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Cryptic speciation in the mesopelagic environment: Molecular phylogenetics of the lanternfish genus *Benthosema*

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ABSTRACT

The presence of morphologically identical individuals that are genetically divergent, known as cryptic species, is a phenomenon that is likely to be of importance at the theoretical level, for speciation studies, and at the applied level, for management purposes. Cryptic species are probably common in the marine environment, and they have been described in a variety of habitats. Here we present the first case of cryptic speciation in the mesopelagic environment for the *Benthosema* lanternfishes. Based on mitochondrial and nuclear sequences, we describe the presence of widely divergent *Benthosema pterotum* cryptic species that diverged at least 5 million years ago. Our results have important theoretical, as well as practical implications, related to developing mesopelagic fisheries.

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1. Introduction

The current loss of biodiversity due to anthropogenic factors has sprung renewed research in conservation biology and on the factors that promote speciation. To this end, the wide use of molecular techniques applied to landscape ecology, systematics, and best exemplified by barcode of life projects, has uncovered a number of surprises. Notably, some groups that are indistinguishable using "classical" morphological characters were found to display large amounts of genetic divergence and were dubbed cryptic species (James et al., 2010). In the ocean in particular, where direct and lengthy observations are difficult, cryptic species are likely to be common (Knowlton, 1993). This may have some very significant theoretical and practical consequences (Bickford et al., 2007). For example, the idea of different genetic entities may challenge commonly accepted ideas of the relative importance of allopatric and sympatric speciation in the marine environment. At a practical level, fisheries that were once managed as a single stock may reveal a complex of previously unidentified species that require modulated enforcement. In either case, it is clear that our understanding of cryptic speciation in the ocean is important but still very limited.

Thus far, much work on the genetics of marine organisms has been relegated to coastal regions and deep-sea vent communities. This translates in the corresponding discovery of cryptic species in these habitats (e.g. Bernardi and Goswami, 1997; Bucciarelli et al., 2002; Vrijenhoek et al., 2008). The presence of cryptic species is not entirely surprising, since habitat discontinuities are likely to promote speciation. A more challenging situation is encountered in the open ocean, where the absence of physical barriers would not restrict gene flow and would, in turn, preclude speciation. Yet, unexpected population structure has been found in open ocean plankton (Unal and Bucklin, 2010), indicating that even in environments with no apparent barriers to gene flow, cryptic speciation may potentially be present. The mesopelagic environment, which corresponds to a mid layer of open ocean water between a 200 m and 1000 m, also lacks apparent barriers to gene flow, and is the habitat of a large guild of fishes, the mesopelagic fishes, which prominently include lanternfishes. Here we focus on evaluating cryptic speciation in a group of lanternfishes of the genus *Benthosema*.

Lanternfishes, family Myctophidae, comprise approximately 250 species that live in the mesopelagic environment, usually between 300 and 1200 m, where sonar reflections on their swim bladder are mostly responsible for the Deep Scattering Layer phenomenon. During the night, they tend to migrate closer to the surface, usually within the first 100 m in depth. Myctophids are very abundant fishes, for instance they form the major part of the biomass in the Antarctic region and play an important ecological role as a major link in its food web

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(Eastman, 1993). Their abundance has also sparked the interest of communities and plans for large scale fisheries is underway in the Arabian Sea (Valinassab et al., 2007).

Lanternfishes in the genus *Benthosema* comprise only 5 recognized species. *Benthosema fibulatum*, ranges widely in the tropical Indo-Pacific, *Benthosema glaciale*, is found in the northern Atlantic and Mediterranean, *Benthosema panamense*, ranges from the northeastern to the tropical eastern Pacific, *Benthosema pterotum*, tends to be most abundant in the northern Indian Ocean, Oman Sea, Arabian Sea and extends in the western Pacific to Japan, and finally *Benthosema suborbitale* which exhibits the widest range by being circumglobal within the 50° latitudes (Valinassab et al., 2007). In general, lanternfishes are fragile and often in such poor shape when brought back from the depths as to make their identification difficult to impossible. Thus, molecular assessment is an important tool for identification purposes.

Recent interest in exploitation of *B. pterotum* in the Arabian Sea region prompted us to study the genetics of this poorly known group of fishes. We decided to examine all species of the genus using an array of nuclear and mitochondrial markers to investigate the relationships between these species. Our goal was first to establish a molecular phylogeny of the genus. Although cryptic species have not yet been described in the mesopelagic environment thus far, considering the difficulty to study the mesopelagic material and the complexity of lanternfish systematics, our second goal was to assess the presumption that cryptic species are likely to be present in this system.

