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Determination and Analysis of the Complete Mitochondrial DNA Sequence of *Octopus dollfusi* (Mollusca: Cephalopoda: Octopodidae) from China

Yunjun Yan, Zhenming Lü, Tianming Wang, Yongjiu Chen, Jingwen Yang, Baoying Guo, Lihua Jiang, Changwen Wu and Liqin Liu*

National Engineering Research Center of Marine Facilities Aquaculture, College of Marine Sciences, Zhejiang Ocean University, Zhoushan, Zhejiang, 316022, China

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ZL, LL and YY designed the study and wrote the arctile. TW and YC helped to analyze the sequence data and submit to the Genbank. BG, LJ, and CW helped to sample and identify species.

Key words
Mitochondrial genome, Octopus dollfusi, Proteins, tRNAs, Phylogenetic relationship.

ABSTRACT

In this study, we have determined the complete nucleotide sequence of the mitochondrial genome of *Octopus dollfusi* (Robson, 1932) collected from the coast of Guangdong Province, China, and analyzed the phylogenetic relationship with other cephalopod species. The results show that the mitochondrial genome of *O. dollfusi* composed of 15843 nucleotide pairs and encodes 13 proteins, 2 ribosomal RNAs (rRNAs), 22 transfer RNAs (tRNAs) and a major long noncoding region (LNCR) of the mitochondrion's own protein synthesizing system. Seven of thirteen proteins, eight tRNAs are encoded by the plus strand, while the other proteins and tRNAs, as well as two rRNAs are encoded by the minus strand. Two (ND4 and ND4L) of the 13 protein coding genes of *O. dollfusi* began with the unorthodox translation initiation codon ATA and all others use the standard ATG. Ten protein-coding genes use TAA as the termination codon and the rest share the termination codon TAG. There are five cases where tRNA genes appear to overlap. The LNCR of *O. dollfusi* was 926 nucleotides and no repeated sequences were found in this LNCR. Phylogenetic analysis of 24 cephalopoda species based on the complete mitochondrial genome showed that the *O. dollfusi* is most closely related to *Amphioctopus aegina*. These results seems to support the recent notion that *O. dollfusi* should be considered as synonym of *Amphioctopus aegina*. More morphologic and molecular evidences should be involved to resolve the taxonomic status of *O. dollfusi* in future's studies.

INTRODUCTION

Cephalopoda is the third largest molluscan class including a great number of families and more than 800 species have been identified with high commercial value as food sources (Lindgren *et al*., 2004; Boyle and Rodhouse, 2007). Octopus, one genus of cephalopod with four pairs of arms, more than 200 species have been recognized and play an increasing important role in the ocean fisheries in recent years (Lindgren *et al*., 2004). So far, the octopus scientific classification system are continuously in revision and consummation (Guzik *et al*., 2005). At present, more and more new genus were separated from traditional genus *Octopus* (Huffard and Hochberg, 2005; Norman and Hochberg, 2005). Although morphology has been successfully employed to identify differences between subspecies or species in most coleoids, there are still many disadvantages about the taxonomic classification of this species based on morphological differences (Roper and Hochberg, 1988; Voight, 1994; Herke and Foltz, 2001).

Analysis of metazoan mitochondrial (mt) genome is a valuable tool for resolving ancient phylogenetic relationships and subtle taxonomic classification between species (Boore and Brown, 1998). Mt genomes are typically circular duplex molecules ranging in size from 14 to 18 kilobases, consisting of 13 protein-coding genes (PCGs), 22 tRNAs and 2 rRNAs (Boore, 1999). The mitochondrial genome has now been extensively used for studying population structure, phylogeography and phylogenetic relationships at various taxonomic levels across animal taxa based on several valuable characteristics including its small size, fast evolutionary rate, relatively conserved gene content and organization, and limited recombination (Avise *et al*., 2003; Liu and Cui, 2011; Xu *et al*., 2011; Huang *et al*., 2017). Some studies on taxonomic classification by using mt genomes have been reported such as the genus *Echinococcus* and *Sotalia* (Caballero *et al*., 2007; Nakao *et al*., 2007). So

