangements. Holothuria scabra, Holothuria forskali, Apostichopus japonicus, Parastichopus nigripunctatus, Parastichopus parvimensis and Parastichopus californicus had the same gene arrangements [3]. Florometra serratissima and P. gracilis also had the same gene arrangement. The gene arrangement of Astropecten polyacanthus and A. planci is the same [9]. The gene order of A. japonicus (class Holothuroidea), S. droebachiensis (class Echinoidea) and P. liuwutiensis had similar changes with the inversion occurring between the genes trnD and trnM (Fig. 6), including the two conserved genetic blocks (cox1-R-nad4L-cox2-K-atp8-atp6-cox3-S2-nad3-nad4-H-S1-nad5-nad6-cob-F-rrnS-E-T-P-Q-N and Y-G-L2-nad1-I-nad2-rrnL-L1-A-W-C-V), which were found in the same order. The gene order of F. serratissima (class Crinoidea) was

Gene	Strand	Sequence location	Size (bp)	Start codon	Stop codon	Intergenic region
trna-pro	+	1–66	66			-4
trna-gln	_	63–132	70			6
trna-asn	+	139–207	69			2
trna-leu(tag)	+	210-282	73			-1
trna-ala	_	282–348	67			1
trna-trp	+	350-419	70			0
trna-cys	+	420–484	65			1
trna-val	_	486–555	70			4
trna-asp	_	560-624	65			57
trna-met	+	682–750	69			7
trna-tyr	+	758–826	69			-1
trna-gly	+	826-896	71			0
trna-leu(taa)	+	897–967	71			0
nad1	+	968–1939	972	GTG	TAA	5
trna-ile	+	1945–2012	68			2
nad2	+	2015-3055	1041	ATG	TAA	0
16S	+	3056-4403	1348			130
cox1	+	4534–6087	1554	ATG	TAA	1
trna-arg	+	6089–6153	65			0
nad4L	+	6154–6450	297	ATG	TAA	0
cox2	+	6451-7140	690	ATG	TAA	2
trna-lys	+	7143-7206	64			0
atp8	+	7207–7371	165	ATG	TAA	-7
atp6	+	7365–8048	684	ATG	TAA	2
сох3	+	8051-8833	783	ATG	TAA	-1
trna-ser(tga)	_	8833-8903	71			39
nad3	+	8943–9287	345	ATG	TAA	3
nad4	+	9291-10 652	1362	ATG	TAG	-10
trna-his	+	10 643–10 710	68			1
trna-ser(gct)	+	10 712-10 778	67			-3
nad5	+	10 776-12 605	1830	TTG	TAA	13
nad6	_	12 619-13 107	489	CTA	CAT	8
cytb	+	13 116–14 255	1140	ATG	TAA	1
trna-phe	+	14 257–14 327	71			0
12S	+	14 328–15 128	801			0
trna-glu	+	15 129–15 196	68			0
trna-thr	+	15 197–15 266	70			0
Putative CR	+	15 267–15 969	703			0

significantly different from that of *P. liuwutiensis*, with more translocations and inversions, and two genes were rearranged (*rrnL* and *V*). Ophiura lutkeni (class Ophiuroidea) produced more translocations. Therefore, these 10 genes (*G*, *rrnL*, *M*, *P*, *E*, *Y*, *D*, *cob*, *T* and *W*) were involved in the gene rearrangement, and five conserved gene blocks (*cox1-R-nad4L-cox2-K-atp8-atp6-cox3-S2-nad3-nad4-H-S1-nad5-nad6*, *C-V*, *L1-A*, *Q-N* and *L2-nad1-I-nad2*) were found in the same order. In contrast, *A. planci* (class Asteroidea) had two parts of a wide range of gene location inversions (*trnY-rrnL* and *trnP-trnV*), and the translocations of these two parts were observed. The only two

conserved genetic blocks (cox1-R-nad4L-cox2-K-atp8atp6-cox3-S2-nad3-nad4-H-S1-nad5-nad6-cob-F-rrnS-E-T and D-M) were found in the same order. Four conserved gene blocks were found (cox1-R, N-L1, nad4Lcox2-K-atp8-atp6-cox3-S2-nad3-nad4-H-S1-nad5-nad6cob-F-rrnS, Y-G-L2-nad1-I-nad2-rrnL) in the gene arrangement of C. miniata (class Holothuroidea), and the inversion occurred between the genes trnD and trnM. In the sea cucumber Benthodytes marianensis (class Holothuroidea), five conserved gene blocks (atp6-cox3-S2-nad3-nad4-H-S1-nad5-nad6-cob-F-rrnS-E, A-W, P-Q-N-L1, C-V-D, L2-nad1-I-nad2-T-rrnL) were identified. Meanwhile, compared with the gene

Table 4. The base composition and preference of mitochondrial gene in P. liuwutiensis.

