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Phylogeography of Sepioteuthis lessoniana (the bigfin reef squid) and Uroteuthis duvauceli (the Indian squid).

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Phylogeography of *Sepioteuthis lessoniana* (the bigfin reef squid) and *Uroteuthis duvauceli* (the Indian squid).

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Abstract

Sepioteuthis lessoniana (the bigfin reef squid) and *Uroteuthis duvauceli* (the Indian squid) are two squid species found in largely overlapping regions in the Indian and Pacific Oceans. While both squids are important to fisheries throughout their ranges, very little taxonomic work has been done on either of them. Previous studies have led scientists to believe that *S. lessoniana* is actually a species complex (for example, there appear to be three species of "*S.* cf. *lessoniana*" in Japanese waters alone). The similarly broad geographic range of *U. duvauceli* suggests that this species could also harbor substantial cryptic genetic diversity. In order to evaluate genetic variation within these two species, regions of two mitochondrial genes—the large subunit ribosomal RNA gene (16S) and the cytochrome oxidase I gene (COI)—from specimens caught in regions throughout the northern Indian and western Pacific Oceans were sequenced and compared. Sequences were obtained by extracting the DNA from tissue samples of both species, amplifying the DNA using the polymerase chain reaction (PCR), determining the sequences of both DNA strands using a automated DNA sequencer, and comparing sequences to one another to establish similarities and differences between geographic locations. To expand the significance of this study, we compared our sequences to data contributed by a collaborator (Samantha H. Cheng, Department of Ecology and Evolutionary Biology, UCLA) and data downloaded from GenBank (an online genetic database). Phylogeographic analyses showed that *Sepioteuthis lessoniana* from southern India represent two very distinct genetic lineages, suggesting that "*S.* cf. *lessoniana*" comprises at least two cryptic species in south Indian waters. For *Uroteuthis duvauceli*, specimens from Iran are genetically distinct from those in Thailand and Japan, which may support the hypothesis of several undescribed species within "*U. cf. duvauceli*". This study is the first attempt to assess genetic diversity across the ranges of these

two species; future work will require additional genetic markers and (most importantly) additional sampling from other geographic regions.

Introduction

Sepioteuthis lessoniana (Figure 1, Roper et al., 1984) and *Uroteuthis duvauceli* (Figure 2, Hoyle, W.E., 1886) are two squid species found in largely overlapping regions in the Indian and Pacific Oceans. *S. lessoniana* (the bigfin reef squid) is found from the Red Sea and the Mediterranean Sea to northern Australia, Japan, and the Hawaiian Islands. *U. duvauceli* (the Indian squid) is found from the Red Sea to the Philippines and South China Sea, including Taiwan (Roper et al., 1984). Both species are important to fisheries throughout their ranges. The bigfin reef squid is captured throughout the year for commercial sale in Southeast Asia. In Sri Lanka, it is the most common cephalopod species caught, and in China, it is one of four species from the family Loliginidae captured and sold (Roper et al., 1984). It is the target of both commercial and subsistence fisheries in the Gulf of Thailand (Flaherty and Karnjanakesorn, 1993). The Indian squid is the second most valuable squid species for trawl fisheries in the Gulf of Aden and one of the major fisheries species in the Gulf of Thailand (Roper et al., 1984). This species is exploited by artisanal subsistence fisheries (Roper et al., 1984) and is a large portion of the bycatch of prawn trawlers off the northeastern South African coast (Fennessy, 1993). Even though these squid are economically important, little taxonomic work has been done on either species.

Previous studies have shown that at least three genetically distinct populations of *S. lessoniana* exist in part of its range. Due to different body color, size, and appearance, three different forms of *S. lessoniana* have long been recognized by Japanese fishermen. Allozyme studies supported the hypothesis that these different forms represent three genetically distinct groups (Izuka, 1996). Furthermore, a recent study has shown that these three species are different from other *S. lessoniana* found around Taiwan and Vietnam (Aoki et al., 2008). Finally, *S. lessoniana* in Australian waters appears to comprise two genetically distinct groups, both of

which are distinct from the Southeast Asian groups described above (Izuka, 1994; Triantafillos and Adams, 2005). These studies show that there could be six or more separate species of "*S. cf. lessoniana*" in total. By contrast, geographic patterns of genetic variation in *U. duvauceli* have not been studied to date, but similar species have been documented to comprise cryptic species and high levels of genetic diversity (Yeatman and Benzie, 1993).

