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# Characterization of Complete Mitochondrial Genome and Phylogeny of *Sepia lycidas* (Sepioidea, Sepiidae)

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**Authors' Contribution**
**BG and ZL conceived the idea. BG, YC and CZ designed the experiments and wrote the article. KX, HP and HS provided the specimens. CZ conducted the experimental work. YC analyzed the sequence data and submitted to the Genbank.**

**Key words**
**Cephalopoda, Sepiida, *Sepia lycidas*, Mitochondrial genome, Phylogeny.**

## ABSTRACT

We sequenced the complete mitochondrial (mt) genome of the kisslip cuttlefish, *Sepia lycidas*, made the comparison with the mt genomes of other cuttlefishes, and constructed phylogenetic trees estimating their relationships. The genome was 16,228 bp and contained 13 protein-coding genes, 22 transfer RNA genes, 2 ribosomal RNA genes, and 2 long-noncoding regions [both in the control regions (CR)]. The composition and order of genes in *S. lycidas* were similar to those of most other invertebrates. The overall base composition of *S. lycidas* is 35.8% T, 14.8% C, 41.3% A, and 8.1% G, with extremely high A+T content (77.1%). Both control regions contain termination-associated sequences and conserved sequence blocks. Maximum likelihood and Bayesian methods were used to build phylogenetic trees based on protein coding mtDNA genes of 37 cuttlefish species. *S. lycidas* have a close relationship with *S. pharaonis*, *S. aculeata*, and *S. esculenta*. This result confirmed the relationships of *S. lycidas* as being similar to the traditional taxonomy. This study plays an important role in the investigation of phylogenetic relationships, taxonomic resolution, and phylogeography for Sepiidae species.

## INTRODUCTION

*Sepia lycidas* (Sepioidea, Sepiidae) is a demersal and neritic species (Dong, 1988), which mainly occurs throughout the East China Sea (including areas near southern Japan), South China Sea, and nearby regions of the Indian and West Pacific Oceans (Carpenter and Niem 1999; Okutani, 2005). Known as the kisslip cuttlefish, it is a common and large cuttlefish (38 cm adult mantle size), and has high nutritional and medicinal value (Dong, 1988). Extensive studies of *S. lycidas* have been focused on breeding, morphological, and biological characteristics (Natsukari and Tashiro, 2009; Nagai *et al*., 2001; Lucky *et al*., 2012), but preliminary studies about the phylogenetic analysis were based on single gene fragment (Montserrat *et al*., 2010; Wen *et al*., 2017). We sequenced the complete mt genome of *S. lycidas* (Genbank accession number: KJ162574) to assess its genomic structure and provide a more robust estimate of its phylogenetic relationships.

Most metazoan species possess a compact, circular mt genome, which varies in size from 15 to 20 kb that typically contain 37 genes, including 13 protein coding genes, two ribosomal RNA (rRNA) genes, and 22 transfer RNA (tRNA) genes necessary for translation of the proteins encoded by the mtDNA (Boore, 1999; Cheng *et al*., 2012). MtDNA has been extensively used for studying phylogenetic and evolutionary relationships among animal species, due to its maternal inheritance, rapid evolutionary rate, and lack of genetic recombination (Zheng *et al*., 2004; Cheng *et al*., 2013; He *et al*., 2016; Liu *et al*., 2016). Partial sequences of mtDNA genes, such as cytochrome oxidase I (COI), cytochrome oxidase III (COIII), and 16S rRNA, have proved to be important tools in intraspecific

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and interspecific phylogenetic studies of Cephalopoda and other mollusks (Akasaki *et al*., 2006; He *et al*., 2016; Mao *et al*., 2016; Wang *et al*., 2016). Compared to partial mt genes, complete mtDNA sequences can uncover gene rearrangements and other variation at the genome level for all phyla, and are especially useful because they have sufficient interspecies sequence variability for resolving species-level relationships (Cheng *et al*., 2013). The levels of variation of different genes vary significantly, making different genes useful for phylogenetic analysis at different taxonomic levels (Zheng *et al*., 2015).

The ability of different cephalopods to efficiently identify members of their own species is very important for breeding. Zheng (2001) showed that there were significant differences in radular structure among nine species of cuttlefish and suggested that radular morphology is informative for their taxonomy. This suggestion has been reinforced by other studies (Ma *et al*., 2016). Radular morphology could be used as a basis for classification of the cuttlefishes in the coastal areas of China. Morphological traits can be difficult to apply for classification of cephalopods due to phenotypic plasticity, overlapping geographical distributions and notable differences during growth and development. In addition, cephalopods are found throughout variable marine habitats and have diverged in response to a variety of ecological pressures (Boyle and Boletzky, 1996; Hanlon and Messenger, 1996; Allen *et al*., 2014). An understanding is needed of the phylogenetic relationships among genera of Sepiidae, especially when studying the differences among species within different groups.

Wakabayashi *et al*. (2012) studied phylogenetic relationships in Family Ommastrephidae, based on mtDNA 16S rRNA and COI genes. The relationships among subspecies in the resulting maximum parsimony trees were consistent with neighbor joining trees produced using allozyme data (Yokawa, 1994). However, these relationships differed from those inferred from analyses of morphological characters (Roeleveld, 1988). The first determination of the whole genome sequence of the human mt genome was made in 1981 (Anderson *et al*., 1981). Since then, genome-level sequencing has provided many new insights into species relationships. Both molecular and morphological analyses are needed to provide the most informative classification within cuttlefish and among higher taxonomic ranks.

