



Morphological and molecular evidence of *Heteroteuthis dagamensis* in the Gulf of Mexico

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ABSTRACT.—Published records indicate that *Heteroteuthis dispar* (Ruppell, 1844) is found in the North Atlantic Ocean and that *Heteroteuthis dagamensis* Robson, 1924 inhabits the South Atlantic Ocean. However, specimens recently collected in the northern Gulf of Mexico ($n = 123$) show that *H. dagamensis* is the only species of the genus common in the Gulf of Mexico based on identification of male specimens. Also, comparison of DNA barcodes for three morphologically similar species of *Heteroteuthis*, *H. dispar*, *H. dagamensis*, and *Heteroteuthis hawaiiensis* (Berry, 1909) confirm that all are distinct species and indicate that *H. dagamensis* and *H. hawaiiensis* are closer genetically than either is to *H. dispar*.

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Heteroteuthinae is a pelagic subfamily of Sepiolidae, the bobtail squids, which are small, broad-bodied decapod cephalopods with a rounded posterior mantle and ear-like fins. The heteroteuthins have a large, visceral photophore, as well as a ventral shield (Young et al. 2007), and are found circumglobally in tropical and warm temperate oceans. Little is known about this group even though they are collected regularly (Rotermund and Guerrero-Kommritz 2010) over wide areas. They appear to be an important food source for fishes, crustaceans, and elasmobranchs (Sartor and De Ranieri 1995, Hoving et al. 2008), and are known from the stomach contents of albacore (*Thunnus alalunga* Bonnaterre, 1788) in the Adriatic Sea (Bello 1997) and of the giant red shrimp (*Aristaeomorpha foliacea* Risso, 1827) from the Strait of Sicily (Bello and Pipitone 2002). They have a 2-yr life cycle (Rotermund and Guerrero-Kommritz 2010) and are believed to lay their eggs on the ocean floor (Young et al. 2014).

Heteroteuthis is one of six genera in the subfamily. Currently, five species are recognized in the genus, the type species *Heteroteuthis dispar* (Ruppell, 1844), *Heteroteuthis nordopacifica* Kubodera and Okutani, 2011, *Heteroteuthis ryukuensis* Kubodera, Okutani and Kosuge 2009, *Heteroteuthis hawaiiensis* (Berry, 1909), and *Heteroteuthis dagamensis* Robson, 1924. The two known specimens of *H. ryukuensis* were found near Japan (Kubodera et al. 2009), *H. nordopacifica* is known only from a single female specimen also taken off Japan, whereas *H. hawaiiensis* is common in the central Pacific Ocean. *Heteroteuthis dispar* and *H. dagamensis* are found in the Atlantic Ocean. Historically, the *Heteroteuthis* species of the Atlantic Ocean were

Table 1. Heteroteuthid collections examined for the present study.

Source	Year collected	Total examined	<i>Heteroteuthis dagamensis</i> male	<i>Heteroteuthis dispar</i> male
WALTER HERWIG survey	1971	39	21	3
US National Museum of Natural History collection	Various	25	0	12
Sperm Whale Acoustics and Prey survey	2010	20	13	0
Offshore Nekton Sampling and Analysis Program survey	2011	39	16	0
Total		123	50	15

thought to be separated into North Atlantic and South Atlantic endemics, with *H. dagamensis* found south of the equator and *H. dispar* found north of the equator, including in the Gulf of Mexico (GOM) (Vecchione 2003, Judkins 2009). *Heteroteuthis hawaiiensis* and *H. dispar* are extremely similar in morphology with no obvious morphological differences distinguishing the species. *Heteroteuthis dagamensis* differs morphologically from *H. dispar* only in the positions of enlarged suckers along ventrolateral arms of mature males (Young et al. 2014). The DNA sequences of *H. dispar*, *H. dagamensis*, and *H. hawaiiensis* have not previously been compared. We present the recent finding of *H. dagamensis* in the Gulf of Mexico and revise the known distribution for *H. dagamensis* and *H. dispar* in the Atlantic Ocean. Also, DNA barcode sequences based on mitochondrial genes for three *Heteroteuthis* species were compared and we provide biogeographic interpretation of divergence among them.

