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Phylogenetic and morphological characteristics of *Sepioteuthis lessoniana* (Cephalopoda: Loliginidae) in the South China Sea and Andaman Sea

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ABSTRACT: A total of 142 samples of bigfin reef squid (*Sepioteuthis lessoniana* FÉRUSSAC IN LESSON, 1831) were collected from the Andaman Sea, Gulf of Thailand, and South China Sea to study their morphological characteristics and assess their genetic divergence. Morphological variations were observed in their body size, number of sucker ring teeth, and the radula. Genetic sequences were obtained using cytochrome c oxidase subunit I (COI) gene and 16S ribosomal RNA (16S rRNA) gene markers. Two size variants (small and medium) were identified, which could be distinguished based on body size, number of sucker ring teeth, and characteristics of the radula. The medium-size form comprised 101 individuals, the small-size form comprised 39 individuals, and there were 2 intermediate individuals. In the genetic analysis, there were 2 distinct genetic lineages based on the 40 haplotypes discovered across the study areas. The small-size form group was dominant in Taiwan Strait, and the medium-size form group was widely dispersed and dominated elsewhere in the South China Sea and Andaman Sea. Results of the morphological analysis indicated a correlation with high level of genetic differentiation clearly separating the 2 size groups and revealing the existence of 2 sub-species of *S. lessoniana* in the study areas that could be recognized by the differences in their DNA sequences and morphometrics.

KEYWORDS: cytochrome c oxidase subunit I gene, 16S rRNA gene, *Sepioteuthis lessoniana*, radula

INTRODUCTION

A cephalopod is one of the marine invertebrates classified in phylum Mollusca because its body is made up of a mantle, gills, and radula, and it also belongs to the Cephalopoda class characterized by its bilateral body symmetry, a prominent head, and a set of appendages (arms and tentacles). In general, the number of appendages is used to classify the cephalopod, for instance, Superorder Octopodiformes, e.g., an octopus, has 8 appendages (arms) whereas Superorder Decapodiformes, e.g., a squid and a cuttlefish, has 10 appendages (8 arms and 2 tentacles). For morphological characteristic, a fin, large eyes, and an elongated soft body are considered the physical characteristics of the squid. Another characteristic is that its body is supported by a rod-like gladius or pen made of chitin along its upper part of the mantle as the internal skeleton construction [1].

Squids of the genus *Sepioteuthis* (family Loliginidae) comprise 3 species that occur widely in coastal waters. *S. lessoniana* (bigfin reef squid) is distributed from East Asia to Australia and New Zealand and from Hawaii to east Africa, Mozambique, and Madagascar [2].

Like other loliginids, *S. lessoniana* comprises different forms that vary in size and shape. It can be assumed that this squid can be divided into 3 forms according to previous studies, namely small, medium, and large; sizes are 104 mm in males and 108 mm in females for the small-size form [3], 146 mm in males and 150 mm in females for the medium-size form [4], and 170 mm in males and 193 mm in females for the large-size form [5]. In Japan, 3 forms differ in appearance [6], egg capsules, and spawning behavior [7], while in Thailand, notable variations have been reported in the number, size, and arrangement of chromatophores on paralarvae [8]. Moreover, the results of a molecular study revealed that *S. lessoniana* comprises multiple genetically distinct lineages that could represent cryptic species [9].

Our group has previously reported on the embryonic development and early morphological characteristics of the sandbird octopus (*Amphioctopus aegina*) which is the moderate-size species of octopuses in the Cephalopoda class [10]. In the present study, morphological characterization and genetic differentiation of the squid *S. lessoniana* from the South China Sea and the Andaman Sea were investigated. This work could be used to distinguish the squid variations in the sampling sites complying with their geographical distances and to examine its distribution, phylogeny, and morphology.

MATERIALS AND METHODS

Specimen collection

Whole specimens and tissue samples of 142 individuals of *S. lessoniana* were randomly collected from 7 coastal locations in the Andaman Sea, Gulf of Thailand, South

Table 1 Seven sampling sites in the Andaman Sea, Gulf of Thailand, South China Sea, and near Taiwan Strait.