Gene	T (%)	C (%)	A (%)	G (%)	G + C (%)	Total (bp)	GC-skew	AT-skew
PCGs	30.20	23.60	34.10	12.10	35.70	11 352	-0.322	0.061
nad6	18.40	22.90	50.51	8.18	31.08	489	-0.474	0.466
nad5	28.96	22.79	38.25	10.00	32.79	1830	-0.390	0.138
nad4L	35.35	23.91	32.32	8.42	32.32	297	-0.479	-0.045
nad4	29.59	25.11	35.17	10.13	35.24	1362	-0.425	0.086
nad3	34.20	23.77	30.14	11.88	35.65	345	-0.333	-0.063
nad2	34.49	21.13	33.14	11.24	32.37	1041	-0.306	-0.020
nad1	34.67	20.88	30.76	13.68	34.57	972	-0.208	-0.060
cytb	28.33	25.70	33.51	12.46	38.16	1140	-0.347	0.084
сох3	29.76	25.93	29.50	14.81	40.74	783	-0.273	-0.004
cox2	30.72	24.20	31.74	13.33	37.54	690	-0.290	0.016
cox1	29.99	23.42	30.24	16.34	39.77	1554	-0.178	0.004
atp8	23.64	16.36	49.09	10.91	27.27	165	-0.200	0.350
atp6	30.70	26.02	32.31	10.96	36.99	684	-0.407	0.026
trna-val	32.86	21.43	30.00	15.71	37.14	70	-0.154	-0.045
trna-tyr	27.54	15.94	37.68	18.84	34.78	69	0.083	0.156
trna-trp	34.29	12.86	44.29	8.57	21.43	70	-0.200	0.127
trna-thr	35.71	11.43	41.43	11.43	22.86	70	0.000	0.074
trna-ser	29.58	26.76	29.58	14.08	40.85	71	-0.310	0
trna-ser(gct)	31.34	17.91	28.36	22.39	40.30	67	0.111	-0.050
trna-pro	36.36	9.09	36.36	18.18	27.27	66	0.333	0.000
trna-phe	25.35	16.90	40.85	16.90	33.80	71	0	0.234
trna-met	33.33	18.84	33.33	14.49	33.33	69	-0.130	0
trna-lys	31.25	17.19	39.06	12.50	29.69	64	-0.158	0.111
trna-leu	34.25	16.44	32.88	16.44	32.88	73	0.000	-0.020
trna-leu(taa)	29.58	16.90	33.80	19.72	36.62	71	0.077	0.067
trna-ile	32.35	19.12	32.35	16.18	35.29	68	-0.083	0
trna-his	32.35	16.18	38.24	13.24	29.41	68	-0.100	0.083
trna-gly	30.99	15.49	40.85	12.68	28.17	71	-0.100	0.137
trna-glu	26.47	19.12	36.76	17.65	36.76	68	-0.040	0.163
trna-gln	31.43	20.00	35.71	12.86	32.86	70	-0.217	0.064
trna-cys	41.54	9.23	33.85	15.38	24.62	65	0.250	-0.102
trna-asp	36.92	21.54	26.15	15.38	36.92	65	-0.167	-0.171
trna-asn	24.64	17.39	37.68	20.29	37.68	69	0.077	0.209
trna-arg	32.31	15.38	38.46	13.85	29.23	65	-0.053	0.087
trna-ala	35.82	13.43	38.81	11.94	25.37	67	-0.059	0.040
16S	27.74	17.36	39.09	15.80	33.16	1348	-0.047	0.170
12S	21.22	21.72	41.57	15.48	37.20	801	-0.168	0.324
CRs	26.60	21.30	42.70	9.40	30.70	703	-0.388	0.232

arrangement of *P. liuwutiensis*, the translocation of three tRNAs (M, G and Y) can be found.

