









Phylogenomics illuminates the evolution of bobtail and bottletail squid (order Sepiolida)

Author	Gustavo Sanchez, Fernando A. Fernandez-Alvarez, Morag Taite, Chikatoshi Sugimoto, Jeffrey Jolly, Oleg Simakov, Ferdinand Marletaz, Louise Allcock, Daniel S. Rokhsar
journal or publication title	Communications Biology
volume	4
number	1
page range	819
year	2021-06-29
Publisher	Nature portfolio
Rights	(C) 2021 The Author(s).
Author's flag	publisher
URL	http://id.nii.ac.jp/1394/00001989/

doi: [info:doi/10.1038/s42003-021-02348-y](https://doi.org/10.1038/s42003-021-02348-y)

Phylogenomics illuminates the evolution of bobtail and bottletail squid (order Sepiolida)

Gustavo Sanchez ^{1,2,8}, Fernando Á. Fernández-Álvarez ^{3,8}, Morag Taite³, Chikatoshi Sugimoto², Jeffrey Jolly², Oleg Simakov ⁴, Ferdinand Marlétaz⁵, Louise Allcock ³ & Daniel S. Rokhsar ^{2,6,7} 

Bobtail and bottletail squid are small cephalopods with striking anti-predatory defensive mechanisms, bioluminescence, and complex morphology; that inhabit nektobenthic and pelagic environments around the world's oceans. Yet, the evolution and diversification of these animals remain unclear. Here, we used shallow genome sequencing of thirty-two bobtail and bottletail squids to estimate their evolutionary relationships and divergence time. Our phylogenetic analyses show that each of Sepiadariidae, Sepiolidae, and the three sub-families of the Sepiolidae are monophyletic. We found that the ancestor of the Sepiolinae very likely possessed a bilobed light organ with bacteriogenic luminescence. Sepiolinae forms a sister group to Rossinae and Heteroteuthinae, and split into Indo-Pacific and Atlantic-Mediterranean lineages. The origin of these lineages coincides with the end of the Tethys Sea and the separation of these regions during the Eocene and the beginning of the Oligocene. We demonstrated that sepiolids radiated after the Late Cretaceous and that major biogeographic events might have shaped their distribution and speciation.

¹Graduate School of Integrated Science for Life, Hiroshima University, Higashi Hiroshima, Hiroshima, Japan. ²Molecular Genetics Unit, Okinawa Institute of Science and Technology Graduate University, Onna, Okinawa, Japan. ³Ryan Institute and School of Natural Sciences, National University of Ireland Galway, Galway, Ireland, UK. ⁴Department of Molecular Evolution and Development, University of Vienna, Vienna, Austria. ⁵Department of Genetics, Evolution and Environment, Centre for Life's Origins and Evolution, University College London, London, UK. ⁶Department of Molecular and Cell Biology, Life Sciences Addition #3200, Berkeley, CA, USA. ⁷Chan-Zuckerberg BioHub, San Francisco, CA, USA. ⁸These authors contributed equally: Gustavo Sanchez, Fernando Á. Fernández-Álvarez. ✉email: dsroksar@gmail.com

Sepioids are small round-bodied cephalopods of around 1–8 cm of dorsal mantle length which live in a range of habitats from shallow coastal waters to mesopelagic environments across the globe¹. These animals are nekto-benthic and pelagic species that have evolved remarkable anti-predatory defense mechanisms. Bobtail squids, for example, use counter-illumination² or discharge luminous secretions³, while bottletail squids secrete toxic substances through their slime⁴. Taxonomically, sepioids comprise the order Sepiolida Keferstein, 1866 which is split into the families Sepiolidae Leach, 1817 and Sepiadariidae Fisher, 1882, commonly known as bobtail and bottletail squid, respectively.

Bobtail and bottletail squids are emerging as model organisms for cephalopods due to their easy culture under laboratory conditions⁵. Most notably, the Hawaiian bobtail squid *Euprymna scolopes* Berry, 1913 is a model for host-symbiont interaction with the bacterium *Allivibrio fischeri* (Bejerinck 1889)⁶, which interacts with the light organ of the animal^{2,7,8}. The luminous organs of bobtail squids emit light of bacteriogenic or autogenic origin; these organs have either bilobed or rounded forms^{3,9}. Genomic resources to study these animals have also increased through the recent sequencing of the nuclear genome of *E. scolopes*¹⁰ and transcriptomes of several different species^{11–14}.

Previous phylogenetic analyses have placed the order Sepiolida as sister to the family Idiosepiidae Appellöf, 1898^{15,16}. The recent study of Anderson and Lindgren 2020¹⁷, however, finds Sepiolida to be sister to a clade containing the Oegopsida Orbigny, 1845 (oceanic squid with no cornea), Myopsida Naef, 1916 (neritic squid with cornea), and Sepiida Zittel, 1895 (cuttlefish) adding a controversy about the phylogenetic position of these animals. Sepiolida forms the third largest group of Decapodiformes after cuttlefishes and oegopsids¹⁸. Bobtail squids are the most diverse of the order with around 68 valid species grouped within three subfamilies, Sepiolineae Leach, 1817, Rossinae Appellöf, 1898, and Heteroteuthinae Appellöf, 1898. In contrast, their relatives, the bottletail squids have only five species grouped into two genera, *Sepiadarium* Steenstrup, 1881 and *Sepioloidea* d'Orbigny 1845. The genus *Euprymna* Steenstrup, 1887 has been recently redescribed using both molecular and morphological analyses¹¹. The systematics of Sepiolineae has recently been reviewed by Bello¹⁹, who proposed subdividing *Euprymna* and other genera into multiple new genera according to variation in the shape of the hectocotylus and other morphological characters. However, more molecular studies with confident taxonomic identifications are required to clarify the systematics, diversity, and evolutionary relationships of Sepiolida.

Recently, transcriptome sequencing provided several nuclear DNA markers and resolved the evolutionary relationships of *Euprymna* and other species formerly classified in the genus *Sepiola* Leach, 1817 (see Bello¹⁹) collected in the shallow waters (up to 20 m depth) of the Ryukyu archipelago and mainland Japan¹¹. Other members of the order Sepiolida, however, are found in oceanic waters (the record is for *Heteroteuthis nordo-pacifica* Kubodera and Okutani, 2011 collected at 1000 m depth, off Toba district in Japan²⁰), and are often caught as by-catch in which the animal does not survive the collection. While this makes the sampling of fresh tissue of these animals for transcriptome sequencing difficult, genomic DNA suitable for sequencing is readily recovered.

While full genome sequencing is costly, low-coverage shotgun sequencing allows the recovery of phylogenetically useful markers, including complete or nearly complete mitochondrial genomes and highly copied nuclear loci such as 18S and 28S rRNA²¹. This approach, sometimes called “genome skimming”, does not require the collection of fresh tissues from the animal. For sepioids, the genome of *E. scolopes*¹⁰ provides a suitable reference to map reads and recover additional nuclear loci.

In this study, we reconstruct the evolutionary relationships of Sepiolida and performed molecular dating to estimate their divergence timeframe. We first characterized our specimens based on morphological characters, and then sequenced their DNA at low coverage to skim for mitochondrial genes and nuclear ribosomal genes, and to define a set of ultraconserved loci based on shared alignments to the reference genome of *E. scolopes*. Our datasets allowed us to reconstruct mitochondrial and nuclear phylogenetic trees that support a sister relationship between Rossinae and Heteroteuthinae, and the split of Sepiolineae into lineages from the Indo-Pacific and Atlantic plus Mediterranean region. We also infer that the ancestor of Sepiolineae possessed bilobed light organ hosting symbiotic bacteria, lost independently in *Iniotheuthis* and *Sepietta* from Sepiolineae. Moreover, the monophyly of Heteroteuthinae confirms a unique origin of the autogenic luminescence in this group, validating its association with pelagic lifestyles in Cephalopoda. In addition, the time-calibrated phylogeny shows that the split of many sepioids is correlated with and was plausibly driven by major biogeographic events after the late Cretaceous.