Site*	Location	Latitude	Longitude	Sampling month (2019)	N	nH	COI Haplotype
AN	Ranong fish market, Ranong, Thailand	9° 56′ 52″	98° 35′ 41″	Feb	20	14	H1–14
GT1	Ao Yai Ai Bay, Chumphon, Thailand	10° 44′ 55″	99° 24′ 0″	Jan	20	7	H4, H15–20
GT2	Fishing village, Rayong, Thailand	12° 38′ 1″	101° 28′ 40″	Jan	20	8	H4, H12, H15–17, H20–23
GT3	Songkhla Municipality Pier, Songkhla, Thailand	7° 11′ 12″	100° 35′ 31″	Feb	24	14	H4, H6, H11, H14–15, H17, H19–20, H24–28
SC1	Hongfa market, Guangxi, China	21° 58′ 56″	108° 37′ 53″	Jul	20	8	H4–5, H14, H37–40
SC2	Yangjiang market, Guangdong, China	22° 5′ 53″	111° 55′ 7″	Dec	15	2	H11, H20
TS	Xipu market, Fujian, China	23° 53′ 31″	117° 24′ 35″	Aug	20	12	H5, H14, H20–21, H29–36

* AN, Andaman Sea; GT, Gulf of Thailand; nH, number of haplotypes; SC, South China Sea; TS, Taiwan Strait.

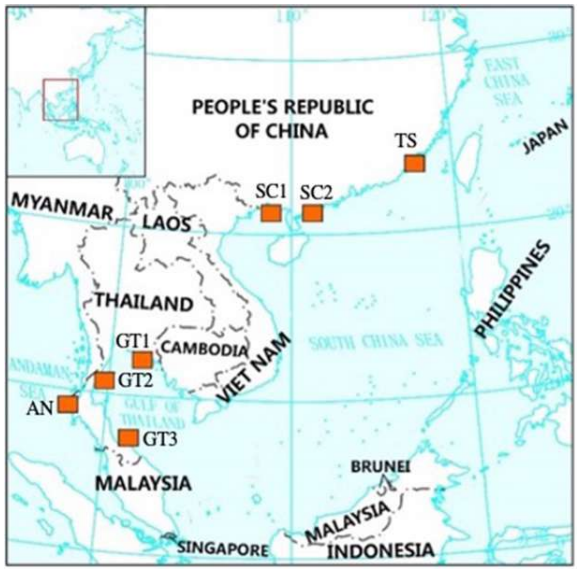

Fig. 1 Map of the 7 sampling sites in the Andaman Sea, Gulf of Thailand, South China Sea, and near Taiwan Strait.

China Sea, and near Taiwan Strait during January to December in 2019 (Fig. 1 and Table 1). Approximately 1–3 cm of tentacle and mantle tissue was collected from fresh specimens and stored in 90% ethanol for DNA analysis [2].

Morphological analyses

Morphological variation was investigated using 5 body measurements: dorsal mantle length (ML), fin width (FW), head length (HL), length of arms I (AI), and length of arms IV (AIV) following the measurement reference of Roper and Voss [11]. The number of arm III sucker ring teeth (ASRT), the number of tentacle sucker ring teeth (TSRT), and radulae were investigated under a stereomicroscope. Three radulae collected randomly from each sampling site were observed and measured using a Hitachi SU8020 scanning electron microscope (SEM, Hitachi High-Technologies Corporation, Japan). The morphometrics of all specimens were adjusted to eliminate size-dependent variations prior to the statistical analysis using the formula from Elliott et al [12] and modified by Xu et al [13]:

$$M_{\text{adj}} = M_0 \left(\frac{L_s}{L_0} \right)^b$$

where M_{adj} denotes the adjusted value of the morphometrics, M_0 is the initial value of the target morphometrics, L_0 represents the ML of individual squid (standard length), and L_S denotes the average standard length of all individuals. The parameter b was estimated as the slope of the regression of $\log M_0$ on $\log L_0$.

The adjusted value datasets for the 5 morphometric variables were used in discriminant function analysis (DFA), which was performed using the Linear Discriminant Analysis (LDA) function via the MASS package in R version 4.0.3 [14], and significant differences of the measurements were determined using ANOVA. The authors confirm that the squid specimens used in this study respect the animal rights and comply with the Institutional Animal Care and Use Committee (IACUC) protocols.

DNA extraction and amplification

Mantle or tentacle muscle was extracted for the genomic DNA using the applied CTAB method [15]. DNA was estimated by NanoDrop™ Spectrophotometers (Thermo Fisher, USA) to determine the DNA concentration and DNA quality. Then, cytochrome c oxidase subunit I (COI) gene primer [16] and 16S ribosomal RNA (16 rRNA) gene primer [17] were amplified from total genomic DNA using the polymerase chain reaction.

