

## **An Initial Analysis into the Timeline of Adaptive Radiation in Cephalopod Species**

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Cephalopoda is class of marine invertebrates including species that are among the most behaviorally and morphologically complex within the phylum Mollusca. Coleoid cephalopods comprise the largest portion of the class and are characterized by a soft outer body due to their shell becoming internalized over millions of years. This group is made up of true squids, cuttlefish, bottle and bobtail squids, and octopus species. In the evolutionary transition from shelled and slow moving to agile yet unprotected, they have developed notably advanced and wildly unique neurological systems as evidenced by their complex cognitive and problem solving abilities, acute eyesight through camera like eyes, and nuanced methods of communication that set them apart from all other species across the invertebrate lineage.

Ancient ancestors and kin of extant cephalopods, namely the ammonites and belemnites, have been well studied thanks to an abundance of examples of these shelled creatures contained within the fossil record. Conversely, because soft tissues do not typically preserve well, or at all in most cases, little information has been able to be inferred regarding the diversification of coleoid cephalopod species through time since they first began to diverge from their shelled origins. Additionally, cryptic diversity plays a role in the challenge of identifying speciation events within this class by morphological means. The rise of Next Generation Sequencing providing an abundance of molecular data in recent decades now enables us to find greater clarity in the relationships between and evolutionary history of this enigmatic class of creatures.

While species and clade specific genomic data is being consistently employed for the advancement of knowledge of phylogenetic relationships within this class, no reference spanning the entire class has yet been published to serve as a foundational study from which to gain more wide reaching clarity of relationships between differing cephalopod clades and families. It is generally accepted that coleoid cephalopods experienced adaptive radiation during the Cambrian period alongside many of their vertebrate counterparts, but due to the lack of fossil record and relative recency of deeper genomic investigation of these creatures, little else is known about the specifics of their diversification and timeline of development.

Cephalopods have evolved to possess many traits that make them seem vertebrate-like but did so without sharing a common ancestor that could explain genetically these parallels. A well resolved phylogeny of the cephalopod radiation will offer new clarity regarding the genetic history of a class of creatures that convergently developed complex cognition, color perception, and highly developed methods of communication. Because these species developed these abilities separately but alongside vertebrates, prioritizing investigation into their genome could prove a critical first step toward beginning to form general rules regarding the genetic basis for advanced cognition. Here we propose a resolved phylogeny of the class Cephalopoda spanning every family within the class and leverage molecular clock analysis to propose new hypotheses of the timeline of adaptive radiation of soft bodied mollusks of the class Cephalopoda.

## Methods

Study UCE's were obtained from research partners at OIST, visualized and inspected for paralogs and outliers using a combination of bash and R scripting, and 455 of these loci were selected to move forward for the purpose of phylogenetic analysis. Alignments were concatenated with the toast R package and phylogeny from this data was curated using IQTree as were all individual locus trees included within the astral tree analysis.