MATERIALS AND METHODS

SPECIMENS EXAMINED.—One-hundred-and-twenty-three *Heteroteuthis* specimens were examined from the Atlantic Ocean and the Gulf of Mexico. Atlantic specimens came primarily from a 1971 WALTER HERWIG cruise in the eastern Atlantic Ocean. Other small collections housed at the US National Museum of Natural History (NMNH), Washington, DC, were also included (Table 1). Gulf of Mexico specimens came from two surveys: the Sperm Whale Acoustics and Prey Survey (SWAPS-2010) and the National Oceanic and Atmospheric Administration (NOAA) Natural Resource Damage Assessment (NRDA) Offshore Nekton Sampling and Analysis Program (ONSAP) surveys (NOAA/NRDA ONSAP-2011).

All specimens were fixed in 10% formalin and then preserved in 50% isopropyl alcohol. Organisms were identified to species when possible.

During the SWAPS-2010 survey, mantle tissue from one *H. dagamensis* was collected for DNA sequencing. For this sample only, the tissue was stored in 95% ethanol prior to specimen fixation.

DNA EXTRACTION AND AMPLIFICATION OF MITOCHONDRIAL CYTOCHROME C OXIDASE SUBUNIT I (COI) GENE.—The mitochondrial cytochrome c oxidase subunit I (COI “DNA barcode”) gene was sequenced from the tissue mentioned above. The tissue sample was dried at 37 °C for 1 hr. DNA was extracted from dried tissue using the DNeasy Blood and Tissue Kit (QIAGEN) following manufacturer’s instructions. The COI gene was then amplified through PCR using the universal COI primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198

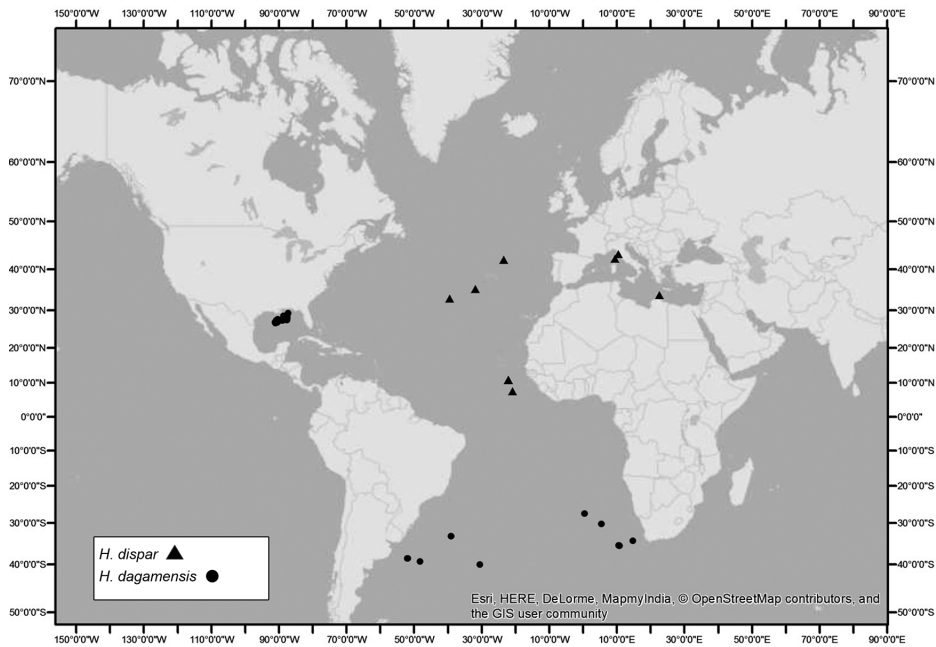


Figure 1. *Heteroteuthis dispar* (triangle) and *Heteroteuthis dagamensis* (circle) distribution in the Atlantic Ocean based on the male specimens examined.

(5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al. 1994). PCR reactions contained the following in 50- μ l solution: 1.5 mM MgCl₂; 1X Apex NH₄ Buffer; 0.5 μ M LCO1490; 0.5 μ M HCO2198; 6% DMSO; 1 μ g/ μ l BSA, 1 U Apex Red Taq DNA Polymerase; and 3 μ l of template DNA. Thermocycling conditions consisted of an initial denaturation at 95 °C for 2 min, followed by 35 cycles of 94 °C for 1 min, 48 °C for 1 min incrementally decreasing the temperature by 0.1 °C on each cycle, and 72 °C for 1 min, with a final extension at 72 °C for 7 min. COI gene PCR products were cloned using the CloneJET PCR Cloning Kit (Thermo Scientific) and commercially sequenced using vector primers.

