Title	Morphological and molecular assessments of bobtail squids (Cephalopoda: Sepiolidae) reveal a hidden history of biodiversity
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Publication Date	2021-01-20
Publication Information	Fernández-Álvarez, Fernando Á., Sánchez, Pilar, & Villanueva, Roger. (2021). Morphological and Molecular Assessments of Bobtail Squids (Cephalopoda: Sepiolidae) Reveal a Hidden History of Biodiversity. Frontiers in Marine Science, 7(1233). doi:10.3389/fmars.2020.632261
Publisher	Frontiers Media
Link to publisher's version	https://doi.org/10.3389/fmars.2020.632261
Item record	http://hdl.handle.net/10379/16494
DOI	http://dx.doi.org/10.3389/fmars.2020.632261

frontiers in Marine Science

ORIGINAL RESEARCH
published: 20 January 2021
doi: 10.3389/fmars.2020.632261

Morphological and Molecular Assessments of Bobtail Squids (Cephalopoda: Sepiolidae) Reveal a Hidden History of Biodiversity

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Specialty section:
This article was submitted to Marine Evolutionary Biology, Biogeography and Species Diversity, a section of the journal Frontiers in Marine Science

Received: *22 November 2020*
Accepted: *22 December 2020*
Published: *20 January 2021*

Citation:
Fernández-Álvarez FÁ, Sánchez P and Villanueva R (2021) Morphological and Molecular Assessments of Bobtail Squids (Cephalopoda: Sepiolidae) Reveal a Hidden History of Biodiversity. Front. Mar. Sci. 7:632261. doi: 10.3389/fmars.2020.632261

Molecular species delimitation assists taxonomic decisions for challenging species, like cryptic species complexes. Bobtail squids (Family Sepiolidae Leach, 1817) are a very diverse group of benthic and nektonic small to medium size cephalopods with many taxonomic questions to solve. In this study we provided new sequence data for 12 out 17 Mediterranean bobtail squid species including all the genera present i n the area. Other relevant species from other parts of the world were used as comparison. The combined use of several molecular species delimitation methods consistently showed a picture of hidden biodiversity within this family which hinders the use of molecular data isolated from morphological characters. On the one hand, those methods provided contrasting results for the number of recognized species of some morphologically well-defined species. We suggest this can be an effect of recent speciation phenomena followed by an intense morphological drift. On the other hand, cryptic biodiversity was detected among members of several monophyletic clades assigned to the same nominal species, pointing to recent speciation phenomena without a parallel morphological evolution. Although Mediterranean bobtail diversity has been extensively studied for more than a century, a new species of *Stoloteuthis* Verrill (1881) was discovered and described here, both using molecular and morphological methods. This new research stresses the necessity of combined morphological and molecular studies to correctly assess cephalopod diversity.

urn:lsid:zoobank.org:act:57AFBB38-18EA-4F80-B1D4-73519C12694F.

Keywords: Mollusca, cryptic species, molecular species delimitation, systematics, taxonomy, new species

INTRODUCTION

The use of molecular species delimitation methods is widespread in modern systematics and taxonomic research. Methods such as the Automatic Barcode Gap Discovery (ABGD, Puillandre et al., 2012), the statistical parsimony networks (e.g., Pons et al., 2006), the Bayesian Poisson Tree Processes model (bPTP, Zhang et al., 2013) and the Generalized Mixed Yule Coalescent approach (Fujisawa and Barraclough, 2013) assist in taking taxonomic decisions for challenging species, like cryptic species complexes (e.g., Fernández-Álvarez et al., 2020) or other morphologically challenging organisms. Molecular species identification methods, such DNA barcoding (Hebert et al., 2003), provide tools for identification of challenging organisms, such as undescribed

ontogenetic stages of known species (e.g., Fernández-Álvarez et al., 2017, Olmos-Pérez et al., 2018a,b; Villanueva et al., 2020).

The family Sepiolidae Leach, 1817, commonly known as bobtail squids, is a very diverse group of benthic and nektonic small to medium size cephalopods. In recent years, the taxonomy and systematics of the family has been dynamic, including a few molecular phylogenetic studies (e.g., Groenenberg et al., 2009; Sanchez et al., 2019), major systematics reviews (Bello, 2019, 2020) and the description of a relatively large number of new species (e.g., de Heij and Goud, 2010; Kubodera and Okutani, 2011; Sanchez et al., 2019). In the last decade, new species have been even discovered in the Mediterranean Sea (Bello, 2013; Bello and Salman, 2015), where bobtail squid diversity has been extensively studied for more than a century (Bello, 2015, 2019). The closure of the Strait of Gibraltar is an historical process that may explain the contemporary Mediterranean sepiolid endemism and high species richness, especially in the western area (Mangold and Boletzky, 1988; Bello, 2003; Rosa et al., 2019). Sepiolid systematics and taxonomy rely mostly on the morphology of the light organs or photophores, the male copulatory organ or hectocotylus, and the female sperm storage organ or bursa copulatrix (Reid and Jereb, 2005; Bello, 2020). Three subfamilies are recognized: Heteroteuthinae Appellöf, 1898, Rossiinae Appellöf, 1898 and Sepiolinae Leach, 1817. Even though hectocotylus morphology is a reliable character, recently it was discovered that the previously recognized intraspecific variability in one species undercovered pseudocryptic biodiversity (Groenenberg et al., 2009; de Heij and Goud, 2010). Moreover, identifications based on early life stages and females are challenging and misidentifications are abundant on GenBank (Groenenberg et al., 2009), hindering identification based only on DNA barcoding.

Here, we examined most of the Mediterranean biodiversity for the family Sepiolidae, performed several molecular delimitation methods in order to assess the actual diversity of this group, and provide a solid molecular framework for future studies based on DNA identification methods. Additionally, we discovered a new species of Heteroteuthinae, which is described here using both molecular sequences and morphological characters.

MATERIALS AND METHODS

Sample Collection

In total, 77 newly collected bobtail squids were analyzed (**Table 1**), covering 12 of the 17 known Mediterranean species and all the genera (Bello, 2015, 2020). Sampled individuals were mostly collected in the Mediterranean coasts of the Iberian Peninsula from Tiñoso Cape in the south to Blanes in the north, including the Balearic Islands, between the years 2006 and 2015. Most of this material was collected during the Spanish research cruises MEDITS 2006 (https://www.sibm.it/SITO%20MEDITS/principaleprogramme.htm) and FORMED 5 (Demestre et al., 2017). Additional material were collected by commercial fishing trawlers from Tarragona and Vilanova i la Geltrú fishing ports and from the "sonsera" littoral artisanal fishery from Blanes (Lleonart et al., 2014), which is a small scale littoral boat seine fishing method aimed to catch sand eels. Four individuals of *Heteroteuthis dispar* (Rüppell, 1844) were collected in NE Atlantic waters near the Canary Islands in the spring of 2015, during the MAFIA research cruise (Olivar et al., 2017). **Figure 1** summarized the collection localities. After collection, the individuals were frozen at −20°C until their study in the lab. In a time lapse ranging from a few weeks to 8 years, the material was defrosted, identified, and photographed. From each specimen, a portion of the mantle was removed for DNA extraction and the rest of the body was preserved as a morphological voucher.

Morphological Identification and Vouchering

Individuals were identified following the morphological key of Bello (1995). In the case of the genera *Sepietta* (Naef, 1912a) and *Sepiola* Leach, 1817, only males were identified based on the hectocotylus morphology to ensure reliable identifications. After removal the tissue for DNA extraction, the specimens were fixed in 4% buffered formalin for 3–10 days and transferred to ethanol 70%. The specimens are deposited in the Biological Reference Collections (CBR-ICM) at the Institut de Ciències del Mar (ICM-CSIC, Barcelona, Spain) under the accession numbers provided in **Table 1** (Guerrero et al., 2020).

The description provided here for a new butterfly squid is based in a careful morphological examination of all the available specimens ($n = 4$). The measurements and morphometric characters of these specimens followed Roper and Voss (1983) as: dorsal mantle length (DML), ventral mantle length (VML), fin width (FW), fin length (FL), fin base (FB), head width (HW), head length (HL), funnel length (FnL), arm I-IV length (AIL-AIVL), tentacle length (TeL), tentacle club length (CL), and web depth A-E (WDA-E). Two additional measurements were taken: the occipital band length (mantle-head fusion) (OBL), defined as the length of the fusion between the head and the mantle, and the ventral shield length (VSL), as the shield length along its midline. All the morphological measurements from this study were performed on formaline-fixed individuals stored in 70% ethanol.

Beaks and radulae were extracted from selected individuals. For the beaks, the upper and lower rostral lengths (URL and LRL, respectively) were measured according to Clarke (1986). The radula was observed under a Hitachi S3500N scanning electron microscope (SEM). At the beginning of SEM preparation, the radulae were dehydrated in an increasing concentration of ethanol (80, 90, and 96%) until they were saturated in absolute ethanol. Each ethanol bath lasted 10 min. After complete dehydration in the ethanol series, the samples were dried to a critical point using CO_2 as the transition liquid. After the drying stage, samples were mounted on stubs with double-sided conductive sticky tape to place them in the preferred position. The mounted samples were sputter coated with gold–palladium before SEM observations.

The spermatophores from selected males were extracted for the assessment of the spermatophore count (SpC), and the spermatophore length (SpL) based on 30 randomly selected spermatophores that were measured according with

TABLE 1 | List of specimens used in this work, indicating the sampling locality, the number of studied sequences (n), and the GenBank Accession Numbers.

Species	Locality	*n*	Morphological voucher	GenBank Accesion Number	References
Subfamily Rossinae (Appellöf, 1898)					
Neorossia caroli (Joubin, 1902)	Off Vilanova i la Geltrú, NW Mediterranean	1	ICMC000330	MW261922	This work
	Alboran Sea, SW Mediterranean Sea	3	ICMC000335-ICMC000337	MW261922-MW261925	This work
Rossia macrosoma (delle Chiaje, 1830)	Off Tarragona, NW Mediterranean Sea	1	ICMC000329	MW261927	This work
	Off Barcelona, NW Mediterranean Sea	3	ICMC000332-ICMC000334	MW261928-MW261930	This work
	Off Cartagena, NW Mediterranean Sea	1	ICMC000331	MW261926	This work
	North Sea	8		KM517929-KM517936	Gebhardt and Knebelsberger, 2015
Semirossia tenera (Verrill, 1880)	Gulf of St. Lawrence, Pacific Ocean	1		AY426436	Nishiguchi et al., 2004
Subfamily Heteroteuthinae (Appellöf, 1898)					
Heteroteuthis dagamensis (Robson, 1924)	Northern Gulf of Mexico, Atlantic Ocean	1		KR606071	Judkins et al., 2016
	North Atlantic	2		MT223185, MT219813	Taite et al., 2020
	New Zealand, SW Pacific	8		MK185916-MK185923	Braid and Bolstad, 2019
Heteroteuthis dispar (Rüppell, 1844)	SW Mediterranean Sea	1	ICMC000394	MW261936	This work
	Off Torrevieja, NW Mediterranean Sea	2	ICMC000393, ICMC000395	MW261935, MW261937	This work
	Ebro Delta, NW Mediterranean Sea	2	ICMC000396-ICMC000397	MW261938-MW261939	This work
	NE Atlantic Ocean, MAFIA stations 11 and 12 (Olivar et al., 2017)	3		MW261940 MW260133-MW260134	This work
Heteroteuthis hawaiiensis (Berry, 1909)		1		AF000044	Carlini and Graves, 1999; Lindgren et al., 2004
		1		AY293728	Nishiguchi et al., 2004
Heteroteuthis ryukyuensis (Kubodera et al., 2009)	Yonaguni Island, NW Pacific Ocean	1		AB591074	Kubodera and Okutani, 2011
Heteroteuthis sp. KER		1		MK185924	Braid and Bolstad, 2019
Sepiolina nipponensis (Berry, 1911)	Tosa Bay, NW Pacific Ocean	1		AY293727	Nishiguchi et al., 2004
	Tosa Bay, NW Pacific Ocean	1		AB591073	Kubodera and Okutani, 2011
Sepiolina petasus Kubodera and Okutani, 2011	Okinawa Island, NW Pacific Ocean	1		AB591071	Kubodera and Okutani, 2011
Stoloteuthis cthulhui **sp. nov.**	Balearic Sea, NW Mediterranean Sea	2	ICMC000164-ICMC000165	MW261931-MW261932	This work
	Alboran Sea, SW Mediterranean Sea	1	ICMC000163	MW261934	This work
	Balearic Sea, NW Mediterranean Sea	1	ICMC000166	MW261933	This work
Stoloteuthis japonica Kubodera and Okutani, 2011	Okinawa Island, NW Pacific Ocean	1		AB591072	Kubodera and Okutani, 2011