**Table I.- Primers used for amplification of the *Sepia lycidas* mitochondrial genome.**

| Primer pair | Primer sequence (5′-3′) | Region | Size (bp) |
|---|---|---|---|
| 1 F | GTRGGWATAGAYGTWGATACACGAGCYTATT | 2934-8397 | 5463 |
| 1 R | TGTGCCAGCATYYGCGGTT | | |
| 2 F | TAAAGAATAATAGGGTATCTAATCCTAGT | 8187-9640 | 1454 |
| 2 R | TTTATAACATCAATATAAYCCGTTCA | | |
| 3 F | GAGGCMTTTAACTGTTAATTAAAT | 9548-11260 | 1713 |
| 3 R | AATTGCVGGDATAACTAAAAGAGC | | |
| 4 F | GATATTATTATTACWCCYAATTGACT | 10964-12598 | 1635 |
| 4 R | AATTGCAGGDTCWATRATTTTAGC | | |
| 5 F | AAWGAYTTAAYATCMCTTTGWCC | 12416-14063 | 1648 |
| 5 R | TGARAATTTTATTCCBGCTAAYCC | | |
| 6 F | GGAATWGAACGTAAAATWGCAT | 13968-15463 | 1496 |
| 6 R | GCTAARWHTTWAAGCTATTGGGTTC | | |
| 7 F | GGRATWGCYGAWACTAAATTAG | 14361-15772 | 1412 |
| 7 R | ATAWGCTMRAGGGATGTTTGAGAG | | |
| 8 F | GGGTATGAACCCAATAGCTT | 15433-16172, 1-808 | 1548 |
| 8 R | GCTTAAATTCGGCCACTTAAT | | |
| 9 F | TCHACYTTYTTTGTAGCTACAGG | 577-2087 | 1511 |
| 9 R | RTGRTTWGTKGAGAAWARTCATCG | | |
| 10 F | CCWAAYWTAACYAAACAAATAACTTG | 1400-3041 | 1642 |
| 10 R | WGAWCCATARATAGTRGCTAATCA | | |

## MATERIALS AND METHODS

*Sample and DNA extraction*

Specimens of *S. lycidas* were collected from the Zhanjiang fishing ground (GuangDong Province, 2011.8). The sample was preserved at -80°C until use. Total DNA was extracted from wrist muscle tissue of *S. lycidas* with the conventional phenol-extraction method (Sam, 1989).

*Polymerase chain reaction (PCR)*

We used 10 pairs of primers that amplify contiguous, overlapping segments of the complete mt genome of *S. lycidas* (Table I). The primers were designed from the complete mt genome sequence of *S. officinalis* (NC007895, Akasaki et al., 2006), *S. japonica* (NC028731, Zheng et al., 2015) and *S. pharaonis* (NC021146, Wang and Wu, 2014). The Long-PCR reaction volume was 50 µl, containing 31.5 µl sterile deionized water, 5.0 µl 10× LA PCR Buffer ($Mg^{2+}$ plus), 8.0 µl dNTPs (2.5 mM each), 1 µl of each primer (25 pmol/ml), 0.5 µl LA Taq DNA polymerase (Takara), and 3 µl DNA template, with the following reaction parameters: 2 min high-denaturation (93°C), followed by 20 cycles of 10 s denaturation (92°C), 30 s annealing (53°C), and a 10 min extension (68°C), with a 7 min final extension (68°C).

**Table II.- The complete mitochondrial genome sequence in this study.**

| Species | Length(bp) | Taxonomic status | | Accession No. |
|---|---|---|---|---|
| *Bathyteuthis abyssicola* | 20,075 | Bathyteuthidae | Oegopsida | NC_016423 |
| *Doryteuthis opalescens* | 17,370 | Loliginidae | Myopsida | KP336703 |
| *Loligo bleekeri* | 17,211 | Loliginidae | Myopsida | NC_002507 |
| *Loliolus beka* | 17,483 | Loliginidae | Myopsida | NC_028034 |
| *Loliolus uyii* | 17,134 | Loliginidae | Myopsida | NC_026724 |
| *Sepioteuthis lessoniana* | 16,694 | Loliginidae | Myopsida | KM878671 |
| *Uroteuthis chinensis* | 17,353 | Loliginidae | Myopsida | NC_028189 |
| *Uroteuthis duvauceli* | 17,413 | Loliginidae | Myopsida | NC_027729 |
| *Uroteuthis edulis* | 17,360 | Loliginidae | Myopsida | NC_017746 |
| *Allonautilus scrobiculatus* | 16,132 | Nautilidae | Nautiloidea | NC_026997 |
| *Nautilus macromphalus* | 16,258 | Nautilidae | Nautiloidea | NC_007980 |
| *Amphioctopus aegina* | 15,545 | Octopodidae | Octopoda | NC_029702 |
| *Cistopus chinensis* | 15,706 | Octopodidae | Octopoda | KF017606 |
| *Cistopus taiwanicus* | 15,793 | Octopodidae | Octopoda | NC_023257 |
| *Octopus bimaculatus* | 16,084 | Octopodidae | Octopoda | KT581981 |
| *Octopus bimaculoides* | 15,733 | Octopodidae | Octopoda | NC_029723 |
| *Octopus conispadiceus* | 16,027 | Octopodidae | Octopoda | NC_029747 |
| *Octopus minor* | 15,974 | Octopodidae | Octopoda | NC_015896 |
| *Octopus ocellatus* | 15,979 | Octopodidae | Octopoda | NC_007896 |
| *Octopus vulgaris* | 15,744 | Octopodidae | Octopoda | NC_006353 |
| *Architeuthis dux* | 20,333 | Architeuthidae | Oegopsida | KC701764 |
| *Watasenia scintillans* | 20,089 | Enoploteuthidae | Oegopsida | KJ845633 |
| *Dosidicus gigas* | 20,324 | Ommastrephidae | Oegopsida | NC_009734 |
| *Illex argentinus* | 20,278 | Ommastrephidae | Oegopsida | NC_026908 |
| *Ommastrephes bartramii* | 20,308 | Ommastrephidae | Oegopsida | NC_020348 |
| *Sthenoteuthis oualaniensis* | 20,306 | Ommastrephidae | Oegopsida | NC_010636 |
| *Todarodes pacificus* | 20,254 | Ommastrephidae | Oegopsida | NC_006354 |
| *Sepia aculeata* | 16,219 | Sepiidae | Sepiida | KF690633 |
| *Sepia apama* | 16,184 | Sepiidae | Sepiida | NC_022466 |
| *Sepia esculenta* | 16,199 | Sepiidae | Sepiida | NC_009690 |
| *Sepia latimanus* | 16,225 | Sepiidae | Sepiida | NC_022467 |
| *Sepia lycidas* | 16,228 | Sepiidae | Sepiida | KJ162574 |
| *Sepia officinalis* | 16,163 | Sepiidae | Sepiida | NC_007895 |
| *Sepia pharaonis* | 16,208 | Sepiidae | Sepiida | NC_021146 |
| *Sepiella inermis* | 16,191 | Sepiidae | Sepiida | KF040369 |
| *Sepiella japonica* | 16,170 | Sepiidae | Sepiida | NC_028731 |
| *Vampyroteuthis infernalis* | 15,617 | Vampyroteuthida | Vampyromorphida | NC_009689 |

