Molecular markers based phylogenetic inferences reveals cryptic lineage within Sepiella inermis species complex

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Abstract

Over the last two decades genetic and phylogeographic studies in cephalopods around the world have identified many species complexes and cryptic species. Indian Ocean, in the tropical region remained least studied one. In the present study *Sepiella inermis* samples were collected from different landing centres along Indian coasts to investigate the distribution of these species in a phylogeographic context. Phylogenetic analyses were performed using the mitochondrial Cytochrome Oxidase I gene and 16S rRNA gene.The currently described species consists of two genetically distinct clades (pair-wise genetic divergence varied in between 7.7 to 9.1%). One clade composed of individuals collected in Arabian Sea and the other from Bay of Bengal (northern and north-eastern part of Indian Ocean). The study led to the identification of potential cryptic speciation within *Sepiella inermis*. Mean intraspecific and interspecific nucleotide distances for COI were 0–2% and 2–7%, respectively, while these values for 16S rRNA sequences were 0–1% and 1–4%. Furthermore, this study also provides evidence of previously undocumented sub-population structuring in the Indian waters.

Introduction

Cuttlefishes are one of the major fishery resources with increasing demand.Cuttlefishes belong to the family Sepiidae which contains two genera, *Sepia* and *Sepiella*. The genus *Sepiella* Grey, 1849 is widely distributed in the tropical and sub-tropical waters. *Sepiella* genus can be easily identified by the presence of gland and gland pore located on the ventral side of the posterior end of the mantle. Only one species of the genus *Sepiella* namely *Sepiella inermis* have been reported in Indian waters by Silas et al. (1985). *S. inermis*, which is widely distributed in the Indo-Pacific region, is one of the commercially important cuttlefish resource form Indian waters. Even though this species is extensively distributed in Indian Ocean, studies on the phylogeography or population genetics of this species are meagre. Most of the studies are focused on the distribution, biology and stock assessment (Sundaram *et al.*, 2011, 2009; Sarvesan, 1996; Kasim, 1988; Silas et al., 1985b; Unnithan, 1982)

The recent ecological and genetic studies identified many cephalopods with considerable genetic differentiation between populations and the occurrence of cryptic species. When a species group can't be differentiated using observable morphological characters, molecular genetic tools used to identify the co-occurring cryptic groups in a species complex. Using this technique, many marine species that were once considered cosmopolitan are actually comprised of many genetically distinct species (Sales et al., 2017, 2013; Cheng et al., 2014; Dai et al., 2012; Anderson et al., 2011; Leite et al., 2008; Triantafillos & Adams 2005).Though, in recent years genetic studies increased to identify new species of cephalopods and phylogeographic patterns, such studies on cuttlefishes from India remained unexplored.

In the present study the patterns of genetic differentiation of Arabian Sea and Bay of Bengal populations of the *Sepiella inermis* were examined by means of DNA analyses of the mitochondrial Cytochrome C Oxidase subunit I (COI) and 16S rRNA (16S) genes. This data is used to assess the phylogenetic status of *Sepiella inermis* species complex.

Materials And Methods

Specimen Collection

Fresh specimens *S. inermis* of different sizes were collected from different landing centres along the East and West coasts and transported to the laboratory in Kochi, Kerala, India (Fig. 1). For each specimen morphometric measurements were taken to the nearest millimetreas recommended by Roper & Voss (1983). A small piece of muscle tissue was excised from the mantle and preserved in 100% ethanol for molecular analysis and the animals were preserved in 4% formalin. These voucher specimens have been stored at PMFGR Centre, ICAR-NBFGR, Kochi, India.

DNA Extraction, PCR & Sequencing

DNA was extracted from mantle tissue by a standard salting-out protocol (Miller et al., 1988). The mitochondrial COI gene region was amplified with the primers HCO2198–LCO1490 (Folmer et al., 1994) and 16S with primers 16Sar: 5'-CGCCTGTTTATCAAAAACAT-3' and 5'-CCGGTCTGAACTCAGATCACGT-3' (Palumbi *et al*., 1991); PCR was set up on a total volume of 25 µl with 0.5 µl primer (forward and reverse primer (10 µM), 12.5 µl TaKaRa Emerald Amp GT PCR Master Mix, 1µlof DNA (20 ng/µL) and 11µlH2O (double distilled). PCR conditions were set as initial denaturation at 94°C for 01 m, followed by 35 cycles of denaturation at 94ºC for 40 s, annealing at 50ºC for 40 s and extension at 72ºC for 1 m, with a final elongation at 72ºC for 10 m for COI gene and initial denaturation at 94°C for 04 m, followed by 35 cycles of denaturation at 94ºC for 30 s, annealing at 52ºC for 40 s and extension at 72ºC for 45 s, with a final elongation at 72ºC for 5 m for 16S gene. Then, 3 µl of each PCR product was checked on 1.5% agarose gel. PCR samples were sequenced by Sanger sequencing.