* Corresponding author: liuliqin-666@163.com
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far, most molluscan mt genomes reported have different gene organizations, and there are large differences in mt gene organization within each class (Kurabayashi and Ueshima, 2000; Grande *et al*., 2002; Tomita *et al*., 2002; Cheng *et al*., 2012). Regarding *Octopus*, complete mtDNAs have been sequenced from some species, such as *O. vulgaris*, *O. bimaculatus*, *O. conispadiceus*, *O. minor* and *O. ocellatus*, which provided useful information for the future research of genetic diversity and phylogenetics (Yokobori *et al*., 2004; Akasaki *et al*., 2006; Cheng *et al*., 2012; Dominguez-Contreras *et al*., 2015; Ma *et al*., 2016). Comparison of mtDNA gene sequences plays an important role in solving the evolutionary history especially those involving deep branching events (Boore and Staton, 2002; Helfenbein and Boore, 2004).

Octopus dollfusi, commonly called as "marbled octopus", is one of the most economically marine cephalopods which belongs to Octopodidae, Incirratai, Coleoidea, Cephalopoda, animalia Mollusca (Dong, 1988; Sundaram and Sawant, 2010). It is distributed mainly along Indo-China and Hong Kong (Lei *et al*., 2006; Sundaram and Sawant, 2010). As a kind of important economic cephalopods, *O. dollfusi* is a seafood with higher protein and lower fat, very high protein nutritive value, more fatty acid and mineral elements (Lei *et al*., 2006). The total production of *O. dollfusi* is very high and consumed heavily by the consumers throughout the world each year. Nevertheless, for this species, there are a lot of debates regarding its taxonomic status, to dates. *O. dollfusi* was firstly discovered and named by Robson after an intensive examination of stored Cephalopod samples in British museum, in 1929 and was considered as valid species, as well as *O. aegina* (Robson, 1932). Voss and Wiliamson (1972) and Dong (1979) supported the valid species of both *O. dollfusi* and *O. aegina,* and described the difference, in detail, of the morphology and life history between these two species (Voss and Wiliamson, 1972; Dong, 1979). However, Norman and Hochberg (2005) removed the *O. dollfusi* from genus octopus and considered it invalid as a synonym of *A. aegina* (Norman and Hochberg, 2005). Nevertheless, Chen and some other authors still regarded *O. dollfusi* and *O. aegina* as two different species when they sampled in their researches (Chen *et al*., 2009; Herrero *et al*., 2012; Gestal *et al*., 2015). The mtDNAs genomic information is very helpful to understand the phylogenetic and taxonomic status of species. In this study, we present the complete sequence of the mtDNA genome of the octopus species *O. dollfusi* (GenBank accession number KX108697). The gene arrangement of *O. dollfusi* shows remarkable similarity to that of Octopodiformes. Phylogenetic analysis of 24 species cephalopoda (Supplementary Table I) based on the complete mitochondrial genome showed that the *O. dollfusi* is most closely related to *A. aegina*. This study will provide important information for taxonomic status of *O. dollfusi.*

MATERIALS AND METHODS

PCR amplification and sequencing of mitochondrial DNA

O. dollfusi were obtained from the Zhanjiang, Guangdong, China and identified based on both the morphologic features and the COX1. According to Dong's research, the morphology of *O. dollfusi* is quite unique, with a smooth body surface, longitudinal pale stripe on dorsal mantle and pale stripe between eyes, which differed from that of *O. aegina* (Dong, 1979). Muscle Tissue samples were reserved in 95% ethanol for molecular analysis and stored at -20°C. Whole genomic DNA was extracted from muscle tissue of individual specimens using the phenol-chloroform method (Sambrook *et al*., 1989). Primers were designed according to the conserved regions of cephalopod mtDNA sequences present in GenBank. Primers were generated by Primer Premier 5 and listed in Supplementary Table II. Sequences were amplified by PCR with long and accuracy Taq (LA-Taq) DNA polymerase (Takara) following the manufacturer's protocol. Then the PCR product sequencing was sequenced. Primer synthesis and PCR product were completed by Thermo Fisher Scientific Company. The complete mtDNA genome were assembled after the amplified regions were individually sequenced.