Polymerase chain reactions (PCR) were performed in 10 µl volumes containing 0.05 µl r*Taq* DNA polymerase (Takara, Japan), 1 µl PCR buffer, 1 µl 0.2 mmol/l dNTP mix, 0.25 µl of each primer set, 6.45 µl of sterilized water, and 1 µl of DNA sample. PCRs were performed by thermal cycler (GeneAmp System 9700, Thermo Fisher) with 3 min at 94 °C,

35 cycles of 1 min at 94 °C, 1 min at the annealing temperature (55 °C for COI primer and 50 °C for 16S rRNA primer) and 1 min at 72 °C, then followed by a 10 min for final extension at 72 °C. The PCR products were resolved through 1% agarose gel with TBE buffer, and ethidium bromide staining was used to visualize the bands. A 100-bp DNA ladder (Invitrogen, USA) was used as reference to determine the allele size. An EZ-10 spin column DNA gel extraction kit (Sangon Biotech, China) was used to purify the PCR products. The purified PCR products were sent to the Sangon Company (Shanghai, China) and sequenced by the walking method using an ABI 3730 XL automatic sequencer (Applied Biosystems, USA). The data supporting the findings of this study are available within this article. The novel data of this study are available in GenBank at https://www.ncbi.nlm.nih.gov, reference numbers of 16S rRNA sequences are MZ007860–MZ008001 and COI sequences are MZ008069–MZ008210.

Genetic divergence and haplotypes

To evaluate the genetic diversity of the samples, multiple sequence alignments, phylogenetic analysis, transitions, transversions, and genetic distances were performed using MEGA 7 version 7.0.26 [18]. The Kimura two-parameter (K2P) distances of the COI and 16S rRNA sequences were determined using the K2P substitution model in MEGA 7 with 10,000 bootstrap replicates of the uniform rates [19].

Mitochondrial COI and 16S rRNA variance parameters including G/C contents, haplotype diversity, the average number of nucleotide differences, and nucleotide diversity were computed using the DNASP version 5.10.01 [20]. The final alignments of COI and 16S rRNA were analyzed using Network software to come up with the median-joining haplotype networks [21].

Phylogenetic relationships

All sequences were compared with the COI and 16S rRNA sequences. Bayesian inference (BI) and maximum likelihood (ML) were used for the phylogenetic analysis. MrBayes version 3.2.0 was used to perform the Bayesian analysis, and the results were displayed with FigTree version 1.4.3. For the maximum likelihood analysis, the 4 gamma-distributed rate categories by the IQTREE Web Server were used, and tree topology searching was applied using NNI moves.

RESULTS

Morphological characteristics

All *S. lessoniana* samples were larger than the size at first maturity (ML 80 mm in females and 100 mm in males), so all were assumed to be mature. The samples could be divided into 2 size groups: a medium-size form comprising 101 individuals and a small-size form comprising 39 individuals; 2 individuals were considered intermediate individuals based on the results of the genetic analysis. In the small-size form, the average ML was 120.9 mm (range: 119.0–130.4 mm), and the average FW was 76.2 mm (range: 62.7–93.0 mm). In the medium-size form, the average ML was 157.1 mm (range: 153.9–167.2 mm), and the average FW was 113.3 mm (range: 94.7–126.4 mm) (Table 2). Based on morphometric characteristics, the small- and medium-size forms could clearly be separated by their ML and FW with a correlation coefficient = 0.638 (Fig. 2A) and from the analysis of the association between genetic species delimitation and morphometrics. The results showed that the discriminant function of the total variance accounted for 99.87% as evaluated for samples used as an aid in classifying these 3 groups, including small-size form, medium-size form, and intermediate form (Fig. 2B).

The TSRT and ASRT of both forms had triangular teeth, whereas the average number of TSRT was 20.7 (range: 16–25) in the small-size form and 16.7 (range: 14–30) in the medium-size form. For the number of ASRT, the average number was 25.9 (range: 19–34) in the small-size form and 21.3 (range: 18–28) in the medium-size form. The result of the pairwise *t*-test showed that the numbers of sucker ring teeth on both TSRT and ASRT were significantly higher in the small-size form than in the medium-size form ($p < 0.05$) (Table 2).

The radulae had 7 transverse rows of teeth comprising one tricuspid rachidian tooth, 2 lateral teeth (the first lateral tooth was tricuspid and the second lateral tooth with unicuspid), and one unicuspid marginal tooth (radula formula: 1+2+R+2+1), and the marginal plate was oval. However, the tooth shape, especially for the rachidian teeth, differed in the radulae of the small-size form and medium-size form; in the small-size form, the rachidian teeth were slender, and both sides of the lateral cusp were less than one-half the size of the central cusp, but in the medium-size form, the rachidian teeth seemed plump with both sides of the lateral cusp more than one-half the size of the central cusp (Fig. 3).