Sequence Editing & Phylogenetic Analysis

Before analysis, sequences were trimmed at the 5' and 3' ends and aligned using the ClustalW multiple alignment tool implemented in BioEdit (Hall, 2004). For phylogenetic analysis, apart from the sequences we generated (Table 1), available COI and 16S gene sequences of *Sepiella inermis, S. japonica and S. maindroni* were retrieved from the NCBI GenBank online (Table 2). COI sequences of *Sepia ramani* (MZ356365), *Sepia elliptica* (MZ363831), *Sepia pharaonis* (MZ356377) and 16S rRNA sequences of *Sepia ramani* (OL714385), *Sepia pharaonis* (OL714384) were considered as outgroups. MEGA 11 (Tamura et al., 2021) was used to generate Maximum Likelihood tree for the COI and 16S sequence data under GTR + G and T92 + G models respectively for 1,000 iterations. Genetic distances were also calculated using MEGA 11.

Table 1
Sepiella inermis sequences generated in this study

Species Name	Location	COI	16S
Sepiella inermis	Paradeep, Odisha	OL456270	OL739295
S. inermis	Paradeep, Odisha	OL456271	OL739296
S. inermis	Paradeep, Odisha	OL456272	OL739297
S. inermis	Paradeep, Odisha	OL456273	OL739298
S. inermis	Kochi, Kerala	OL468768	OL719311
S. inermis	Kochi, Kerala	OL468769	OL719312
S. inermis	Kochi, Kerala	OL468770	
S. inermis	Kochi, Kerala	OL468771	
S. inermis	Kochi, Kerala	OL468772	
S. inermis	Tuticorin, Tamil Nadu	OL519126	OL739294
S. inermis	Tuticorin, Tamil Nadu	OL519127	
S. inermis	Mandapam, Tamil Nadu	OL519128	
S. inermis	Veraval, Gujarat	OL469283	OL714394
S. inermis	Veraval, Gujarat	OL469284	OL714395
S. inermis	Veraval, Gujarat	OL469285	
S. inermis	Veraval, Gujarat	OL469286	
S. inermis	Digha, West Bengal	OL468800	OL757135
Sepia ramani	Tuticorin, Tamil Nadu	MZ356365	OL714385
Sepia elliptica	Kochi, Kerala	MZ363831	
Sepia pharaonis	Tuticorin, Tamil Nadu	MZ356377	OL714384

Table 2
Sepiella species included in the phylogenetic analyses and their GenBank accession numbers.

Species	Location	COI	16S
S. inermis	Indonesia	LC504754	
S. inermis	China	KF040369	
S. inermis	China	HQ846081	
S. inermis	Indonesia		LC121070
S. inermis	China		LC121069
S. inermis	China		HQ845992
S. inermis	China		EU735190
S. inermis	China		AF369960
S. inermis	Iran		KX984286
Sepiella sp.	Iran		KX984287
S. japonica	Taiwan		AM088001
S. japonica	China		KP699648
S. japonica	Taiwan		AY616978
S. japonica	China		HQ845993
S. japonica	China		LC121071
S. japonica	China	HQ846082	
S. japonica	China	KC900380	
S. japonica	Taiwan	AB430415	
S. japonica	Japan	AB645082	
S. maindroni	Japan	AF346853	
S. maindroni	China	KR912215	
S. maindroni	China	JF700180	
S. maindroni	China	AF361361	
S. maindroni	China		EU234590

Results

Morphological Analysis

We were able to assign the collected specimens to the species *Sepiella inermis* based on morpho-meristic studies. *Sepiella inermis* is spine less cuttle fish. Mantle is oblong, posterior gland and gland pore pigmented reddish, located on the ventral side of the posterior end of the mantle. Hectocotylus have 10 rows of reduced suckers proximally, Tentacular club with 12 to 24 suckers in transverse rows, Cuttlebone outline oval, broad, strongly convex in lateral view; Dorsal mantle has more than 7 reddish patches adjacent to base of fins.