**Table III.- Mitochondrial genome characteristics of *S. lycidas.***

| Gene | Position | | Size (bp) | | Codon | | Intergenic nucleotidesa | Strand |
|---|---|---|---|---|---|---|---|---|
| | From | To | Nucleotide | Amino acid | Initiation | Stop | | |
| CO3 | 1 | 780 | 780 | 259 | ATG | TAA | 0 | L |
| tRNALys | 791 | 858 | 68 | | | | 10 | L |
| tRNAAla | 857 | 923 | 67 | | | | -2 | L |
| tRNAArg | 923 | 989 | 67 | | | | -1 | L |
| tRNASer | 993 | 1058 | 66 | | | | 3 | L |
| ND2 | 1059 | 2090 | 1032 | 343 | ATG | TAA | 0 | L |
| CO1 | 2068 | 3600 | 1533 | 510 | ATG | TAA | -23 | L |
| CO2 | 3603 | 4289 | 687 | 228 | ATG | TAA | 2 | L |
| ATP8 | 4354 | 4548 | 195 | 64 | ATG | TAA | 64 | L |
| ATP6 | 4511 | 5203 | 693 | 230 | ATG | TAG | -38 | L |
| tRNAPhe | 5230 | 5293 | 64 | | | | 26 | L |
| ND1 | 5295 | 6233 | 939 | 312 | ATG | TAA | 1 | H |
| tRNALeu | 6234 | 6306 | 73 | | | | 0 | L |
| tRNALeu | 6307 | 6381 | 75 | | | | 0 | L |
| 16S rRNA | 6382 | 7655 | 1274 | | | | 0 | L |
| tRNAVal | 7656 | 7726 | 71 | | | | 0 | L |
| 12S rRNA | 7727 | 8707 | 981 | | | | 0 | L |
| tRNACys | 8708 | 8770 | 63 | | | | 0 | L |
| tRNATyr | 8769 | 8832 | 64 | | | | -2 | L |
| tRNAGln | 8834 | 8898 | 65 | | | | 1 | L |
| tRNAGly | 8905 | 8977 | 73 | | | | 6 | L |
| Control region | 8982 | 9558 | 577 | | | | 4 | L |
| tRNAAsn | 9561 | 9626 | 66 | | | | 2 | H |
| tRNAIle | 9628 | 9692 | 65 | | | | 1 | L |
| ND3 | 9693 | 10,046 | 354 | 117 | ATG | TAA | 0 | L |
| tRNAAsp | 10,052 | 10,117 | 66 | | | | 5 | H |
| ND5 | 10,129 | 11,850 | 1722 | 573 | ATG | TAA | 11 | H |
| tRNAHis | 11,851 | 11,915 | 65 | | | | 0 | L |
| ND4 | 11,914 | 13,275 | 1362 | 453 | ATA | TAA | -2 | H |
| ND4L | 13,272 | 13,568 | 297 | 98 | ATG | TAA | -4 | H |
| tRNAThr | 13,575 | 13,639 | 65 | | | | 6 | L |
| tRNASer | 13,640 | 13,705 | 66 | | | | 0 | H |
| Cyt b | 13,705 | 14,844 | 1140 | 379 | ATG | TAG | -1 | H |
| ND6 | 14,837 | 15,349 | 513 | 170 | ATG | TAG | -8 | H |
| tRNAPro | 15,351 | 15,418 | 68 | | | | 1 | L |
| tRNAMet | 15,419 | 15,486 | 68 | | | | 0 | L |
| tRNATrp | 15,489 | 15,555 | 67 | | | | 2 | L |
| tRNAGlu | 15,557 | 15,650 | 94 | | | | 1 | L |
| Control region | 15,652 | 16,228 | 577 | | | | 1 | L |

The PCR products were electrophoresed on a 1% agarose gel and stained with ethidium bromide for band characterization via ultraviolet transillumination. The products were recovered and concentrated using a Gel Extraction Kit (CW2302, CWBIO) according to the manufacturer's protocol.