(Continued)

TABLE 1 | Continued

Species	Locality	*n*	Morphological voucher	GenBank Accesion Number	References
Stoloteuthis leucoptera (Verrill, 1878)	Gulf of Maine, NW Atlantic Ocean (M. Vecchione, pers. comm.)	1		AF000068	Carlini and Graves, 1999; Nishiguchi et al., 2004
"Heteroteuthidinae" sp	Off Vigo, NE Atlantic Ocean	2		MG407056-MG407057	Olmos-Pérez et al., 2018b
Subfamily Sepiolinae (Leach, 1817)					
Rondeletiola minor (Naef, 1912c)	Off Tarragona, NW Mediterranean Sea	2	ICMC000380-ICMC000381	MW261945-MW261946	This work
	Off Torrevieja, NW Mediterranean Sea	1	ICMC000382	MW261947	This work
	Off Ibiza Island, NW Mediterranean Sea	6	ICMC000383-ICMC000384 ICMC000386-ICMC000389	MW261948-MW261949MW261951-MW261954	This work
	Palos Cape, SW Mediterranean Sea	1	ICMC000385	MW261950	This work
	Valencia Gulf, W Mediterranean Sea	3	ICMC000390-ICMC000392	MW261955-MW261957	This work
	Off Banyuls-sur-mer, NW Mediterrean Sea	1		AY293725	Nishiguchi et al., 2004
	Off Vigo, NW Atlantic Ocean	8		MG407030-MG407034, MG407038-MG407042	Olmos-Pérez et al., 2018b
Sepietta neglecta (Naef, 1916)	Off Tarragona, NW Mediterranean Sea	1	ICMC000356	MW261975	This work
	Off Denia, NW Mediterranean Sea	1	ICMC000357	MW261976	This work
	North Sea	8		FJ231301, FJ231312, FJ231324-FJ231329	Groenenberg et al., 2009
	North Sea	1		KM517940	Gebhardt and Knebelsberger, 2015
Sepietta obscura (Naef, 1916)	Off Tarragona, NW Mediterranean Sea	1	ICMC000355	MW260131	This work
	Off Banyuls-sur-mer, NW Mediterrean Sea	1		AY293723	Nishiguchi et al., 2004
Sepietta oweniana (Férussac and d'Orbigny, 1834–1848)	Off Tarragona, NW Mediterranean Sea	14	ICMC000338-ICMC000351	MW261977-MW261991	This work
	Tiñoso Cape, SW Mediterranean Sea	2	ICMC000352-ICMC000353	MW261992- MW261993	This work
	Gulf of Valencia, W Mediterranean Sea	1	ICMC000354	MW261994	This work
	Off Banyuls-sur-mer, NW Mediterrean Sea	2		AY293722, AY293724	Nishiguchi et al., 2004
	North Sea	3		FJ231298-FJ231300	Groenenberg et al., 2009
	Baltic Sea and Northern Sea	4		KM517941-KM517944	Gebhardt and Knebelsberger, 2015
		1		AF036912	Nishiguchi et al., 1998
Sepiola affinis (Naef, 1912c)	Off Blanes, NW Mediterranean Sea	1	ICMC000358	MW261964	This work
	Off Arenys de Mar, NW Mediterranean Sea	2	ICMC000359-ICMC000360-	MW260132, MW261965	This work

(Continued)

TABLE 1 | Continued

Species	Locality	*n*	Morphological voucher	GenBank Accesion Number	References
cf. *Sepiola affinis*	Adelaide, Pacific Ocean (?)	1		DQ646730	Jones et al., 2006
Sepiola atlantica d'Orbigny, 1842 in (Férussac and d'Orbigny, 1834–1848)	North Sea	2		KM517945-KM517946	Gebhardt and Knebelsberger, 2015
	North Sea	11		FJ231303, FJ231304, FJ231308-FJ231311, FJ231314, FJ231316-FJ231318, FJ231322	Groenenberg et al., 2009
	Off Vigo, NE Atlantic Ocean	1		AY293721	Nishiguchi et al., 2004
	Off Vigo, NE Atlantic Ocean	1		MG407043	Olmos-Pérez et al., 2018b
Sepiola intermedia (Naef, 1912c)	Off Tarragona, NW Mediterranean Sea	8	ICMC000361-ICMC000368	MW261966-MW261973	This work
	Ebro Delta, NW Mediterranean Sea	1	ICMC000369	MW261974	This work
	Off Banyuls-sur-mer, NW Mediterrean Sea	1		AY293720	Nishiguchi et al., 2004
Adinaefiola ligulata (Naef, 1912c)	Off Tarragona, NW Mediterranean Sea	1	ICMC000370	MW261941	This work
	Off Torrevieja, NW Mediterranean Sea	1	ICMC000371	MW261942	This work
	Gulf of Valencia, NW Mediterranean Sea	2	ICMC000372-ICMC000373	MW261943-MW261944	This work
	Off Banyuls-sur-mer, NW Mediterrean Sea	1		AY293717	Nishiguchi et al., 2004
	Off Vigo, NE Atlantic Ocean	1		MG407045	Olmos-Pérez et al., 2018b
Adinaefiola pfefferi (Grimpe, 1921)	North Sea	1		KM517947	Gebhardt and Knebelsberger, 2015
	North Sea	4		FJ231292, FJ231293, FJ231295, FJ231296	Groenenberg et al., 2009
	Off Vigo, NE Atlantic Ocean	5		MG407046, MG407050-MG407053	
Sepiola robusta (Naef, 1912c)	Off Tarragona, NW Mediterranean Sea	5	ICMC000374-ICMC000378	MW261959-MW261963	This work
	Off Alicante, NW Mediterranean Sea	1	ICMC000379	MW261958	This work
	Off Banyuls-sur-mer, NW Mediterrean Sea	1		AF035710	Nishiguchi et al., 1998
	Off Banyuls-sur-mer, NW Mediterrean Sea	3		AY293716, AY293718, AY293719	Nishiguchi et al., 2004
	Off Vigo, NE Atlantic Ocean	1		AF035707	Nishiguchi et al., 1998
	Atlantic Ocean	1		AF035713	Nishiguchi et al., 1998
Sepiola tridens (de Heij and Goud, 2010)	North Sea	16		KM517948-KM517963	Gebhardt and Knebelsberger, 2015
	North Sea	9		FJ231305-FJ231307, FJ231313, FJ231315, FJ231319-FJ231321, FJ231323	Groenenberg et al., 2009

(Continued)

TABLE 1 | Continued

Species	Locality	*n*	Morphological voucher	GenBank Accesion Number	References
	Off Vigo, NE Atlantic Ocean	2		MG407054-MG407055	Olmos-Pérez et al., 2018b
Sepiolinae sp. 1		1		AY545194	Strugnell et al., 2004
Sepiolinae sp. 2		1		AY557523	Lindgren et al., 2004
Lusepiola birostrata (Sasaki, 1918)	Tosa Bay, NW Pacific Ocean	2		AY293710, AY293715	Nishiguchi et al., 2004
Euprymna berryi (Sasaki, 1929)	Tosa Bay, NW Pacific Ocean	1		AY293711	Nishiguchi et al., 2004
Euprymna hyllebergi (Nateewathana, 1997) (OTU 1)	Rayong, W Pacific Ocean	3		DQ646710-DQ646712	Jones et al., 2006
	Gulf of Thailand, W Pacific Ocean	1		AY293714	Nishiguchi et al., 2004
Euprymna hyllebergi (Nateewathana, 1997) (OTU 2)	Off Phuket, NE Indian Ocean	7		DQ646703-DQ646709	Jones et al., 2006
Euprymna scolopes (Berry, 1913) (OTU1)	Off Hawaii Island, central Pacific Ocean	10		DQ646731-DQ646740	Jones et al., 2006
	Kaneohe Bay, Hawaii Island, central Pacific Ocean	1		AF035714	Nishiguchi et al., 1998
Euprymna scolopes (Berry, 1913) (OTU2)	Off Paiko, Honolulu Island, central Pacific Ocean	1		AY293712	Nishiguchi et al., 2004
Euprymna tasmanica (Pfeffer, 1884) (OTU 1)	Off NW Australia, E Indian Ocean	1		DQ646729	Jones et al., 2006
Euprymna tasmanica (Pfeffer, 1884) (OTU 2)	Off SE Australia, SW Pacific Ocean	6		DQ646722-DQ646727	Jones et al., 2006
Euprymna tasmanica (Pfeffer, 1884) (OTU 3)	Off NW Australia, E Indian Ocean	1		DQ646728	Jones et al., 2006
Euprymna tasmanica (Pfeffer, 1884) (OTU 4)	Off SW Australia, SW Pacific and SE Indian Oceans	9		DQ646713-DQ646721	Jones et al., 2006
	Off Melbourne, SW Pacific Ocean	1		AY293713	Nishiguchi et al., 2004
Outgroup					
Sepiadariidae Fischer, 1882 in 1880–1887 (Fischer, 1880–1887)					
Idiosepius pygmaeus (Steenstrup, 1881)		1		AF000046	Carlini and Graves, 1999; Lindgren et al., 2004

Nigmatullin et al. (2003). The specimen ICMC000165 had intact spermatophores, while in the remaining studied animals the spermatophoric reaction was triggered (Marian, 2015). Sixteen spermatophores from these males were used to make the following spermatophore measurements: head (SpH), ejaculatory tube (SpE), cement body (SpCe), seminal reservoir (SpS), and posterior empty part (SpEm). As comparative morphological material, eight males and seven females of *Stoloteuthis leucoptera* (Verrill, 1878), collected off Namibia (Villanueva and Sánchez, 1993) and deposited at the CBR-ICM under the accession numbers ICMC000167-ICMC000181, were examined.

Following the recommendation number 11 from the Appendix B of the International Code of Zoological Nomenclature (International Commission on Zoological Nomenclature, 1999), we set up four abbreviations to unambiguously refer to the four genera starting with "S" mentioned in this work: "S." for *Sepiola*, "St." for *Sepietta*, "Sl." for *Stoloteuthis* and "Se." for *Sepiolina* (Naef, 1912b).