Molecular Analysis

Three strongly supported subclades within *S. inermis* were observed in the phylogenetic analysis. Clade A includes *S. japonica* species, clade B, *S. inermis* species from Bay of Bengal and final clade C includes *S. inermis* species from Arabian Sea. In the phylogenetic tree, these clusters were supported by high bootstrap values (> 75%) (Fig. 2, 3). It was noted that the interspecies distance between the Arabian Sea and Bay of Bengal specimens was 0.066 (7%). The intra specific distance within Arabian Sea *S. inermis* was 0.007 and within Bay of Bengal specimens it was 0.003.Newly generated sequences have been submitted to GenBank .

Discussion

In our study the *Sepiella* specimens collected from Indian waters were morphologically identified as *Sepiella inermis* and are found different from the other closely related species within this genus. Three strongly supported geographically demarcated clades were observed in the COI and 16S gene phylogenies.The phylogenetic analysis performed here clearly support population differentiation between Arabian Sea and Bay of Bengal. Our results suggest that *Sepiella inermis* is a cryptic species, and a thorough taxonomic revision of this species complex is necessar

In the phylogenetic analysis using COI gene, three well supported clades were formed. Sequences of specimens we identified as *S. inermis* from the north east and south east Arabian Sea form clade A. *Sepiella japonica* forms a well-supported clade (clade B) and sequences of *S. inermis* (clade A) were observed as a sister to clade B. The type locality of spine less Japanese cuttle fish *S. japonica* is Toyoma Bay and is distributed in north western Pacific Ocean. Since Zeng *et al.* (2015) noted that *S. japonica* and *S. maindroni* are closely related and cluster B includes sequences from Japan (AB675082, AF236853) (Cheng *et al*., 2013), we are confident that the cluster B is *S. japonica*. Clade C includes sequences from specimens we identified as *S. inermis* from Bay of Bengal together with sequences of *S. inermis* from Indonesia (LC304754) and China (KF040369, HQ846081). Since most of the *S. inermis* sequences are from the type locality, we consider this clade is of Bay of Bengal population. Intra-specific genetic distance within both the Arabian Sea clade and Bay of Bengal clade is 0.004. Interspecific distance between these clades is 0.07. In the case of *S. japonica* clade, the intraspecific difference is 0.006 and inter-specific difference with Arabian Sea clade of *S. inermis* clade and Bay of Bengal clad of *S. inermis* is 0.7 and 0.6 respectively.

Phylogenetic analysis using 16S rRNA gene also revealed three well supported clades. Clade A includes sequences of specimens we identified as *S. inermis* from the Bay of Bengal, along with sequences of *S. inermis* from China (LC121069, HQ845992 and EU735190), Indonesia (LC121070) and one *S. maindroni* (AF369960) from China. Clade B includes *S. japonica* sequences and one *S. maindroni* (EU234590) sequence also from China .Clade C includes *S. inermis* sequences from Arabian Sea generated in this study along with *S. inermis* sequences (KX984286, KX984287) from Iran. The intraspecific difference within clade A, B and C were 0.004, 0.001 and 0.003 respectively. Interspecific distance between Bay of Bengal clade and Arabian Sea clade was 0.022. The *Japonica* clade showed 0.02 and 0.03 genetic difference with *S. inermis* of Bay of Bengal clade and *S. inermis* of Arabian Sea clade respectively. Within the *Sepiella* genus, genetic data of only three species is available i.e. S. *inermis, S. japonica and S. maindroni.* In our study, phylogenetic analysis using both the genes confirmed the presence of two strongly supported clades within the *S. inermis* species originated from Arabian Sea and Bay of Bengal. *S. japonica* is not yet been reported from the north western Indian Ocean. Since the waters in the western Indian Ocean is warmer (Saraswat et al., 2007) than the seas around Japan, the possibility of occurrence of *S. japonica* species western Indian Ocean is rare. The type locality of *S. inermis* species is Indian Ocean and it is widely distributed in the Indo-Pacific region. Hence we suggest the possibility of cryptic speciation for *Sepiella inermis* within Indian waters.