*Cloning and sequencing of the PCR products*

The PCR products were ligated into pMD 18-T vectors and cloned using a TOPO TA Cloning Kit (Invitrogen, USA) according to the manufacturer's instructions. Each cloned DNA fragment was sequenced according to the manufacturer's protocol with TOPO vector inner primers T7 and M13R (Invitrogen, USA). Labeled fragments were analyzed on an ABI Prism 3730 DNA Sequencer (Applied Biosystems, USA).

*Sequence analysis*

The characterization of the mt genome of *S. lycidas* was performed in comparison with other cuttlefish mt genomes (Akasaki *et al.*, 2006; Yokobori *et al.*, 2007; Wang *et al.*, 2014). MEGA 6.06 software (Tamura *et al.*, 2007) was used for sequence splicing and analyses. Concatenated nucleotide sequences were loaded into DNAStar 7.1 statistical software packages to analyze the full mtDNA sequence, nucleotide content, codon usage, and amino acids. Tandem Repeats Finder (http://tandem.bu.edu/trf/trf.html; Benson, 1999; Huang *et al*., 2017) was used to analyze sequences that were rich with AT-repeat regions. Finally, we used the online mapping software OGDraw v1.2 (http://ogdraw.mpimp-golm.mpg.de/index.shtml) to diagram the *S. lycidas* mtDNA circular structure. Protein coding genes were analyzed by ORF Finder using the invertebrate mitochondrial code. Base composition and codon usage were calculated with DNAStar software.

*Phylogenetic analysis*

All 13 protein-coding gene sequences of mtDNA were used to estimate phylogenetic relationships among 37 species in Cephalopoda, using maximum likelihood (ML) and Bayesian inference (BI). The mtDNA protein-coding regions were concatenated and aligned using the ClustalW algorithm with default parameters implemented in MEGA 6.06 (Tamura *et al*., 2013). In order to discuss phylogenetic relationships within Sepiidae and with other families of Cephalopoda (Ommastrephidae, Loliginidae, Vampyroteuthidae, and Octopodidae) thirty-seven species in Genbank were included (Table II). *Allonautilus scrobiculatus* (NC. 026997.1) and *Nautilus macromphalus* (NC. 007980.0) were used as outgroup taxa. The ML tree was evaluated with 1000 bootstrap replicates in MEGA 6.06.

MrBayes ver 3.1.2 (Huelsenbeck *et al*., 2001) was used for Bayesian analyses under the Mtzoa-F + C4 model to partition different amino acids positions. Two runs with four chains of MCMC iterations were performed for 100,000 generations, sampling trees every 100 generations.

## RESULTS

*Genome organization and composition*

The complete mt genome of *S. lycidas* is 16,228 bp in length and consists of 13 protein-coding genes, 22 transfer RNA genes (tRNA), 2 ribosomal RNA genes (rRNA), and 2 long-noncoding regions (CR). The structural organization of the complete mt genome is given in Figure 1 and Table III. The base composition is 35.8% T, 14.8% C, 41.3% A, and 8.1% G. As seen in other invertebrate mt genomes, A+T content (77.1%; Table IV) is much higher than the G+C content.

*Protein-coding genes and codon usage*

The 13 protein-coding genes of *S. lycidas* can be classified into two categories: COIII, ND2, COI, COIII, ATP8, ATP6, and ND3 are encoded by the light strand, whereas ND1, ND5, ND4, ND4L, Cyt b, and ND6 are encoded by the heavy strand (Table III). The overall A+T content of the protein genes is 76.2%, with the highest in ATP8 (83.1%), and the lowest in COI (70.1%). AT and CG skew of the 13 protein-coding genes is 0.087 and -0.294, respectively (Table IV).

Most of the protein-coding genes start with an ATG initiation codon, except for ND4, which starts with ATA. Ten protein-coding genes (COI-III, ND1-5, ATP8, and ND4L) use TAA as the termination codon, while the other three (ATP6, Cyt b, and ND6) share the termination codon TAG. The pattern of codon usage of *S. lycidas* mtDNA is shown in Table V. The most frequently used amino acids are Leu (15.7%), followed by Ile (10.2%), Phe (8.5%), Met (7.9%), Ser (7.3%), and Gly (6.0%). The least frequently used amino acid is Gln (1.6%). The most frequently used codon in *S. lycidas* and other cuttlefish is UUA (appearing 456 times).

*Transfer RNA genes and RNA genes*

A total of 22 transfer RNA genes are interspersed in the *S. lycidas* mt genome and range from 63 bp to 94 bp, with a base composition of 39.2% T, 12.6% C, 39.2% A,

**Table IV.- Nucleotide composition in mitochondrial genome of *S. lycidas.***

| Species | | Size (bp) | A% | T% | C% | G% | A+T% | C+G% |
|---|---|---|---|---|---|---|---|---|
| *S. lycidas* | Whole genome | 16,228 | 41.3 | 35.8 | 14.8 | 8.1 | 77.1 | 22.9 |
| | 13 PCGs | 11,174 | 41.4 | 34.8 | 15.4 | 8.4 | 76.2 | 23.8 |
| | tRNA | 1,501 | 39.2 | 39.2 | 12.6 | 9.0 | 78.4 | 21.6 |
| | rRNA | 2,255 | 42.0 | 37.6 | 13.7 | 6.7 | 79.6 | 20.4 |
| | 2 LNCRs | 1,154 | 41.6 | 36.0 | 14.4 | 8.0 | 77.6 | 22.4 |