A better understanding of genetic variation in these species will provide better protection from fishery overexploitation. Genetic techniques have already been used in many finfish fisheries (Ryman and Utter, 1987; Carvalho and Pitcher, 1995) and offer the same benefits to invertebrate fisheries such as identifying stocks for fisheries and conservation; learning how genetics affect growth rate, survival, and disease resistance; developing strains for captive breeding; and understanding the structure of populations (Thorpe et al., 2000). Understanding genetic relationships within these species will give insight into stocks—genetic breeding units—that help fisherman and scientists determine sustainable catch levels for different species (Bohnsack and Ault, 1996). If populations of these species from different regions are genetically distinct, overfishing of a single population could result in extinction of a distinct species. Captive breeding has been implemented for endangered species such as the white surgeon (*Acipenser transmontanus*) to prevent extinction. While this hopefully is a last resort, it does serve as a protection plan in which genetic techniques are used to ensure fish are genetically similar to wild stocks before they are released (Ireland et al., 2002) In order to be able to use this measure, however, scientists must have an understanding of what genes they are trying to preserve. In short, fishery pressure in certain areas may threaten as-yet-undiscovered species which could be protected through greater genetic understanding.

In order to evaluate genetic variation in these two species, two regions of mitochondrial DNA from specimens caught off the coasts of Iran and India were compared to sequences of specimens from different regions in the northern Indian and western Pacific Oceans generated in previous studies. Similar research has been done on *Sepia pharaonis*, another commercially important cephalopod species in this region, and distinct groups from different geographical regions have been distinguished (Anderson et al., 2007; 2011.) Due to the success of these studies on similar cephalopod species, sequences from *S. lessoniana* and *U. duvauceli* were mapped and compared in order to provide information that could be useful to fisheries scientists and cephalopod researchers.

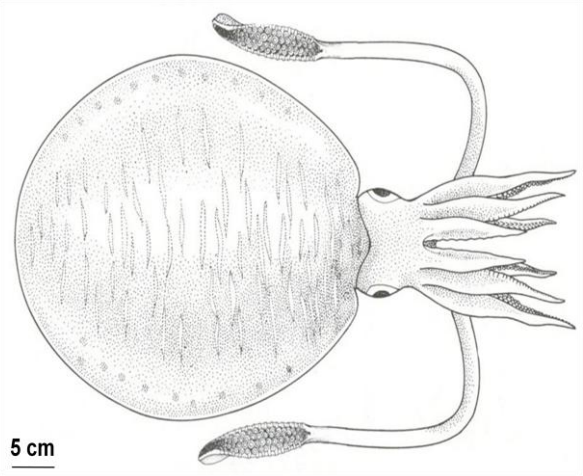

Figure 1 *Sepioteuthis lessoniana* (Roper et al., 1984)

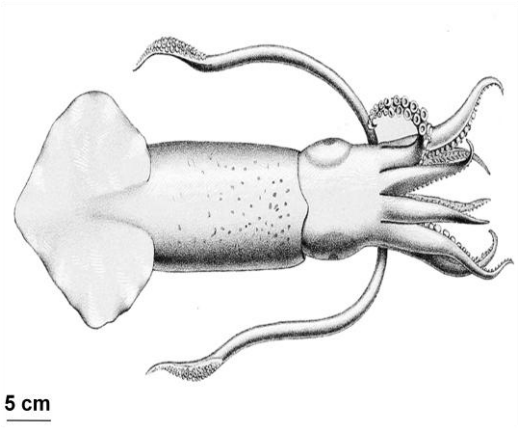

Figure 2 *Uroteuthis duvauceli* (Hoyle, W.E., 1886)

Methods

Tissue samples from the fins of thirteen *U. duvauceli* specimens from the Oman Sea (25°00’N-25°10'N, 59° 00’E- 59°30'E) and six from the Persian Gulf (27°40'N - 29°00'N, 50°30'E - 52°30'E) as well as seven *S. lessoniana* individuals from India (two from Mangalore Bay, five from off the coast of Pamban Island, and two from Palk Bay; Figure 3; Table 1), were sent to Southern Illinois University Carbondale in 80-100% ethanol by collaborators of Dr. Frank E.