Gene annotation and analysis

Sequences were assembled using Geneious 4.5.3 (http://www.geneious.com). PCGs and rRNAs in mtDNA were identified based on a comparison with mtDNA sequences of *O. vulgaris* (Accession No. NC_006353.1), *O. minor* (Accession No. NC_015896.1), *O. ocellatus* (Accession No. NC_007896.1) and *A. aegina* (Accession No. NC_029702.1). tRNAs sequences were verified in sequences between protein and rRNA genes by their ability to fold into the typical cloverleaf structures characteristic of mt-tRNA genes, and from the trinucleotide in the anticodon position of these structures either using the tRNAscan-SE Search Server (http://lowelab.ucsc.edu/tRNAscan-SE/) and corrected through alignment with other cephalopod sequences. Sequence alignment was deduced by BioEdit 7.0 using ClustalW alignment. The complete nucleotide sequence has been submitted to GenBank (Accession number: KX108697).

Phylogenetic analysis

To estimate phylogenetic relationships, phylogenetic tree was constructed depending on the Neighbor-Joining (NJ) Method of Molecular Evolutionary Genetics Analysis (MEGA 6.0) based on the 24 complete mitochondrial genome sequences of cephalopods. The bootstrap value was repeated 1000 times to obtain the confidence value for the analysis (Kimura, 1980).

Table I.- Mitochondrial genome characteristics of *O. dollfusi*.

Gene	Position		Size(bp)		Codon		Intergenic nucleotides[a]	Strand
	From	To	Nucleotide	Amino acid	Initiation	Stop		
COIII	1	780	780	259	ATG	TAA	0	L
tRNA^{Lys}	790	855	66				9	L
tRNA^{Ala}	854	919	66				-2	L
tRNA^{Arg}	919	985	67				-1	L
tRNA^{Asn}	986	1052	67				0	L
tRNA^{Ile}	1053	1119	67				0	L
ND3	1120	1470	351	116	ATG	TAA	0	L
tRNA^{Ser}	1469	1537	69				-2	L
ND2	1538	2575	1038	345	ATG	TAA	0	L
COI	2547	4079	1533	510	ATG	TAA	-29	L
COII	4085	4771	687	228	ATG	TAA	5	L
tRNA^{Asp}	4774	4840	67				2	L
ATP8	4842	4997	156	51	ATG	TAA	1	L
ATP6	4999	5691	693	230	ATG	TAG	1	L
tRNA^{Phe}	5716	5783	68				24	H
ND5	5739	7475	1737	579	ATG	TAA	-45	H
tRNA^{His}	7476	7540	64				0	H
ND4	7544	8887	1344	447	ATA	TAA	3	H
ND4L	8884	9189	306	101	ATA	TAA	-4	H
tRNA^{Thr}	9184	9247	64				-6	L
tRNA^{Ser}	9250	9315	66				2	H
CYTB	9315	10454	1140	379	ATG	TAA	-1	H
ND6	10447	10959	513	170	ATG	TAG	-8	H
tRNA^{Pro}	10961	11027	67				1	H
ND1	11031	11969	939	312	ATG	TAG	3	H
tRNA^{Leu}	11970	12040	71				0	H
tRNA^{Leu}	12040	12106	67				-1	H
16S rRNA	12105	13408	1304				-2	H
tRNA^{Val}	13410	13478	69				1	H
12s rRNA	13480	14440	961				1	H
tRNA^{Met}	14442	14509	68				1	H
tRNA^{Cys}	14513	14577	65				3	H
tRNA^{Tyr}	14576	14641	66				-2	H
tRNA^{Trp}	14642	14707	66				0	H
tRNA^{Gln}	14708	14775	68				0	H
tRNA^{Gly}	14779	14847	69				3	H
tRNA^{Glu}	14846	14917	72				-2	H
Control region	14918	15843	926				0	H

[a]Numbers correspond to the nucleotides separating adjacent genes. Negative numbers indicate overlapping nucleotides.