DNA Extraction, Amplification, and Sequencing

Tissues for molecular analysis were fixed in 96% ethanol. Total genomic DNA was extracted from an ethanol-fixed piece of the mantle using the NZY Tissue gDNA isolation kit (NZYTech, Lisbon, Portugal), following the manufacturers' protocol and resuspended in a final volume of 100 μL. A negative control that contained no sample was included in every isolation round to check for contamination during the experiments. Sequences from the partial mitochondrial cytochrome *c* oxidase I (COI) gene were amplified, using the primer pair LCO1490 and HCO2198

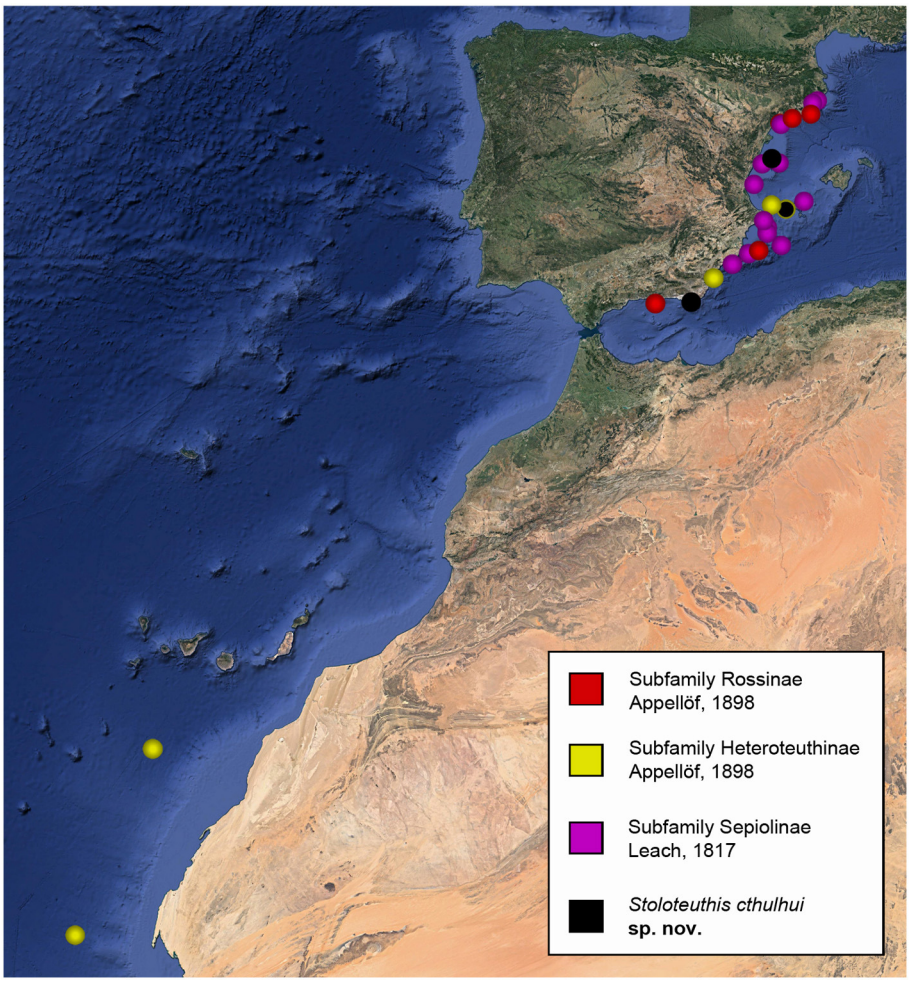

FIGURE 1 | Sampling localities of the specimens sequenced in this work sorted by subfamilies. Sampling localities of *Stoloteuthis cthulhui* **sp. nov**. are also indicated. Modified from Google Earth Pro.

(Folmer et al., 1994). Standard PCR reactions were performed using the NZYTaq Green PCR Master Mix (NZYTech, Portugal) following the manufacturer's protocol in a total volume of 25 mL, which included 0.5 μM of each primer, 25 ng of template DNA and PCR-grade water up to 25 μL. PCRs consisted of an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 45 s, with a final extension of 5 min at 72°C. The amplified products were sequenced using both forward and reverse PCR primers on an ABI 3730xl. DNA sequence data were edited and aligned with Geneious 8.1.5 (http://www.geneious.com). The GenBank Accession numbers of the sequences used in this work together with the morphological voucher Accession numbers are summarized in **Table 1**.

Phylogenetic Analyses

The new sequences obtained in this work together with selected sequences available from GenBank were analyzed (**Table 1**). *Idiosepius pygmaeus* (Steenstrup, 1881) was selected as outgroup for the phylogenetic analyses. Some sequences coming from GenBank were shorter, so Ns were added to align them with the complete sequences. The final alignment contained 245 sequences and 658 positions. As an initial analysis, a Maximum Likelihood tree was obtained through the IQTree portal (Trifinopoulos et al., 2016) [available at http://iqtree.cibiv.univie.ac.at] using the automatic model selection feature. The selected model was TIM2+F+I+G4 according to both Akaike and Bayesian Informative Criteria. The support of the branches was calculated after 2,000 ultrafast bootstrap generations.

Several molecular species delimitation analyses were performed. The online version of software Automatic Barcode Gap Discovery (ABGD, Puillandre et al., 2012) [available at https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html] was used to check the distribution and size of a potential barcoding gap. Previous empirical studies have shown that individuals assigned to a statistical parsimony network with a 95% probability for COI usually correspond to species (Pons et al., 2006; Hart and Sunday, 2007; Bond and Stockman, 2008;

TABLE 2 | Interspecific mean *p*-distance percenteages (%) between sepiolid taxa.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
Neorossia caroli (1)																																				
Rossia macrosoma (2)	11.1																																			
Semirossia tenera (3)	4.11	12																																		
Heteroteuthis dagamensis (4)	12.9	13.6	15.2																																	
Heteroteuthis dispar (5)	14.2	15.1	16.4	4.28																																
Heteroteuthis hawaiinensis (6)	13.4	14.2	15.7	4.36	3.27																															
Heteroteuthis ryukuensis (7)	14.9	14.2	15.8	11.5	12.8	11.7																														
Heteroteuthis sp. KER (8)	12.7	13.7	15.2	3.85	2.87	2.54	11.8																													
Sepiolina nipponensis (9)	14.4	15.3	15.4	11.8	14	13.6	9.53	13.8																												
Sepiolina petasus (10)	14.9	16	15.6	12	13.2	13.3	10.8	12	14																											
Stoloteuthis cthulhui **sp. nov.** (11)	13.5	14.3	14.5	12.7	13.5	12.9	13.1	12.6	12.5	14.7																										
Stoloteuthis leucoptera (12)	13.4	13.5	14.8	12.8	14.2	12.7	11.2	13.2	11.6	14.4	3.5																									
Stoloteuthis japonica (13)	13.6	14.9	14.6	12	13.4	12.7	9.13	12.8	2.43	12.8	12.9	11.6																								
"Heteroteuthidinae" sp. (14)	13.3	13	13.9	13	12.9	11.7	12.7	11.9	13.7	13.9	13.4	12.5	13.1																							
Rondeletiola minor (15)	14	15.3	15.3	15.8	16.6	15.9	13.7	15.8	13.8	14.7	16	15.1	13.1	14.8																						
Sepietta neglecta (16)	14.7	14	14.8	13.4	15.1	14.6	12	14.2	11.6	12.6	13.6	12.6	11.6	12.1	11.3																					
Sepietta obscura (17)	14.4	15.1	15.5	11.3	12.1	11.8	9.43	11.9	9.63	10.8	13.8	12.1	9.63	12.2	12	8.64																				
Sepietta oweniana (18)	14.7	13.8	16.3	11	12.2	11.8	9.78	11.4	11.4	10.9	12.8	12	11	11.4	10.4	7.57	7.65																			
Sepiola affinis (19)	14.2	14	14.8	11.1	12.2	11.7	9.89	11.7	10.2	10.5	13.1	11.3	9.48	11.4	11.6	9.96	7.3	8.46																		
Sepiola atlantica (20)	14.3	13.8	14.6	11.2	12.2	11.8	9.94	11.8	9.94	10.3	13.5	11.4	9.53	11.3	11.7	10.6	7.2	8.36	0.81																	
Sepiola intermedia (21)	13.8	13.4	14.1	10.8	11.8	11.4	9.55	11.4	9.51	9.92	13.1	11	9.51	10.9	12	10.1	6.82	7.93	1.45	1.08																
Adinaefiola ligulata (22)	13.2	15.3	13.8	12.6	13.8	13.2	11.8	13.2	11	12	13.1	12.4	10.3	12.7	13.8	10.8	10.2	11.8	10.1	10.3	10.3														
Adinaefiola pfefferi (23)	12.1	13	13.3	11.6	13.2	12.6	8.58	12.4	9.27	9.07	11.1	9.8	8.74	11.1	13	9.17	8.15	8.58	8.48	8.38	7.94	8.3														
Sepiola robusta (24)	14.5	14.4	15.1	12.5	13.7	13.9	12.8	13.1	12.5	11.4	15.1	14.9	12.4	12.7	13.8	11.9	11.6	10.2	10.5	10.4	9.96	10.8	9.26													
Sepiola tridens (25)	13.2	14.1	13.8	10.7	12	11.4	8.93	11.4	8.51	9.14	12.7	11	8.31	10.5	12.9	9.95	6.4	8.35	3.56	3.46	3.48	9.73	7.33	9.41												
Sepiolinae sp. 1 (26)	15	15.6	16.4	13.4	14.6	14.1	13.6	13.8	13.8	13	13.9	13.8	14	14.5	15.9	13.6	12.1	12.4	14	14	13.5	12.8	12.9	14.7	13.2											
Sepiolinae sp. 2 (27)	13	13.9	14.4	11.1	12.4	12.1	11.8	12	11.4	12.2	13.1	12.8	11	13.1	13.7	12.4	9.84	9.46	9.48	9.53	9.55	11.6	9.66	11.5	8.53	13.4										
Lusepiola birrostrara (28)	12.8	13.4	13.7	13.4	13.9	13.7	13.5	12.8	13.9	11.1	14	13.9	13.7	13.2	14.1	11.7	11.3	10.9	10.8	10.7	10.2	11.1	10.2	12.8	10.3	14.1	12.3									
Euprymna berryi (29)	14.9	15.2	15.8	13.8	13.8	13.1	12.6	13.2	15	13	14.4	14	14.4	13.9	16.1	14.4	14.1	13.8	13.3	13.4	12.9	11.8	12	14.9	13.4	12	14	12.3								
Euprymna hyllebergi (OTU 1) (30)	16.4	16.9	17.8	15.8	15.6	16.3	12.7	15.4	14.4	14.6	16.6	15.4	12.9	14.7	16.3	14.8	14.1	12.8	13.9	14	13.7	12.7	11.5	14.8	13.3	12.3	14.4	15.5	11.3							
Euprymna hyllebergi (OTU 2) (31)	14.6	16.8	16.4	13.9	15.3	15	11.6	14.1	13.3	14.3	15.9	15	12.2	14.2	16.1	14.5	12.8	12.1	13.6	13.6	13.6	12.9	10.7	14.1	12.6	11.9	13.5	14.5	10.9	3.99						
Euprymna scolopes (OTU 1) (32)	15	14.9	16.4	14.4	15.4	14.5	13.8	13.5	16	15.3	15.1	15.1	15.3	14.2	14.8	14.4	14.6	12	13.5	13.5	13.5	15.9	13.6	15	13.5	13.7	12.9	13.6	10.9	12.7	12				
Euprymna scolopes (OTU 2) (33)	14.9	14.8	16.2	14.2	15.2	14.3	13.8	13.4	15.8	15.2	15	15	15.2	14.1	15	14.2	14.5	11.8	13.3	13.4	13.4	15.8	13.4	14.9	13.4	13.6	12.8	13.5	10.8	12.6	12	0.3			
Euprymna tasmanica (OTU 1) (34)	15.5	16.2	16.6	12.7	13.4	13.3	12.2	12.6	13.8	13	14.3	14.2	12.8	14.3	16	13.4	12.1	12	13.5	13.6	13.2	13.8	11.3	13.3	12.4	10.8	13.4	13.9	11.2	13.1	12	11	12		
Euprymna tasmanica (OTU 2) (35)	16.9	17.6	17.2	15.8	15.7	15.5	13.2	15.4	14.8	14.7	16.3	15.5	14.3	15.5	15.6	13.6	13.9	13.4	14.6	14.6	14.3	14.7	12.9	14.8	14.2	13.7	14	15	12.6	12.6	12	14	14	12	
Euprymna tasmanica (OTU 3) (36)	15.5	17.5	15.8	14.6	15.6	15.5	12.4	14.8	14	14	15	14.6	14.2	15.1	15.8	12.8	13.5	13.1	13.2	13.4	13	13.4	11.8	14.5	13.8	11.6	14.6	13.9	11.2	10.9	9.6	12	12	10	8	
Euprymna tasmanica (OTU 4) (37)	15	16.6	15.1	14.8	15.8	15.6	11.8	15.1	13.7	13.7	15.4	13.9	13.5	15.5	16.3	12.8	12.6	12.7	13.6	13.3	12.9	13.9	10.9	13.3	12.7	12	13.7	14.2	12.2	10.8	9.2	12	12	9.7	9	4.4

Euprymna hyllebergi, Euprymna scolopes, and Euprymna tasmanica were splitted in several OTUs (see Material and Methods section for more details).