Anderson, Zoology Department Associate Professor and REACH mentor—K. Sunil Mohamed (Central Marine Fisheries Research Institute, Cochin, India) and Tooraj Valinassab (Iranian Fisheries Research Organization, Tehran, Iran). DNA was extracted from these samples using a DNEasy Extraction Kit (QIAGEN). A HotStarTaq Master Mix (QIAGEN) was used to amplify two regions of the mitochondrial cytochrome c oxidase subunit I (COI) gene and the large subunit ribosomal RNA gene (16S) via PCR, the polymerase chain reaction, following manufacturer's protocols (half-reactions). These gene regions were chosen because they have been used to study the population genetics in *S. pharaonis* (Anderson et al., 2011). The primers used for the CO1 gene were (Folmer et al., 1994): COI-H: 5´-TAAACTTCAGGGTGACCAAAAAATCA-3´ and COI-L: 5´-GGTCAACAAATCATAAAGATATTGG-3´, and for the 16S gene, the primers were (Geller et al., 1997) D16SAR 58-CGCCTGTTTAHYAAAAACAT-3´ and D16SBR 5´-CCGGTCTGAACTCAGMTCAYGT-3´. These primers each bind to a conserved region of the gene and allow enzymatic replication of the region between the primer binding sites. The DNA was amplified using a thermocycler which cycles the temperature for efficient replication as follows: denaturation: 94°C for 2 minutes; amplification: 35 cycles of 94°C denaturing for 1 minute and 72°C extension for 1.5 minutes; extension: 72°C for 7 minutes.

The PCR product was run on an electrophoresis gel against a standard ladder to evaluate the length, quality, and quantity of the PCR products. 16S gene regions often resulted in faint bands and required the sample to be QIAQuick Gel purified. These samples were run on a 1% agarose gel with TAE buffer gel and put under an ultraviolet light to cut the separated DNA out of the gel. The DNA in the gel slices were purified using a QIAQuick Gel extraction Kit (QIAGEN) following manufacturer's protocols. The PCR product of CO1 and the gel-purified

product of 16S were sequenced using a BigDye™ Terminator Cycle Sequencing Ready Reaction Kit v3.0 (one-eighth reactions), and the sequencing reaction product was purified using Sephadex spin columns. The purified sequencing reactions were then run out on an ABI Prism 3730 XL automated DNA sequencer. Sequences were checked and edited using Sequencher 4.1, combined with data from congeneric individuals downloaded from GenBank and data from additional *Sepioteuthis* populations (provided by Samantha Cheng, UCLA, Table 1), and aligned with Muscle v3.8.31 under default settings (Edgar, 2004). The resulting alignments were concatenated using Mesquite v. 2.75 (Maddison and Maddison, 2011). Maximum likelihood analyses were performed using the rapid bootstrapping function coupled with a more thorough ML tree search (analysis option –f a) in RAxML 7.0.4 (Stamatakis, 2006). This included running 500 bootstraps under the general time-reversible (GTR) model with a discrete gamma approximation to account for among-site rate variation using the following RAxML command: (-f a -p <parsimony random number seed> -x <bootstrap random number seed> -# 500 -m GTRGAMMA -s <data file name> -n <output file name>). Ancestral states for one morphological character—photophores (absent or present)—were estimated in Mesquite (Maddison and Maddison, 2011) via maximum likelihood using the Mk1 model (Lewis, 2001) and the asymmetric 2-parameter model on the maximum-likelihood topology.