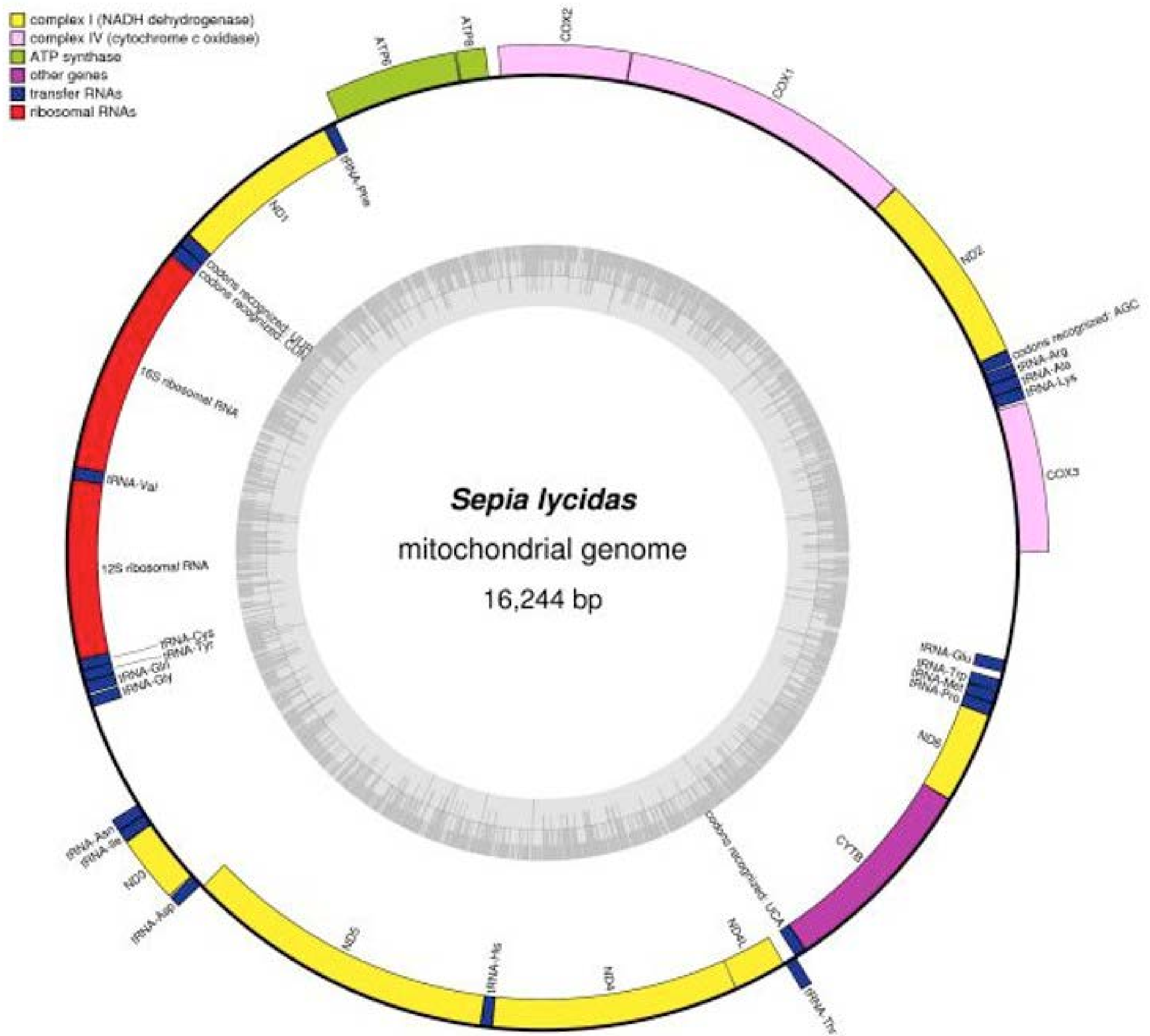


Fig. 1. Gene map of *S. lycidas* mitochondrial genome. Genes encoded on the heavy or light strands are shown outside or inside the circular gene map, respectively. The inner ring indicates the GC content. The figure was initially generated with Organellar Genome DRAW (OGDRAW) and modified manually.

and 9.0% G (Table IV). In addition, tRNA$^{Ser}$ and tRNA$^{Leu}$ both appear twice; the other 18 types of tRNA appear only once. Nineteen of the tRNA are encoded by the light strand and three are encoded by the heavy strand. The overall AT and CG skew of the 22 tRNA genes is 0 and -0.167, respectively. The CG skew of tRNA$^{Arg}$ (CGR), tRNA$^{Asp}$ (GAD), and tRNA$^{Ser}$ (AGS) is zero.

*S. lycidas* has two ribosomal RNA genes, 12S rRNA (981 bp) and 16S rRNA (1274 bp). They are located between tRNA$^{Leu}$ and tRNA$^{Cys}$ genes, and separated by the tRNA$^{Val}$ gene. The base composition of the two rRNAs gene sequences is 42.0% A, 37.6% T, 13.7% C, and 6.7% G. The overall AT and CG skew of the rRNA genes is 0.055 and -0.343. The A+T content of 12S rRNA and 16S rRNA is 80.0% and 79.3%, respectively (Table IV).