RESULTS AND DISCUSSION

Organization of the mitochondrial genome

Gene content and organization of the mtDNA molecule of *O. dollfusi* were given in Table I. Moreover, the mitochondrial gene map of *O. dollfusi* was also shown in Figure 1. The size of *O. dollfusi* molecule is 15843 nucleotide pairs, which is similar with that of the other octopuses (Cheng *et al*., 2012; Dominguez-Contreras *et al*., 2015; Zhang *et al*., 2015; Ma *et al*., 2016). As in almost all metazoan mtDNAs, the *O. dollfusi* mtDNA molecule contains 13 PCGs, 22 tRNAs, 2 rRNAs, and a major non-coding region (926 in length) of the mitochondrion's own protein synthesizing system. The mitogenome base composition was A: 42.15%, T: 33.93%, C: 16.33%, and G: 7.59%, with A+T content (76.08%) was much

higher than the G+C content, which is in common with other invertebrate mitogenomes (Zhang *et al.*, 2015) (Supplementary Table III). 13 PCGs can be classified into two parts: COX3, ND3, ND2, COX1, COX2, ATP8, and ATP6 were encoded by the plus strand (light-strand), the rests including ND1, ND5, ND4, ND4L, CYTB and ND6 were encoded by the minus strand (heavy strand). The two rRNA genes, 16SrRNA (1304 bp) and 12SrRNA (960 bp), are located between tRNA-Leu and tRNA-Met and are separated by tRNA-Val gene. In addition, there are three nucleotide overlaps between ND2 and COI, ND4 and ND4L and Cytb and ND6, respectively. The largest overlap is 29 nucleotides, between ND4 and COI genes (Table I).