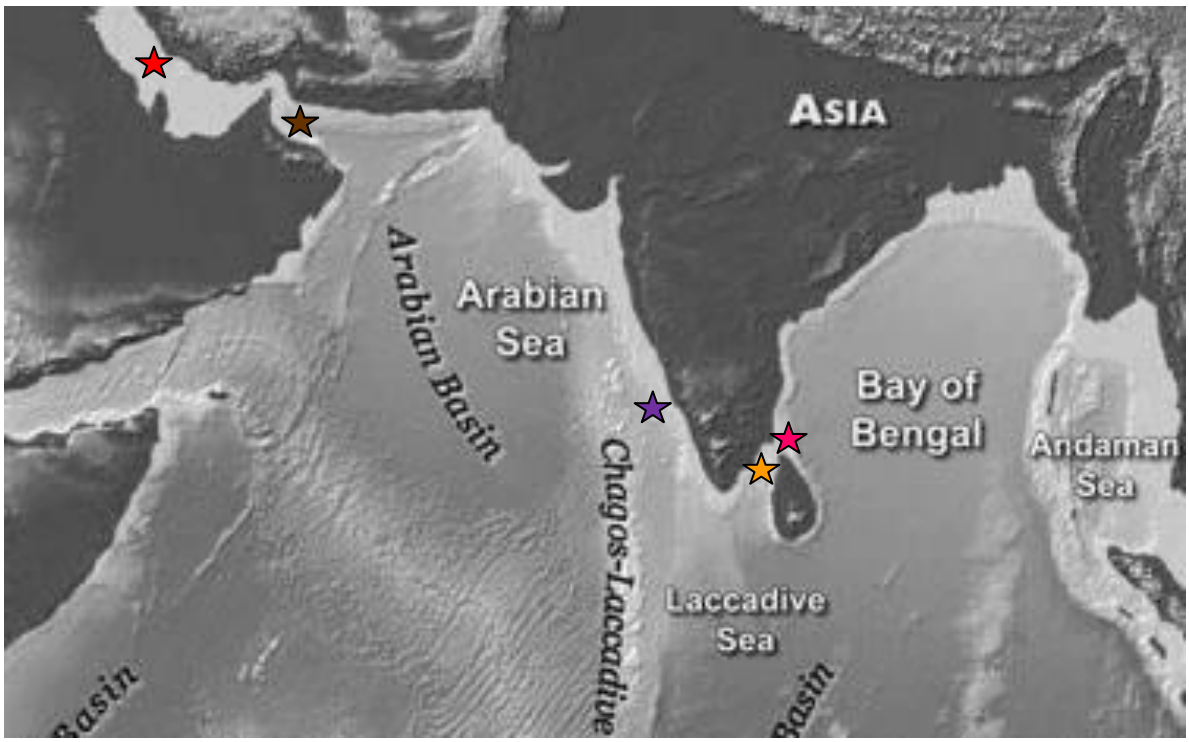

Figure 3: Sample locations for *Uroteuthis duvauceli* and *Sepioteuthis lessoniana* collected by collaborators of Frank E. Anderson

Results

Phylogenetic trees for the *U. duvauceli* data set of combined COI/16S sequences strongly supported monophyly of *U. duvauceli* but revealed that the *U. duvauceli* specimens sampled from Iran were genetically distinct from *U. duvauceli* sampled from the Andaman Sea near Thailand and the western Pacific (China) (Figure 3, Table 1). Sequences from specimens from the Persian Gulf and Oman Sea (Figure 4) were indistinguishable from one another. One specimen (7CH) was identified as *U. duvauceli* by T. Valinassab but was found to be more closely related to another species, *Uroteuthis edulis*. For *Sepioteuthis lessoniana*, specimens grouped distinctly into three forms. Two of the forms, the "Australian form" and the "Indo-Pacific form", appear to be broadly distributed from at least India in the west to the Philippines

in the east, while the third (a Sumatran form) may be more geographically restricted. Figure 5 shows the maximum likelihood bootstrap support values ranging from 94 to 100, supporting a close evolutionary relationship between specimens from India, Indonesia, Philippines, and China that fall either in the Australian or Indo-Pacific form.