Sequence variation and genetic structure

The mitochondrial COI gene (634 bp) and 16S rRNA gene (477 bp) of all 142 samples were used for molecular analysis. The transition/transversion (Ts/Tv) ratio was 5.2 for the COI gene and 2.7 for the 16S rRNA gene.

The pairwise ΦST based on COI had relatively high values of 0.8665 between the small-size form and medium-size form groups and 0.9890 between the small-size form and intermediate form group (Table 3). When the medium-size form population was compared with the intermediate individuals, a negative value (−0.0073) was obtained.

The pairwise ΦST based on 16S rRNA was rela-

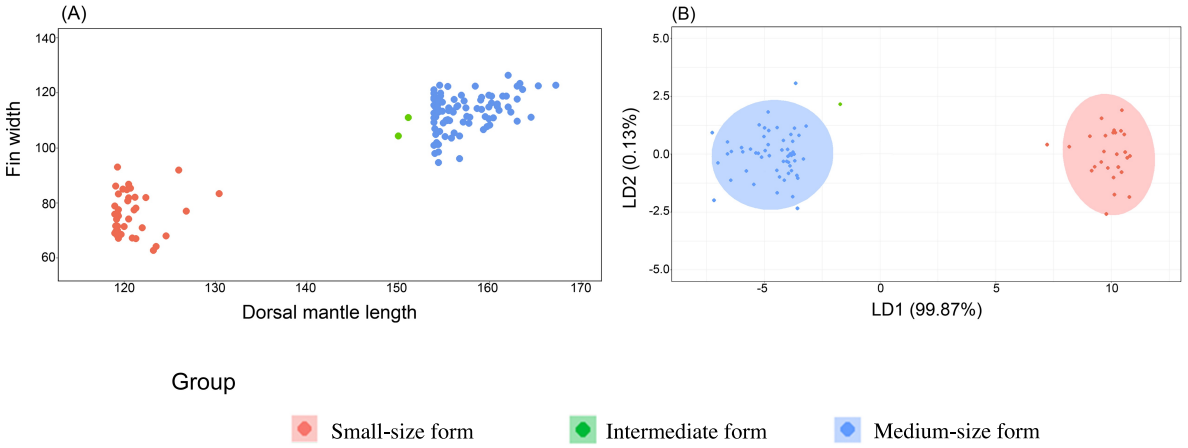

Fig. 2 Scatterplot indicating the discrimination of 3 groups using discriminant function analysis performed by adjusted measurements of small-size form (red), medium-size form (blue), and intermediate form (green). (A) Relationships between ML and FW of the *S. lessoniana*. (B) Analysis of the association between genetic species delimitation and morphometrics of *S. lessoniana*, LD is the abbreviation of Linkage Disequilibrium.