# Discussion

Phylogenetics studies the evolutionary relationships of organisms, and the phylogenetic tree is the topological structure that describes the evolutionary order of various groups of organisms [25,26]. However, with the development and application of molecular biology, our understanding of genes and proteins has been constantly increasing, and a theoretical method has been gradually developed to study the evolutionary relationship of species based on the genetic information of biological macromolecules, such as DNA or protein sequence. Because most of the mitochondrial genes are matrilineal and the sequence variation is rapid, they are widely used to study the phylogenetic evolution [27,28]. The most basic problem solved by phylogenetic research is to determine the taxonomic status of species [29,30]. However, determining the relationship of species can further infer the biological characteristics of unknown species, which, of course, requires a lot of phylogenetic studies to prove [31]. Each newly

Table 5. The codon number and RSCU in P. liuwutiensis mitochondrial protein-coding genes.

Codon	Count	RSCU									
UUU(F)	171	1.14	UCU(S)	162	1.83	UAU(Y)	212	1.39	UGU(C)	30	1.07
UUC(F)	130	0.86	UCC(S)	121	1.36	UAC(Y)	92	0.61	UGC(C)	26	0.93
UUA(L)	169	1.76	UCA(S)	97	1.09	UAA(*)	244	1.71	UGA(W)	74	0.52
UUG(L)	61	0.64	UCG(S)	18	0.20	UAG(*)	111	0.78	UGG(W)	32	1.00
CUU(L)	134	1.40	CCU(P)	141	1.50	CAU(H)	96	1.19	CGU(R)	19	0.50
CUC(L)	73	0.76	CCC(P)	99	1.05	CAC(H)	65	0.81	CGC(R)	16	0.42
CUA(L)	112	1.17	CCA(P)	107	1.14	CAA(Q)	151	1.43	CGA(R)	27	0.71
CUG(L)	27	0.28	CCG(P)	29	0.31	CAG(Q)	60	0.57	CGG(R)	12	0.31
AUU(I)	124	1.15	ACU(T)	81	1.01	AAU(N)	153	1.13	AGU(S)	57	0.64
AUC(I)	74	0.69	ACC(T)	101	1.26	AAC(N)	118	0.87	AGC(S)	77	0.87
AUA(M)	126	1.17	ACA(T)	104	1.30	AAA(K)	340	1.53	AGA(S)	94	2.46
AUG(M)	71	1.00	ACG(T)	34	0.43	AAG(K)	104	0.47	AGG(S)	61	1.60
GUU(V)	43	1.29	GCU(A)	40	1.23	GAU(D)	76	1.11	GGU(G)	25	0.83
GUC(V)	27	0.81	GCC(A)	55	1.69	GAC(D)	61	0.89	GGC(G)	12	0.40
GUA(V)	55	1.65	GCA(A)	31	0.95	GAA(E)	127	1.53	GGA(G)	66	2.20
GUG(V)	8	0.24	GCG(A)	4	0.12	GAG(E)	39	0.47	GGG(G)	17	0.57



Fig. 3. RSCU in P. liuwutiensis mitogenome.

sequenced species enriches the database, providing more information for studying the phylogeny between species.

In this study, we conducted a preliminary study on the mtDNA of *P. liuwutiensis*. Mitochondrial genomes are maternal mtDNA and do not recombine DNA. Therefore, individuals with the same mtDNA sequence are descended from the same female ancestor [32]. Consequently, mtDNA sequences can be used to determine the relationships between species. In this study, we aim to characterize the mtDNA of *P. liuwutiensis*, as well as to determine the taxonomic relationship between *P. liuwutiensis* and other echinoderm species.

The results showed that the contents of base A and base T were higher, the content of A + T was higher compared with G + C, and the lowest content of A + T was 56.34% (A. planci), indicating the characteristics of mtDNA sequence in invertebrates [33]. The CR was found to be the main noncoding region of the mtDNA, which is necessary for the initiation of mtDNA transcription and replication of metazoa [34,35]. Its size and nucleotide sequence greatly varied. Most metazoa mtDNA contain only one CR, whereas in some sea cucumbers there are two duplicate CRs or two independent CRs with the same or highly similar nucleotide sequences [33]. The **mtDNA** of





*P. liuwutiensis* was found to contain a CR between  $tRNA^{thr}$  and  $tRNA^{pro}$ . Three mechanisms may contribute to mtDNA with duplicate CRs: tandem duplication, dimerization and illegitimate recombination. Many studies support the idea that mitogenome with repetitive CRs may replicate more efficiently than mitogenome with a single CR [36,37]. However, because only two species of sea cucumber

(*B. marianensis* and *Cucumaria miniate*) have been shown to have duplicate CRs, additional mitotic genomes are needed to elucidate the mechanism that causes this phenomenon [3].