Table 1 Location of collection by collaborators of Frank E. Anderson of samples that were sequenced, compared, and mapped in Figure 4 and 5.

Species	**Specimen**	**Locality**	**Country**	**Lat/Long**
Uroteuthis duvauceli	1CH	Oman Sea	Iran	59°00´E - 59°30´E, 25°00´N - 25°10´N
	2CH	Oman Sea	Iran	59°00´E - 59°30´E, 25°00´N - 25°10´N
	3CH	Oman Sea	Iran	59°00´E - 59°30´E, 25°00´N - 25°10´N
	4CH	Oman Sea	Iran	59°00´E - 59°30´E, 25°00´N - 25°10´N
	5CH	Oman Sea	Iran	59°00´E - 59°30´E, 25°00´N - 25°10´N
	6CH	Oman Sea	Iran	59°00´E - 59°30´E, 25°00´N - 25°10´N
	7CH	Oman Sea	Iran	59°00´E - 59°30´E, 25°00´N - 25°10´N
	8CH	Oman Sea	Iran	59°00´E - 59°30´E, 25°00´N - 25°10´N
	9CH	Oman Sea	Iran	59°00´E - 59°30´E, 25°00´N - 25°10´N
	10CH	Oman Sea	Iran	59°00´E - 59°30´E, 25°00´N - 25°10´N
	11CH	Oman Sea	Iran	59°00´E - 59°30´E, 25°00´N - 25°10´N
	12CH	Oman Sea	Iran	59°00´E - 59°30´E, 25°00´N - 25°10´N
	13CH	Oman Sea	Iran	59°00´E - 59°30´E, 25°00´N - 25°10´N
	BO2	Persian Gulf	Iran	50°30´E - 52°30´E, 27°40´N - 29°00´N
	BO3	Persian Gulf	Iran	50°30´E - 52°30´E, 27°40´N - 29°00´N
	BO4	Persian Gulf	Iran	50°30´E - 52°30´E, 27°40´N - 29°00´N
	BO5	Persian Gulf	Iran	50°30´E - 52°30´E, 27°40´N - 29°00´N
	BO6	Persian Gulf	Iran	50°30´E - 52°30´E, 27°40´N - 29°00´N
	BO7	Persian Gulf	Iran	50°30´E - 52°30´E, 27°40´N - 29°00´N
Sepioteuthis lessoniana	GM1 IF	Pamban, Gulf of Mannar	India	
	GM2 IF	Pamban, Gulf of Mannar	India	
	GM3 IF	Pamban, Gulf of Mannar	India	
	GM1 AF	Pamban, Gulf of Mannar	India	
	GM2 AF	Pamban, Gulf of Mannar	India	
	MNG1	Mangalore Bay	India	
	MNG2	Mangalore Bay	India	
	PB1 IF	Mandapam, Palk Bay	India	
	PB1 AF	Mandapam, Palk Bay	India	
	SUM00201	Sumatra (Banda Aceh)	Indonesia	5°53'19.85" N, 95°20'46.00" E