Genbank accession records, AF035713 (*H. dispar*), AF000044, and AY293728 (*H. hawaiiensis*) were used for comparison purposes for sequence similarity among species (<http://www.ncbi.nlm.nih.gov/genbank>). Pairwise identities between the different species were calculated as $1 - (M/N)$, where M is the number of mismatching nucleotides and N the total number of positions along the alignment at which neither sequence has a gap character, using the publicly available Species Demarcation Tool software (SDT version 1.2; <http://web.cbio.uct.ac.za/~brejnev/>). The pairwise alignments were performed using the MUSCLE algorithm implemented in SDT over approximately 560 base pairs to match the shortest reference sequence (*H. dispar*).

RESULTS

Of the 123 *Heteroteuthis* specimens examined, only males could be identified confidently (Table 1). The distribution map of the 65 male specimens (Fig. 1) show that *H. dispar* was found in the eastern North Atlantic, whereas *H. dagamensis* was found

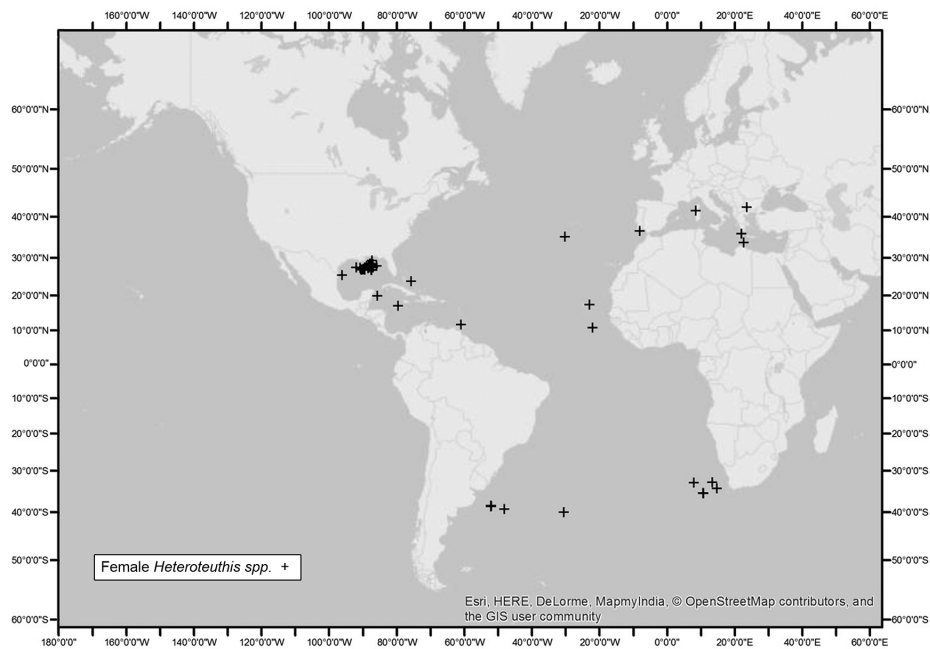


Figure 2. Distribution of female *Heteroteuthis* spp. (+) examined from the Atlantic Ocean.

in the Gulf of Mexico as well as the South Atlantic. Figure 2 displays the distribution of female specimens ($n = 58$).

The molecular sequence of the mitochondrial COI region of a *Heteroteuthis* specimen collected from the Gulf of Mexico SWAPS revealed that the barcode was different from GenBank records of both *H. dispar* (1 record, 86.5% pairwise identity) and *H. hawaiiensis* (2 records, approximate 95% pairwise identity) (Table 2). Based on the sequence differences and morphological characteristics, this specimen was identified as *H. dagamensis*. All 29 male specimens examined from the Gulf of Mexico cruises in the same region as the sequenced specimen were morphologically consistent with *H. dagamensis*.