Results and discussion

Genome skimming provides robust phylogeny. Pioneering molecular phylogenetic studies in Sepiolida that used short regions of a few mitochondrial and nuclear genes failed to resolve the relationship of major clades^{9,22,23}. To increase the number of phylogenetically informative sites, Sanchez et al.¹¹ sequenced and analyzed the transcriptomes of multiple species of *Euprymna* Steenstrup, 1887, related bobtail squids including *Sepiola parva* Sasaki, 1913 and *Sepiola birostrata* Sasaki, 1918, and several bottletail squids. They found that *S. parva* grouped with the *Euprymna* species to the exclusion of *S. birostrata*, and further morphological analysis led to the formal redefinition of the genus *Euprymna* and the reassignment of *S. parva* Sasaki, 1913 to *Euprymna parva*¹¹. The following year, in an exhaustive study of hectocotylus structure, Bello¹⁹ proposed that *Euprymna* be split back into the original *Euprymna* Steenstrup, 1998 and a newly defined genus, *Eumandya* Bello 2020 that contains *E. parva* Sasaki, 1913 and *E. pardalota* Reid 2011, two taxa whose arms have two rows of suckers rather than four as in other *Euprymna* species. Similarly, Bello introduced a new genus, *Lusepiola* Bello, 2020 that has the effect of renaming *Sepiola birostrata* Sasaki, 1918 as *Lusepiola birostrata*. For clarity, we adopt the finer-grained nomenclature of Bello below, but happily note that *E. parva* and *E. pardalota* have the same abbreviations in both the notation of Sanchez et al.¹¹ and Bello¹⁹.

Sanchez et al.¹¹ also emphasized the need for more taxon sampling, careful species assignment, and the inclusion of more informative sites when studying this group of cephalopods. However, the distribution and lifestyle of many lineages of Sepiolida makes the collection of fresh tissue for RNA sequencing very challenging. To overcome this limitation, we sequenced the genomic DNA of several Sepiolida species at shallow coverage up to 3.6× and accessed by this way several mitochondrial and nuclear loci. Most of our samples were carefully identified at the species level based on morphological characters.

We recovered the mitochondrial genomes of the species targeted in this study and annotated the 13 protein-coding genes, 22 tRNAs, and two rRNAs (although only the conserved region of the large and small rRNA was obtained for *Rondeletiola minor* Naef, 1912).

Additionally, we also downloaded the complete mitochondrial genomes of *S. austrinum* and *Idiosepius* sp., and the transcriptome of *E. tasmanica* available in the NCBI database. The transcriptome of *E. tasmanica* was used to extract its complete set

of mitochondrial protein-coding genes. We could reconstruct the mitochondrial gene order for all species with complete mtDNA genomes, but we observed no re-arrangement for members of Sepiolidae, and only *Sepiadarium austrinum* deviated from the arrangement seen in all other Sepiadariidae (Fig. S1).

To complement the mitochondrial-based evolutionary history, we also annotated several nuclear loci. As ribosomal gene clusters are present in numerous copies, they were successfully retrieved for almost all the species, except for 28S of the Sepiadariidae sp. specimen, which appeared problematic and was excluded.

By mapping reads to the reference genome of *E. scolopes*, we obtained 3,279,410 loci shared between at least two species and further selected 5215 loci presented in most of our Sepiolidae species, but allowed some missing data in the *Euprymna* + *Eumandya* clade. This was done because the phylogenetic relationships of the *Euprymna* + *Eumandya* species were previously described in detail in Sanchez et al.¹¹ using transcriptome data. Out of the 5215 loci, 5164 loci had a per-site coverage ranging between two and five. After trimming and removal of regions without informative sites, 577 loci remained. These ultraconserved loci had lengths ranging between 10 and 690 base pairs (bp), with an average of 65 bp. Our alignment matrix had a length of 37,512 bp and consisted of 16,495 distinct site patterns, and variable sites between 1 and 130 bp with an average value of 7 bp. We expected a low value of variable sites because these regions are highly conserved.

We considered resolved nodes to be those with the ultrafast bootstrap support and posterior probability larger than 95% and 0.9, respectively. Only the very unresolved nodes were found based on the mito_nc matrix (Fig. 1). However, among the species in these nodes, *Adinaefiola ligulata* Naef, 1912 was well supported with amino acid sequences from mitochondrial genes (posterior probability of 1 and 94% bootstrap support) and partially by the ultraconserved loci (posterior probability of 1, but only 85% bootstrap support) as sister to the *Sepiola* clade (Figs. 2 and S2). Moreover, compared to the mito_nc matrix and with identical topology, mito_aa and UCEbob fully resolved the relationship of the Indo-Pacific and Mediterranean Sea Sepiolinae. The tree generated by the nuclear rRNA produces a topology with most nodes unsupported (Fig. S3), suggesting these markers are too conserved for assessing the relationships among this group.

Using the UCEbob matrix, the topology and supported relationships of *Euprymna* + *Eumandya* species resemble those reported in Sanchez et al.¹¹ using transcriptome sequences, proving our protocol valid when using low coverage sequencing and when a reference genome of the closest related species is available.

The position of *R. minor* showed discordance between mitochondrial and nuclear datasets. Using the mitochondrial matrices, *R. minor* rendered the *Sepietta* Naef, 1912 clade paraphyletic, whereas using the UCEbob and rRNA_nc matrices, *R. minor* appeared sister to the *Sepietta* clade. These relationships were resolved in both mitochondrial and nuclear-based trees and require further investigation with more DNA markers and a wider population sampling.

Molecular systematics of Sepiolida clades. Using the complete mitochondrial genome, ribosomal nuclear genes, and ultra-conserved loci, we recovered the monophyly of the two families of the order Sepiolida—Sepiadariidae and Sepiolidae^{9,24}—and the monophyly of the three described subfamilies of the family Sepiolinae. However, contrary to what is proposed based on morphology in Young²⁴, the Rossinae is not sister to all the remaining Sepiolidae but rather is sister to Heteroteuthinae,

although this is unresolved in the UCE phylogeny. With the lack of systematic work on these subfamilies, our robust phylogenetic backbone in Sepiolida using new samples carefully identified by morphology and with museum vouchers, represents a notable advance to clarify the evolution of morphological traits in major clades within the family.

Based on morphological characters of the hectocotylus, Bello¹⁹ recently split the polyphyletic *Sepiola* Leach 1817 into *Lusepiola*, *Adinaefiola*, and *Boletzkyola*, reserving *Sepiola* for the *S. atlantica* group *sensu* Naef 1923. These newly defined clades are consistent with our molecular phylogeny here and in Sanchez¹¹, who also noted the polyphyly of *Sepiola* in the Indo-Pacific lineage.

We find that Sepiolinae can be robustly split into two geographically distinct tribes: one that comprises species with known distribution in the Indo-Pacific region (tribe Euprymini *new tribe*, defined as Sepiolinae with a closed bursa copulatrix, type genus *Euprymna*) and the other including all the Mediterranean and Atlantic species (tribe Sepiolini Appellof, 1989, defined here as Sepiolinae with an open bursa copulatrix, type genus *Sepiola*). Our molecular relationship is consistent with 13 of the 15 apomorphies used in the cladogram shown in Fig. 21 in Bello¹⁹. The other two proposed apomorphies in Bello (his apomorphic characters 4 and 6) group two IP lineages, *Lusepiola* and *Iniotheuthis*, in a clade with species from the Mediterranean and Atlantic. Such relationships contradict our Euprymini-Sepiolini sister relationship. Moreover, according to our phylogeny, apomorphy 6 of Bello, characterized by the participation of ventral and dorsal pedicels in the formation of the hectocotylus copulatory apparatus, implies that the male ancestor of Sepiolinae had a more developed hectocotylus that was simplified in the *Euprymna* and *Eumandya* clades.

Among euprymins, we confirmed the monophyly of *Euprymna* Steenstrup 1887 as found previously by transcriptome analysis¹¹. We also support the monophyly of *Eumandya* Bello, 2020 (Figs. 1 and 2), grouping the type species *E. pardalota* with *E. parva* along with the unnamed “Type 1” Ryukyuan species of Sanchez et al.¹¹, for which only hatchlings were available. The phylogenomic grouping of Ryukyuan “Type 1” with *Eumandya* suggests that when its adults are found (or hatchlings are raised to maturity), its arms will carry two rows of suckers. We also found an adult of a Ryukyuan “Type 4” (extending the notation of Sanchez et al.¹¹ in the coastal waters of Kume Island, that groups with *E. scolopes* from Hawaii, suggesting a divergence based on geographic isolation in the North Pacific. We also find that *Lusepiola birostrata* (formerly *Sepiola birostrata*) is grouped with *Iniotheuthis japonica* as sister to a clade containing *Euprymna*, *Eumandya*, and an unnamed sepioline from Port Kembla, at the northeast of Martin Island in Australian waters.