In order to identify cryptic species, we needed to use a predefined species concept. Reviewing species definitions is beyond the scope of this study, suffice to say that a large number of concepts have been proposed, with the biological species concept (BSC) and the phylogenetic species concept (PSC) being arguably the two most generally used definitions (Coyne and Orr, 2004; De Queiroz and Gauthier, 1994). Mesopelagic fishes tend to be fragile and the transit in deep trawls from deeper water to the surface generally results in specimens that are either dead or in poor physical condition. This means that breeding is not possible, preventing experimental work on mating compatibilities, thus precluding direct testing of the BSC. In this study, we have therefore chosen to define species using the PSC. Importantly, two types of cryptic species may be present. When a barrier to gene flow is first established, cryptic species represent the very early stages of speciation, with the resulting cryptic species being sister species (von der Heyden et al., 2011). Alternatively, divergence may occur between species and morphological differences may or may not be produced, resulting in cryptic species that are not sister species. The presence of intervening species that are morphologically identifiable, supports evidence of bona fide separate species. In both situations, concordant phylogenetic patterns among several molecular markers are consistent with the PSC.

2. Materials and methods

2.1. Collections and DNA samples

Sampling sizes and locations are listed in Table 1. Sampling was performed with mid-water trawls, after collection specimens were immediately placed in 95% ethanol. All five recognized *Benthosema* species were obtained. *B. fibulatum* samples were obtained from India, *B. glaciale* from Florida, *B. panamense* from the Gulf of California in Guaymas, Mexico, and the Tropical Eastern Pacific, *B. pterotum* from Japan and Iran, and *B. suborbitale* from Japan. Two 16S RNA sequences from *B. glaciale*, and *B. suborbitale* deposited in GenBank were retrieved and added to our analysis. Phylogenetic relationships of lanternfishes are poorly known, thus the choice of the most closely related outgroup is difficult to make (Yamaguchi et al., 2000). For mitochondrial markers, we chose two lanternfish belonging to the same subfamily (Myctophidae), *Myctophum affine* and *Diaphus splendidus*, because their entire mitochondrial genome is available in GenBank, thus ensuring that the same individual was used for all mitochondrial molecular markers. For nuclear markers, we used the only available GenBank sequence for rhodopsin from *Electrona antarcticum.*

Table 1
Collection localities for *Benthosema* spp. Columns represent the number of individuals included in the study.

Species	Sampling site	n	Label	Source
Benthosema fibulatum	India, Arabian Sea	2	PH 14, 17	NIO, Goa, India
Benthosema glaciale	USA, Florida	2	FLO	T. Sutton, HBOI, Florida
	Norway	1	DQ532843	GenBank
Benthosema panamense	Mexico, Guaymas	4	GUA	U. Markaida, CICESE, Mexico
	Tropical E. Pac.	1	TEP	William Walker, NOAA, USA
Benthosema pterotum	Japan	2	JAP	C. Sassa, Nagasaki, Japan
	Oman Sea	6	IRA	Tooraj Valinassab, Iran
Benthosema suborbitale	Japan	4	JAP	C. Sassa, Nagasaki, Japan
	Japan	1	AB042179	GenBank

After collection, samples were immediately placed in 95% ethanol and stored at ambient temperature in the field, and then at 4 °C in the lab. Muscle or liver tissue was later dissected from these samples. Total genomic DNA was prepared from 75 to 150 mg of muscle or liver tissue by proteinase K digestion in lysis buffer (10 mM Tris, 400 mM NaCl, 2 mM EDTA, 1% SDS) overnight at 55 °C. This was followed by purification using chloroform extractions and alcohol precipitation (Sambrook et al., 1989).

2.2. PCR amplification and sequencing

Amplification of the mitochondrially encoded 12S and 16S ribosomal gene regions was accomplished with the primers 12SAL and 12SBH, and 16SAR and 16SBR (Kocher et al., 1989). Amplification of the mitochondrial cytochrome b used GLUDG-L and CB3H primers (Palumbi, 1996). Amplification of the nuclear rhodopsin marker (Rod) followed published nested amplification protocols (Sevilla et al., 2007) with RHO30F and RHO 319R for the first set of primers and Rho F2x and RhoR4n for the second set of primers. After purification following the manufacturer's protocol (ABI, Perkin-Elmer), sequencing was performed with the primers used in the PCR amplification on an ABI 3100 automated sequencer (Applied Biosystems, Foster City, CA).