Kang et al., 2015), so the software TCS v.1.21 (Clement et al., 2000) was used to construct haplotype networks with a maximal connectivity limits of 95, 98, and 99%. Bayesian Poisson Tree Processes (bPTP; Zhang et al., 2013) was applied through the bPTP portal [http://species.h-its.org/ptp/]. The initial tree was the Maximum Likelihood tree obtained by IQTree, and the bPTP portal was used with the default parameters. For the Generalized Mixed Yule Coalescent approach (GMYC, Fujisawa and Barraclough, 2013), a Bayesian analysis under a lognormal relaxed clock was performed with Beast v1.10.4 (Drummond and Rambaut, 2007) in the CIPRES server (Miller et al., 2010) using the TN93 gamma model for 100 million generations sampled each 10,000 generations. Tracer v1.6 (Rambaut and Drummond, 2003–2009) was used to check whether the parameter had reached values of effective sample size over 100 and a burn-in of 25% was applied through TreeAnnotator v.1.10.4 (Drummond and Rambaut, 2007). The resulting maximum credibility tree was visualized and converted to Newick format with FigTree v1.4.4 (Rambaut, 2006–2009) and submitted to the GMYC web server [http://species.h-its.org/gmyc/] by both the single and multi-threshold methods.

Uncorrected genetic distances (*p*-distances) between and within species (excluding the outgroup taxa) were calculated with MEGA6 (Tamura et al., 2013). The specific labels employed here were defined according with the morphological identification of the morphological vouchers or the taxonomic labels provided in the original work (**Table 1**). *Euprymna hyllebergi* (Nateewathana, 1997), *Euprymna scolopes* (Berry, 1913) and *Euprymna tasmanica* (Pfeffer, 1884) showed intraspecific distances larger than those found at an interspecific level between other species of the dataset and identified as different species by some of the molecular species delimitation methods. Therefore, they were, respectively, split into two to four Operational Taxonomic Units (OTUs) in the genetic divergence analyses (**Tables 1**–**3**).

When possible, species identifications of the clades were based on voucher specimens. Several sequences uploaded to GenBank from previous works were originally based on misidentified specimens. The correct identifications, the original identifications, their GenBank Accession numbers and the original references are summarized in **Table 4**.

TABLE 3 | Intrapecific *p*-distance percenteages (%).

Species	Mean	Range	*n*
Neorossia caroli	0.1	0–0.2	4
Rossia macrosoma	0.75	0–1.4	13
Semirossia tenera	N/A	N/A	1
Heteroteuthis dagamensis	0.38	0–2.2	11
Heteroteuthis dispar	0.05	0–0.2	8
Heteroteuthis hawaiinensis	1.42	N/A	2
Heteroteuthis ryukyuensis	N/A	N/A	1
Heteroteuthis sp. KER	N/A	N/A	1
Sepiolina nipponensis	0	N/A	2
Sepiolina petasus	N/A	N/A	1
Stoloteuthis cthulhui **sp. nov.**	0.24	0–0.3	4
Stoloteuthis japonica	N/A	N/A	1
Stoloteuthis leucoptera	N/A	N/A	1
"Heteroteuthidinae" sp.	0.2	N/A	2
Rondeletiola minor	0.44	0–1.2	14
Sepietta neglecta	0.04	0–0.2	11
Sepietta obscura	0.2	N/A	2
Sepietta oweniana	0.24	0–0.6	28
Sepiola affinis	0.34	0–0.8	3
Sepiola atlantica	0	0	14
Sepiola intermedia	0.12	0–0.6	10
Adinaefiola ligulata	0	0–0.5	5
Adinaefiola pfefferi	0.3	0	5
Sepiola robusta	0.27	0–1.8	12
Sepiola tridens	0.02	0	25
Sepiolinae sp 1	N/A	N/A	1
Sepiolinae sp 2	N/A	N/A	1
Lusepiola birostrata	0.2	N/A	2
Euprymna berryi	N/A	N/A	1
Euprymna hyllebergi (OTU 1)	0.1	0–0.3	4
Euprymna hyllebergi (OTU 2)	0.41	0–0.6	7
Euprymna scolopes (OTU 1)	0.28	0–0.8	11
Euprymna scolopes (OTU 2)	N/A	N/A	1
Euprymna tasmanica (OTU 1)	N/A	N/A	1
Euprymna tasmanica (OTU 2)	0.18	0.2–0.9	6
Euprymna tasmanica (OTU 3)	N/A	N/A	1
Euprymna tasmanica (OTU 4)	0.35	0–0.8	10

Euprymna hyllebergi, Euprymna scolopes, and Euprymna tasmanica were split in several OTUs (see section Material and Methods for more details). N/A, not available.

RESULTS

Molecular Species Delimitation

Thirty seven different clades of bobtail squids were identified based on molecular and morphological data (**Figure 2**). The individuals examined in the present study and identified based on morphological characters always clustered together in a single clade, but some inconsistencies were detected on previously published sequences. Some of them are amended according with our identifications over other members of their respective clades (**Table 4**). Sepiolinae sp. 1 was not originally identified at the species level (Strugnell et al., 2004) and the only sequence available did not match with any correctly identified clade. Sepiolinae sp. 2 was originally identified as *Sepiola affinis* (Naef, 1912c) (Lindgren et al., 2004; **Table 4**), but this sequence did not group with the correctly identified *S. affinis*. The sequences AY293710 and AY293715 were described as *Lusepiola birostrata* (Sasaki, 1918) or *Euprymna morsei* (Verrill, 1881) and their divergence is compatible with an intraspecific distance. Sanchez et al. (2019) assigned those sequences to *Sepiola* (= *Lusepiola*) *birostrata*. *Euprymna hyllebergi*, *E. scolopes,* and *E. tasmanica* showed 2–4 highly divergent clades.

The species delimitation methods provided conflicting results. The eight partitions of the ABGD identified from 26 to 41 groups depending of the prior maximal distance. It identified 28 groups with a maximal intragroup distance of 2.1%, 33 groups with maximal distances of 0.04–1.2% and 41 groups

TABLE 4 | Misidentifications from GenBank.

Species	Originally identified as	GenBank Accession number	References
Sepietta oweniana	*Sepietta neglecta*	AY293722	Nishiguchi et al., 2004
Sepietta oweniana	*Euprymna stenodactyla*	AF035704*	Nishiguchi et al., 1998
Sepietta oweniana	*Sepietta obscura*	AF036912	It is not included in Nishiguchi et al. (1998) although it is indicated as such in GenBank.
cf. *Sepiola affinis*	*Euprymna tasmanica*	DQ646730	Jones et al., 2006
Sepiola intermedia	*Sepiola affinis*	AF035706*	Nishiguchi et al., 1998
Sepiola intermedia	*Sepiola rondeleti*	AY293720	Nishiguchi et al., 2004
Sepiola robusta	*Sepiola atlantica*	AF035707	Nishiguchi et al., 1998
Sepiola robusta	*Adinaefiola ligulata*	AF035710	Nishiguchi et al., 1998
Sepiola robusta	*Heteroteuthis dispar*	AF035713	Nishiguchi et al., 1998
Sepiola robusta	*Sepiola affinis*	AY293716	Nishiguchi et al., 2004
Sepiola robusta	*Sepiola intermedia*	AY293718	Nishiguchi et al., 2004
Sepiolinae sp. 2	*Sepiola affinis*	AY557523	Lindgren et al., 2004
Euprymna scolopes	*Rondelentiola minor*	AF035714	Nishiguchi et al., 1998
Euprymna scolopes	*Euprymna morsei*	AF035702	Nishiguchi et al., 1998

**The sequence presented stop signals. They were considered pseudogenes and excluded from the phylogenetic analyses. The correct species, the original identification, the GenBank Accesion, and the original reference numbers are indicated.*

for 0.2–0.1 maximal distances. Based on the distribution of the distances of the whole dataset, no discrete barcoding gap was detected. **Figure 2** represents the species assemblages based on the results from ABGD with a maximal distance prior of 1.2%, since it is closer to the observed maximal intra-specific distance observed in the dataset. The TCS analysis with 95% of maximum connectivity identified 34 networks. *Sepiola affinis*, *Sepiola atlantica* d'Orbigny, 1842 in Férussac and d'Orbigny, 1834–1848, and *Sepiola intermedia* (Naef, 1912d) formed a single network, while *E. hyllebergi*, *E. scolopes,* and *E. tasmanica* were split in 2–4 networks (**Figure 2**). The TCS analyses at 98 and 99% of divergence differed in the relations between *S. affinis*, *S. atlantica*, *S. intermedia* and the sequence DQ646730 (identified as *E. tasmanica*). In the 99% analysis, the three species and DQ646730 formed four independent networks, while in the 98% analysis *S. affinis*, *S. atlantica,* and DQ646730 formed a single network. Both 98 and 99% analyses over-split *Rossia macrosoma* (delle Chiaje, 1830), *Heteroteuthis dagamensis* (Robson, 1924), *Heteroteuthis hawaiinensis* (Berry, 1909), and *Sepiola robusta* (Naef, 1912c) in two networks each. The Maximum Likelihood solution of the bPTP analysis recovered 38 species majorly consistent with the results of the TCS 95% analysis and the morphological and molecular assignations of the Maximum Likelihood tree (**Figure 1**). However, two species were detected for *E. scolopes* OTU 1 and three for *H. dagamensis* (one including all New Zealand specimens and the two others included North Atlantic specimens); and *S. affinis*, *S. atlantica,* and *S. intermedia* were recognized as a single species. The highest Bayesian supported solution recognized 83 species, and *S. intermedia* was recognized as a different species than *S. affinis* and *S. atlantica* (results not shown). The single threshold method of the GMYC identified 28 clusters (confidence interval 20–32) and 41 entities (confidence interval 26–48) with a significant Likelihood Ratio test (LR = 4.312508e-07). *Sepiola affinis* including the sequence DQ646730, *S. atlantica* and *S. intermedia* were recognized as three independent species. Two species were recognized for *H. dagamensis*: one including all the Atlantic individuals and another formed by the New Zealand specimens. *Rossia macrosoma* and *Rondeletiola minor* (Naef, 1912c) were split in two species each. The multi-threshold method revealed 31 clusters (confidence interval 24–31) and 43 entities (confidence interval 31–43) also with a significant result (LR = 1.928034e-08). In this analysis *S. affinis* including the sequence DQ646730 and *S. intermedia* were recognized as independent species, but *S. atlantica* was recognized as two species. *Rossia macrosoma*, *E. hyllebergi* OTU 2 and *Sepietta oweniana* d'Orbigny in Férussac and d'Orbigny, 1834–1848 were also recognized as two species each, while *E. tasmanica* OTUs 3 and 4, and *Stoloteuthis japonica* Kubodera and Okutani, 2011 and *Sepiolina nipponensis* (Berry, 1911) were merged in single species. It also split Atlantic and New Zealand *H. dagamensis* in two different species.