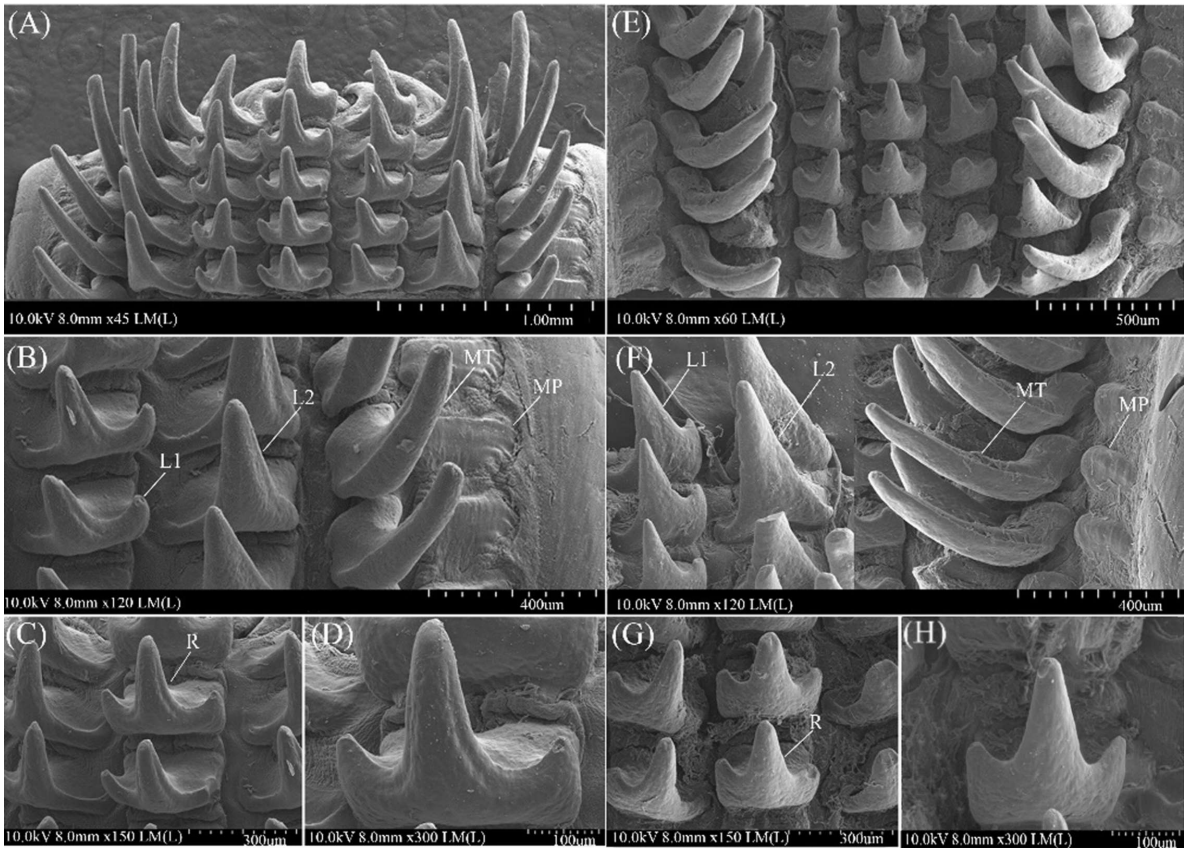

Fig. 3 Radulae of *S. lessoniana* under an SEM: (A)–(D) Radulae of small-size form. (A) Each transverse row comprising 7 teeth. (B) Lateral teeth, marginal teeth, and marginal plate under × 120LM. (C) Rachidian teeth under × 150LM. (D) Rachidian tooth under × 300LM. (E)–(H) Radulae of medium-size form. (E) Each transverse row comprising 7 teeth. (F) Lateral teeth, marginal teeth, and marginal plate under × 120LM. (G) Rachidian teeth under x150LM. (H) Rachidian tooth under × 300LM. L, lateral teeth; MP, marginal plate; MT, marginal teeth.

Table 2 Morphological measurements of *S. lessoniana*. * $p < 0.05$.

Index†	Small-size form					Medium-size form					Intermediate form				
	N	Mean	SD	Range		N	Mean	SD	Range		N	Mean	SD	Range	
				Min	Max				Min	Max				Min	Max
ML	39	120.9*	2.5	119.0	130.4	101	157.1	16.0	153.9	167.2	2	150.5	0.8	150.0	151.1
FW	39	76.2*	7.6	62.7	93.0	101	113.3	12.9	94.7	126.4	2	107.6	4.8	104.3	111.0
HL	39	36.9	9.6	17.9	50.8	101	41.6*	12.1	26.9	84.9	2	37.7	3.2	35.4	39.9
AI	39	36.4*	7.1	20.7	52.8	101	52.8	11.0	36.9	87.7	2	52.0	10.9	44.3	59.7
AIV	39	70.0*	13.4	49.5	101.1	101	86.1	15.0	55.5	123.4	2	80.1	2.7	78.2	82.1
ASRT	39	25.9*	5.5	19.0	34.0	101	21.3	2.1	18.0	28.0	2	22.0	0.0	22.0	22.0
TSRT	39	20.7*	2.6	16.0	25.0	101	16.7	2.8	14.0	30.0	2	16.5	3.5	14.0	19.0

† AI, length of arms I; AIV, length of arms IV; ASRT, number of arm sucker ring teeth; FW, fin width; HL, head length; ML, mantle length; TSRT, number of tentacle sucker ring teeth.

Table 3 The pairwise ΦST (below diagonal: COI; above diagonal: 16S rRNA) among groups. The label (*) indicates the ΦST value at $p < 0.05$.

	Small-size form	Medium-size form	Intermediate form
Small-size form		0.8919*	−0.0456
Medium-size form	0.8665*		0.8668*
Intermediate form	0.9890*	−0.0073	

tively high between the small-size form and medium-size form (0.8919) and between the medium-size form and intermediate individual group (0.8668) (Table 3). Moreover, a negative value was observed when the small-size form was compared with the intermediate form (−0.0456). Both COI and 16S rRNA analysis revealed 2 distinct forms with high significant levels of Fu's Fs and Tajima's D values. The values of the Fu's Fs and Tajima's D based on COI and 16S rRNA genes were negative, except for the Fu's Fs of the small-size form, which was positive (Table S2).