*Non-coding regions*

The complete mt genome of *S. lycidas* has two long, noncoding regions that are both control regions, regulating replication and transcription. Their overall base composition is rich in A and T (Jing et al., 2016). Nucleotide composition across the non-coding regions is 42.0% A, 37.6% T, 13.7% C, and 6.7% G. The A+T content is 79.6%. AT and CG skew is 0.055 and -0.343, respectively (Table IV).

Two non-coding regions are both 577 bp in length and contain termination-associated sequences and conserved sequence blocks: one is located between $tRNA^{Gly}$ and $tRNA^{Asn}$, another is located between $tRNA^{Glu}$ and the protein-coding gene COIII. The control region in *S. lycidas* exhibits the typical tripartite structure with an extended termination-associated sequence domain (ETAS), central conserved sequence block domain (CD), and conserved sequence block domain (CSB). The possible function of the LNCR as the control region is deduced from the potential stem and loop structures (Tomita et al., 2002). The termination-associated sequence (TAS) domains, which are thought to act as a signal for the termination of heavy strand elongation, were identified in ETAS. This is a hypervariable domain that may be useful for analyzing interspecies variation within *S. lycidas* (Southern et al., 1988).

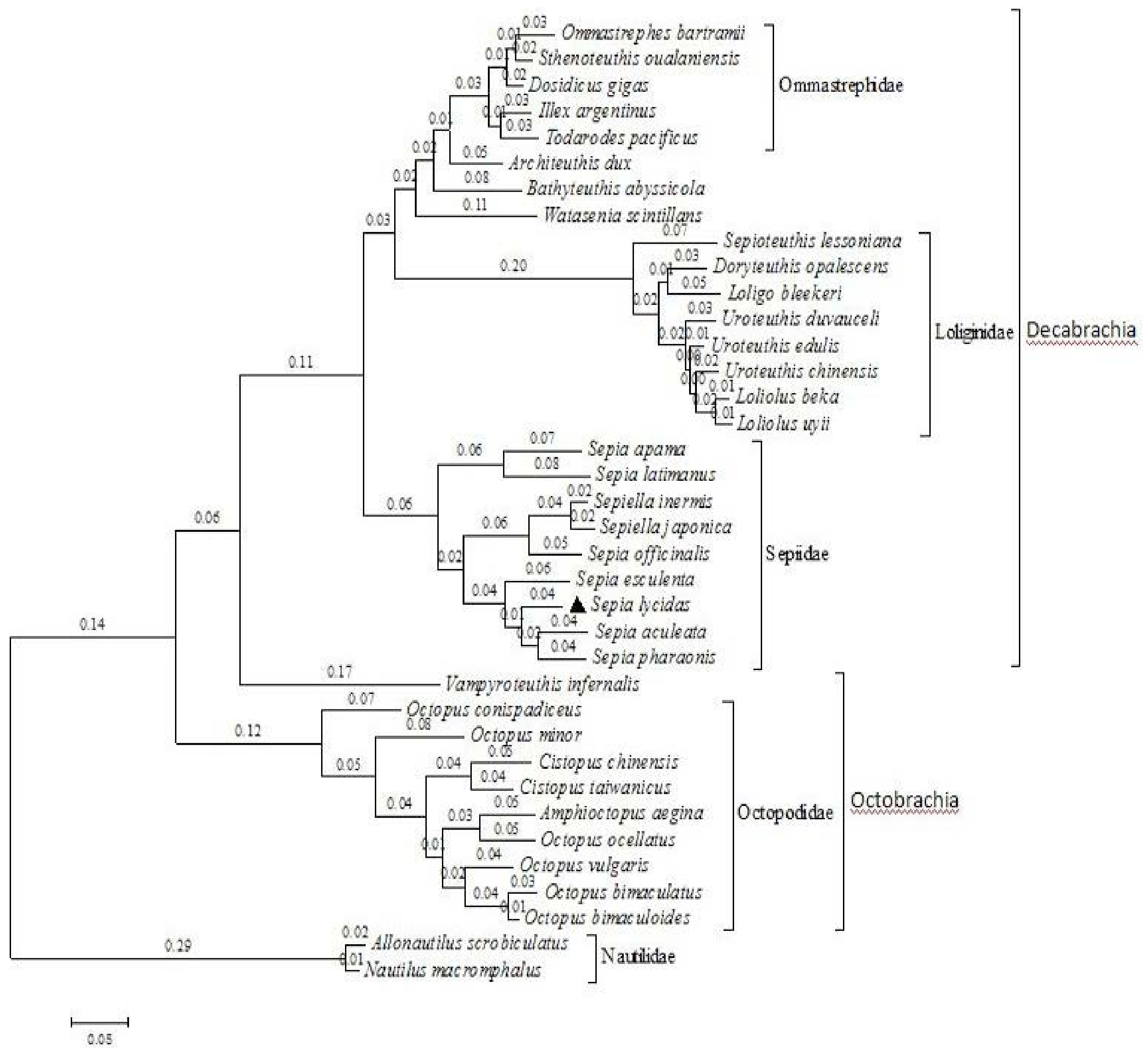


Fig. 2. The maximum-likehood phylogenetic tree reconstructed by the amino acid analysis under the ML method with JTT model.