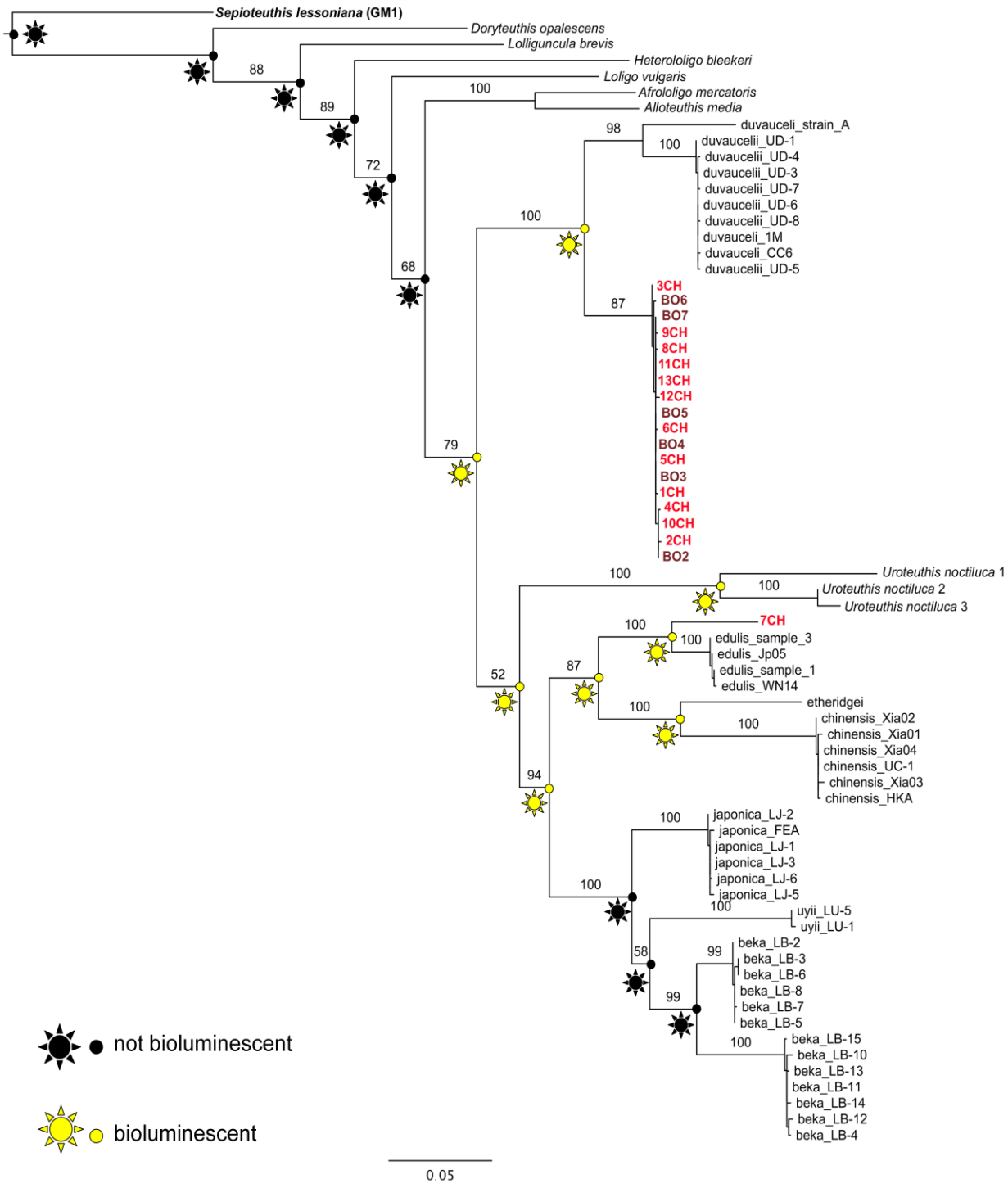

Figure 4. Maximum-likelihood phylogram for *U. duvauceli*, with presence/absence of bacterial bioluminescent organs (inferred via MP and ML ancestral state reconstruction in Mesquite) shown. Samples collected in this study are highlighted in red and brown text. A *Sepioteuthis lessoniana* haplotype found in this study (shown in **bold**) was used as an additional outgroup.