Among the sepiolines, we confirm the monophyly of *Sepietta* (only for nuclear-genome-based trees, see below). *Adinaefiola*, another genus erected by Bello¹⁹, with *Sepiola ligulata* Naef 1912 as its type species; was found sister to the *Sepiola* clade, but only in the tree based on amino acid mitochondrial sequences (mito_aa matrix) with a bootstrap value of 94% and a posterior probability of 1 (Fig. S2).

Outside the sepiolines, members of the subfamily Heteroteuthinae are the most elusive and underrepresented in studies of cephalopod systematics due to their oceanic lifestyles. The placement of several heteroteuthin remains controversial. Lindgren et al.⁹, with six nuclear and four mitochondrial genes downloaded from GenBank found that *Sepiolina* Naef, 1912 was sister to *Heteroteuthis* Gray, 1849 + *Rossia* Owen, 1834 + *Stoloteuthis* Verrill, 1881; rendering the subfamily Heteroteuthinae polyphyletic. In contrast, our work supports the monophyly of Heteroteuthinae by including *Stoloteuthis* and *Heteroteuthis* in this subfamily, while *Rossia* was placed within the Rossinae

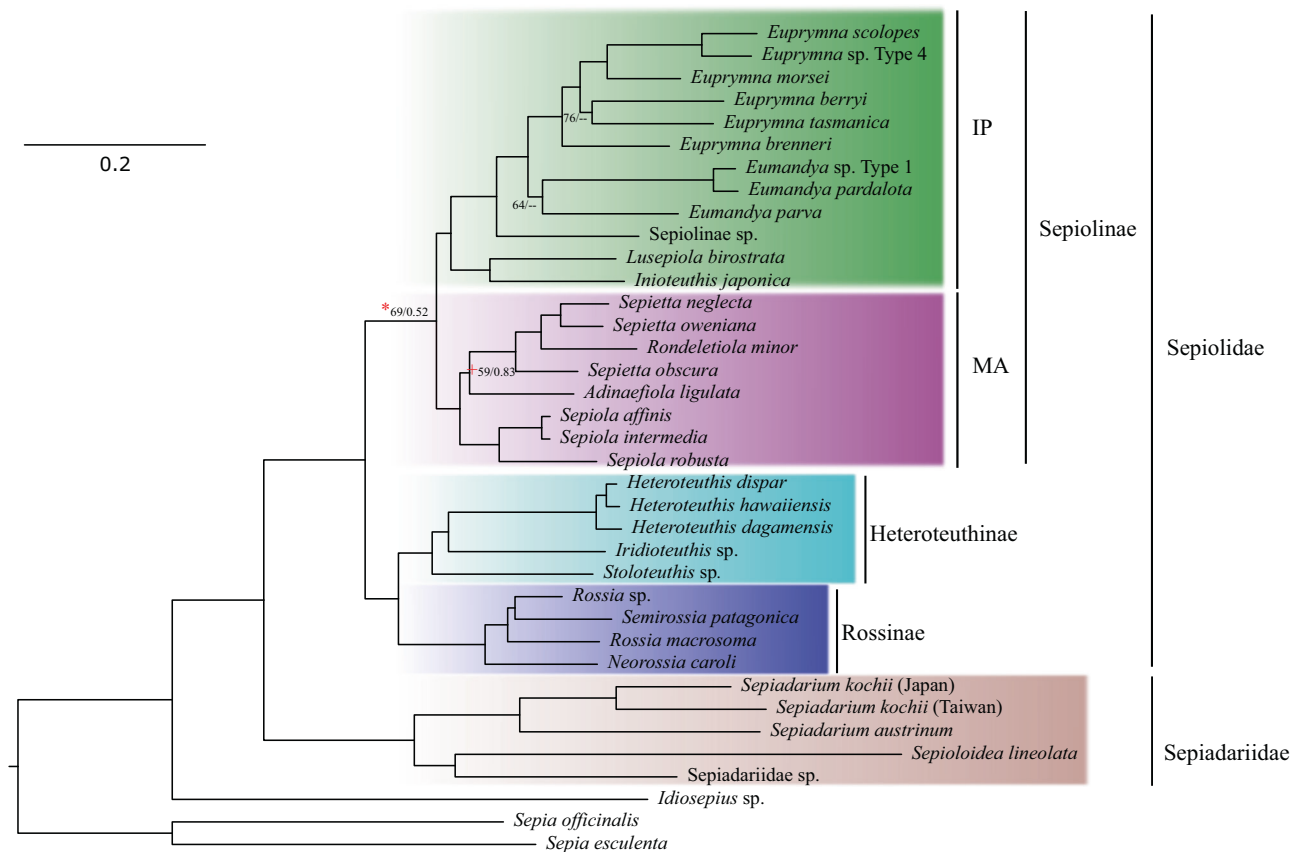


Fig. 1 Phylogeny of Sepioida based on nucleotide sequences from the mitochondria (mt_nc matrix). The topology of the maximum likelihood tree is shown. Numbers by the nodes indicate bootstrap support and the Bayesian posterior probabilities. Values of bootstrap support and posterior probabilities above 95% and 0.95, respectively, are not shown. (*) indicates that the node was resolved with the mito_aa and UCEbob matrices. (+) indicate that *A. ligulata* is sister to *Sepioloidea* using mito_aa with ultrafast bootstrap support of 94% and a posterior probability of 1. Abbreviations: IP, Indo-Pacific Ocean; MA, Mediterranean Sea, and the Atlantic Ocean.

(Figs. 1, 2, S2). Members of Heteroteuthinae included in this study formed a sister group to a monophyletic Rossinae (Figs. 1, 2, S2). *Semirossia*, however, rendered the *Rossia* clade paraphyletic. Further discussion about the position of *Semirossia* is difficult because of the lack of information about the original source of this specimen in Kawashima et al.²⁵.

The light organ and luminescence evolution. Bobtail squids are thought to use the bioluminescence of their light organ to camouflage them from predators while foraging and swimming at night through a mechanism called counter-illumination. This has been researched extensively using *E. scolopes* as a model system^{26–28}. Unfortunately, the limited number of sequences available and the misidentification of bobtail squids in the GenBank database^{11,29,30} have hindered our understanding of the light organ evolution in the whole taxon.

Our robust phylogeny and Bayesian reconstruction of ancestral bioluminescence clarify how the light organ and its luminescence have evolved in the family Sepioidae. Members of Sepiolinae comprise neritic and benthic adults with bilobed light organs, except for two genera: *Iniotheuthis* from the Indo-Pacific region, and the *Sepietta* species from the Mediterranean Sea and the Atlantic waters. The ancestor of the Sepiolinae very likely possessed a bilobed light organ that harbored luminescent symbiotic bacteria (Fig. 3). This character persisted until the ancestor of the euprymnins and sepiolins. Assuming that *R. minor* is sister to the *Sepietta* clade (as shown with the nuclear-based dataset, Fig. 2), it is clear that the bilobed light organ was

lost once in *Iniotheuthis* and *Sepietta*, and simplified to a rounded organ in *R. minor*. The alternative scenario, where *R. minor* renders the *Sepietta* clade paraphyletic (based on mitochondrial matrices, Fig. 1), is less plausible as it implies that the light organ was lost twice in the *Sepietta* group, once in *S. obscura* and then in the ancestor of *S. neglecta* and *S. oweniana*; or alternatively that it was lost in the ancestor of *Sepietta-Rondeletiola* followed by a reversion of this character in the lineage of *Rondeletiola*.

The light organ is also present in all members of Heteroteuthinae. These bobtails are pelagic as adults, and their light organ appears as a single visceral organ rather than the bilobed form found in nekto-benthic Sepiolinae. In contrast to the bacteriogenic luminescence of the light organ in *E. scolopes*³¹, previous studies in *H. dispar*³ failed to detect symbiotic bacteria and suggested that the luminescence has an autogenic origin. Thus, it seems plausible that the monophyly of Heteroteuthinae found in our study supports the findings in Lindgren et al.⁹ for convergent evolution of autogenic light organs associated with pelagic lifestyle in many squid, octopus, and *Vampyroteuthis* Chun, 1903^{9,32}.