Uncorrected *p*-distances across species ranged from 0.81 to 18% (**Table 2**; mean: 12.7%) and from 0 to 2.3% (**Table 3**; mean: 0.3%) at an intraspecific level. Regarding interclade distances, it should be noted that most interspecific distances were values above 3% [in accordance to the values for the family Sepiolidae published by Gebhardt and Knebelsberger (2015)], but the distances were lower between *Se. nipponensis* and *Sl. japonica* (2.4%); *H. dispar, Heteroteuthis* sp. KER [from the Kermadec Islands, see Braid and Bolstad (2019)] and *H. hawaiinensis* (2.5–2.8%); and between *S. intermedia*, *S. atlantica,* and *S. affinis* (1.45%). The identification of the last three species is assured since it was confirmed morphologically (Groenenberg et al.,

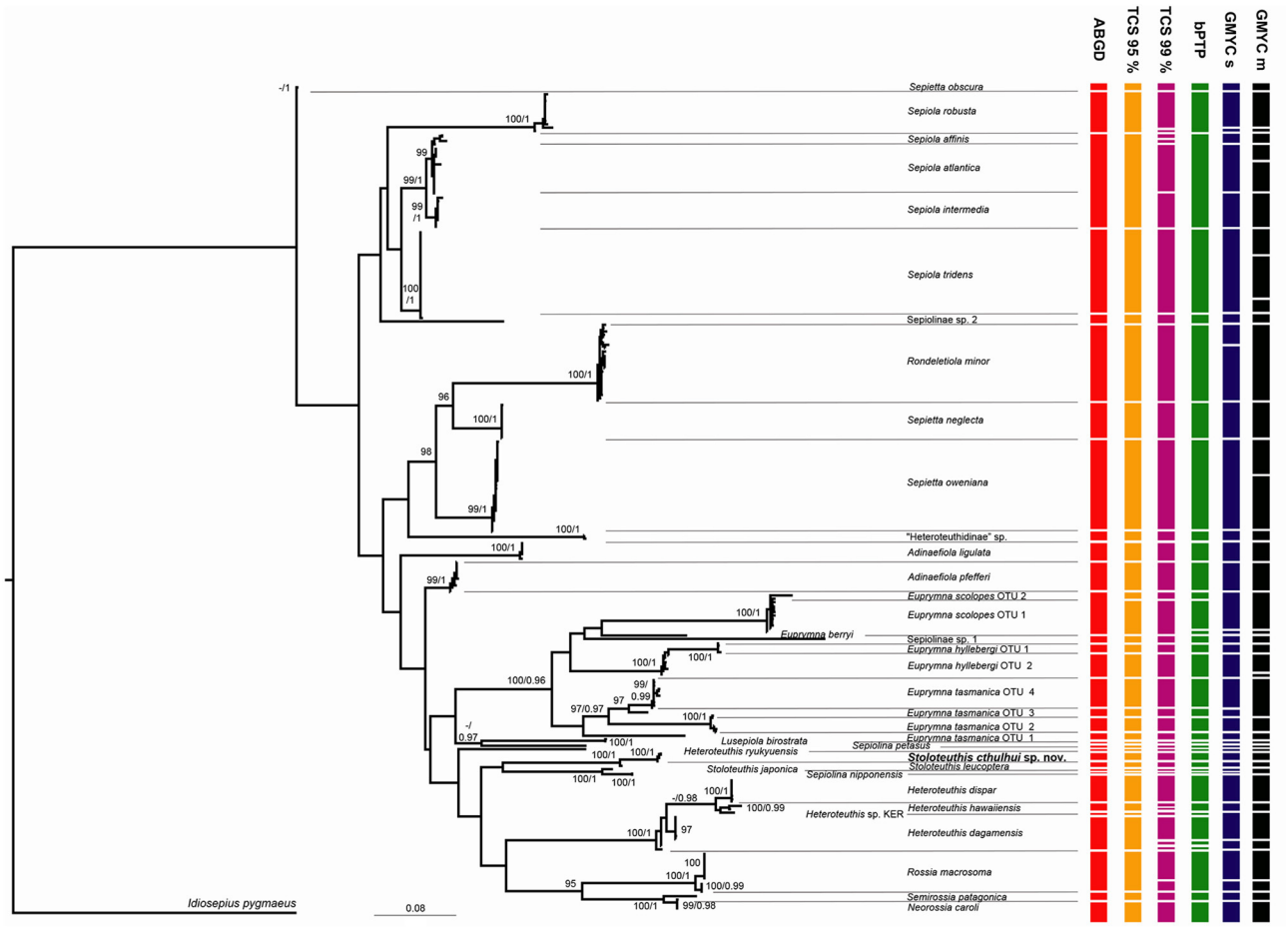

FIGURE 2 | Maximum Likelihood tree obtained with the software IQTree. Numbers on the nodes signal bootstrap percentages after 2,000 ultrafast bootstrap generations and posterior probabilities from the Beast analysis. Only support values above 95% and 0.95 are indicated. Results of molecular species delimitation methods (ABGD, TCS 95%, TCS 99%, bPTP, GMYC single threshold and GMYC multi-threshold, the last two abbreviated as GMYC s and GMYC m, respectively) are summarized.

2009 for *S. atlantica*; this work for *S. affinis* and *S. intermedia*, **Figure 3**). Regarding intraclade distances, in *H. dagamensis* distances above 2% were reported (**Table 2**).

Among the analyzed Rossinae species [*Neorossia caroli* (Joubin, 1902), *Rossia macrosoma* (delle Chiaje, 1830), and *Semirossia tenera* (Verrill, 1880)] interspecific distances ranged from 4 to 12.2% (**Table 2**) and at intraspecific level ranged between 0 and 1.4%. Among *R. macrosoma* two different clades diverging 1.4% were identified. One of them was formed by four Mediterranean individual while the other one was formed by one Mediterranean and eight North Sea individuals. Among Heteroteuthinae, interspecific *p*-distances ranged from 2.4 to 17.8% (**Table 2**) and the intraspecific *p*-distances range was 0–2.2% (**Table 3**). Between the Mediterranean *Stoloteuthis* individuals and *Sl. leucoptera* the distance levels were typical for interspecific distances. Among Sepiolinae, the interspecific *p*-distances ranged from 0.81 to 15.8%, but if *S. affinis*, *S. atlantica* and *S. intermedia* are excluded, the lowest intraspecific distance detected is 3.4% and there is no overlapping between intra- and interclade *p*-distances (**Tables 2**, **3**), as occurs in the other available sequences of the other two subfamilies. In the genus *Euprymna* (Steenstrup, 1887), cryptic biodiversity was found in *E. hyllebergi* and *E. tasmanica*, formed by 2 and 4 highly divergent clades, respectively (**Figure 2**, **Tables 2**, **3**). Between the two clades of *E. hyllebergi*, a distance of 3.9% was reported. Distances of 4.3–11.6% are found between the four divergent clades of *E. tasmanica*. The TCS and bPTP analyses found cryptic biodiversity in *E. scolopes*, although the ABGD, the GMYC, and the *p*-distance analyses did not find it.

Systematics

Subfamily Heteroteuthinae Appellöf, 1898

Genus *Stoloteuthis* Verrill, 1881

Stoloteuthis cthulhui

sp. nov.

(**Figure 4**, **Tables 5**, **6**)

Stoloteuthis leucoptera— Orsi Relini and Massi (1991)

Stoloteuthis leucoptera— Volpi et al. (1995)

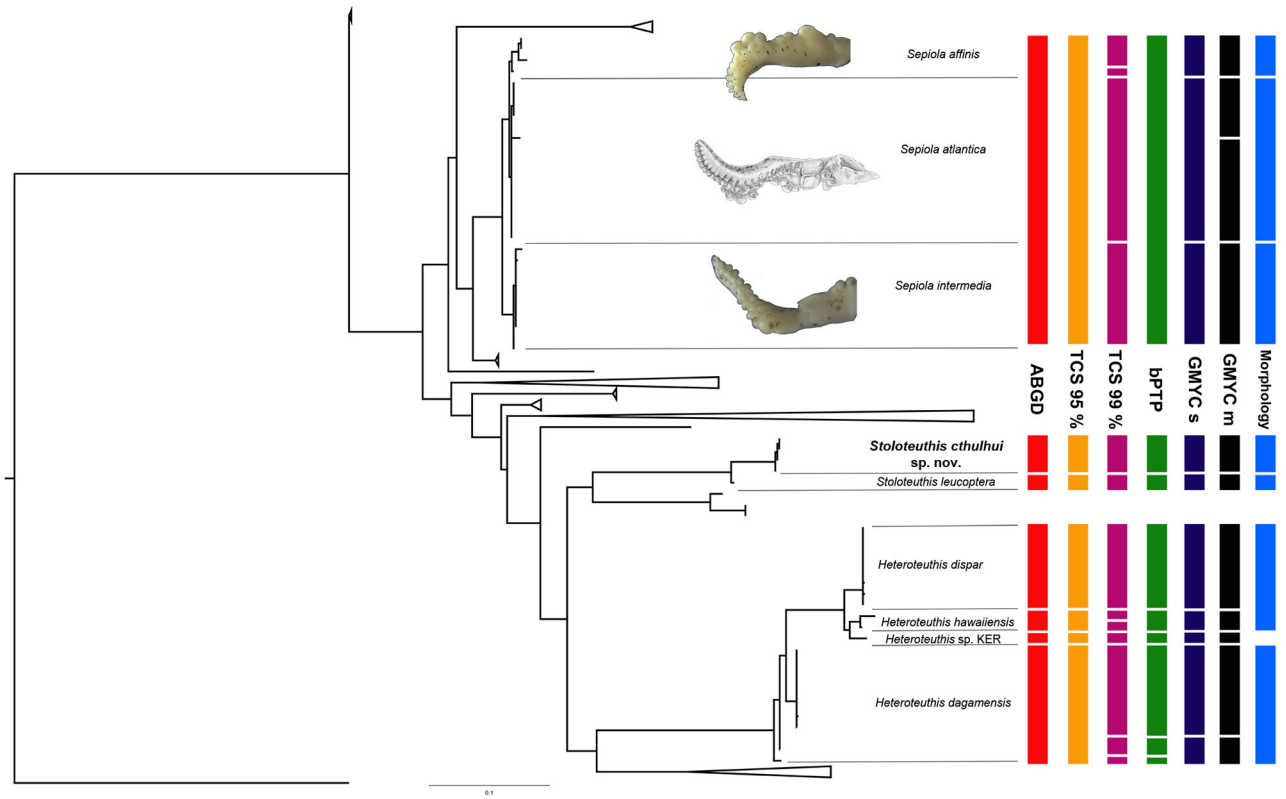

FIGURE 3 | Selected clades of the Maximum Likelihood tree depicted in **Figure 2**. A summary of the species delimitation based on molecular methods (ABGD, TCS 95%, TCS 99%, bPTP, GMYC single threshold, and GMYC multi-threshold, the last two abbreviated as GMYC s and GMYC m, respectively) and morphology is provided at the right of the phylogenetic tree. Morphological diferences for the depicted species of the genus *Sepiola* is based in our material and de Heij and Goud (2010); for *Stoloteuthis cthulhui* **sp. nov.** and *Stoloteuthis leucoptera* based on the results of this study; and for *Heteroteuthis* spp. based on Vecchione and Young (2007), Judkins et al. (2016), and Braid and Bolstad (2019). *Sepiola atlantica* hectocotylus reproduced with permission from de Heij and Goud (2010).