Genetic differences and haplotype patterns

The distance of the Kimura two-parameter (K2P) pairwise based on COI clades between the small-size form and medium-size form was 11.68; 1.04% for the small-size form clade and 1.24% for the medium-size form clade. The K2P pairwise distance based on 16S rRNA clades between the small-size form and the medium-size form was 2.86%; 0.05% for the small-size form clade and 0.19% for the medium-size form clade. The K2P pairwise distances are shown in Table S1.

The COI sequences from the 7 sample sites yielded 40 haplotypes, of which 12 were found in multiple sampling sites and 28 were found only at one sampling site. Conversely, the 16S rRNA sequences yielded only 11 haplotypes showing 2 clusters of abundant haplotypes with simple distribution (Fig. 4).

Phylogenetic analysis

Results of the similar topology in 2 phylogenetic trees (Fig. 5) showed that the phylogenetic tree of COI had 4 well-supported major clades: *S. australis*, *S. sepioidea*, *S. lessoniana* small-size form, and *S. lessoniana* medium-size form. The *S. lessoniana* medium- and small-size forms contained 2 well-supported sub-lineages inconsistent with the geographical distance. Also, the phylogenetic analyses based on 16S rRNA dataset revealed 4 major clades. Moreover, these phylogenetic trees revealed 2 individuals (GT2-20 and SC1-20), which were suspected to be intermediate individuals. In Fig. 5, the red star represents the potential intermediate individuals.

DISCUSSION

Phylogeny of *Sepioteuthis lessoniana*

In this study, *S. lessoniana* was divided into 2 lineages with clearly different genetic and morphological results. Compared with the results of previous studies of the *S. lessoniana* that used the mtDNA COI (COI) + 16S rRNA gene and was sampled in the Indian Ocean and Indo-West Pacific Ocean [19], they reported 3 lineages (A, B, and C) of *S. lessoniana* inconsistent with the geographical distance, but the results of our genetic analysis indicated that the small-size form corresponding to "Lineage B" and the medium-size form to "Lineage C". It is apparent that both sub-species are commonly sympatric throughout the Indian and Indo-Pacific region.

For species delimitation, the genetic distance of *S. lessoniana* in our study area was high compared with K2P distances in other regions [19], and the results of phylogenetic trees showed the relationships between 2 lineages were also high. In addition, the Bayesian inference and maximum likelihood values revealed that the China Sea and Andaman Sea clades of *S. lessoniana* comprised 2 different monophyletic lineages, especially in the COI phylogenetic tree.