DISCUSSION

The distribution of *H. dagamensis* reported previously in cephalopod literature is in the southeastern Atlantic Ocean and southwestern Indian Ocean (Reid and Jereb 2005). *Heteroteuthis dispar* has been documented in the North Atlantic Ocean and the Mediterranean Sea (Reid and Jereb 2005). Rotermund and Guerrero-Kommritz (2010) recently extended the range of *H. dispar* in the North Sea and the reported *H. dagamensis* in the southwest Atlantic Ocean near the coast of Brazil.

The Gulf of Mexico has recently been the focus for many deep water studies. Prior to 2010, there were very few records of *H. dispar* reported in the Gulf of Mexico (Voss 1956, Passerella 1990, Judkins 2009). Our study reports on 59 *Heteroteuthis* specimens from the Gulf of Mexico, of which all males are *H. dagamensis*. This contradicts the statement of Rotermund and Guerrero-Kommritz (2010) that their results “show that the Atlantic Ocean can clearly be separated into the northern hemisphere,

Table 2. Pairwise identities among mitochondrial cytochrome c oxidase subunit I (COI) genes of identified squid species and reference sequences from Genbank.

Species	1	2	3	4
1. <i>Heteroteuthis hawaiiensis</i> _AF000044	100.0			
2. <i>Heteroteuthis hawaiiensis</i> _AY293728	98.8	100.0		
3. <i>Heteroteuthis dagamensis</i> KR606071	95.0	95.4	100.0	
4. <i>Heteroteuthis dispar</i> _AF035713	85.6	85.8	86.5	100.0

where *H. dispar* occurs and the southern hemisphere where *H. dagamensis* occurs.” The morphological characters of *H. dispar* and *H. dagamensis* are very similar, with no way to identify females to species besides location collected.

The mitochondrial DNA barcode was compared to confirm species separation for three species, *H. dispar* and *H. dagamensis* in the Atlantic Ocean, and very similar *H. hawaiiensis* from the central Pacific Ocean. The latter was included because of the possibility that it may be synonymous with *H. dispar* (Young et al. 2014). The DNA barcode comparison indicated that the three taxa examined are indeed separate species, with *H. dispar* as the most distinct of the three (Table 2). However, the genetic distance of *H. dispar* from congeners presumed to be closely related was surprisingly high. Subsequent communications with L Allcock (National University of Ireland Galway, pers comm, October 2015) on a separate project indicated that the *H. dispar* sequence in GenBank was very likely a misidentified *Sepiolo*. Therefore, no confidently identified sequence for *H. dispar* is currently available. Because *H. dispar* is morphologically indistinguishable from *H. hawaiiensis*, we expect that the sequence for true *H. dispar* is very similar to that of *H. hawaiiensis*. Comparison of two specimens of *H. hawaiiensis* is consistent with within-species variability. The difference between *H. hawaiiensis* and *H. dagamensis* is consistent with distinct species. Until a barcode sequence for a confidently identified *H. dispar* is available, we conclude tentatively that the morphological difference between males of *H. dagamensis* and *H. dispar* is indicative of species-level distinction. *Heteroteuthis hawaiiensis* and *H. dagamensis* most likely diverged with the emergence of the Isthmus of Panama. The complete emergence of the Isthmus of Panama closed the Inter-American Seaway by the middle Miocene, based on new geological and biological evidence (Bacon et al. 2015, Montes et al. 2015). Vicariance speciation resulting from the emergence of the Isthmus of Panama is well known. Some examples include echinoderms (McCartney et al. 2000, Coppard et al. 2013), octocorals (Vargas et al. 2008), and bivalves (Marko and Jackson 2001). Our barcode results suggest that a speciation event for two species of *Heteroteuthis* (*H. dagamensis* and *H. hawaiiensis*) took place after the Inter-American Seaway closed (Smith et al. 2003, Jackson and O’Dea 2013).

A question that requires further specimen collection is “where is the ecotone for *H. dispar* and *H. dagamensis*?” A possible answer might be the Caribbean Sea; Caribbean *Heteroteuthis* specimens are scarce in collections, and the material examined for the present study (Fig. 2) are unidentifiable females. A study examining the beaks of the female heteroteuthids could shed light on possible differences within beak structure for this morphologically identical group of squids. Sampling in the Caribbean Sea would be an important next step to assess possible distributional overlap of *H. dispar* and *H. dagamensis*.

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