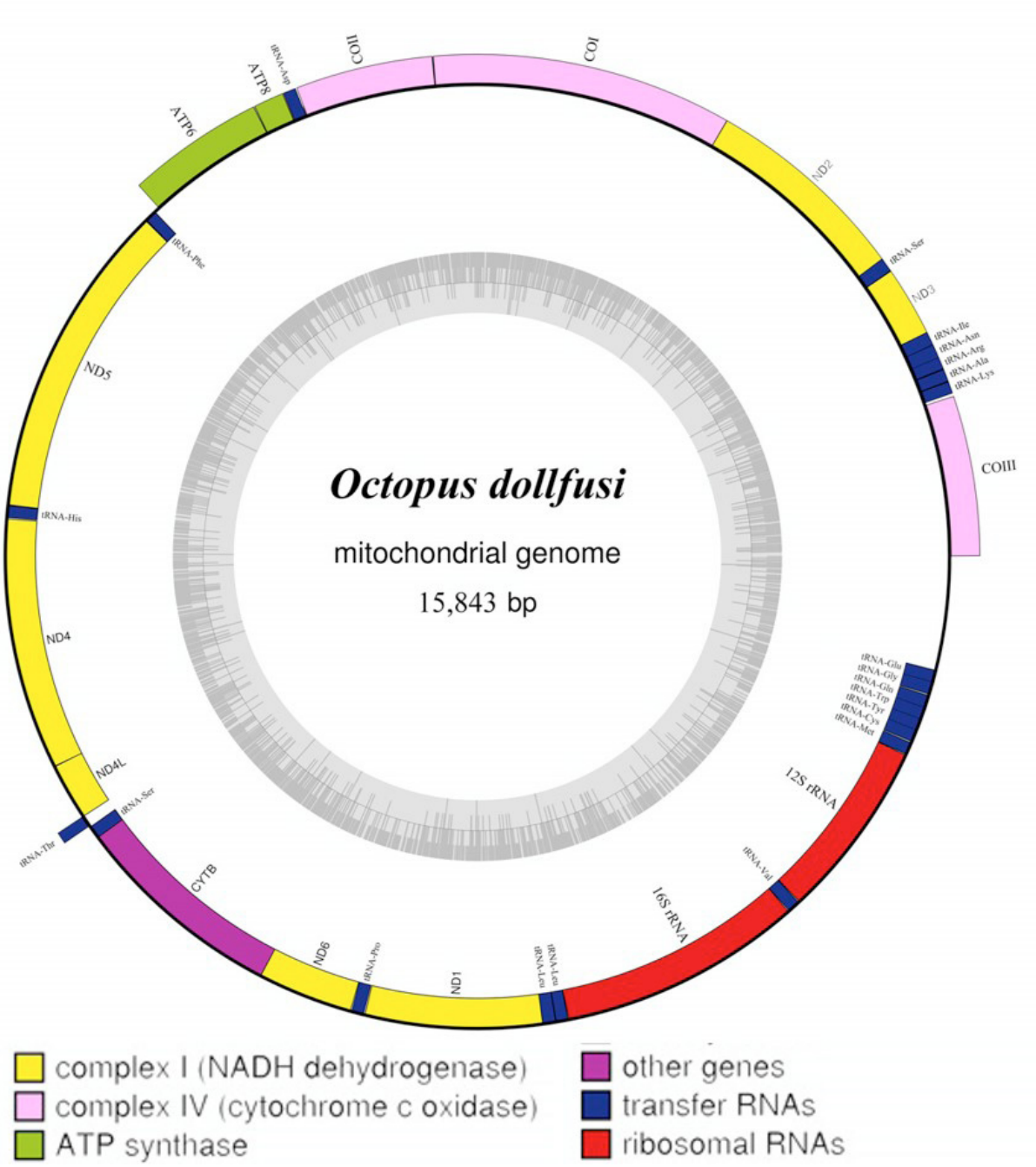

Fig. 1. Mitochondrial gene map of the cephalopod mollusk *O. dollfusi*.

Table II.- Codon usage in the 13 mt-PCGs of *O. dollfusi*.

Phe	TTT	306	Ser2	TCT	82	Tyr	TAT	153	Cys	TGT	57
	TTC	44		TCC	24		TAC	18		TGC	4
Leu1	TTA	375		TCA	121	TER	TAA	10	Trp	TGT	87
	TTG	80		TCG	6		TAG	3		TGG	12
Leu2	CTT	35	Pro	CCT	63	His	CAT	58	Arg	CGT	14
	CTC	9		CCC	14		CAC	17		CGC	0
	CTA	53		CCA	46	Gln	CAA	52		CGA	33
	CTG	2		CCG	0		CAG	7		CGG	5
Ile	ATT	325	Thr	ACT	57	Asn	AAT	150	Ser1	AGT	57
	ATC	38		ACC	11		AAC	33		AGC	4
Met	ATA	280		ACA	60	Lys	AAA	88		AGA	58
	ATG	43		ACG	4		AAG	14		AGG	26
Val	GTT	97	Ala	GCT	59	Asp	GAT	60	Gly	GGT	74
	GTC	1		GCC	14		GAC	10		GGC	3
	GTA	87		GCA	43	Glu	GAA	55		GGA	106
	GTG	21		GCG	9		GAG	23		GGG	39

The number of occurrences of each codon in the 13 mt-PCGs of *O. dollfusi* is given. Assumed modifications relative to the standard genetic code are AGA and AGG specify serine; ATA specific methionine and TGA specific tryptophan.

So far, complete mtDNA sequences have been determined at least 30 species in cephalopods, a very small sampling compared to those available for molluscs. In recent two years, more and more cephalopod complete mtDNA sequences were revealed such as *A. aegina*, *Allonautilus scrobiculatus*, *O. bimaculatus*, *Illex argentines*, *Loligo duvaucelii*, *Loliolus uyii*, *Sepiella maindroni* (Domínguez-Contreras *et al*., 2015; Groth *et al*., 2015; Jiang *et al*., 2015, 2016; Zhang *et al*., 2015). The mtDNA of the *O. dollfusi* is fairly typical in many respects, with a size, gene content, and A+T-richness similar to those most common for animal mtDNAs. The mtDNA of *O. dollfusi* shares the same structure and characteristics with other cephalopods, which corresponds to the fact the structure and arrangement order of protein-coding genes and rRNAs of cephalopods are relatively conservative compared to other molluscs.

Codon usage of PCGs

Codon usage among the 13 PCGs was given in Tables I and II. All amino acids specifying codons and the two termination codons, TAA and TAG are used for *O. dollfusi*. Non-standard initiation codons are often used in mitochondrial genomes (Wolstenholme, 1992). As shown in Table I, ND4 and ND4L start with ATA as an initiation codon and the last protein-coding genes start with an ATG initiation codon. Except for Ten protein-coding genes (COX3, ND3, ND2, COX1, COX2, ATP8, ND4, ND4L, ND5 and CYTB) use TAA as the termination codon, the rest three protein-coding genes (ATP6, ND6, ND1,) share the termination codon TAG. Overlapping nucleotides in protein are commonly observed for mtDNAs, which are thought to be translated as a bicistron. For example, ND2 is inferred to overlap COI by 29 nucleotides; ND4L is inferred to overlap ND4 by 4 nucleotides and ND6 is inferred to overlap Cytb by 8 nucleotides.