Though species are congeneric and ecologically similar, there are chances of substantially different phylogeographic patterns between sympatric species, (Crandall et al., 2008). Avise (1992) proposed that in species with wide distribution range, the changes in the biogeographic boundaries will affect population genetic structure by natural selection or by reducing gene flow. Recognition of taxonomic boundaries is necessary to understand the biogeographical and ecological distribution of an organism. Though less work has been done on Indian Ocean cephalopod populations, some studies have found evidence of a phylogeographic break between the eastern and western Indian Ocean (Ridgway &Sampayo, 2005). Jereb *et al.* (2005) suggested that *S. inermis* can possibly be a species complex. Our results suggest the possibility of cryptic speciation within *Sepiella 'inermis'* species complex. The type locality of *S. inermis* is in Indian Ocean and the exact location is not mentioned. Our study was conducted in Arabian Sea and Bay of Bengal which are in the northern and north-eastern part of the Indian Ocean respectively. Even though many phylogeographic studies have done on Indian Ocean species, phylogeography Arabian Sea species and Bay of Bengal species were not much reported. Additional research is called for to investigate relationships between different populations of this species.Furthermore, additional specimens from different locationsof the range of the S. *inermis* must be evaluated for the further clarification about the status of the species.

Declarations

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Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with animals performed by any of the authors

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Figures

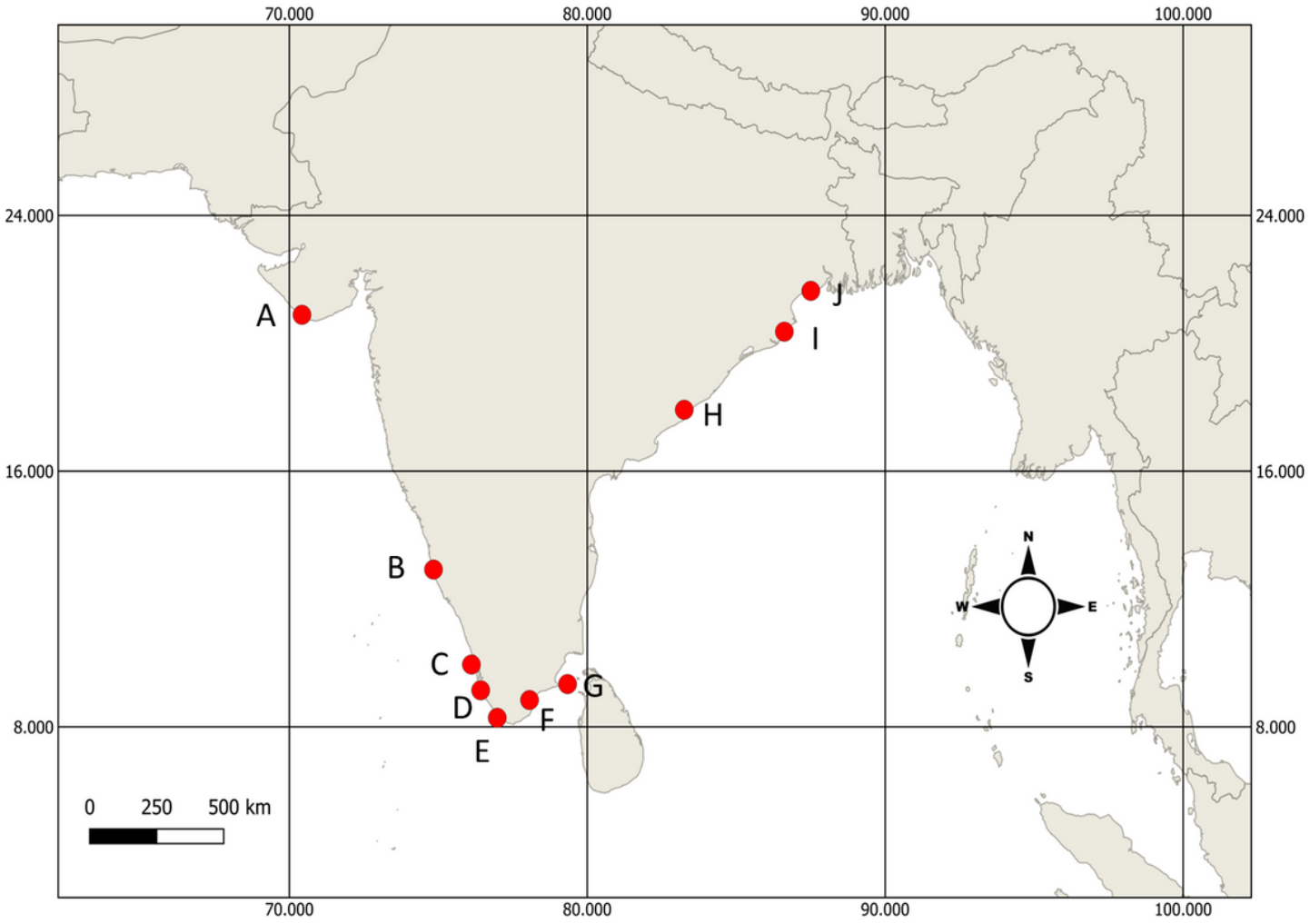

Figure 1

Sampling locations for *Sepiella inermis* sourced in this study (shown in red) generated using QGIS software. Acronyms represent the following localities: A- Veraval; B- Mangalore; C- Cochin; D- Shakthikulangara; E- Vizhinjam; F- Thoothukudi; G- Rameswaram; H- Vishakhapatnam; I- Paradeep; J- Digha