Divergence time of Sepioida. The absence of fossils for this group limited our calculations of divergence time to the use of secondary calibrations. These calibrations can provide more accurate estimates depending on the type of primary calibrations that are used³³. We retrieved secondary calibrations from previous estimations in Tanner et al.¹⁵, who used eleven fossil records spanning from coleoids to gastropods in transcriptome-

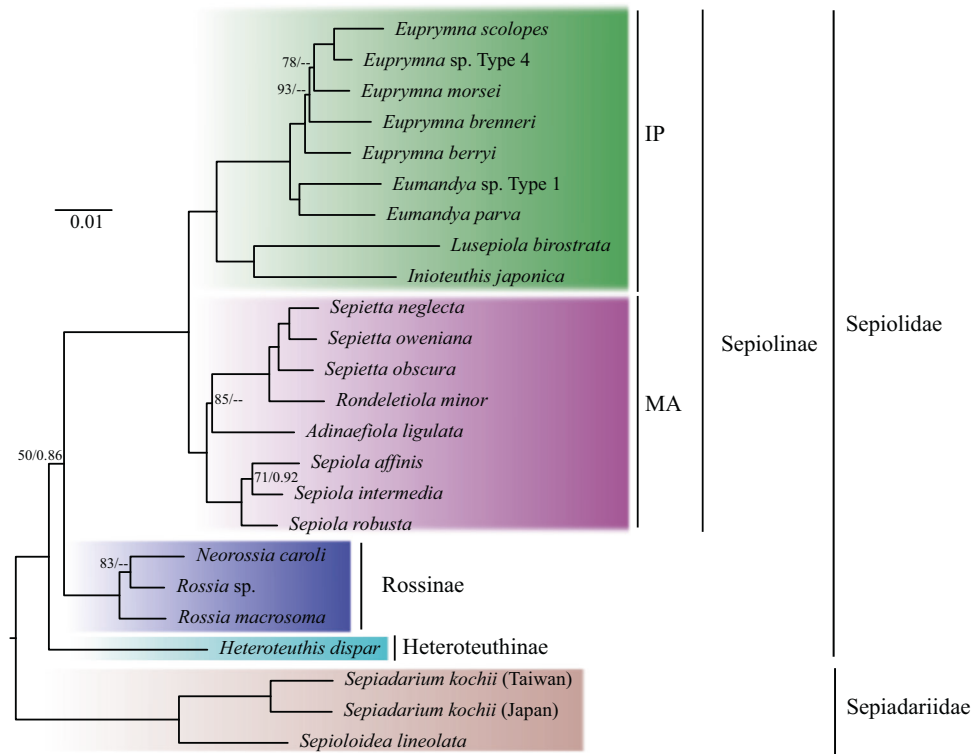


Fig. 2 Phylogenetic tree of Sepioida based on conserved nuclear loci (UCEbob matrix). The topology of the maximum likelihood tree is shown. Numbers in by the nodes indicate the bootstrap support and the Bayesian posterior probability. Values of bootstrap support and posterior probabilities above 95% and 0.95, respectively, are not shown. IP, Indo-Pacific Ocean; MA, Mediterranean Sea, and the Atlantic Ocean.

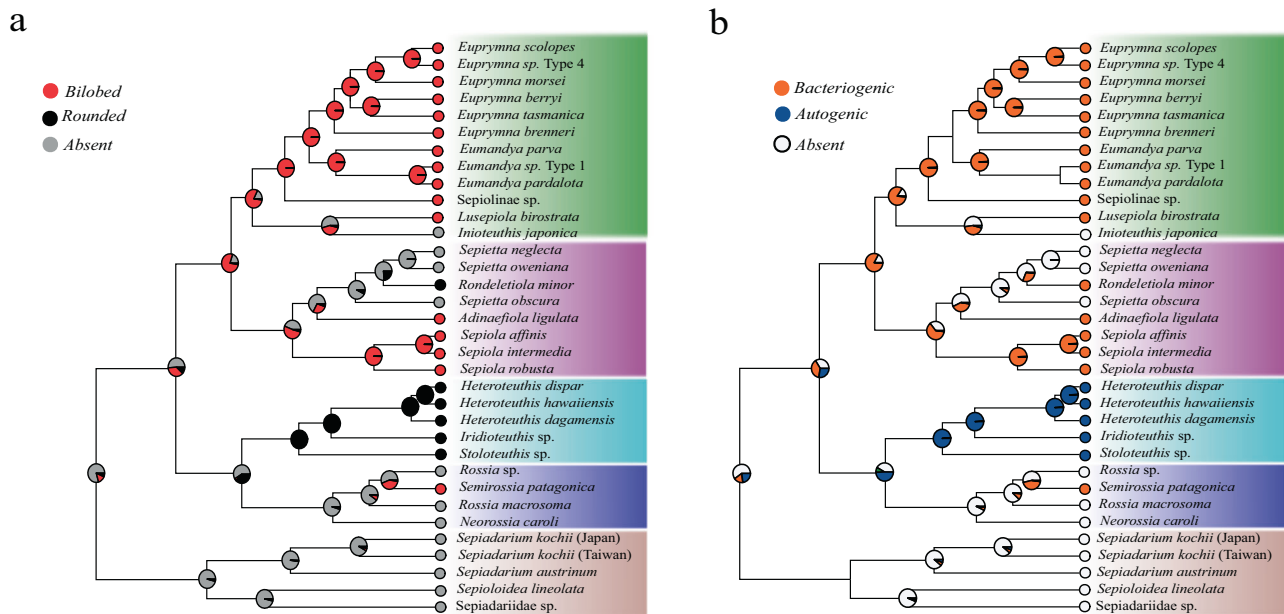


Fig. 3 Ancestral character reconstruction (ASR). ASR of (a) the shape of the light organ and (b) the origin of luminescence in the Sepioida. The posterior probability of each state is shown as a pie chart, mapped tree generated in BEAST (based on mito_nt matrix, see below), with the outgroups removed.

based phylogenetic trees. Specifically, we used the time for the splits of *Sepia esculenta* and *S. officinalis* (~91 Mya), Idiosepiidae, and Sepioida (~132 Mya) and the origin of the Decapodiformes (root age, ~174 Mya) (Fig. 4). These calibrations and our robust phylogenetic trees allow us to investigate the events that shape the divergence of some clades of the order Sepioida (Figs. 4, S4).

Sepioida appeared before the Cretaceous-Paleogene extinction event³⁴, during the middle Mesozoic around 94 Mya (95% HPD =

60.61–130.72). This time frame coincides with the rapid diversification of several oegopsida lineages^{15,35}. Our molecular estimates also indicate that radiation of Sepiolidae and Sepidiariidae occurred around the Cretaceous-Paleogene boundaries and is concurrent with the rapid diversification of modern marine percomorph fishes around the globe, after the extinction of Mesozoic fishes^{36,37}.

Among the species of Sepiolinae collected in the Mediterranean Sea for this study, only *Sepioloidea robusta* Naef, 1912, and *Sepioloidea*

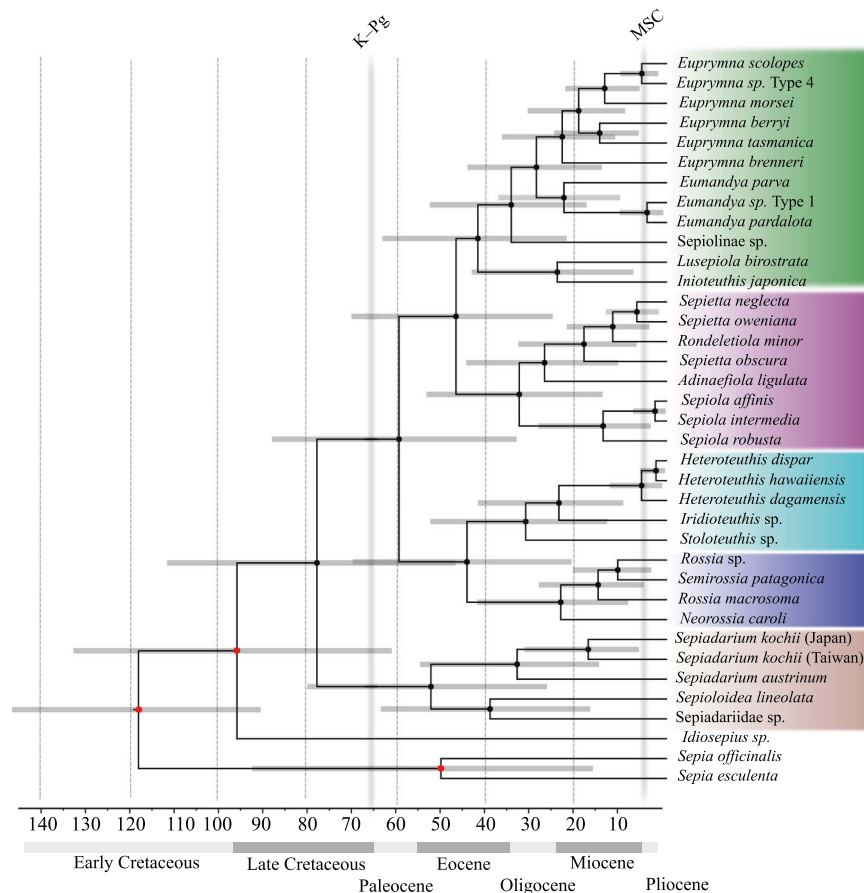


Fig. 4 A chronogram of sepiolids using complete mitochondrial genes. Red dots indicate the nodes with secondary calibrations. K-Pg, refers to the Cretaceous-Paleogene boundary and MSC, to the Messinian salinity crisis.