Based on the basic assumption that shared genetic arrangements imply a common ancestor, it is highly unlikely that the same sequence of genes will emerge independently in different lineages [9]. Therefore,



Fig. 5. Phylogenetic trees based on the concatenated amino acid of 13 PCGs. The Balanoglossus carnosus (NC001887.1) is used as outgroup. The red name highlights the species sequenced in this study. P. liuwutiensis (MN198190), Amphiura digitula (MH791160.1), Apostichopus japonicus (FJ986223.1), P. nigripunctatus (AB525762.1), Parastichopus parvimensis (KU168761.1), Parastichopus californicus (KP398509.1), Stichopus sp. SF-2010 (HM853683.2), Stichopus horrens (HQ000092.1), Holothuria scabra (KP257577.1), Holothuria forskali (FN562582.1), Cucumaria miniata (AY182376.1), Benthodytes marianensis (MH208310.1), Freyastera benthophila (MG563681.1), Astropecten polyacanthus (AB183560.1), Acanthaster planci (AB231475.1), Echinaster brasiliensis (MG636999.1), Heliocidaris crassispina (KC479025.1), Heterocentrotus mammillatus (KJ680292.1), Strongylocentrotus droebachiensis (EU054306.1), Hemicentrotus pulcherrimus (KC490911.1), Florometra serratissima (NC001878.1), Phanogenia gracilis (DQ068952.1), Ophiura lutkeni (AY184223.1), Peniagone sp. YYH-2013 (KF915304.1), Amphipholis squamata (FN562578.1) and Astrospartus mediterraneus (NC013878.1).

comparative gene alignment may be a useful tool for phylogenetic studies, especially when some ancestral relationships are concerned. Over the past two decades, many studies have been reported on the mitochondrial gene sequence in echinoderms [38,39]. There are four possible mechanisms for genome rearrangement: inversion (reversals), transposition, reverse transposition and tandem duplication random losses [3]. It is of great significance to explore the evolutionary history of mitochondrial gene rearrangement in echinoderms by comparing the gene arrangement in the mtDNA of echinoderms and studying the common sequence among different individuals [40,41]. In this study, the gene arrangement of P. liuwutiensis was very similar to that of the other five echinoderm species, which all contained conserved gene blocks of 15 genes. In particular, A. japonicus and S. droebachiensis had an inversion of only two genes compared with P. liuwutiensis. Shen et al. [9] have hypothesized that the inclusion of a consensus nonavian vertebrate gene order does support the echinoid mtDNA gene order as the most likely representative of the echinoderm ground pattern. Genes and the environment act together on biological traits, with genes playing a dominant role. Therefore, the study

of genes can infer the adaptability of species to the environment. Mu *et al.* [3] have studied the adaptation of sea cucumber to the deep-sea environment through the mtDNA of sea cucumber, and predicted that *nad2* and *nad4* might be important candidate genes for the further study on the adaptation of *B. marianensis* to the deep-sea environment. The different gene order may be related to certain ecological or morphological features of the species. Genes may produce different effects in different results. These assumptions need to be tested by further research.

# Conclusions

In this study, we characterized the structure of the mtDNA of *P. liuwutiensis*, and the results showed that the mtDNA (15 969 bp) encoded 37 genes, including 13 PCGs, 22 tRNA genes and 2 rRNA genes. One putative CR was found in the mitogenome of *P. liuwutiensis*. The mtDNA of *P. liuwutiensis* was clustered together with *C. miniate*. Moreover, the gene arrangement of *P. liuwutiensis* was also described in detail.



**Fig. 6.** Linear representation of gene rearrangements of *P. liuwutiensis, Apostichopus japonicus, Florometra serratissima, Strongylocentrotus droebachiensis, Ophiura lutkeni, Acanthaster planci, Cucumaria miniata and Benthodytes marianensis.* Gene segments are not drawn to scale. All genes are transcribed from left to right except those indicated by underlining, which are transcribed from right to left. The circling arrows indicate inversions. tRNA genes are represented by the corresponding single-letter amino acid code, especially S1 (AGN), S2 (UCN), L1 (CUN) and L2 (UUR). *rrnL* and *rrnS* are the large and small rRNA subunits, respectively.

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# **Conflict of interest**

The authors declare no conflict of interest.

# **Data accessibility**

All original sequence data in this study were submitted to the NCBI database under accession number MN198190.

# **Author contributions**

QY and QL designed and supervised the research. FY performed most of the experiments and wrote the paper with assistance from C. Zhou, NTT, ZS, JW, HG, ZL, C. Zhong and ZZ. All authors made contributions to the final version of this manuscript. All authors read and approved the final manuscript.

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