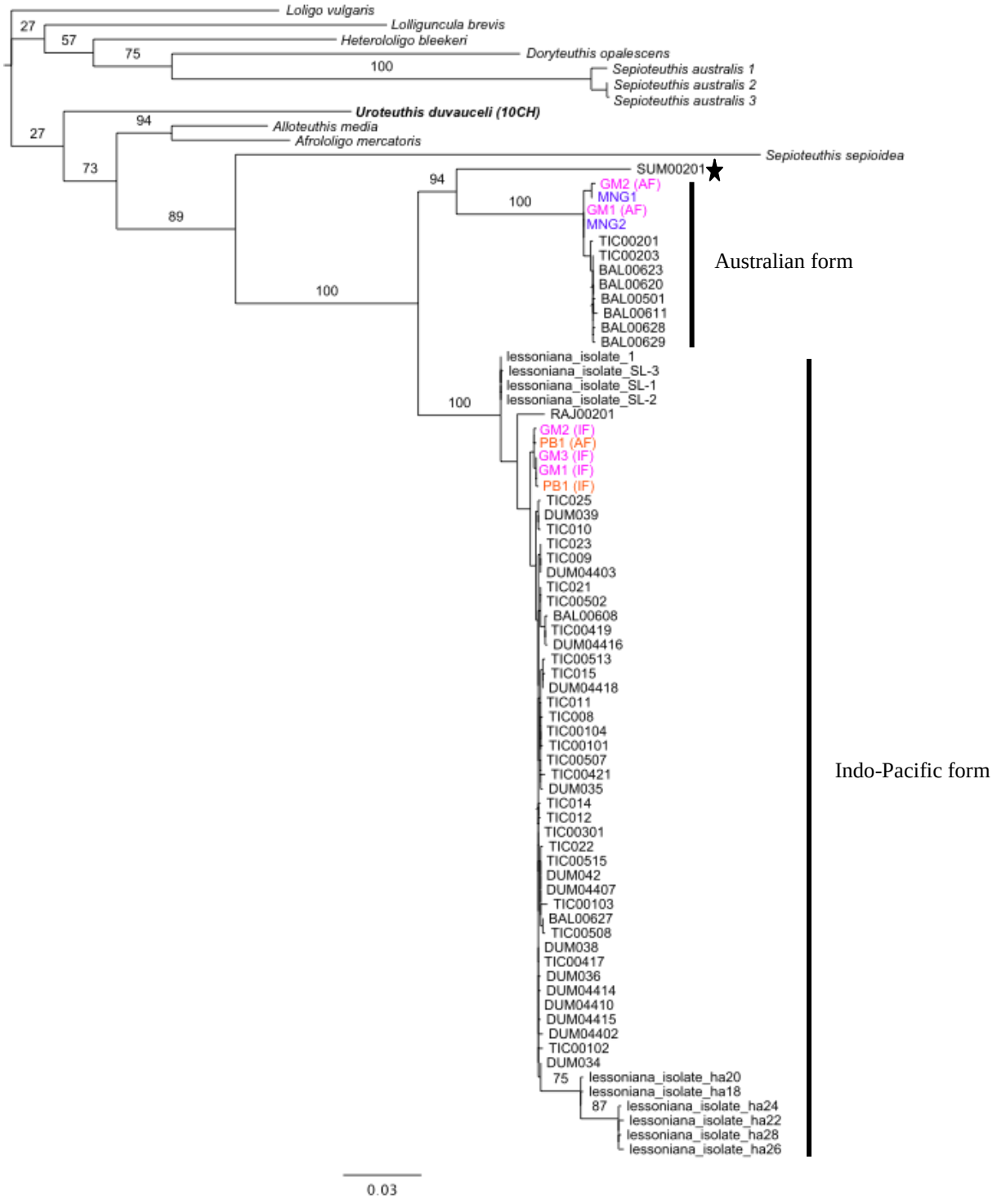

Figure 5. Maximum-likelihood phylogram for *S. lessoniana.* Samples collected in this study are highlighted in purple (Mangalore), pink (Gulf of Mannar) and orange (Palk Bay) text. The genetically distinct Sumatran haplotype is highlighted with a **star** (★). A *Uroteuthis duvauceli* haplotype found in this study (shown in **bold**) was used as an additional outgroup.