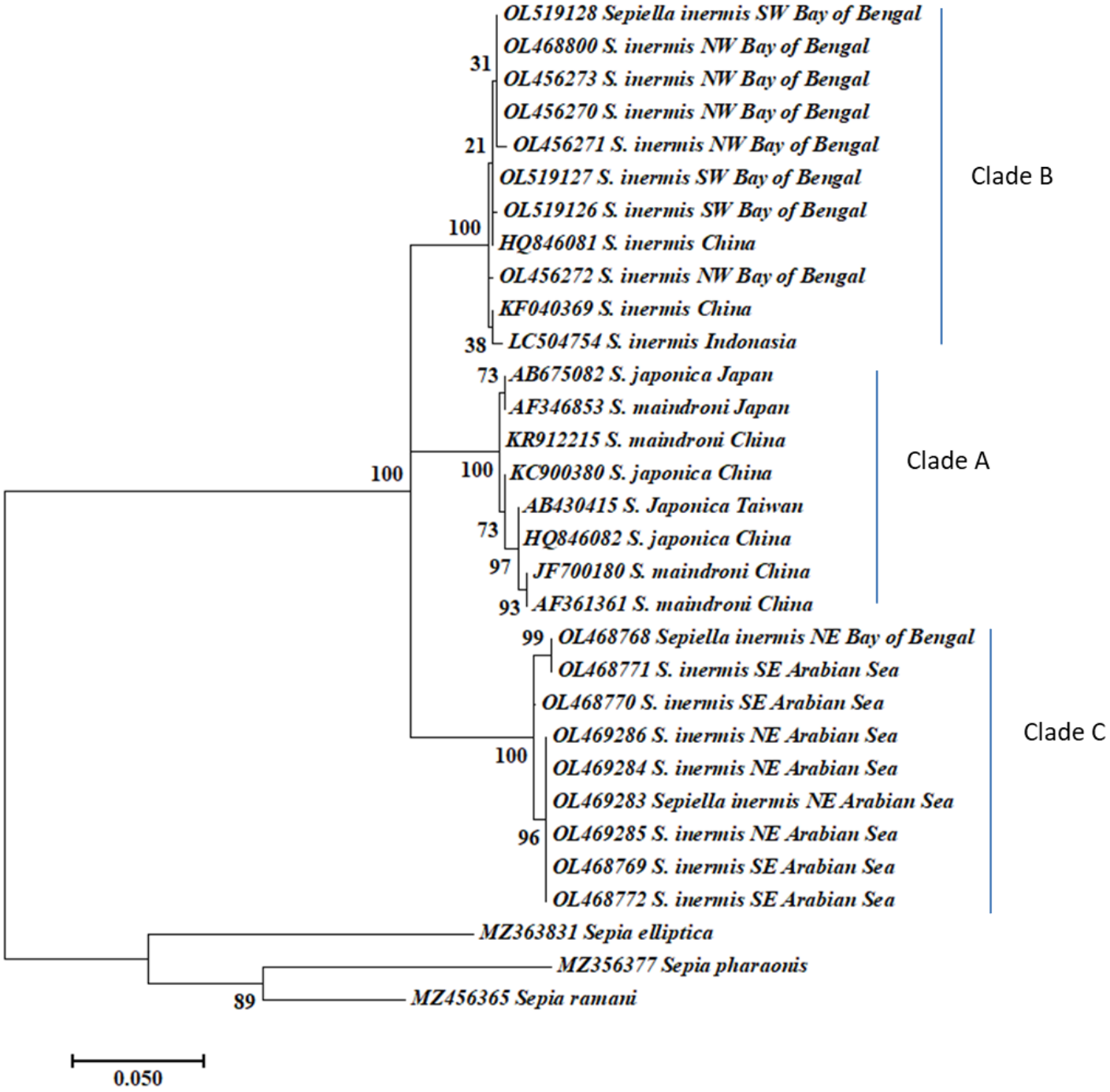

Figure 2

Maximum likelihood topology depicting phylogenetic relationship within the genus *Sepiella* using GTR+G model. Analysis is based on partial mitochondrial COI gene showing bootstrap values >70. Node labels refect locations represented by individuals contributing to node (SW= south west, NW= North West, NE= north east)