Stoloteuthis leucoptera— Würtz et al. (1995)
Stoloteuthis leucoptera— Sánchez et al. (1998)
Stoloteuthis leucoptera— Giordano and Carbonara (1999)
Stoloteuthis leucoptera— Biagi et al. (2002)
Stoloteuthis leucoptera— Cuccu et al. (2010)
Stoloteuthis leucoptera— Fanelli et al. (2012)
Stoloteuthis leucoptera— Quetglas et al. (2013)
Stoloteuthis leucoptera— Bello (2003 and references therein)
Stoloteuthis leucoptera— Zaragoza et al. (2015)
Stoloteuthis leucoptera— Keller et al. (2017)
Stoloteuthis leucoptera— Bello et al. (2020 and references therein).

Diagnosis

Stoloteuthis with a maximum size of 18 mm of mantle length; with a narrow occipital band; with a ventral shield of around 80% of the ventral mantle surface; with a wide head; tentacles representing 251–379% of the mantle length; males with glands in the first two thirds of both dorsal and ventral margins of arms I; males with rows 2–4 of ventral suckers slightly enlarged, ventral, and dorsal rows 5–6 enlarged to the same level in arms II; males with 3–4 series of suckers at the tip of arms IV.

For a diagnosis of the genus *Stoloteuthis* see Verrill (1881, Appendix:417).

Type Material

Holotype. ICMC000164, mature male, 13.9 mm ML, 22 May 2006, 38.85783°N 0.98°E, between 451 and 457 m depth. Paratypes: ICMC000165, mature male, 17.9 mm ML, 22 May 2006, 38.85783°N 0.98°E, between 451 and 457 m depth; ICMC000163, mature female, 12.3 mm ML, 11 May 2006, 36.63767°N 2.45833°W, between 324 and 326 m depth; ICMC000166, mature male, 16.7 mm ML, 25 May 2006, 40.1671667°N 0.7645°E, between 77 and 81 m depth.

Type Locality

SW off Ibiza Island, Mediterranean Sea. 38.85783°N, 0.98°E, between 451 and 457 m depth.

Etymology

The specific epithet *cthulhui* was erected in honor to the fiction cosmic horror entity "Cthulhu" created by Howard Phillips Lovecraft (1890–1937), which holds both cephalopod-like tentacles and wings (Lovecraft, 1928), resembling the pair of fins of this new species of butterfly squid. Cthulhu was described

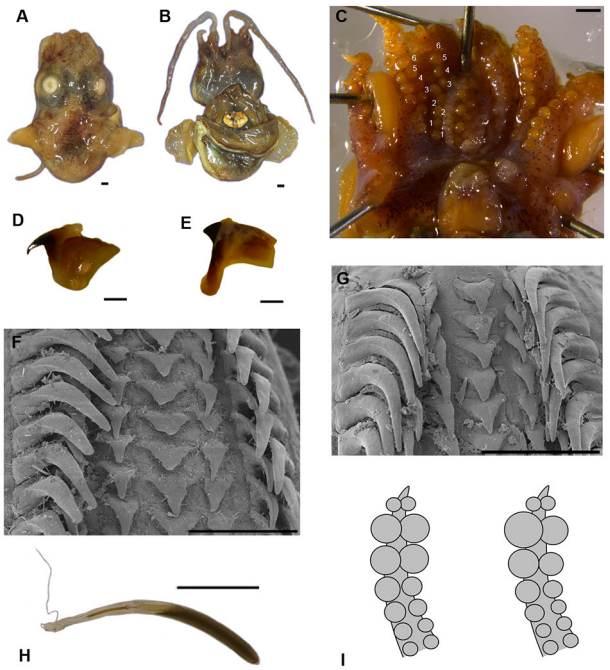

FIGURE 4 | *Stoloteuthis cthulhui* **sp. nov**. **(A)** Dorsal view of the mature female specimen 12.3 mm ML ICMC000163. **(B)** Ventral view of the mature male specimen 13.9 mm ML ICMC000164 (holotype). **(C)** Dissected arm crown showing the arrangement of the suckers of the arms of the mature male 17.9 mm ML ICMC000165. Numbers above the suckers of the right arm II depict the rows of suckers. **(D)** Lower beak of the mature specimen 16.7 mm ML ICMC000166. **(E)** Upper beak of the specimen ICMC000163. **(F)** Radula of the specimen ICMC000165. **(G)** Radula of the *Stoloteuthis leucoptera* specimen ICMC000175, mature female 13.3 mm ML. **(H)** Spermatophore of the specimen ICMC000166. **(I)** Arrangement and relative size of arm II suckers of the male of *Stoloteuthis cthulhui* (left) and *Stoloteuthis leucoptera* (right). Scale bars: A–E, H, 1 mm; F,G, 200 μm.

in the literature as male, so a male gender genitive suffix was applied. Pronunciation: /kəˈθu:lu:/.

Proposed Vernacular Names

Cthulhu's butterfly squid (English), globito de Cthulhu (Spanish), morralet de Cthulhu (Catalan).

Description

Butterfly squid up to 18 mm DML (**Figures 4A,B**). The body is muscular with a rounded posterior end. The mantle dorsally is fused directly with the head forming a narrow commissure of 5–8 mm and is heavily and uniformly pigmented. Ventral mantle is anteriorly bilobed with roughly the same extension of dorsal mantle or slightly larger, with a ventral shield of dark brown pigment and an iridescent greenish hue roughly representing nearly 80% of the ventral mantle surface. This ventral shield is surrounded by a shallow yellowish bright band. Fins are large, oval, and less pigmented than the mantle, attached dorsally in the lateral mantle. The mantle component of the funnel/mantle locking-apparatus is straight with a low ridge at the mantle edge and the funnel component is a straight simple groove. The funnel reach the anterior edge of the eyes and the pigmentation forms a diamond patch from the funnel tip toward the mantle; near the tip of the funnel there is a round patch devoid of chromatophores, and the funnel sections covered by the lobes of the mantle also lack of chromatophores.

The head is bulbous, wide and uniformly pigmented, representing from 88 to 145% of the ML. Eyes are large and occupy most part of the head. Olfactory organs are prominent. Arms are short and muscular with a well-developed keel in arms III. Arm formula: II ≈ III > I > IV. Two rows of suckers in both arms, but in males the arms IV might appear to have four rows. Female arm crown lacks of any enlarged suckers. Arm suckers in all arms more developed in males than in females. Arms I of mature males with well-developed glands in the first two thirds of both dorsal and ventral margins. Arms II suckers from rows 2–6 are slightly enlarged according with the following pattern: rows 2–4 of ventral suckers are slightly enlarged and the ventral and dorsal rows 5–6 have the same degree of enlargement (**Figure 4C**). A well-developed web unites the arms until approximately the last third of the arm length from arms I-III, arms IV united by a shallower web reaching less than a quarter of the arm length or absent (individual ICMC000165). Tentacles range from 44 to 46 mm length, representing 251–379% of ML. The distal 5–6 mm of the tentacles are occupied by the club. The tentacle organ slightly overlaps the tentacle club, formed by 10–11 transversal rows of suckers, which are larger in both proximal and terminal rows of the club.

The light organ has the typical shape for Heteroteuthinae (**Figure 4B**), with a round morphology and two pores in the midline. The visceral mass was not dissected. The upper beak has a long and curved rostrum of 0.8–0.9 mm length with a sharp pointed rostral tip (**Figure 4D**), representing ∼4.9% of the ML. Beak angle of ∼90°, with a smooth edge; specimen ICMC000165 has an irregular tooth. Hood is small and fragile. The rostrum and the shoulders are darkly pigmented, while the hood and the lateral wings are moderately pigmented. Lower beak has a short and blunt rostrum of 0.6–0.7 mm length with a rounded rostral tip (**Figure 4E**), representing ∼3.6–3.7% of the ML. The lower beak angle is of ∼130°, without teeth. The hood is narrow and the wings are wide. The rostrum and the hood are darkly pigmented, while the wings and lateral walls are moderately pigmented and almost transparent at the edges. The male specimens ICMC000165 and ICMC000166 and the female specimen ICMC000165, 17.9, 16.7, and 12.3 mm ML, respectively, were used for measuring the radulae. The radula has seven transverse rows of teeth displaying a typical homodont morphology (**Figure 4F**). Rhachidean teeth are 96–126 μm length, with a wide base, sharply pointed and slightly curved shape. First lateral teeth are 88–130 μm length, with a narrower base, sharply pointed and slightly curved shape. The second lateral and marginal teeth are narrow and strongly curved, of 98–164 μm and 176–240 μm length, respectively. All the spermatophores of specimens ICMC000165 and ICMC000166 were extracted and counted, and a random selection of 30 were used for measuring their length. ICMC000165, 17.9 mm ML, had 236 spermatophores of 2.6 ± 0.1 mm length (range 2.5–3.0 mm).

TABLE 5 | Measurements (in mm) of the type material of *Stoloteuthis cthulhui* **sp. nov**.

Character	Individual			
	ICMC000163	ICMC000164 (holotype)	ICMC000165	ICMC000166
Sex	Female	Male	Male	Male
DML	12.3	13.9	17.9	16.7
VML	19.4	13	16.3	–
FW	32.4	21.2	32	23.1
FL	10.1	9.8	8	9
FB	6.5	6	6.8	9
OBL	7.6	6.8	4.9	10.3
VSL	–	10.3	15	–
HW	17.8	12.3	17.2	16.8
HL	9.8	12	12.7	10.3
FnL	9.4	7.2	8.6	7.9
AIL	6.8	7.5	6.8	7.2
AIIL	8.2	8.4	8.6	7.8
AIIIL	9.3	7.5	8.2	9.7
AIVL	7.6	5.6	7	7.3
TeL	46.5	46	44.9	46.4
CL	6.6	5.4	4.6	5.8
WDA	4	3.7	2.9	5.4
WDB	2.9	3.9	3.6	4.7
WDC	3.3	2.8	4.6	4.2
WDD	3.7	3.2	2.6	–
WDE	2.3	1.2	–	0
URL	0.88	–	0.88	0.82
LRL	0.74	–	0.66	0.61
SpC	N/A	–	236	11,645
SpL	N/A	–	2.6 ± 0.1	1.9 ± 0.2
SpH	N/A	–	0.28 ± 0.01	–
SpE	N/A	–	0.76 ± 0.03	–
SpCe	N/A	–	0.64 ± 0.05	–
SpS	N/A	–	1.32 ± 0.05	–
SpEm	N/A	–	0.0 ± 0.0	–

DML, dorsal mantle length; VML, ventral mantle length; FW, fin width; FL, fin length; FB, fin base; OBL, head occipital band lenght; VSL, ventral shield length; HW, head width; HL, head length; FnL, funnel length; AIL-AIVL, arm I-IV length; TeL, tentacle length; CL, tentacle club length; WDA-E, web depth A-E; URL, upper beak rostral length; LRL, lower beak rostral length; SpC, spermatophore count; SpL, spermatophore length index; SpH, spermatophore head; SpE, spermatophore ejaculatory tube; SpCe, spermatophore cement body; SpS, spermatophore seminal reservoir; SpEm, spermatophore posterior empty part; N/A, not applicable. SpL was estimated based on 30 randomly selected spermatophores from the spermatophoric sac; SpH, SpE, SpCe, SpS, and SpEm was estimated based on 16 randomly selected spermatophores. For the spermatophore measurements, mean ± SD are indicated.