affinis Naef, 1912 are endemic to the Mediterranean Sea³⁸. The distribution of the other species includes the Mediterranean Sea, North Atlantic Ocean, East Atlantic Ocean, and/or up to the Gulf of Cadiz. The confidence intervals for the split between the Mediterranean-Atlantic and Indo-Pacific lineages, and their diversification, overlap during the early Eocene to the beginning of the Oligocene (Figs. 4 and S4). This time interval coincides with the end of the Tethys Sea, which separated the Indo-Pacific from the Mediterranean and Atlantic region through the Indian-Mediterranean Seaway^{39,40}. This separation also influenced the divergence of loliginid clades, coinciding with the split between the Eastern Atlantic plus Mediterranean clade (*Loligo*, *Afrololigo*, *Alloteuthis*) and Indo-Pacific clade (*Uroteuthis* and *Loliolus*) (~55 Mya based on Fig. 2 in Anderson and Marian⁴¹).

Our chronogram indicates that the ancestor of Sepiolinae arose prior to the early Eocene around 46 Mya (95% HPD = 25.16–69.49) (Fig. 4), already possessing a bilobed light organ hosting luminescent bacteria (Fig. 3). We estimate that the split between *S. affinis* and *S. intermedia* occurred around 2.62 Mya (95% HPD = 0.3–7.4) (Fig. 4) during the end of the Zanclean period, when the Atlantic Ocean refilled the Mediterranean after the Messinian salinity crisis^{42,43}. While *S. affinis* is a coastal species with a narrow depth limit, *S. intermedia* inhabits a wider range of deeper waters. It is possible that two populations of their ancestor, each adapted to a different ecological niche and diverged sympatrically in Mediterranean waters, and, after the speciation, *S. intermedia* extended its distribution outside the Mediterranean to the Gulf of Cadiz⁴⁴.

We also estimate that the split between *H. dispar* Rüppell, 1844 and *H. hawaiiensis* (Berry, 1909) occurred around 2.4 Mya (95%

HPD = 0.46–5.88), coinciding with the closure of the Isthmus of Panama around 2.8 Mya⁴⁵. Surveys of these species found *H. hawaiiensis* in the North Pacific and *H. dispar* in the North Atlantic Ocean and Mediterranean Sea⁴⁶. A recent speciation event might be the reason for the lack of morphological differences between the two species⁴⁶. Thus, these species may be rendered as cryptic species, a phenomenon increasingly reported in oceanic cephalopods⁴⁷. The sister species of this cryptic species complex, *H. dagamensis* Robson, 1924, appeared before, around 6 Mya, and is reported with broad distribution in the South Atlantic Ocean off South Africa, the Gulf of Mexico, North Atlantic Ocean between Ireland and Newfoundland in Canada, and the South Pacific Ocean off New Zealand^{48–50}.

The origin of the *Heteroteuthis* ancestor of *H. dispar*, *H. hawaiiensis*, and *H. dagamensis* can be placed in the Pacific Ocean. After the formation of the Isthmus of Panama, the northern population of *Heteroteuthis* might have split into *H. hawaiiensis* in North Pacific and *H. dispar* in the Atlantic Ocean (from where it also migrated to the Mediterranean Sea). Meanwhile, the formation of the equatorial currents isolated the southern population of *Heteroteuthis* and gave rise to *H. dagamensis*. Then, *H. dagamensis* extended its distribution from the Southern Pacific to the South Atlantic Ocean, the North Atlantic waters, and the Gulf of Mexico. Analysis of molecular species delimitation, however, suggests that *H. dagamensis* includes cryptic lineages among Atlantic and New Zealand populations³⁰.

While the origin of *Heteroteuthis* might also be in the Atlantic Ocean, the higher diversity of heteroteuthins in the Pacific (*H. hawaiiensis*, *H. dagamensis*, *H. ryukyensis* Kubodera, Okutani and

Kosuge, 2009, *H. nordpacific* Kubodera and Okutani, 2011, and an unknown *H. sp.* KER (only known from molecular studies⁴⁹)) than at the Atlantic (*H. dispar* and *H. dagamensis*), make its origin at the Atlantic less plausible. Moreover, the Atlantic *Heteroteuthis* were found nested within Heteroteuthinae species from the Pacific, supporting Pacific Ocean origin (Figs. 1, 4).

By sequencing the genomic DNA of sepiolids at low coverage, we recovered complete mitochondrial genomes and nuclear ribosomal genes for most of our collections. Furthermore, mapping reads to the reference genome of *E. scolopes* allowed us to retrieve additional nuclear-ultraconserved regions. We demonstrate that these nuclear and mitochondrial loci are useful to reconstruct robust phylogenetic trees, especially when the transcriptomes of specimens are difficult to collect, as for sepiolids inhabiting oceanic environments. Finally, our study integrated genomic DNA sequencing with confident morphological identification, which helped to reconstruct the ancestral character of the light organ and its luminescence in sepiolids, and clarify how major lineages have evolved, establishing the existence of distinct Indo-Pacific and Mediterranean-Atlantic subfamilies of Sepiolinae. Our collections and genomically anchored phylogenies will provide a reliable foundation classification of sepiolids for future studies.

Materials and methods

Specimens and data collection. For this study, we sampled 32 different species of bobtail and bottletail squids. Samples were collected in different locations of the Japan Sea, Western North Pacific Ocean, Atlantic Ocean, Mediterranean Sea, Tasman Sea, Great Australian Bight, Banda Sea, and South Pacific Ocean. Specimens comprised members of the family Sepiariidae and all the valid subfamilies of Sepiolidae (Sepiolinae, Heteroteuthinae, and Rossinae). For each individual, a small piece of tissue from the mantle was stored in 70% ethanol for DNA analyses and the remaining animals were stored in museums. Details of our specimens are found in Table S1.

Individuals collected off the coast of mainland Japan and the Ryukyu Archipelago were previously identified in Sanchez et al.¹¹. Mediterranean species were identified based on general morphology and/or the hectocotylus following Bello⁵¹. We also retrieved the full mitochondrial genome of *Semirossia patagonica* (E. A. Smith, 1881) (AP012226) and *Sepiadarium austrinum* Berry, 1921 (KX657686), mitochondrial genes from the transcriptome of *Euprymna tasmanica* (Pfeffer, 1884) (SRR2984339), and the nuclear reads of *Euprymna scolopes* (PRJNA470951) from the GenBank database. For the outgroups, we retrieved the complete mitochondrial genome of *Sepia esculenta* Hoyle, 1885 (NC_009690), *Sepia officinalis* Linné, 1785 (NC_007895) and *Idiosepius* sp. (KF647895), the complete 18S rRNA gene of *Idiosepius pygmaeus* Steenstrup, 1881 (AY557477.1), and a partial region of the 28S rRNA gene of *Idiosepius pygmaeus* (AY293684.1). While the proximal outgroup of the Sepiolidae remains unclear (with different tree topologies found based on transcriptome sequences in Tanner et al.¹⁵, Lindgren and Anderson⁵², and Anderson and Lindgren¹⁷), we believe the inclusion of Sepiariidae should prevent any conflict relationships within Sepiolidae.

DNA isolation, library preparation, and data filtering. We isolated the genomic DNA of our specimens using the DNeasy Blood and Tissue kit (Qiagen). Genomic libraries were constructed using Illumina TruSeq DNA LT Sample Prep Kit. Libraries were size selected to 300 bp, pooled, and sequenced on an Illumina HiSeq 4000 (2 × 150 bp paired-end run). We generated around 1.6–3.6X coverage per species (assuming the total number of reads, the read length, and a genome size of 5 GB reported for *Euprymna scolopes*¹⁰ (Table S1)).