### Phylogenetic Tree of the Class Cephalopoda:

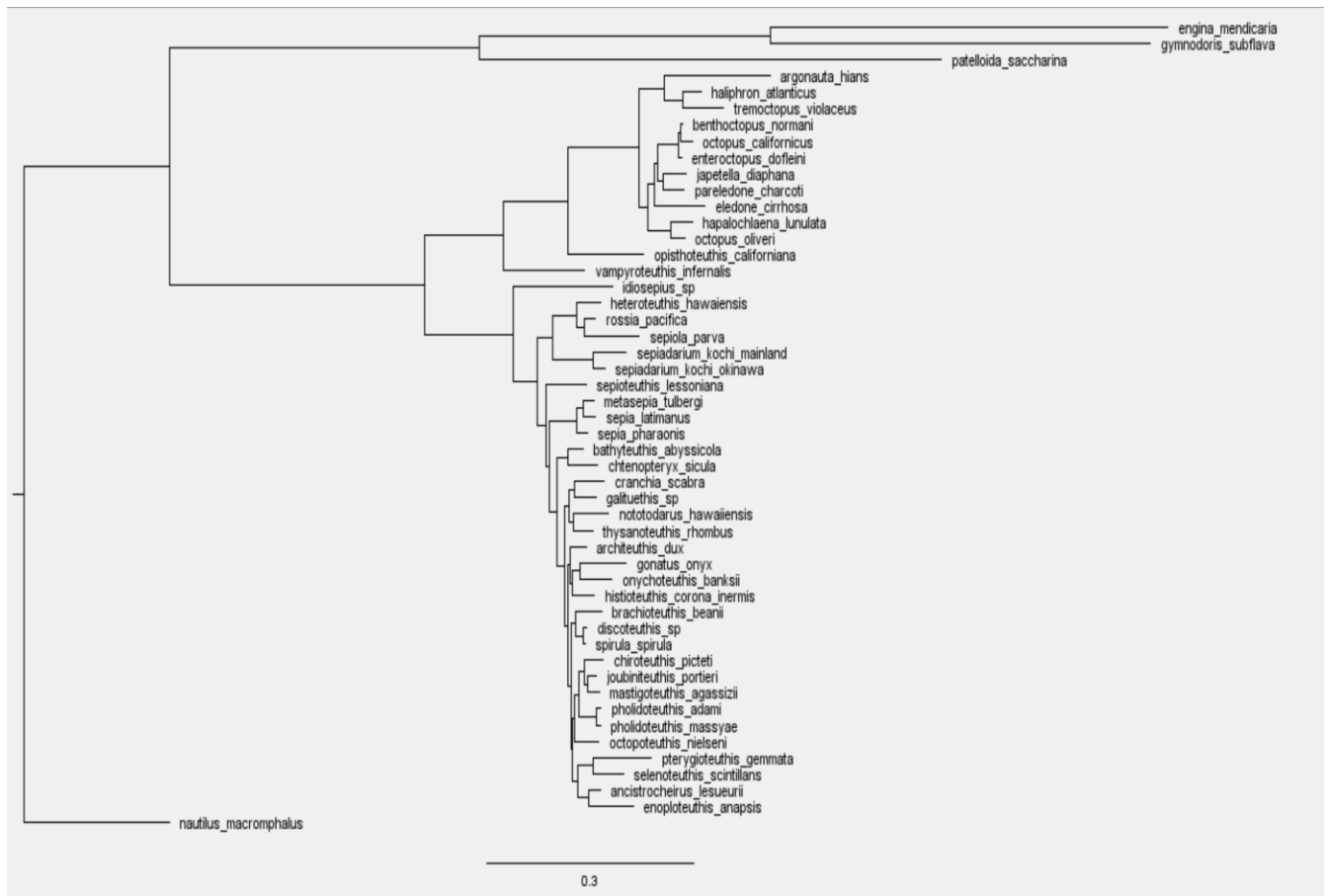


Figure 1: The first known phylogenetic reference spanning all clades and families within the Class Cephalopoda. Due to size of the data set, bootstrapping values were generally close to 100% at all nodes.