**Table V.- Frequency and count for genetic codons and codon usage in *Sepia lycidas* mitochondrial genome.**

| Codon /1000(C) | Codon /1000(C) | Codon /1000(C) | Codon /1000(C) |
|---|---|---|---|
| UUU 280(74.9) | UCU 84(22.5) | UAU 153(41.0) | UGU 61(16.3) |
| Phe | Ser | Tyr | Cys |
| UUC 38(10.2) | UCC 15(4.0) | UAC 24(6.4) | UGC 7(1.9) |
| Phe | Ser | Tyr | Cys |
| UUA 456(122.1) | UCA 94(25.2) | UAA 10(2.7) | UGA 81(21.7) |
| Leu | Ser | Stop | Trp |
| UUG 47(12.6) | UCG 4(1.1) | UAG 3(0.8) | UGG 17(4.6) |
| Leu | Ser | Stop | Trp |
| CUU 39(10.4) | CCU 64(1.7) | CAU 70(18.7) | CGU 23(6.1) |
| Leu | Pro | His | Arg |
| CUC 4(1.1) | CCC 4(1.1) | CAC 18(4.8) | CGC 5(1.3) |
| Leu | Pro | His | Arg |
| CUA 39(10.4) | CCA 49(13.1) | CAA 55(14.7) | CGA 19(5.1) |
| Leu | Pro | Gln | Arg |
| CUG 0 | CCG 1(0.3) | CAG 6(1.6) | CGG 6(1.6) |
| Leu | Pro | Gln | Arg |
| AUU 355(95.0) | ACU 75(20.1) | AAU 162(43.4) | AGU 64(17.1) |
| Ile | Thr | Asn | Ser |
| AUC 27(7.2) | ACC 12(3.2) | AAC 21(5.6) | AGC 18(4.8) |
| Ile | Thr | Asn | Ser |
| AUA 260(69.6) | ACA 64(17.1) | AAA 91(24.4) | AGA 47(12.6) |
| Met | Thr | Lys | Ser |
| AUG 35(.4) | ACG 0 | AAG 9(2.4) | AGG 16(4.3) |
| Met | Thr | Lys | Ser |
| GUU 90(24.1) | GCU 75(20.1) | GAU 60(16.0) | GGU 128(34.3) |
| Val | Ala | Asp | Gly |
| GUC 2(0.5) | GCC 15(4.0) | GAC 10(2.7) | GGC 5(1.3) |
| Val | Ala | Asp | Gly |
| GUA 97(26.0) | GCA 40(10.7) | GAA 73(19.5) | GGA 60(16.1) |
| Val | Ala | Glu | Gly |
| GUG 11(2.9) | GCG 3(0.8) | GAG 15(4.0) | GGG 31(8.3) |
| Val | Ala | Glu | Gly |

Total codon number: 3736.

*Phylogenetic status of* S. lycidas

Many systematic and population genetic studies have been based on genetic markers in the mt genomes at either the nucleotide or amino acid level (Zou *et al*., 2011). Phylogenetic relationships inferred from ML and Bayesian analyses were basically consistent with each other (Figs. 2 and 3) and with the existing morphological classification.

The use of long DNA sequences can help to resolve major phylogenetic relationships and provide resolution of closely related species. In our study, the phylogenetic trees were inferred using protein sequences. In both phylogenetic trees, the ten sampled species of Sepiidae were divided into three clades. *S. lycidas* forms a clade with *S. pharaonis*, *S. aculeata* and *S. esculenta*.

Both the ML and BI analyses divided Decapodiformes into three parts: Oegopsida, Myopsida, and Sepiida; the Oegopsida consisted of Ommastrephidae, Architeuthidae, Bathyteuthidae, and Enoploteuthidae; the Myopsida only had one family and the Loliginidae contained eight species. Octobrachia can be divided into two suborders: Cirrina and Incirrina. However, there are no complete mt genomes available for Cirrina, and so our analyses only include Incirrina. *Octopus conispadiceus* is separated from other Octopodidae in both trees (Tanner et al., 2017).

The position of *Ommastrephes bartramii* differed between the ML and BI trees. In the ML tree, *O. bartramii* was sister to *Sthenoteuthis oualaniensis*, whereas in the BI tree *O. bartramii* was sister to the remainder of Ommastrephidae (Figs. 2 and 3).