Illumina reads were demultiplexed, and low-quality reads were filtered using Trim Galore v. 0.4.0 (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) and adapter-trimmed with *cutadapt*⁵³.

Genome skimming. Bioinformatic analyses were performed on the Okinawa Institute of Science and Technology (OIST) HPC cluster and the National Institute of Genetics cluster.

Mitochondrial genes. We assembled the mitochondrial genome *de novo* for all our collections using NOVOplasty 4.2⁵⁴ with the cytochrome oxidase I gene of each species or the closest relative species available on the GenBank as a seed and the complete mitochondrial genome of *Idiosepius* sp. Reid and Strugnell 2018 (KF647895) as a reference. The FASTA files recovered were annotated using the standalone ORFfinder (<https://www.ncbi.nlm.nih.gov/orffinder/>). We selected protein-coding genes per species based on the best hit of their amino-acid sequences with the amino-acid sequences retrieved from the mitochondrial

genome of *Idiosepius* sp. using the blastp in the standalone NCBI BLAST v2.10 (<https://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/LATEST/>). We used the MITOS Web Server⁵⁵ to annotate ribosomal and transfer genes. The information on the gene order from each mitochondrial genome was obtained from the overall results of the MITOS Web Server and ORFfinder.

Protein coding genes were codon-based aligned using MUSCLE 3.8⁵⁶ implemented in AliView⁵⁷ and the borders were trimmed manually. Ribosomal RNAs were aligned using R-coffee⁵⁸ and poorly aligned regions were deleted through Gblocks v0.91b⁵⁹. Transfer RNAs were concatenated for further analyses.

Nuclear genes. We assembled the nuclear ribosomal genes 28S and 18S using NOVOplasty. The seed used was a conserved region from multiple 18S and 28S sequences retrieved from GenBank and our alignment. The contigs retrieved were annotated in RNAmmer 1.2 Server⁶⁰. Alignment and trimming were performed following the same methodology as the mitochondrial ribosomal genes.

Ultraconserved loci. We only considered 24 species sequenced with a depth of coverage higher than 2X. Illumina reads were aligned to the reference genome of *Euprymna scolopes* using the *bwa mem* algorithm from the Burrows-Wheeler Aligner (BWA) v0.7.17⁶¹ with the default mode. We filtered bam files with reads mapped with a quality of at least 40 using Samtools v1.9⁶² and marked duplicate reads with Sambamba v0.7.1⁶³. Using these bam files, we generated a list of regions shared between at least two species using PHYLUCE⁶⁴. This list was used to generate a dataset of loci (UCEbob) that are shared in a minimum of 14 species (58%) to recover enough polymorphic sites for the phylogenetic analyses (see below).

For UCEbob, we first created VCF files for each species per shared loci using *freebayes* v.1.3.2⁶⁵ with the settings: -min-coverage 2, -report-monomorphic, -limit-coverage 5, and -use-best-n-alleles 1. Then, VCF files were used to reconstruct aligned FASTA files of DNA sequences using custom scripts. For each locus, we removed sites with gaps presented in more than 50% of the species using Gblocks v0.91b⁵⁹.

Phylogenetic analysis. Apart from the UCEbob matrix, we also created different alignment matrices: (1) *mito_aa*: with amino acid sequences from mitochondrial genes, (2) *mito_nc*: with nucleotide sequences from all coding and non-coding mitochondrial genes, (3) *nuclear_rRNA*: with the nuclear ribosomal genes.

For each matrix, we performed maximum likelihood (ML) phylogeny in IQ-TREE⁶⁶ with the best model and partition scheme selected by ModelFinder⁶⁷, and 1000 replicates of ultrafast likelihood bootstrap⁶⁸. Additionally, we performed a phylogenetic inference with a Bayesian approach in Exabayes v.1.5⁶⁹ for all matrices using a partition per gene and the GTR + Gamma model of substitution. Two independent chains of more than 1.5 × 10⁶ generations were run in parallel, sampling every 1000 generations. The chains' convergence was validated when the average standard deviation from split frequencies was less than 0.5% and the ESS more than 200. Finally, we discarded the first 25% generations as burn-in, and the 50% majority consensus tree was summarized using the Consensus script from Exabayes.

Ancestral character reconstruction. We used BayesTraits v3⁷⁰ with the multi-state model of discrete traits to obtain the posterior probability of the light organ shape and the luminescence origin of ancestral sepiolids. We coded the shape of the light organ as bilobed, rounded, and absent; and luminescence origin as autogenic, bacteriogenic, and absent. For taxa with no information about the state of the trait analyzed, we assume such a state is the same as its congeneric species.

We applied the reversible jump MCMC model with hyper prior exponential distribution of interval from 0 to 30, as this prior produces the best acceptance rate (average of 30%). As the input trees, we used 24335 trees generated from our divergence time analysis in BEAST (see below) after 25% of burn-in. We run BayesTraits with 1 million interactions, sampling every 1000th iteration; and plot the mean probabilities of each character in the ultrametric tree generated with the *mito_nt* matrix using the R package *ape*⁷¹.

Divergence time. Divergence time for several lineages within Sepiolida was estimated using a Bayesian approach implemented in BEAST v2.6⁷² with nucleotide sequences of the mitochondrial gene (the *mito_nc* matrix). The analysis in BEAST was run with the best model of substitution for each partition selected by *brmodeltest*⁷³, the Yule model of speciation, and the fast relaxed lognormal clock. We used internal calibrations estimated in Fig. S4 of Tanner et al.¹⁵ as priors with the normal distribution and the mean in real space as follows: Decapodiformes Leach, 1817 (mean: 174.22 Mya; SD: 26), Sepiida Zittel, 1895 (mean: 91.13 Mya; SD: 20; and “use originate”), and for the split of Idiosepiidae Appellof, 1898 and Sepiolida (mean: 132.37 Mya; SD: 26; and “use originate”). The analysis was run twice for 160 million generations, sampling every 5000 generations. The convergence of ESS > 200 was evaluated in Tracer v.1.7.1⁷⁴ and the chronogram was annotated in TreeAnnotator using the topology of the maximum likelihood tree constructed with *mito_nc* matrix, with a burn-in of 10% and mean node heights.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Input and output for analyses in BayesTraits, and alignments, best model and partition scheme for each matrix can be found in FigShare (<https://figshare.com/s/1e0dec1d073a34fee2a>). Raw reads can be found in the GenBank database under the BioProject number PRJNA640585.

Received: 23 December 2020; Accepted: 13 May 2021;