2.3. Phylogenetic analyses

We used the computer program MAFFT (Katoh et al., 2002) implemented by the Geneious software package (Drummond et al., 2010) to align the DNA sequences. Phylogenetic relationships were assessed by Maximum Likelihood (ML, GARLI software, (Zwickl, 2006)), Maximum Parsimony (MP, PAUP* software (Swofford, 2003)), and Neighbor-Joining (NJ, PAUP software), methods. For Maximum Likelihood topologies, we conducted 10 independent runs in GARLI, using default settings and the automated stopping criterion, terminating the search when the ln score remained constant for 20,000 consecutive generations. The best likelihood of those runs was retained and is presented here (Fig. 1). Maximum Parsimony searches included 100 random addition replicates and TBR branch swapping with the Multrees option. Neighbor-Joining reconstructions used distances based on substitution models obtained with Modeltest (Posada and Crandall, 1998). Statistical confidence in nodes was evaluated using 2000 non-parametric bootstrap replicates (Felsenstein, 1985) (100 replicates for Maximum Likelihood in GARLI, using the automated stopping criterion set at 10,000 generations). Topological differences were tested using a Shimodaira and Hasegawa test (Shimodaira and Hasegawa, 1999) implemented by PAUP, based on resampling of estimated log-likelihoods tests (RELL, 1000 replicates).

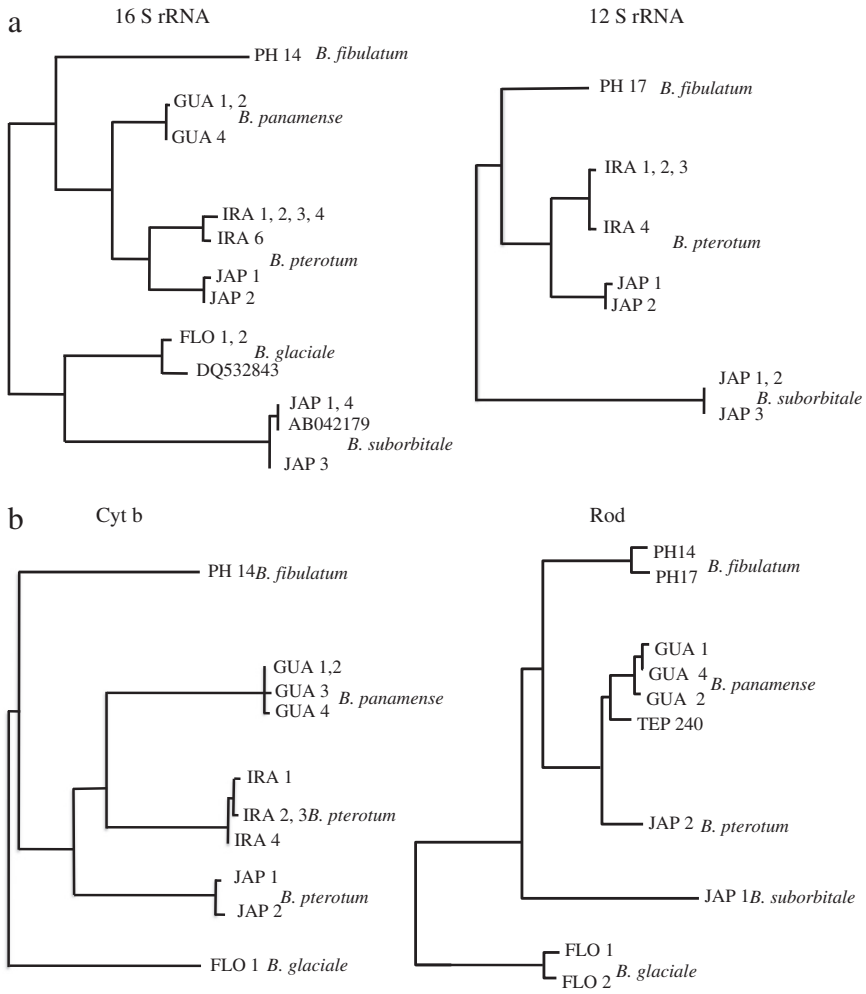

Fig. 1. Phylogenetic relationships of *Benthosema* spp. based on mitochondrial and nuclear markers (names of markers are above each tree). *Myctophum affine* and *Diaphus splendidus* were used as outgroups for the mitochondrial trees, *Electrona antarcticum* for the nuclear tree. Maximum Likelihood, Maximum Parsimony and Neighbor-Joining methods were used and resulted in similar topologies, Maximum Likelihood is presented here. Bootstrap values and statistical support are presented next to the nodes.