Discussion

Uroteuthis duvauceli is a mobile squid species that can be found at depths from 0-170 m, whereas *Sepioteuthis lessoniana* prefers coral reefs and shallower waters (0-100 m) (Norman, 2000). These niche preferences would suggest that *U. duvauceli* might exhibit more gene flow across its range, while *S. lessoniana* would have more distinct forms throughout its range. The data, however, suggest otherwise. While geographic sampling is limited for *U. duvauceli*, our data show that *U. duvauceli* from Iranian waters are genetically distinct from specimens caught in Thai and Chinese waters, a phylogeographic pattern similar to that seen in *Sepia pharaonis*, another neritic cephalopod found throughout the Indo-Pacific (Anderson et al., 2007; 2011). One specimen from Iran provisionally identified as *U. duvauceli* (7CH) was found to group instead with *U. edulis*. *U. edulis* was long believed to be absent from the western Indian Ocean, but Jereb and Roper (2006) recently suggested that the range of *U. edulis* includes the Oman Sea. In addition to sharing habitat, *U. edulis* and *U. duvauceli* are both medium-sized squid species and share some similar characteristics. Commercial fisheries commonly catch *U. edulis* ranging in size from 150-250 mm which includes the most common size of *U. duvauceli* (150 mm). *U. edulis* is described as moderately stout by Jereb and Roper (2010), and according to Okutani (2005), *U. duvauceli* has also been recorded as a chubby/stout form in the eastern Indo-Pacific region. Additionally, a large form of *U. duvauceli* was reported in the Gulf of Aden and the Arabian Sea (Nesis, 1982/1987), waters near where our 7CH sample was collected. With these similarities in appearance, these species have the potential to be confused. Adding to the confusion, both species are known to be polymorphic, and *U. edulis* is often misidentified as other species such as *U. sibogae* and *U. chinensis* (Jereb and Roper, 2010).

Intriguingly, inclusion of *Loliolus* sequences in our analysis strongly suggests that *Uroteuthis* is paraphyletic. *Loliolus* appears to be nested deep within *Uroteuthis. Uroteuthis* species are the only loliginids that possess bacterial bioluminescent organs on the ventral side of their ink sacs (Nesis, 1987). Evolutionarily, this is believed to be a defense mechanism that makes them less visible to predators through counterillumination of their ink sac (Young, 1977; Moynihan, 1983; Jones & Nishiguchi, 2004). Ancestral state reconstruction of photophore evolution using maximum likelihood and maximum parsimony on the tree in Figure 4 strongly suggests that bacterial bioluminescence was present in the common ancestor of *Uroteuthis* and *Loliolus* but was lost in the ancestor of *Loliolus*. This hypothesis is supported by previous studies that noted a pair of papillae in a similar position on males of *Loliolus affinis* and *Loliolus hardwickei* as photophores on *Uroteuthis*. Lu et al. (1985) suggested that these may represent vestigial photophores that *Loliolus* at one time possessed but evolutionarily lost. This was further supported by Bayesian and maximum-likelihood ancestral state reconstructions that suggest photophores arose once in Loliginidae, an ancestor to Loliolini, and were lost in *Loliolus* descendants later on (Anderson et al., 2013). Many questions still remain on this topic and require genetic analysis of more specimens to fully understand this discovery.

Both the Indo-Pacific and Australian forms of *S. lessoniana* appear to be widely distributed geographically (Figure 5). The Australian form specimens sampled from Indian, Indonesian, and Philippine waters were genetically very similar to one another. Similarly, Indo-Pacific form specimens sampled from Indian, Chinese, Taiwanese, Indonesian and Philippine waters were genetically very similar. A third genetically distinct lineage was sampled from Sumatra (Banda Aceh) by our collaborator Samantha Cheng. This form may be more localized than the other forms revealed in our phylogeny, but further sampling will be necessary to

confirm this. One Australian form specimen from India—PB1 AF—grouped with the Indo-Pacific form specimens. This could be mislabeling due to similar body forms. It also could be due from hybridization between the forms, given that these forms overlap in their distribution.

In summary, ***Uroteuthis duvauceli*** **and** ***Sepioteuthis lessoniana*** **both appear to comprise multiple genetically distinct lineages that could represent cryptic species**. Our results require additional phylogenetic evaluations in order to better preserve these species for the fishery industry that relies on them.

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