Published online: 29 June 2021

References

- Vecchione, M. & Young, R. E. Sepioida Keferstein, 1866. Version 26 March 2019 (In press). <http://tolweb.org/Sepioida/23872/2019.03.26> in The Tree of Life Web Project, <http://tolweb.org/> (2019).
- Boettcher, K. J., Ruby, E. G. & McFall-Ngai, M. J. Bioluminescence in the symbiotic squid *Euprymna scolopes* is controlled by a daily biological rhythm. *J. Comp. Physiol. A* **179** (1996).
- Dilly, P. N. & Herring, P. J. The light organ and ink sac of *Heteroteuthis dispar* (Mollusca: Cephalopoda). *J. Zoo.* **186**, 47–59 (1978).
- Caruana, N. J., Strugnell, J. M., Faou, P., Finn, J. & Cooke, I. R. Comparative Proteomic Analysis of Slime from the Striped Pyjama Squid, *Sepioloidea lineolata*, and the Southern Bottletail Squid, *Sepiadarium austrinum* (Cephalopoda: Sepiariidae). *J. Proteome Res.* **18**, 890–899 (2019).
- Neff, E. P. Considering the cephalopod. *Lab Anim.* **48**, 19–22 (2019).
- Urbanczyk, H., Ast, J. C., Higgins, M. J., Carson, J. & Dunlap, P. V. Reclassification of *Vibrio fischeri*, *Vibrio loeii*, *Vibrio salmonicida* and *Vibrio wodanis* as *Aliivibrio fischeri* gen. nov., comb. nov., *Aliivibrio loeii* comb. nov., *Aliivibrio salmonicida* comb. nov. and *Aliivibrio wodanis* comb. nov. *Int. J. Syst. Evol. Microbiol.* **57**, 2823–2829 (2007).
- McFall-Ngai, M. J. The importance of microbes in animal development: lessons from the squid-vibrio symbiosis. *Annu. Rev. Microbiol.* **68**, 177–194 (2014).
- Ruby, E. G. Lessons from a cooperative, bacterial-animal association: the *Vibrio fischeri*–*Euprymna scolopes* light organ symbiosis. *Annu. Rev. Microbiol.* **50**, 591–624 (1996).
- Lindgren, A. R., Pankey, M. S., Hochberg, F. G. & Oakley, T. H. A multi-gene phylogeny of Cephalopoda supports convergent morphological evolution in association with multiple habitat shifts in the marine environment. *BMC Evol. Biol.* **12**, 129 (2012).
- Belcaid, M. et al. Symbiotic organs shaped by distinct modes of genome evolution in cephalopods. *Proc. Natl Acad. Sci. U.S.A.* **116**, 3030–3035 (2019).
- Sanchez, G. et al. New bobtail squid (Sepioidae: Sepiolineae) from the Ryukyu islands revealed by molecular and morphological analysis. *Commun. Biol.* **2**, 465 (2019).
- Pankey, M. S., Minin, V. N., Imholte, G. C., Suchard, M. A. & Oakley, T. H. Predictable transcriptome evolution in the convergent and complex bioluminescent organs of squid. *Proc. Natl Acad. Sci. U.S.A.* **111**, E4736–E4742 (2014).
- Caruana, N. J. et al. A combined proteomic and transcriptomic analysis of slime secreted by the southern bottletail squid, *Sepiadarium austrinum* (Cephalopoda). *J. Proteom.* **148**, 170–182 (2016).
- Caruana, N. J. et al. Quantitative proteomic analysis of the slime and ventral mantle glands of the Striped Pyjama Squid (*Sepioloidea lineolata*). *J. Proteome Res.* **19**, 1491–1501 (2020).
- Tanner, A. R. et al. Molecular clocks indicate turnover and diversification of modern coleoid cephalopods during the Mesozoic Marine Revolution. *Proc. R. Soc. B* **284**, 20162818 (2017).
- Strugnell, J. & Nishiguchi, M. K. Molecular phylogeny of coleoid cephalopods (Mollusca: Cephalopoda) inferred from three mitochondrial and six nuclear loci: a comparison of alignment, implied alignment and analysis methods. *J. Mollusca Stud.* **73**, 399–410 (2007).
- Anderson, F. E. & Lindgren, A. R. Phylogenomic analyses recover a clade of large-bodied decapodiform cephalopods. *Mol. Phylogenet. Evol.* **156**, 107038 (2021).
- Allcock, A. L., Lindgren, A. & Strugnell, J. M. The contribution of molecular data to our understanding of cephalopod evolution and systematics: a review. *J. Nat. Hist.* **0**, 1–49 (2014).
- Bello, G. Evolution of the hectocotylus in Sepiolineae (Cephalopoda: Sepioidae) and description of four new genera. *EJT* (2020) <https://doi.org/10.5852/ejt.2020.655>.
- Kubodera, T. & Okutani, T. New additions of luminous bobtail squids to the Japanese Cephalopod Fauna (Sepioida: Sepioidae: Heteroteuthinae). *Venus. J. Malacol. Soc. Jpn.* **69**, 145–161 (2011).
- Dodsworth, S. Genome skimming for next-generation biodiversity analysis. *Trends Plant Sci.* **20**, 525–527 (2015).
- Sanchez, G. et al. Genus-level phylogeny of cephalopods using molecular markers: current status and problematic areas. *PeerJ* **6**, e4331 (2018).
- Nishiguchi, M. K., Lopez, J. E. & Boletzky, S. V. Enlightenment of old ideas from new investigations: more questions regarding the evolution of bacteriogenic light organs in squids. *Evol. Dev.* **6**, 41–49 (2004).
- Young, R. E. Sepioidae Leach 1817. Version 26 March 2019 (In press). <http://tolweb.org/Sepioidae/19988/2019.03.26> in The Tree of Life Web Project, <http://tolweb.org/> (2019).
- Kawashima, Y. et al. The complete mitochondrial genomes of deep-sea squid (*Bathyteuthis abyssicola*), bob-tail squid (*Semirossia patagonica*) and four giant cuttlefish (*Sepia apama*, *S. latimanus*, *S. lycidas* and *S. pharaonis*), and their application to the phylogenetic analysis of Decapodiformes. *Mol. Phylogenet. Evol.* **69**, 980–993 (2013).
- Jones, B. W. & Nishiguchi, M. K. Counterillumination in the Hawaiian bobtail squid, *Euprymna scolopes* Berry (Mollusca: Cephalopoda). *Mar. Biol.* **144**, 1151–1155 (2004).
- Lupp, C. & Ruby, E. G. *Vibrio fischeri* uses two quorum-sensing systems for the regulation of early and late colonization factors. *J. Bacteriol.* **187**, 3620–3629 (2005).
- Verma, S. C. & Miyashiro, T. Quorum sensing in the squid-*Vibrio* symbiosis. *Int. J. Mol. Sci.* **14**, 16386–16401 (2013).
- Groenenberg, D. S. J., Goud, J., De Heij, A. & Gittenberger, E. Molecular phylogeny of North Sea Sepiolineae (Cephalopoda: Sepioidae) reveals an overlooked *Sepiola* species. *J. Mollusca Stud.* **75**, 361–369 (2009).
- Fernández-Álvarez, F. Á., Sánchez, P. & Villanueva, R. Morphological and molecular assessments of bobtail squids (cephalopoda: Sepioidae) reveal a hidden history of biodiversity. *Front. Mar. Sci.* **7**, (2021).
- McFall-Ngai, M., Heath-Heckman, E. A. C., Gillette, A. A., Peyer, S. M. & Harvie, E. A. The secret languages of coevolved symbioses: insights from the *Euprymna scolopes*-*Vibrio fischeri* symbiosis. *Semin. Immunol.* **24**, 3–8 (2012).
- Herring, P. J. Luminescent Organs. *The Mollusca: Form and Function* (eds E. R. Trueman & M. R. Clarke), **11**, 449–489. (New York, NY: Academic Press, 1988).
- Powell, C. L. E., Waskin, S. & Battistuzzi, F. U. Quantifying the error of secondary vs. distant primary calibrations in a simulated environment. *Front. Genet.* **11**, 252. <https://doi.org/10.3389/fgene.2020.00252> (2020).
- Friedman, M. Explosive morphological diversification of spiny-finned teleost fishes in the aftermath of the end-Cretaceous extinction. *Proc. Biol. Sci.* **277**, 1675–1683 (2010).
- Pardo-Gandarillas, M. C., Torres, F. I., Fuchs, D. & Ibáñez, C. M. Updated molecular phylogeny of the squid family Ommastrephidae: insights into the evolution of spawning strategies. *Mol. Phylogenet. Evol.* **120**, 212–217 (2018).
- Alfaro, M. E. et al. Explosive diversification of marine fishes at the Cretaceous–Palaeogene boundary. *Nat. Ecol. Evol.* **2**, 688–696 (2018).
- Sibert, E. C. & Norris, R. D. New age of fishes initiated by the Cretaceous–Paleogene mass extinction. *Proc. Natl Acad. Sci. U.S.A.* **112**, 8537–8542 (2015).
- Bello, G. The Mediterranean Sepioidae (Mollusca Cephalopoda) diversity. *Biodivers. J.* **10**, 389–404 (2019).
- Cowman, P. F. & Bellwood, D. R. The historical biogeography of coral reef fishes: global patterns of origination and dispersal. *J. Biogeogr.* **40**, 209–224 (2013).
- Rögl, F. Palaeogeographic considerations for Mediterranean and Paratethys Seaways (Oligocene to Miocene). *Ann. Naturhist. Mus. Wien.* **99**, 279–310 (1997).
- Anderson, F. E. & Marian, J. E. A. R. The grass squid *Pickfordiateuthis pulchella* is a paedomorphic loliginid. *Mol. Phylogenet. Evol.* **147**, 106801 (2020).
- García-Castellanos, D. et al. Catastrophic flood of the Mediterranean after the Messinian salinity crisis. *Nature* **462**, 778–781 (2009).
- Krijgsman, W., Hilgen, F. J., Raffi, I., Sierro, F. J. & Wilson, D. S. Chronology, causes and progression of the Messinian salinity crisis. *Nature* **400**, 652–655 (1999).
- Guerra, Á. Cefalópodos capturados en la campaña ‘Golfo de Cádiz-81’. (1982).
- O’Dea, A. et al. Formation of the Isthmus of Panama. *Sci. Adv.* **2**, e1600883 (2016).
- Young, R. E., Vecchione, M. & Roper, C. F. E. *Heteroteuthis hawaiiensis* Berry 1909. Version 26 December 2007 (In press). http://tolweb.org/Heteroteuthis_hawaiiensis/20055/2007.12.26 in The Tree of Life Web Project, <http://tolweb.org/> (2007).
- Fernández-Álvarez, F. Á. et al. Global biodiversity of the genus *Ommastrephes* (Ommastrephidae: Cephalopoda): an allopatric cryptic species complex. *Zool. J. Linn. Soc.* **190**, 460–482 (2020).
- Judkins, H. L., Vecchione, M. & Rosario, K. Morphological and molecular evidence of *Heteroteuthis dagamensis* in the Gulf of Mexico. *Bull. Mar. Sci.* **92**, 51–57 (2016).