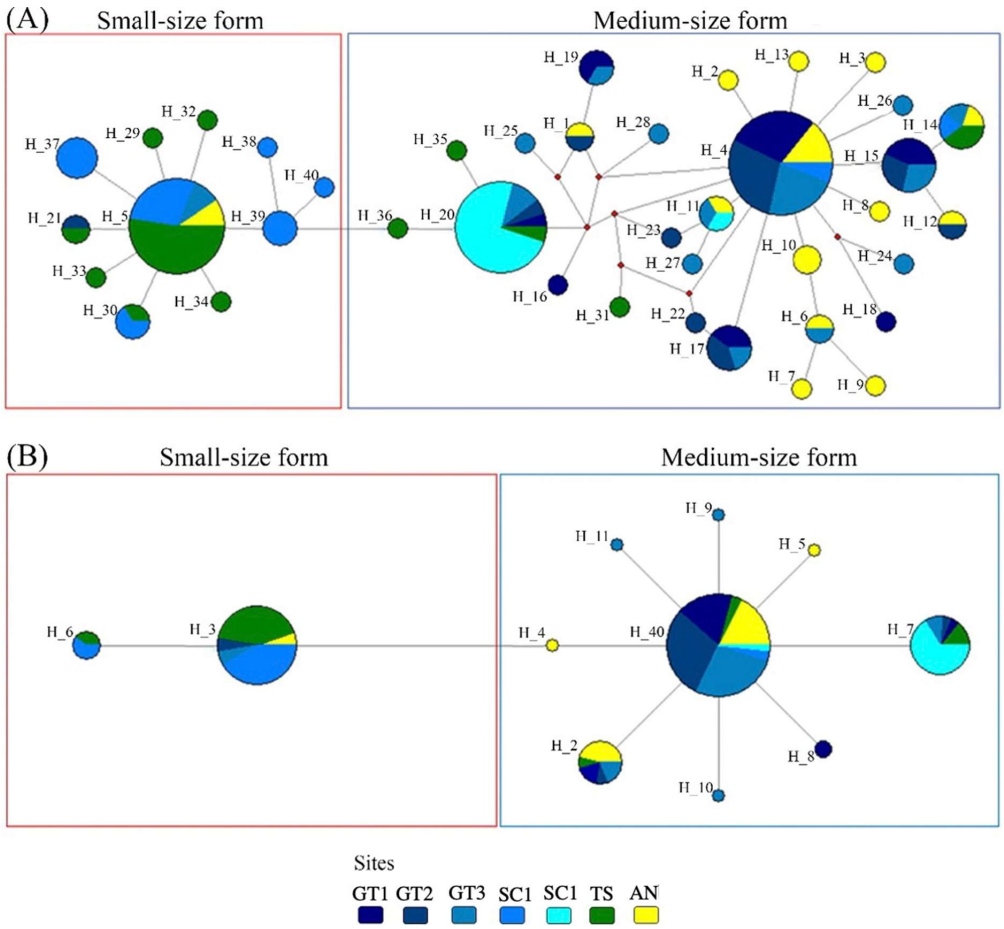

Fig. 4 The median-joining haplotype network of: (A) 40 haplotypes of COI sequences and (B) 11 haplotypes of 16S rRNA sequences of *S. lessoniana*. The circle sizes corresponding to the frequency of each haplotype. H_1–40 indicating haplotypes 1–40. The red dot representing missing haplotype.

This study found 2 intermediate individuals of *S. lessoniana*. One possible reason for the intermediate individuals in this study is that the *S. lessoniana* small-size form male mated with the *S. lessoniana* medium-size form female due to the mitochondrial DNA (mtDNA) inherited from the female, supported with a previous study which showed that mature *S. lessoniana* moved seasonally for mating and spawning [22]. As a result, the small- and medium-size forms might meet and mate in the South China Sea. Moreover, a phylogenetic study of purpleback flying squid (*Sthenoteuthis oualaniensis*) in the South China Sea also found intermediate forms in this region [13]. So, it is possible to find the intermediate forms of the cephalopods in the South China Sea region. These findings would be able to raise our awareness about the conservation of the squid biodiversity and community, especially in the study area; however, this study also has limitations as the sampling sites did not cover all the Andaman Sea and South China Sea. If the sampling sites covered all these areas, more clades or variations of this squid might have been found and able to provide more information to clarify the systematic relationships of *S. lessoniana* in these regions.

Haplotypes and biogeographic patterns of *S. lessoniana*

The total of 40 haplotypes were found across the 7 sampling sites. Specific haplotypes such as haplotypes 5 and 14 (Table 1 and Fig. 4) were found from the Andaman Sea (Ranong, Thailand) to near Taiwan Strait (Fujian, China), which are more than 2,700 km apart, indicating that *S. lessoniana* is a widely dispersed squid. More medium-size forms were found in the South China Sea and Andaman Sea areas than the small-size forms, indicating that there are wide distribution and dominance of this squid in these regions.

The results of Fu's FS negative values indicated genetic drift [23], and the negative value of Tajima's D referred to population expansion or purifying selection [24]. These results suggest that there are geographical barriers in this area. However, this study