The frequencies of nucleotides in codon third positions are higher for T (44.05%) and A (41.56%) and lower for G (7.86%) and C (6.53%). This differed obviously with the base content in the sense strands of the PCGs (T, 43.02%; A, 31.68%; G, 14.03%; C, 11.28%). The overall A +T content in the sense strands of the PCGs (74.70%) shows the obvious bias in the AT nucleotide composition. Statistics found that the A+T content in the sense strands of metazoan mt-PCGs is from 83.3% (Apis mellifera) to 54.9% (human) (Anderson *et al*., 1981; Crozier and Crozier, 1993). The A+T content in the sense strands of the PCGs (74.70%) of *O. dollfusi* is within the range of values.

As shown in Table II, the content of Arg is the lowest and there are 5 amino acids (from high to low: Leu, Ser, Ile, Phe, Met) used in a high frequency. In particular, the content of Leu is the highest, which is a common phenomenon in cephalopods. Some scholars suggested that Leu may effectively participate in transmembrane transport as hydrophobic amino acids since most of the proteins encoded by mt-PCGs are transmembrane proteins (Obuchi *et al*., 2001).

rRNA genes

The *O. dollfusi* mt-rRNA genes (16S and 12S) were identified from nucleotide sequence similarities to the corresponding genes in other cephalopod species. As in mammalian and some other mtDNAs, the two mt-rRNA

genes are separated from each other by a single tRNA gene (Bibb *et al*., 1981; Clary and Wolstenholme, 1985). As shown in Table I and Figure 1, 16S and 12S genes are separated from each other by tRNA-Val in *O. dollfusi* mtDNA. The nucleotides adjacent to the tRNA-Leu and tRNA-Val are tentatively identified as the 5' and 3' ends of the 16S gene, and the nucleotides adjacent to the tRNA-Val and tRNA-Met are tentatively identified as the 5' and 3' ends of the mt-12S gene (Pont-Kingdon *et al*., 1994; Beagley *et al*., 1998). The 16S and 12S genes thus defined are 1304 and 961 ntp, respectively in *O. dollfusi.* Compared to other Octopodidae species, the length of *O. dollfusi* rRNAs are in the normal range and closest to *A. aegina* (Supplementary Table IV).

tRNA genes

Sequences were identified according to the potential secondary structures. The 22 *O. dollfusi* mt-tRNA genes vary in size from 64 nucleotides to 72 nucleotides (Table I). There is strict conservation of the size of the amino-acyl stem (7 ntp), the anticodon stem (5 ntp), and the anticodon loop (7 nucleotides) (Supplementary Fig. S1). The variable loop is 4 or 5 nucleotides lied between the anticodon and the TΨC arms (Supplementary Fig. S1). Within the TΨC arm, the stem varies from 3 to 6 ntp, and the loop varies from 3 to 10 nucleotides (Supplementary Fig. S1).

There are five cases where tRNA genes appear to overlap (Table I). As follows: tRNA-Ala appears to overlap tRNA-Lys by two nucleotides, GG; tRNA-Arg appears to overlap tRNA-Ala by one nucleotide, A; tRNA-leu (TAA) appears to overlap tRNA-leu (TAG) by one nucleotides, T; tRNA-Tyr appears to overlap tRNA-Cys by two nucleotides, CC; tRNA-Glu appears to overlap tRNA-Gly by two nucleotides, AT. It appears for each case that cleavage to form a complete downstream tRNA followed by polyadenylation of the upstream tRNA would yield fully formed, well-paired structures for all (Yokobori and Pääbo, 1995).