Spermatophores were intact and the spermatophoric reaction was not triggered (**Figure 4H**). Spermatophore threads were short and in most cases they were broken. Spermatophores head measures 0.28 ± 0.01 mm and holds three loops of the ejaculatory ducts. The ejaculatory apparatus is 0.76 ± 0.03 mm long and the spiral filament occupies ∼¾ of its length. The cement body is relatively long (0.64 ± 0.05 mm) and approximately half of the length of spermatophore is occupied by the seminal reservoir (1.32 ± 0.05 mm). There is no posterior empty part, but in those spermatophores fixed during the first steps of the spermatophoric reaction, a posterior empty part is observed. Specimen ICMC000166, 16.7 mm ML, had a strongly enlarged spermatophoric sac with a massive amount of spermatophores (11,645) ranging from 1.4 to 2.4 mm length. Smaller spermatophores usually look similar to the larger ones, but with reduced seminal reservoirs. Other spermatophores of this specimen were empty and/or deformed and should be considered as tentative spermatophores sensu Nigmatullin et al. (2003).

Distribution

Western Mediterranean Sea. Some authors consider the presence of this species (cited as *Sl. leucoptera*) as a recent range expansion from Atlantic waters: see Bello et al. (2020) for a systematic review of these opinions.

TABLE 6 | Comparison of the indices (in percentage) of morphological measurements between *Stoloteuthis cthulhui* **sp. nov**.

	DML	VMLI	FWI	FLI	FBI	OBLI	VSLI	HWI	HLI	FnLI	AlLI	AlILI	AlIILI	AlVLI	TeLI	CLI	WDAI	WDBI	WDCI	WDDI	WebEI	URLI	LRLI	SpLI
***Stoloteuthis cthulhui* sp. nov.**																								
Mean	15.2	114	183	63	47	50	79	107	75	56	48	55	58	46	309	38	27	25	24	23	9	5.7	4.5	13.0
SD	2.6	38	56	17	8	16	7	25	11	14	8	10	13	11	57	12	8	4	3	8	9	1.3	1.3	2.0
Range	12.3–17.9	91–157	138–263	45–82	38–54	27–62	74–84	88–145	62–86	47–76	38–55	47–67	46–76	39–62	251–378	26–54	16–33	20–28	20–27	15–30	0–19	4.9–7.1	3.6–6.0	11.6–14.4
Stoloteuthis leucoptera																								
Mean	13.7	106	196	79	48	48	91	84	56	51	51	57	61	52	195	33	34	28	31	33	14	6.2	4.4	15.7
SD	2.4	11	26	24	8	7	10	9	11	7	9	11	6	9	54	9	9	7	10	9	8	0.9	1.0	2.7
Range	9.0–18.7	92–126	160–236	51–141	36–61	36–57	75–108	68–96	40–80	40–62	31–64	41–75	52–71	37–66	115–299	19–51	20–56	17–40	18–50	17–45	0–28	4.5–7.3	2.7–5.7	13.7–17.6

(n = 4, 3 males and 1 female; beaks n = 3, 1 female and 2 males; spermatophores, n = 2) and Stoloteuthis leucoptera (n = 16, 8 males and 8 females; beaks, n = 10, 2 females and 8 males; spermatophores, n = 2). DML, dorsal mantle length; VMLI, ventral mantle length index; FWI, fin width index; FLI, fin length index; FBI, fin base index; OBLI, occipital band lenght index; VSLI, ventral shield length index; HWI, head width index; HLI, head length index; FnLI, funnel length index; AlLI-AlVLI, arm I-IV length index; TeLI, tentacle length index; CLI, tentacle club length index; WDAI-WDEI, web depth A-E index; URLI, upper beak rostral length index; LRLI, lower beak rostral length index; SpLI, spermatophore length index. Mean, standard deviation (SD) and range are provided.

Molecular Data

The COI *p*-distances analyses (**Tables 2**, **3**) show a 3.5% divergence between *Sl. cthulhui* **sp. nov.** and its sister species, *Sl. leucoptera*. This level of divergence was reported by Gebhardt and Knebelsberger (2015) and Groenenberg et al. (2009) between different closely-related bobtail squid species. Moreover, the same level of divergence was found between different well-established species in our analysis (**Table 2**).

Remarks

Morphological differences between *Sl. cthulhui* **sp. nov.** and *Sl. leucoptera* are subtle but still consistent. The morphology of the second pair of arms of the mature male differs in the assemblage of the enlarged suckers. In *Sl. cthulhui* **sp. nov.**, the pattern is less evident than in *Sl. leucoptera*: the first row of suckers is unmodified, rows 2–4 of ventral suckers are slightly enlarged and ventral and dorsal rows 5–6 of suckers showed the same level of enlargement, whereas in *Sl. leucoptera*, the first two rows of suckers are unmodified and ventral suckers from rows 3–6 are progressively enlarged (**Figure 4I**, see also Vecchione and Young, 2013). In some *Sl. leucoptera* specimens the fifth ventral sucker is larger than the sixth or dorsal suckers 5–6 might be larger than the ventral ones. Although the range of values overlaps in all cases, some differences also exist in some morphometric indexes (**Table 6**): the fin length and shield length are larger in *Sl. leucoptera* than in *Sl. cthulhui* **sp. nov.**, while the head is narrower (68–96% of ML) and the tentacles are shorter (115–299% of ML). The rhachidean teeth of the *Sl. leucoptera* radulae are smaller, with 41–85 μm length, measured in two mature females and two mature males 13.3–15.5 and 11.5–13.3 mm ML, respectively (**Figure 4G**). Although the remaining teeth of *Sl. leucoptera* also tend to be smaller, the length ranges overlap with *Sl. cthulhui* **sp. nov**. Other characters, such as other morphometric measures, the beaks morphology and the spermatophores did not show any other relevant morphological differences between the two species.

Stoloteuthis leucoptera was described in the Gulf of Maine, 30 miles east Cape Ann (North-western Atlantic Ocean) (Verrill, 1878). Previously to the present work, *Sl. leucoptera* was thought to be distributed in NW Atlantic Atlantic to Namibian and Mediterranean waters (Reid and Jereb, 2005; Vecchione and Young, 2013). We did not find any morphological differences between Namibian *Sl. leucoptera* (Villanueva and Sánchez, 1993; this work) and the descriptions from the literature for NW Atlantic *Sl. leucoptera* (e.g., Vecchione and Young, 2013). The individuals described by Orsi Relini and Massi (1991: **Table 1**) and Cuccu et al. (2010: **Table 1**) had a HWI of 105–130%, consistent with *Sl. cthulhui* **sp. nov.** and not with *Sl. leucoptera*. We consider previous records of Mediterranean *Sl. leucoptera* as *Sl. cthulhui* **sp. nov.** (Orsi Relini and Massi, 1991; Volpi et al., 1995; Cuccu et al., 2010; Quetglas et al., 2013; Zaragoza et al., 2015).

DISCUSSION

All of the genera and twelve sepiolid species, covering 70% of the known specific biodiversity of the family in the Mediterranean

Sea, have been barcoded and vouchered (**Table 1**). All the studied individuals were successfully linked with their taxonomic name and no inconsistencies arose among the newly sequenced material. Nevertheless, wrong identifications of bobtail squids are relatively frequent in GenBank, as previously reported by Groenenberg et al. (2009) and Sanchez et al. (2019). The work of Groenenberg et al. (2009) on DNA barcoding on vouchered individuals has solved some of the previously misidentified sequences. However, this work was based on animals from NE Atlantic waters and some species not studied by them remained unsolved. Here, we provided a correct barcode for many of these species, solving some of the previous problematic sequences (**Table 4**). Solving those problematic sequences based on morphologically identified animals is extremely important to ensure the quality of the identification based only on molecular data.

For most of the species studied here, there is a tendency of intraspecific distances below 2% and interspecific distances above 2.4–3% (**Tables 2**, **3**). However, this pattern is not universal within the family and some exceptions occur. In fact, no clear barcode gap (Meyer and Paulay, 2005) was identified between intra- and interspecific distances, due to the presence of challenging groups, such as the clade formed by *S. affinis*, *S. atlantica* and *S. intermedia*. The interspecific distances of these three species are the smallest among our dataset (0.81–1.45%). In fact, the largest intraspecific *p*-distances among Sepiolinae (1.8% in *S. robusta* and 2.3% in *E. scolopes*, **Table 3**) are larger than the interspecific distances between *S. affinis*, *S. atlantica* and *S. intermedia*, thus existing an overlapping between intra- and interspecific distances which complicates the use of DNA barcoding methods based on genetic distances for this group of animals. The three species have very distinctive hectocotylus morphology with discrete morphologies (**Figure 3**): the morphological variation of those species does not overlap (Bello, 1995; Reid and Jereb, 2005; de Heij and Goud, 2010). As far as we know, no hybrids have been described among them. While *S. atlantica* is allopatric in reference with the other two species, *S. affinis* and *S. intermedia* both occur in Mediterranean waters (Reid and Jereb, 2005), pointing out to the presence of effective reproductive isolation mechanisms acting at least between the two Mediterranean species. For this clade, only the single threshold approach of the GMYC among all the tested molecular species delimitation methods provided the same results as the morphology (**Figure 3**). If molecular identifications were carried out with no further morphological information, this level of interspecific distances might be mistaken as intraspecific variation. This low level of interspecific distances might be due to recent phenomena of speciation with a fast morphological drift of key morphological characters. Recently, Costa et al. (2021) found that two morphologically different species of coastal squids that diverged in recent times were recognized as a single species by molecular species delimitation methods. Their study and ours highlights the importance of combining studies based on molecular identifications with careful morphological examinations.