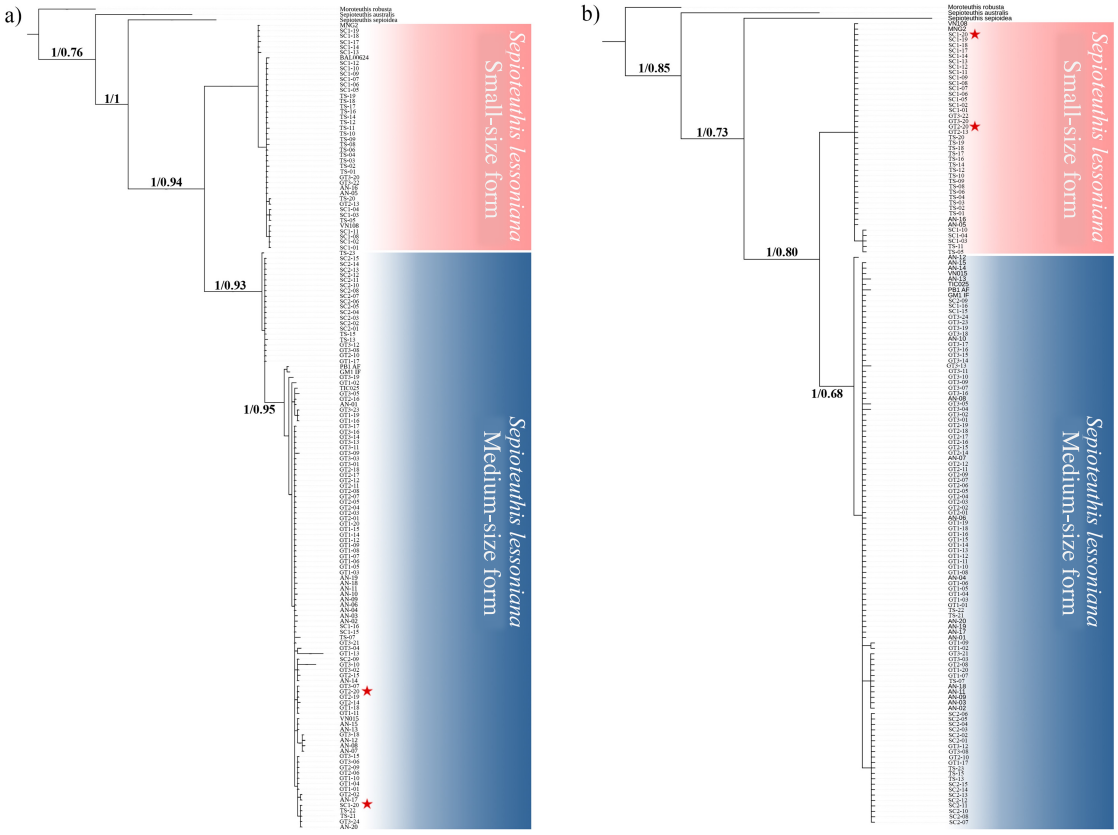

Fig. 5 Phylogenetic tree of *Sepioteuthis* with: (A) COI sequences (B) 16S rRNA sequences using Bayesian inference (BI) and maximum likelihood (ML). Numbers on the nodes referring to the probabilities support values of Bayesian posterior and ML, the values below 0.6 not displayed. The red star represents the intermediate individuals.

was unable to determine the main factors involved in the wide distribution of this squid although water currents are the most plausible reason. In this region, monsoon winds affect the seasonal circulation and currents, especially through Taiwan Strait between the East China Sea and South China Sea and similarly through Malacca Strait between the South China Sea and Andaman Sea [25].

Phenotypic forms of *S. lessoniana*

The clearest morphometric characteristic that can be used to distinguish the 2 forms of this squid is the shape of radula and average body size. Moreover, the discovery of different types of radulae in the small-size form and medium-size form (Fig. 3) is an interesting result since the radula is an important morphological characteristic for classifying many cephalopod species [26]. The difference was in the shape of the teeth, especially the rachidian tooth. In the small-size form, the rachidian teeth have high lateral cusps (more than 50% of the central cusp), but in medium-size form of *S. lessoniana*, the rachidian teeth have short lateral cusps (less than 50% of the central cusp). So, it is possible to classify the small- and medium-size forms into 2 sub-species due to the above-mentioned reasons.

CONCLUSION

Our results showed that the 142 individuals of *S. lessoniana* sampled from 7 sampling sites in the Andaman Sea, Gulf of Thailand, and South China Sea comprised at least 2 groups (small- and medium-size forms) that could clearly be separated based on body size, number of sucker ring teeth, and radular characteristics. Similarly, the results of the phylogenetic analysis showed clear separation based on the 2 clades of these 2 forms. The medium-size form was widely dispersed and dominant in the Andaman Sea and South China Sea whereas the small-size form was dominant near Taiwan Strait. The genetic analysis also found evidence of intermediate individuals between the 2 forms in the South China Sea where their distribution ranges overlap.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2023.005.

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Appendix A. Supplementary data

Table S1 Genetic diversity between/within the cytochrome c oxidase subunit I (COI) clades and 16S ribosomal RNA (16S rRNA) clades of genus *Sepioteuthis* evaluated by Kimura's two-parameter distance (median, in %).

Phenotypic form	Cytochrome c oxidase subunit I			
	S. australis	*S. sepioidea*	*S. lessoniana* small-size form	*S. lessoniana* medium-size form
S. australis				
S. sepioidea	18.24			
S. lessoniana small-size form	20.51	19.62	1.04	
S. lessoniana medium-size form	21.63	19.85	11.68	1.24
	16S ribosomal RNA			
	S. australis	*S. sepioidea*	*S. lessoniana* small-size form	*S. lessoniana* medium-size form
S. australis				
S. sepioidea	13.54			
S. lessoniana small-size form	11.32	12.07	0.05	
S. lessoniana medium-size form	12.53	12.40	2.86	0.19

Table S2 Neutrality test based on COI and 16S rRNA sequences among groups with 10,000 replicates.

	Group	Fu's fs	Tajima's D
COI	Small-size form	−4.4260	−1.4425
	Medium-size form	−4.7390	−1.0552
16S rRNA	Small-size form	0.4310	−0.0432
	Medium-size form	−4.9890	−1.3747