3. Results

3.1. DNA sequences

We used 25 individuals that included all 5 nominal species of *Benthosema*. PCR amplifications of lanternfishes were challenging and some reactions did not produce sequences good enough for proper alignment, resulting in datasets slightly different depending on the genes used for the analyses. The poor PCR performance in these species is most likely not due to intrinsic template characteristics (such as the presence of mutations in the primer regions or pseudogenes), but rather the presence of large quantities of lipids in the tissues, making DNA extractions difficult (Catul et al., 2010; Haque et al., 1981; De Koning and Evans, 1991). All sequences were deposited in GenBank with Accession numbers JX133737-JX133784 (provided upon publication).

Amplifications of the mitochondrial 12S rRNA, 16S rRNA, cytochrome b, and the nuclear Rod fragments yielded products of 369 bp, 524 bp, 697 bp, and 412 bp respectively. No insertions or deletions were detected. Variable sites corresponded to 129, 171, 258, and 133 bp respectively, while phylogenetically informative ones corresponded to 59, 134, 207, and 95 bp. None of the samples showed any heterozygous position at the nuclear locus, making the direct reading of the sequence straightforward and cloning unnecessary.

4. Phylogenetic relationships

The different phylogenetic methods yielded topologies that were not statistically significantly different (SH test, $p > 0.5$), thus we decided to present results based on the Maximum Likelihood method (Fig. 1). Where GenBank sequences were available, for *B. glaciale* and *B. suborbitale*, exact matches were found with our samples, which confirmed the idea that, while identification is very difficult in this group, consistent results were found.

As mentioned in the Materials and methods, sequences were difficult to obtain, resulting in datasets that were slightly different between different loci. Yet, for the portion of the trees that were common to all datasets, results were not statistically different (SH test, $p > 0.1$).

The genus *Benthosema* separated into two major clades. The first clade included the sister species *B. suborbitale* (from Japan) and *B. glaciale* (from Florida, and Norway). The second clade included the remaining three species, *B. fibulatum*, *B. pterotum* and *B. panamense*. Yet, for *B. pterotum*, samples grouped in two very divergent clades, one including all the samples from the Oman Sea (Iran), and one including the samples from the Pacific Ocean (Japan). Bootstrap support was high (>90%) for all the relationships discussed above (Fig. 1), except for the second clade, where support was lower (Fig. 1).

4.1. Genetic distances and divergence times

For 16S rRNA, where datasets were most complete, sequence divergence among species ranged between 19.0% and 4.4%. The distance between *B. pterotum* and *B. panamense* was 4.4%, while the distance between the two *B. pterotum* clades was 4.1%, a difference that was not statistically significant. Genetic distance between the two *B. pterotum* clades at the cytochrome b locus was 12.7%. Molecular clock work has been done at this locus, where analyses consider a mutation rate of 1.5 to 2.5% per million year a conservative estimate (e.g. Domingues et al., 2005). When using such a mutation rate, the two *B. pterotum* clades would have diverged approximately 8.5 to 5.0 Mya.

5. Discussion

Understanding the mechanisms that maintain biodiversity and promote speciation is an important scientific endeavor at the theoretical level, but also at a practical level at times where the loss of habitats threatens entire ecosystems. Recent advances in genetic investigations have uncovered cryptic species that are morphologically indistinguishable but genetically distinct. In the ocean, where observations are difficult, such cryptic species are likely to be widespread. Cryptic species have been described in a variety of habitats such as rocky reefs, coral reefs, the Antarctic, and in invading organisms. Yet, little is known about the mesopelagic environment. The open ocean, which was long thought as an area of large panmictic populations, has recently become the focus of genetic research on plankton and microorganisms that reveal significant genetic discontinuities (Hood et al., 2006; Unal and Bucklin, 2010). Here, we show that in the lanternfish genus *Benthosema*, cryptic species, closely related to *B. pterotum* are present. Importantly, our sampling was relatively limited, and it is likely that more cryptic species in this group of fishes is yet to be discovered.

The presence of unknown genetic stocks in the mesopelagic environment raises unique theoretical questions, as to the nature of breeding barriers in the open ocean, that have been maintained for a very long time when considering our divergence time estimates. Our results also warn managers about considering single management units when dealing with mesopelagic fish stocks.

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