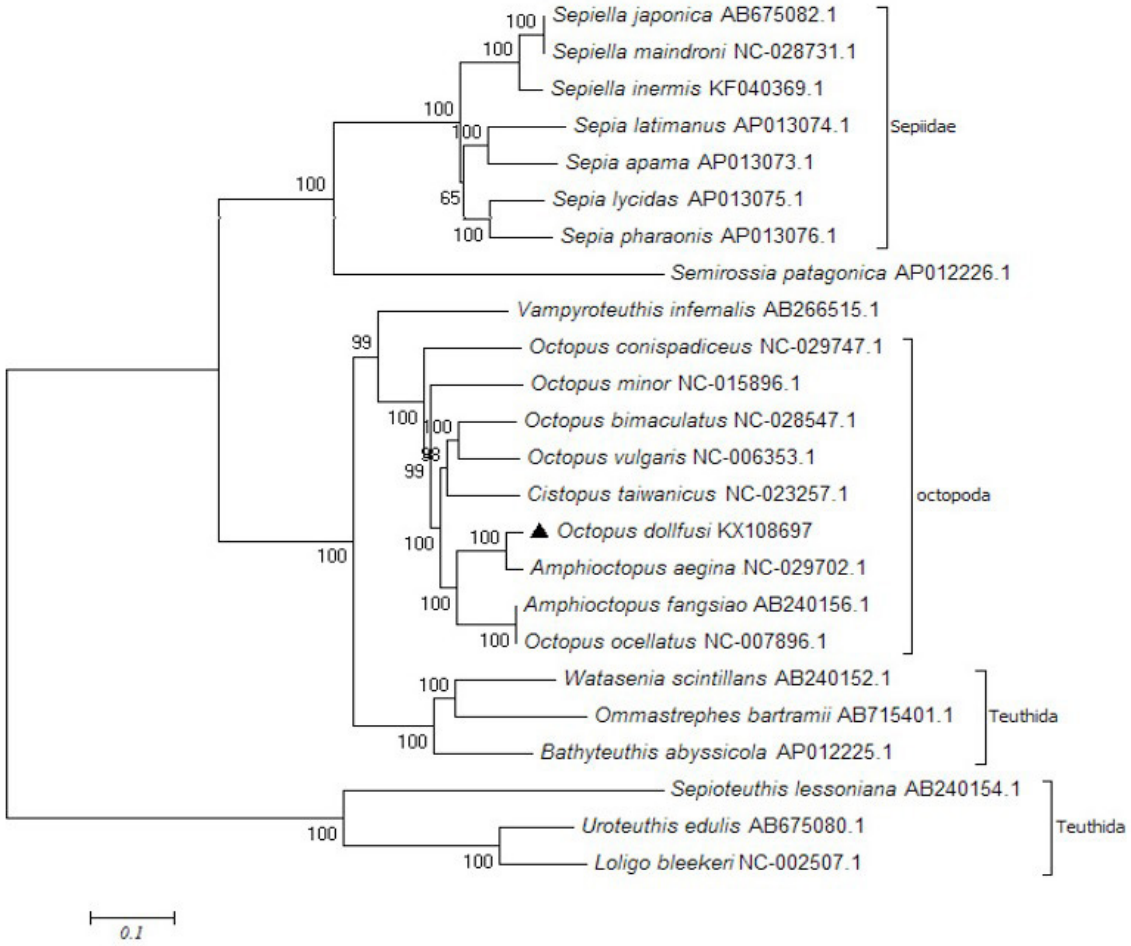

Fig. 2. Phylogenetic tree based on the mitochondrial genome sequences.

Except for at the junctions of ND2/COI, COI/COII, ATP8/ATP6, ND4/ND4L and Cytb/ND6, tRNAs are interspersed between protein and rRNA encoding sequences (Table I). It has been suggested that short sequences at 5' end of some proteins genes with secondary structure potential may serve as signals for transcript processing in the absence of tRNA (Bibb *et al*., 1981). In cases where tRNAs are the intervening sequences they may serve as signals for processing larger transcripts from which proteins are translated (Battey and Clayton, 1981; Ojala *et al*., 1981).