Another important phenomenon hindering the direct use of bobtail squid DNA sequences for species identifications is the presence of cryptic biodiversity. Although cryptic biodiversity is an increasingly reported phenomenon in cephalopod biodiversity studies (e.g., Anderson et al., 2007, Cheng et al., 2014, Fernández-Álvarez et al., 2020), this phenomenon is comparatively unknown in bobtail squids. This is especially true for *E. hyllebergi* and *E. tasmanica*, with interspecific distances between different OTUs ranging from 3.9 to 11.6%. Remarkably, the two *E. hyllebergi* OTUs occur allopatrically in both Indian and Pacific coasts of the Thailand Peninsula, while *E. tasmanica* OTUs might occur sympatrically. It is known that the hatchling size and mode of life has an important effect on the distribution range of cephalopod species, since species with larger benthic hatchlings tend to have smaller distribution areas (Villanueva et al., 2016). Information on the way of life of bobtail squid during their first days of life is scarce (Villanueva et al., 2016: Tables 1, 2). Available information points out that Rossinae and most Sepiolinae tend to have large benthic hatchlings, although some early stages of some species reported as benthic throughout their lives can be also be found in the water column (Olmos-Pérez et al., 2018b). It is particularly remarkable that the species with larger hatchlings from the subfamily Sepiolinae, *E. tasmanica* (5 mm ML, Villanueva et al., 2016: Table 1), has more cryptic lineages with larger interspecific distances, accounting for four different OTUs with a divergence of 4.3–11.6% (**Table 2**). Interestingly, *R. macrosoma* has slightly larger benthic hatchlings (5.5 mm ML) and also has genetic structure, being taken as two species by several species delimitation methods. Comparisons between the sympatric geographic patterns of *E. tasmanica* lineages with the allopatric pattern found in *Euprymna hyllebergi*, a species with smaller planktonic hatchlings (2.2 mm ML), suggest that large hatchlings with direct benthic development have a more intense effect on the dispersal capacity and communication between distant cephalopod populations, and it might be one of the triggers that increase the opportunities for speciation. Rossinae are mostly exclusively benthic species, and Sepiolinae usually are usually reported as benthic species (Reid and Jereb, 2005), but they can also be found in the water column (Bello and Biagi, 1995). Absence of ontogenetic migrations have been suggested for some Sepiolinae species (e.g., Villanueva, 1995), which suggests that this restrictions to movement limit dispersal, contributing to slow down the gene flow between distant populations. This putative lower dispersal during both young and adult stages combined by the fact that some species inhabit relatively non-overlapping bathymetric ranges might have helped to trigger speciation in the Mediterranean Sea and increase their endemic bobtail species (Bello, 2019). Bobtail squids are among the cephalopods with higher tolerances to salinity changes (Mangold and Boletzky, 1988) and young stages can be found in estuarine systems (e.g., Olmos-Pérez et al., 2018b), and adults in the intertidal regions (Fernández-Álvarez, *pers. obs.*). Thus, it seems that salinity it is not a great limitation to bobtail squid dispersal. The Mediterranean Sea Sepiolinae fauna is characterized by the high number of endemic species (Bello, 2019), while many Atlantic species does not distribute also in the Mediterranean. It is possible that the effect of some well-known oceanographic barriers to genetic exchange, such as the Strait of Gibraltar (Pascual et al., 2017), had an important

effect on the evolutionary history and current distribution of European Sepiolinae, while the differences in salinity between the Mediterranean and the Atlantic (Mangold and Boletzky, 1988) might have a comparatively smaller effect. Future population genetic studies focused on those species can answer this question.

Members of the subfamily Heteroteuthinae are exclusively nektonic and benthic-pelagic species (e.g., Orsi-Relini, 1995). Although it is known that oceanic currents can isolate populations of some pelagic squids and lead them to speciation (e.g., Fernández-Álvarez et al., 2020), the opportunities to dispersal and population connectivity are comparatively larger in oceanic environments than in shallow benthic ecosystems. Thus, large distribution areas on wide oceanic basins are commonly reported in Heteroteuthinae species (Reid and Jereb, 2005). According with Vecchione and Young (2007), there are no known morphological differences between *H. dispar* and *H. hawaiinensis*, while all the molecular species delimitation methods performed here support their treatment as different species. In the absence of known morphological differences, these two species shall be considered as members of a cryptic species complex. A third undescribed species with a not yet described morphology, *Heteroteuthis* sp. KER (Braid and Bolstad, 2019), form a clade with *H. dispar* and *H. hawaiinensis*. *Heteroteuthis dagamensis* have a large distribution ranging from the Gulf of Mexico and the South Atlantic (Judkins et al., 2016) to New Zealand (Braid and Bolstad, 2019) and North Atlantic (Taite et al., 2020). Four out six of the molecular species delimitation analyses recognized cryptic biodiversity within this species (**Figure 3**). The large distribution range of the species in combination with the large intraspecific divergence between New Zealand and the specimens coming from other latitudes (>2%, **Table 3**) suggests that some processes of speciation in its early stage might be taking place. Between those described cryptic *Heteroteuthis* species (*H. dispar* and *H. hawaiinensis*), undescribed new species (*Heteroteuthis* sp. KER) and cryptic lineages (*H. dagamensis*) some well-known oceanic and terrestrial barriers exists, such as the Panama Isthmus and the currents that creates the main oceanographic gyres. New combined morphological and molecular studies are necessary for solving this taxonomic problem.

It is remarkable the fact that a closer relationship exists between *Sl. japonica* and *Se. nipponensis* (2.4%) rather than between *Sl. japonica* and other congeneric species (12.7% with *Sl. cthulhui* **sp. nov.** and 14.3% with *Sl. leucoptera*). Divergence between *Sepiolina petasus* Kubodera and Okutani, 2011 and *Se. nipponensis* is 13.9%, similar to that reported between species of the genera *Sepiolina* and *Heteroteuthis*. These data suggest that the current generic assignations to genera in Heteroteuthinae species are not fully molecularly supported and should be revised in future combined morphological and molecular studies, as already suggested by Allcock et al. (2014).

All molecular species delimitation analyses consistently identified *Sl. leucoptera* and *Sl. cthulhui* **sp. nov.** as different species (**Figure 3**), also the COI divergence of 3.5% is typical for different species in bobtail squids. This difference is consistent with interspecific levels in many other invertebrates, such as nemerteans (Fernández-Álvarez and Machordom, 2013), land planarians (Lago-Barcia et al., 2015), crustaceans (Robles et al., 2007), and other cephalopods (Gebhardt and Knebelsberger, 2015; Fernández-Álvarez et al., 2020). Besides, the morphological comparisons between *Sl. leucoptera* and *Sl. cthulhui* **sp. nov.** showed a few differences in key morphological characters that until now remained overlooked, such as the length of the tentacle, the width of the head and slight differences in the sexual modifications of arm II in mature males. In general, it seems that the modifications of the arm II in *Sl. cthulhui* **sp. nov.** are less pronounced than in *Sl. leucoptera*. Even so, the differences between both species are few, likely representing a shallow morphological drift and a recent speciation phenomenon. Species involved in those first steps are commonly referred as in the "gray speciation zone" (Roux et al., 2016). As we showed with *S. affinis*, *S. atlantica* and *S. intermedia*, as well as the molecular data provided for the descriptions of *Sl. japonica* and *Sp. nipponensis*, these situations are likely to be frequent in bobtail squids. Based on this observation, the specific status of distant populations with mild levels of genetic divergence, which are typically identified as intraspecific levels of divergence, should be taken with care.

We took special care of including as many morphological characters as possible to avoid overlooking possible morphological differences between the congeneric *Sl. cthulhui* **sp. nov.** and *Sl. leucoptera*. That is the case of beak and radulae morphologies, which are rarely used in bobtail squid taxonomy (e.g., Kubodera and Okutani, 2011; Sanchez et al., 2019), and spermatophores. The use of spermatophores in cephalopod taxonomy should be taken with caution, as our study also shows. It is known that spermatophore size depends on the size of the male that produces it and since they can accumulate spermatophores for long periods of time, the same individual can hold spermatophores of a huge range of sizes and morphologies, according with the somatic size of the squid when each spermatophore was formed (Hoving et al., 2010; Cuccu et al., 2014). The number of spermatophores stored by the male would vary according with when it mated the last time (if anything at all) and so will do the size of those structures. In the present study we found 353–358 spermatophores in the spermatophoric sacs of *Sl. leucoptera*, while 236 (ICMC000165) and 11,645 (ICMC000166) spermatophores were found in the two examined specimens of *Sl. cthulhui* **sp. nov**. It is interesting to point the fact that the specimen ICMC000165, with a lower number of spermatophores, was also the same individual with a narrowest range of spermatophore size. Particularly, the specimen ICMC000166 not only showed the largest range of spermatophore size: it also showed empty and aberrant spermatophores. It is known that in the beginning of their reproductive life, cephalopod males produce tentative spermatophores (Nigmatullin et al., 2003) and in this case both functional and tentative spermatophores were present in the spermatophoric sac. It is not known to us if this was related with the absence of mating events by this individual for any circumstance or if it is just an aberrant individual. Pelagic cephalopods might have difficulties to find conspecifics to mate (Hoving et al., 2012), or just the opposite situation (Fernández-Álvarez et al., 2018; Hoving et al., 2019), so species

with promiscuous mating systems are expected to hold a less variable morphology in the spermatophores stored in the spermatophoric sac. Special attention should be taken to the characteristics of a cephalopod species sex life if spermatophores are going to be used as a taxonomic character.

CONCLUSIONS

In this study we provided new sequence data for most of the Mediterranean bobtail squid species and added other relevant sequence data from species from other parts of the world. The combined use of several molecular species delimitation methods consistently showed a picture of hidden biodiversity. On the one hand, most of those methods failed to accurately assess the actual biodiversity of some morphologically different species, hindering the use of molecular data for species identification in the absence of morphological data. On the other hand, cryptic biodiversity was detected among members of the same nominal species, pointing to the fact that in some cases, speciation phenomena might be occurring without a parallel morphological evolution. It is also possible that the comparatively low number of taxonomists working on bobtail squids has hindered the discovery of morphological differences between them or that some morphological differences had been overlooked, as it happened with the new *Stoloteuthis* species described in this work. The Mediterranean Sea is one of the more diverse and the better studied areas for members of the family Sepiolidae, with a literature production spanning through more than a century (Bello, 2015). Despite this intense biodiversity and taxonomic research, two new Mediterranean species were described in the last few years (Bello, 2013; Bello and Salman, 2015). Here, a new species of Mediterranean *Stoloteuthis*, previously misidentified, was discovered and described, both using molecular and morphological methods. It is also remarkably that Olmos-Pérez et al. (2018b) found a species ("Heteroteuthinidae" sp.) in European waters whose sequences cannot be assigned to any sequenced species of bobtail squid, while Sanchez et al. (2019) discovered two new species of the genus *Euprymna* in Pacific waters. All these recent new species discoveries stress the need of new taxonomic studies in both benthic and pelagic bobtail squids on a worldwide basis.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: NCBI GenBank (accession: MW260131-MW260134).

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because the cephalopods we worked with for this study were all dead before research began.

AUTHOR CONTRIBUTIONS

FÁF-Á, PS, and RV conceived the study, participate in sample collection and identification, and performed morphological examinations. FÁF-Á performed the phylogenetic analyses, data curation, and prepared the first draft. All authors contributed to the manuscript preparation and revision and read and approved the submitted version.

FUNDING

FÁF-Á was supported by an Irish Research Council—Government of Ireland Post-doctoral Fellowship Award (Ref. GOIPD/2019/460) and by a predoctoral fellowship of the Spanish Ministry of Economy and Competitiveness (MINECO, BES-2013-063551). Some specimens were collected on board the RV Hespérides during the research project MAFIA (CTM2012-39587-C04-03), funded by the MINECO (FEDER/EU), and on board the RV García de Cid during the research cruise FORMED (CGL2012-33989, MINECO). This study was partially funded by the Spanish Ministry of Science, Innovation and Universities (OCTOSET project, RTI2018-097908-B-I00, MCIU/AEI/FEDER, EU) and the European Commission (SUMMER project, GA-817806).

ACKNOWLEDGMENTS

We thank Nuria Zaragoza, Alfredo García de Vinuesa, Anabel Colmenero (ICM-CSIC) and the crew of the fishing trawlers Peret de Fava (Tarragona) and La Seu (Vilanova i la Geltrú) for collecting samples. Thanks to Elisabeth Cuesta-Torralvo (University of Barcelona), Catarina P. P. Martins (Justus Liebig University of Giessen) and Oscar Escolar (ICM-CSIC) for assistance in the lab. Thanks to Mr. J. M. Fortuño (Electron Microscopy Service, ICM-CSIC) for his dedication and kindness during the SEM sessions. Elena Guerrero (ICM-CSIC) performed the curation of the morphological vouchers under the CBR-ICM. We thank the two reviewers for their helpful comments. DNA extractions, PCRs, and sequencing were performed in AllGenetics (A Coruña, Spain). We thank Ate de Heij and Jeroen Goud for granting permission to use the *Sepiola atlantica* hectocotylus drawing by Bas Blankevoort (Naturalis, Netherlands).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.