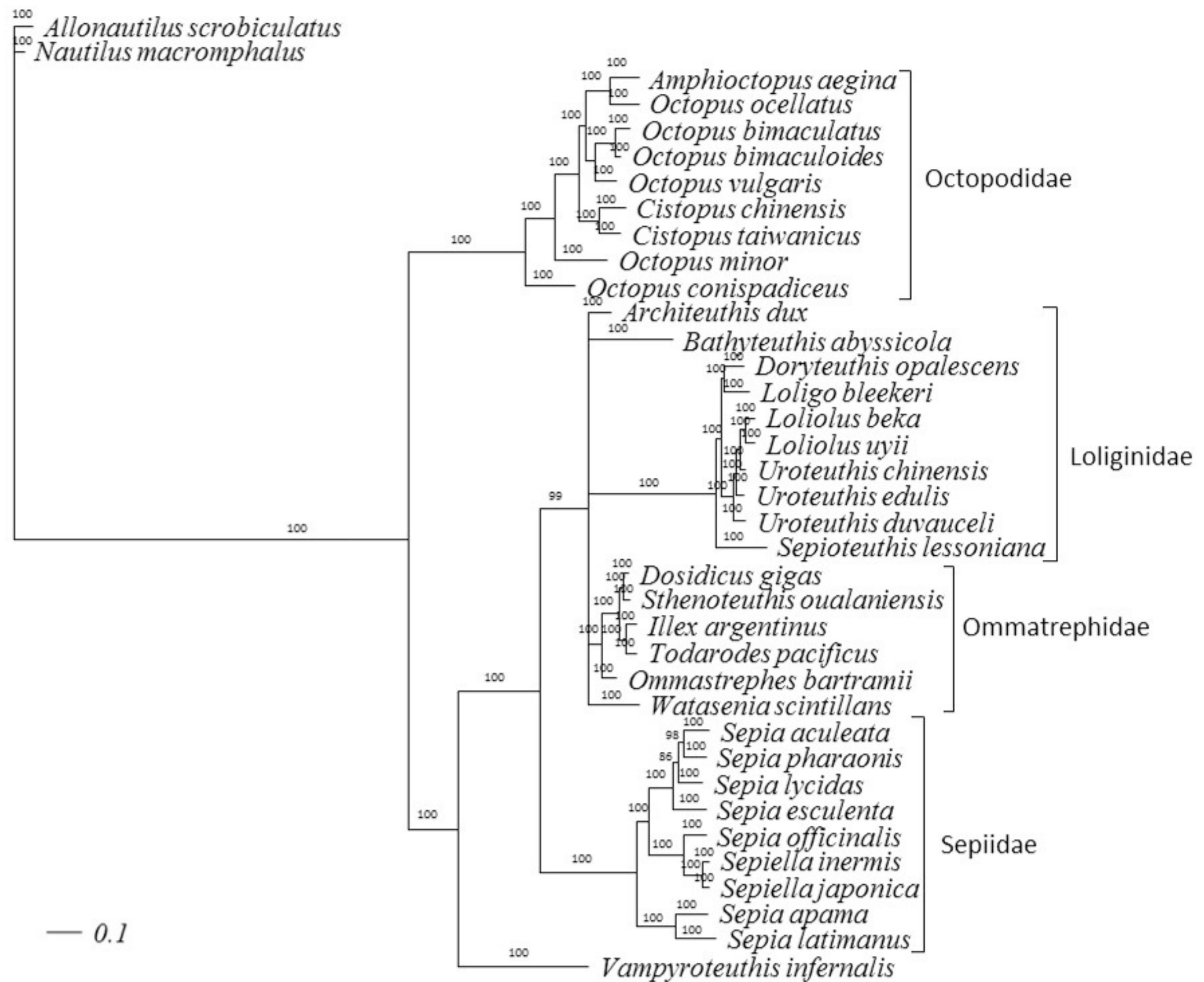


Fig. 3. Phylogenetic tree reconstructed by amino acid analysis under the Bayesian method with MtZoa-F + C4 model.

## DISCUSSION

*Gene arrangement*

The mt genomes of Ommastrephidae and Enoploteuthidae contain six duplicated genes, including COI, COII, COIII, ATP 6 and 8, and $tRNA^{Asp}$ (Fig. 3). These genes occur between 12S RNA and 16S RNA (Yokobori *et al*., 2007). Each gene in the mt genomes for these taxa possesses specific functions. The level of variation between duplicate genes is low, and every gene copy contains several insertions and deletions (Kawashima *et al*., 2013). These duplicated genes likely have lost their function, but the secondary structures and anticodon positions of functional genes would be disrupted without them (Eda *et al*., 2010). These duplications are characteristic of all members of Ommatrephida. It is likely that the octopus-type mt genome is most similar to the ancestral state, this state being maintained from at least the Cephalopoda ancestor to the common ancestor of Oegopsida, Myopsida and Sepiolida (Kawashima *et al*., 2013).

*Phylogenetic analysis*

The previous classification for cephalopoda was based solely on morphological traits (Chen *et al*., 2009). The three sampled members of the family Ommastrephidae (*Dosidicus gigas*, *O. bartramii*, and *S. oualaniensis*) belong to the subfamily Ommastrephinae and were monophyletic in the ML tree. *O. bartramii* is sister to *S. oualaniensis* in our ML tree, but is placed more distantly in our BI tree and in an ML tree base on COI (Wakabayashi *et al*., 2012; Tanner *et al*., 2017).

The largest difference between our results and some of the previous studies is in the Octopodidae. *Vampyroteuthis infernalis* was separated from the remainder of Octopodidae

in our phylogenies, as was also found by Allcock *et al*. (2011). However, other phylogenetic analyses of the mt genomes of *V. infernalis* and other Octopodidae suggested a very close relationship (Kawashima *et al*., 2013). In both the ML and BI trees, *Amphioctopus aegina* and *O. ocellatus* are sister species despite belonging to different genera in the traditional taxonomy (Chen *et al*., 2009). Additional data from the nuclear genome are needed to further test the placement of these species.

The relationships that we inferred within family Sepiidae (Figs. 2 and 3) are consistent with the traditional morphological classification (Chen *et al*., 2009). We found a close relationship between *S. apama* and *S. latimanus*, as was also seen by Akasaki *et al*. (2006). However, our results differ from some previous studies in the position of *S. officinalis* and the relationships among *S. esculenta*, *S. aculeata*, *S. lycidas*, and *S. pharaonis*. Yokobori *et al*. (2007) produced phylogenies based on the DNA sequences of 16S, 12S, and COI, and showed a further relationship between *S. lycidas* and *S. esculenta*. In comparison, our trees place *S. lycidas* sister to an *S. aculeata* + *S. pharaonis* clade, which was same as the ML phylogenetic relationships based on nucleotide and amino acid sequence data (Zhang *et al*., 2015; Groth *et al*., 2015; Strugnell *et al*., 2017). Our MP and BI analyses of amino acid sequences allowed inferences of phylogenetic relationships of different families with results that were consistent with Kawashima *et al*. (2013).

To fully resolve relationships and provide a robust classification, the valuable and extensive information available in mt genomes should be combined with other sources of data, such as nuclear genes and morphological and ecological characteristics.

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*Statement of conflict of interest*

Authors have declared no conflict of interest.

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