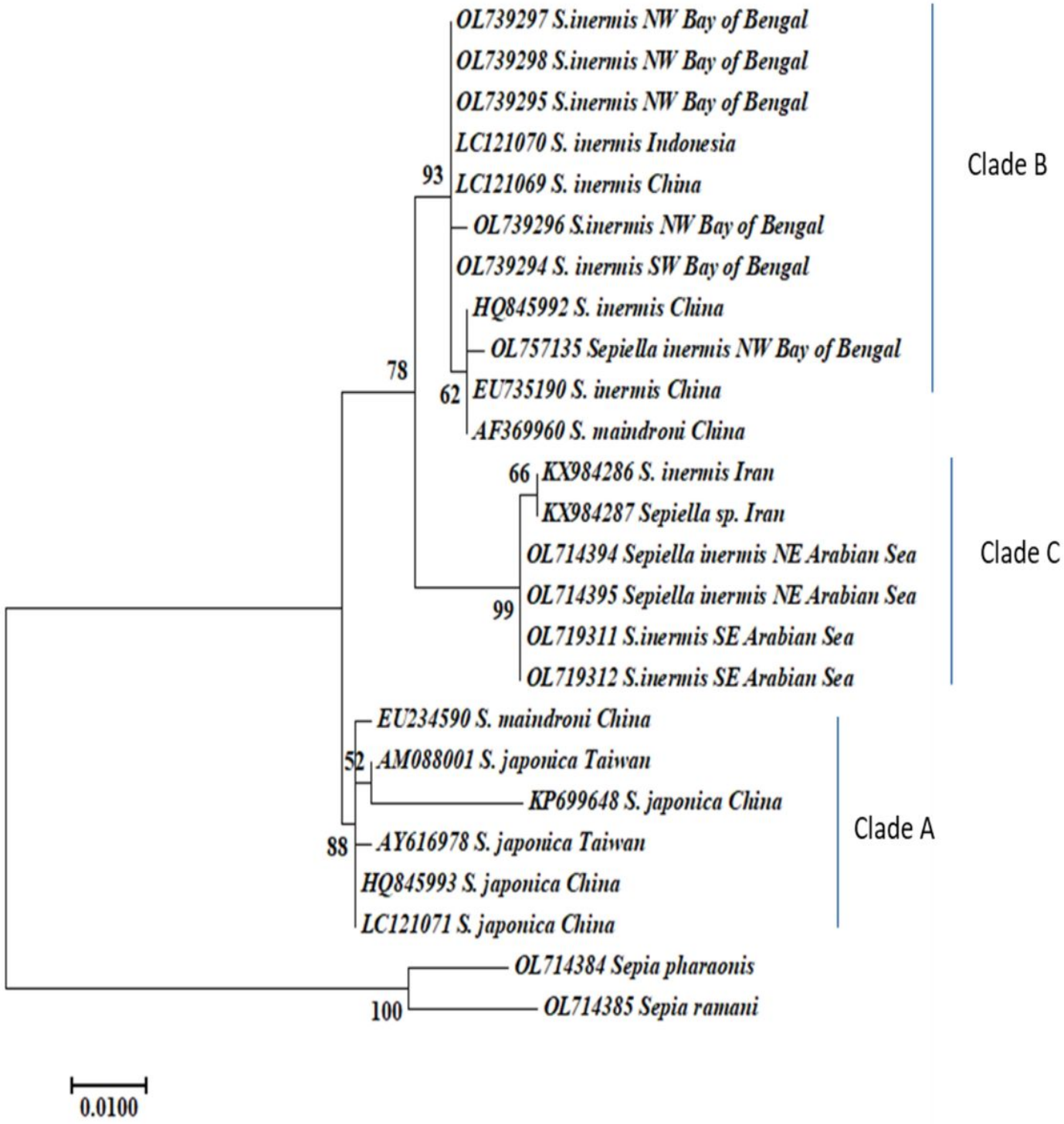

Figure 3

Maximum likelihood topology depicting phylogenetic relationship within the genus *Sepiella* using GTR+G model. Analysis is based partial mitochondrial 16S rRNA gene showing bootstrap values >70. Node labels reflect locations represented by individuals contributing to node (SW= south west, NW= North West, NE= north east)