49. Braid, H. E. & Bolstad, K. S. R. Cephalopod biodiversity of the Kermadec Islands: implications for conservation and some future taxonomic priorities. *Invertebr. Syst.* **33**, 402–425 (2019).
50. Taite, M., Vecchione, M., Fennell, S. & Allcock, L. A. Paralarval and juvenile cephalopods within warm-core eddies in the North Atlantic. *Bull. Mar. Sci.* **96**, 235–262 (2020).
51. Bello, G. A key for the identification of the Mediterranean sepiolids (Mollusca: Cephalopoda). *Bull. Inst. Oceanogr.* **16**, 41–56 (1995).
52. Lindgren, A. R. & Anderson, F. E. Assessing the utility of transcriptome data for inferring phylogenetic relationships among coleoid cephalopods. *Mol. Phylogenet. Evol.* **118**, 330–342 (2018).
53. Martin, M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet. J.* **17**, 10–12 (2011).
54. Dierckxens, N., Mardulyn, P. & Smits, G. NOVOPlasty: de novo assembly of organelle genomes from whole genome data. *Nucleic Acids Res.* **45**, e18 (2017).
55. Bernt, M. et al. MITOS: improved de novo metazoan mitochondrial genome annotation. *Mol. Phylogenet. Evol.* **69**, 313–319 (2013).
56. Edgar, R. C. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **32**, 1792–1797 (2004).
57. Larsson, A. AliView: a fast and lightweight alignment viewer and editor for large datasets. *Bioinformatics* **30**, 3276–3278 (2014).
58. Wilm, A., Higgins, D. G. & Notredame, C. R-Coffee: a method for multiple alignment of non-coding RNA. *Nucleic Acids Res.* **36**, e52 (2008).
59. Castresana, J. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol. Biol. Evol.* **17**, 540–552 (2000).
60. Lagesen, K. et al. RNAMmer: consistent and rapid annotation of ribosomal RNA genes. *Nucleic Acids Res.* **35**, 3100–3108 (2007).
61. Li, H. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. arXiv preprint arXiv:1303.3997 (2013).
62. Li, H. et al. The sequence Alignment/Map format and SAMtools. *Bioinformatics* **25**, 2078–2079 (2009).
63. Tarasov, A., Vilella, A. J., Cuppen, E., Nijman, I. J. & Prins, P. Sambamba: fast processing of NGS alignment formats. *Bioinformatics* **31**, 2032–2034 (2015).
64. Faircloth, B. C. PHYLUCE is a software package for the analysis of conserved genomic loci. *Bioinformatics* **32**, 786–788 (2016).
65. Garrison, E. & Marth, G. Haplotype-based variant detection from short-read sequencing. arXiv preprint arXiv:1207.3907 (2012).
66. Minh, B. Q. et al. IQ-TREE 2: new models and efficient methods for phylogenetic inference in the genomic era. *Mol. Biol. Evol.* **37**, 1530–1534 (2020).
67. Kalyaanamoorthy, S., Minh, B. Q., Wong, T. K. F., von Haeseler, A. & Jermini, L. S. ModelFinder: fast model selection for accurate phylogenetic estimates. *Nat. Methods* **14**, 587–589 (2017).
68. Hoang, D. T., Chernomor, O., von Haeseler, A., Minh, B. Q. & Le, S. V. UFBoot2: improving the Ultrafast Bootstrap Approximation. *Mol. Biol. Evol.* **35**, 518–522 (2017).
69. Aberer, A. J., Kobert, K. & Stamatakis, A. ExaBayes: massively parallel Bayesian tree inference for the Whole-Genome Era. *Mol. Biol. Evol.* **31**, 2553–2556 (2014).
70. Pagel, M., Meade, A. & Barker, D. Bayesian estimation of ancestral character states on phylogenies. *Syst. Biol.* **53**, 673–684 (2004).
71. Paradis, E., Claude, J. & Strimmer, K. APE: analyses of phylogenetics and evolution in R language. *Bioinformatics* **20**, 289–290 (2004).
72. Bouckaert, R. et al. BEAST 2: a software platform for Bayesian evolutionary analysis. *PLoS Comput. Biol.* **10**, e1003537 (2014).
73. Bouckaert, R. R. & Drummond, A. J. bModelTest: Bayesian phylogenetic site model averaging and model comparison. *BMC Evol. Biol.* **17**, 42 (2017).
74. Rambaut, A., Drummond, A. J., Xie, D., Baele, G. & Suchard, M. A. Posterior summarization in bayesian phylogenetics using tracer 1.7. *Syst. Biol.* **67**, 901–904 (2018).

Acknowledgements

This project was funded by the Okinawa Institute of Science and Technology (OIST) to the Molecular Genetic Units. D.S.R. is also grateful for the generous support of the

Marthella Foskett-Brown Chair in Biology and from the Chan-Zuckerberg BioHub. We wish to thank the staff of the OIST DNA sequencing section for the library preparation and sequencing. G.S. and M.T. wish to especially thank Amanda Reid from the Australian Museum Research Institute for her help in identifying *Rossia* sp., *Iniotheuthis japonica*, and *Euprymna* Type 4; and for providing samples of *Euprymna pardalota*, *Iridoteuthis* sp., *Sepiolinae* sp., and *Heteroteuthis hawaiiensis* used in this study. M.T. also wants to thanks to Professor Kat Bolstad, head of the AUT Lab for Cephalopod Ecology & Systematics at the Institute for Applied Ecology New Zealand for kindly sharing the specimens of *Heteroteuthis dagamensis* and *Sepiadiariidae* sp., and to Professor Jan Strugnell from the James Cook University in Australia for *Stoloteuthis* sp.. F.Á.F-Á wants to thank Elena Guerrero (ICM-CSIC) for performing the curation of some morphological vouchers in the Biological Reference Collections (CBR-ICM) at the Institut de Ciències del Mar (ICM-CSIC), Barcelona, Spain. S.C. wishes thanks to Dr. Hiroshi Miyagai who through the Fisheries Cooperative Association of Hamasaka in Hyogo Prefecture, collected samples of *Rossia* sp. and to Dr. Hideo Takahara from the Fisheries and Oceans Hakodate for samples from Niigata Prefecture. J.J. and S.C. also wish to thank Ryo Minemizu from Ryo Minemizu Photo Office for the collection of *Euprymna* sp. Type 4. F.Á.F-Á was supported by the Irish Research Council-Government of Ireland Post-doctoral Fellowship Award (Ref. GOIPD/2019/460). M.T. is funded by a Ph.D. fellowship from the Irish Research Council and is supported by the Dr. Tony Ryan Research Fund.

Author contributions

G.S. and D.S.R. conceived and designed the project. G.S. performed computational analyses with input from D.S.R. and F.M.. G.S. and F.Á.F-Á. interpreted phylogenetic trees. G.S., O.S., C.S., and J.J. collected samples from Japanese waters. F.Á.F-Á. collected and identified samples of the Atlantic Ocean and the Mediterranean Sea. M.T. and L.A. collected and sequenced samples from the South Pacific Ocean. G.S., F.Á.F-Á. and D.S.R. wrote the manuscript. All authors provided comments on the paper.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s42003-021-02348-y>.

Correspondence and requests for materials should be addressed to D.S.R.

Peer review information *Communications Biology* thanks the anonymous reviewers for their contribution to the peer review of this work.

Reprints and permission information is available at <http://www.nature.com/reprints>

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2021