Long-noncoding regions (LNCRs)

In cephalopod, species in Decapodiformes carry two or three near identical LNCRs, while species in Octopodiformes and Nautiloida carry only one LNCR in the mitochondrial genomes (Yokobori *et al*., 2004, 2007; Akasaki *et al*., 2006; Boore, 2006; Cheng *et al*., 2011). In LNCRs, the control region (CR) or displacement loop region (D-loop) plays the very essential role in controlling elements and/or start points of replication and transcription (Wolstenholme, 1992). The control region is also called A+T-rich region because of its high content of adenine and thymine. The length of CR in *O. dollfusi* was 926 nucleotides, which was not an unusually large number (Supplementary Table IV). The previous study found that three of the five species with only one CR carry repeated sequences and these repeats were the major cause for the elongation of CR (Yokobori *et al*., 2004; Akasaki *et al*., 2006; Boore, 2006). Such nucleotide repeats were not found in the CR of *O. dollfusi* as well as *A. aegina, O. minor* and *V. infernalis* (Yokobori *et al*., 2007; Cheng *et al*., 2011; Zhang *et al*., 2015).

Phylogenetic analysis

Phylogenetic tree was produced by the MEGA 6.0 using ClustalW alignment with 24 complete mitochondrial genome sequences of cephalopods (Fig. 2). Phylogenetic analysis indicated that *O. dollfusi* is most closely related to *A. aegina*. The group including *O. dollfusi* and *A. aegina* is sister to *A. fangsiao* and *O. ocellatus*. In Octopoda, *O. conispadiceus* was farthest to *O. dollfusi* and *O. minor* came second. The morphological and ecological data also supported the phylogenic result. Both *O. dollfusi* and *O. ocellatus* belong to the short wrist type octopus and the hectocotylized arms are similar with each other while the hectocotylized arms of *O. minor* differs obviously with that of the other two octopus species. It also suggested a monophyletic status for the Octopoda with strong support.

Vampyroteuthis infernalis is the sister group of Octopoda, which is consistent with Cheng's result used LNCRs and PCGs (Cheng *et al*., 2011). Since the phylogenetic researches based on morphological data have largely avoid the genus Octopus, the molecular phylogenetic analyses of octopus would play more important roles. The mt gene organization of *V. infernalis* and Octopoda have more similar to the proposed ancestral cephalopod mt gene organization than the Decapoda (Cheng *et al*., 2011). Combined phylogenetic analysis of both molecular and morphological data, Vampyromorpha has been inferred to be the sister group to Octopoda (Lindgren *et al*., 2004).

Phylogenetic results seemed to support Norman's notion that *O. dollfusi* should be considered as synonym of *A. aegina*. To further confirm this notion, we compared the mt genome sequence of *O. dollfusi* with all the available partial sequence either under the name *O. dollfusi*, *O. aegina,* or under the name *A. aegina* submitted by authors from around the world. The results showed that all these partial sequence has quite high homology with the mt genome sequence of *O. dollfusi*. All the sequences seemed to form a monophyly, suggesting likely belonging to a single species. Otherwise, at least two lineages should be recognized if *O. dollfusi* and *A. aegina* are valid different species. However, we still argue that it is far from jumping a conclusion that *O. dollfusi* should be regarded as synonym *of A. aegina*. In our another attempt to study the genetic variation of different population of *O. dollfusi* along the coast of China, we found that there likely existed cryptic or subspecies in *O. dollfusi* (data unpublished). What the possible cryptic or subspecies means to *O. dollfusi,* and *A. aegina* still awaiting further study and more solid evidences.

CONCLUSION

In conclusion, this study determined the complete mtDNA sequence for *O. dollfusi*. The availability of the complete mitochondrial genome sequence of the *O. dollfusi* provides useful information for further investigations of phylogenetic relationship, taxonomic resolution and phylogeography of the cephalopod and other cephalopods. Either phylogenetic analysis using whole mt genome sequence or nucleotide BLAST analysis using partial mt genes disclosed high sequence homology between *O. dollfusi* and *A. aegina*, supports the notion that *O. dollfusi* is the synonym *of A. aegina*. More efforts should be made to resolve the taxonomic status of *O. dollfusi* in the future.

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Supplementary material

There is supplementary material associated with this article. Access the material online at: http://dx.doi.org/10.17582/journal.pjz/2018.50.2.463.472

Statement of conflict of interest

The authors report no conflicts of interest.

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