## Astral Tree of Cephalopod Species:

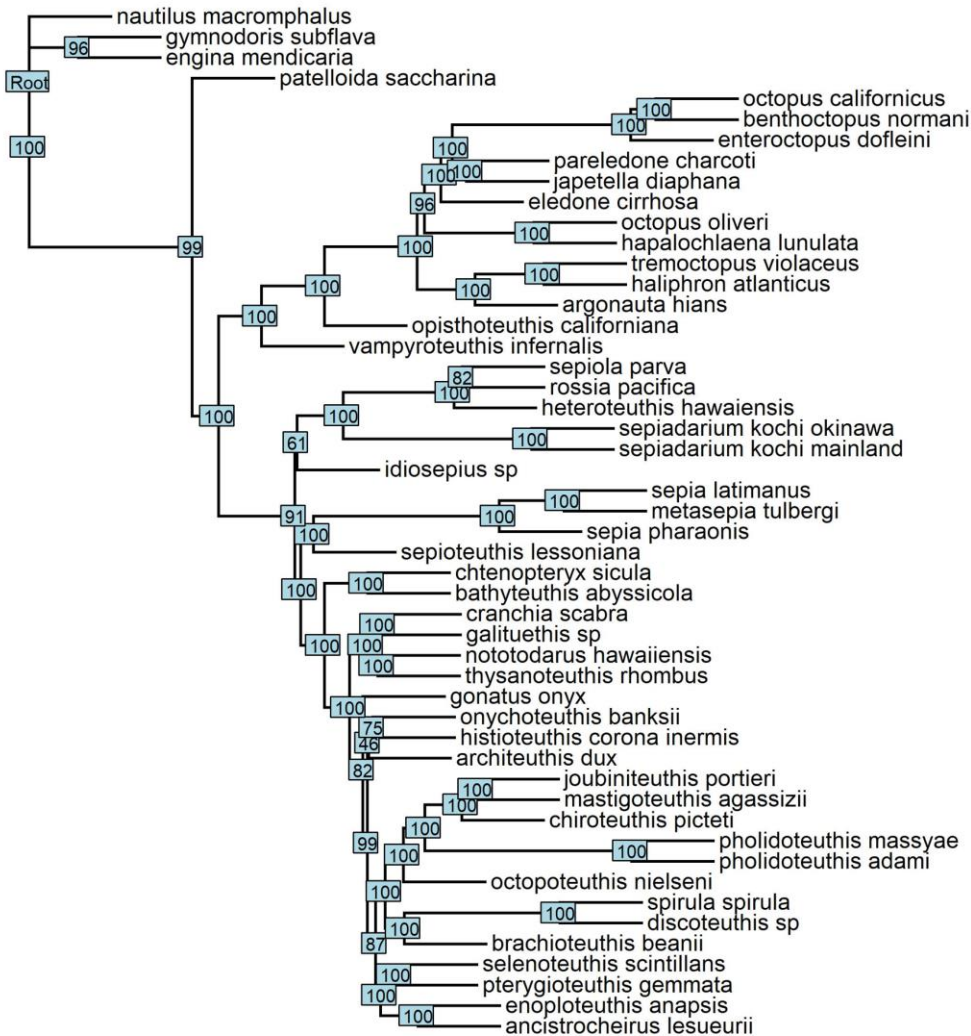


Figure 2: This tree was based upon individual ultra-conserved-element trees and was created using astral software and visualized in R. While largely congruent to figure 1, some discrepancy exists, most notably in the species for which the least amount of overlapping data was present, such as the gastropod outgroups and select other species.

## Do the two trees differ?

```
> treedist(astral, CephTree)
      symmetric.difference  branch.score.difference  path.difference  quadratic.path.difference
      14.000000          9.009037          47.675990          214.248821
> |
```

The `treedist()` function in R was used to quantify distinction between the two analyses. The non-zero number of symmetric differences indicates the presence of some phylogenetic incongruence between them and suggests a need for further investigation regarding the conflicting clades.

Because we used enriched UCE data taken from samples of various origin, the overlapping segments of genetic information did not share consistent taxa across all loci. The outgroup, and particularly the gastropod species of the outgroup, were represented in only a fraction of the loci and were rarely found together. To locate a subset of the loci that held the largest and most consistent variety of species, a python script was created to record taxa at each loci and cross reference them across all others. Loci were selected if they contained the species of interest for which node calibration data was collected in addition to a large variety of other species of interest to ensure that all clades are well represented in the study. The final data set used in the following analysis contains 43 species of interest across 6 UCEs.

Molecular Clock Analysis was run using MrBayes software with nodes and calibrations set as follows:

```
begin mrbayes;
  set autoclose=yes nowarn=yes;
  execute CephConcat.nex;
  charset uce-12368.txt=1-251;
  charset uce-16542.txt=252-906;
  charset uce-29580.txt=907-1101;
  charset uce-30321.txt=1102-1286;
  charset uce-35495.txt=1287-1469;
  charset uce-4691.txt=1470-1653;
  partition byLocus = 6:uce-12368.txt,uce-16542.txt,uce-29580.txt,uce-30321.txt,uce-35495.txt,uce-4691.txt;
  set partition=byLocus;
  lset applyto=(all) nst=6 rates=propinv;
  unlink statefreq=(all) revmat=(all) shape=(all);
  prset applyto=(all) ratepr=variable;
  Outgroup nautilus_macromphalus;
  constraint Outgroup = 23;
  constraint Octos = 3 5 10-11 14-15 19 26-29 42;
  constraint Spirula = 40;
  constraint BBSquids = 16 33 37-38;
  constraint Cuttlefish = 22 35-36 39;
  calibrate Outgroup = truncatednormal(100, 415, 100);
  calibrate Octos = truncatednormal(100, 240, 100);
  calibrate BBSquids = truncatednormal(20, 128, 20);
  calibrate Spirula = truncatednormal(20, 95, 20);
  calibrate Cuttlefish = truncatednormal(20, 88, 20);
  prset nodeagepr = calibrated;
  prset topologypr = constraint(Outgroup, BBSquids, Octos, Spirula, Cuttlefish);
  prset brienspr = clock:uniform;
  prset treeagepr = truncatednormal(100, 550, 100);
  prset clockratepr = lognorm(-5.5,0.5);
  prset clockvarpr = igr;
  mcmc ngen=10000000 samplefreq=100 printfreq=1000;
  sump relburnin=yes burninfrac=0.25;
  sumt relburnin=yes burninfrac=0.25;
end;
```

Figure 3: Node calibrations were selected by referencing recent research studies specific to species *spirula spirula* in relation to other decapodiformes, *vampyroteuthis infernalis* in relation to other octopodiformes, species of the family Sepiidae (cuttlefish) in relation to bobtail squid, and our outgroup *nautilus macomphalis*.

## Results

Molecular clock analysis using MrBayes software yielded two resulting files suggesting equal likelihood between two hypotheses. One of the hypotheses finds the age of the Cephalopod class to be approximately 475 million years old with origins in the Ordovician Period whereas the other finds the age of the group to be around 550 million years old with origins in the Neoproterozoic Era, among other notable distinctions.

# Molecular Clock Analysis Tree 1:

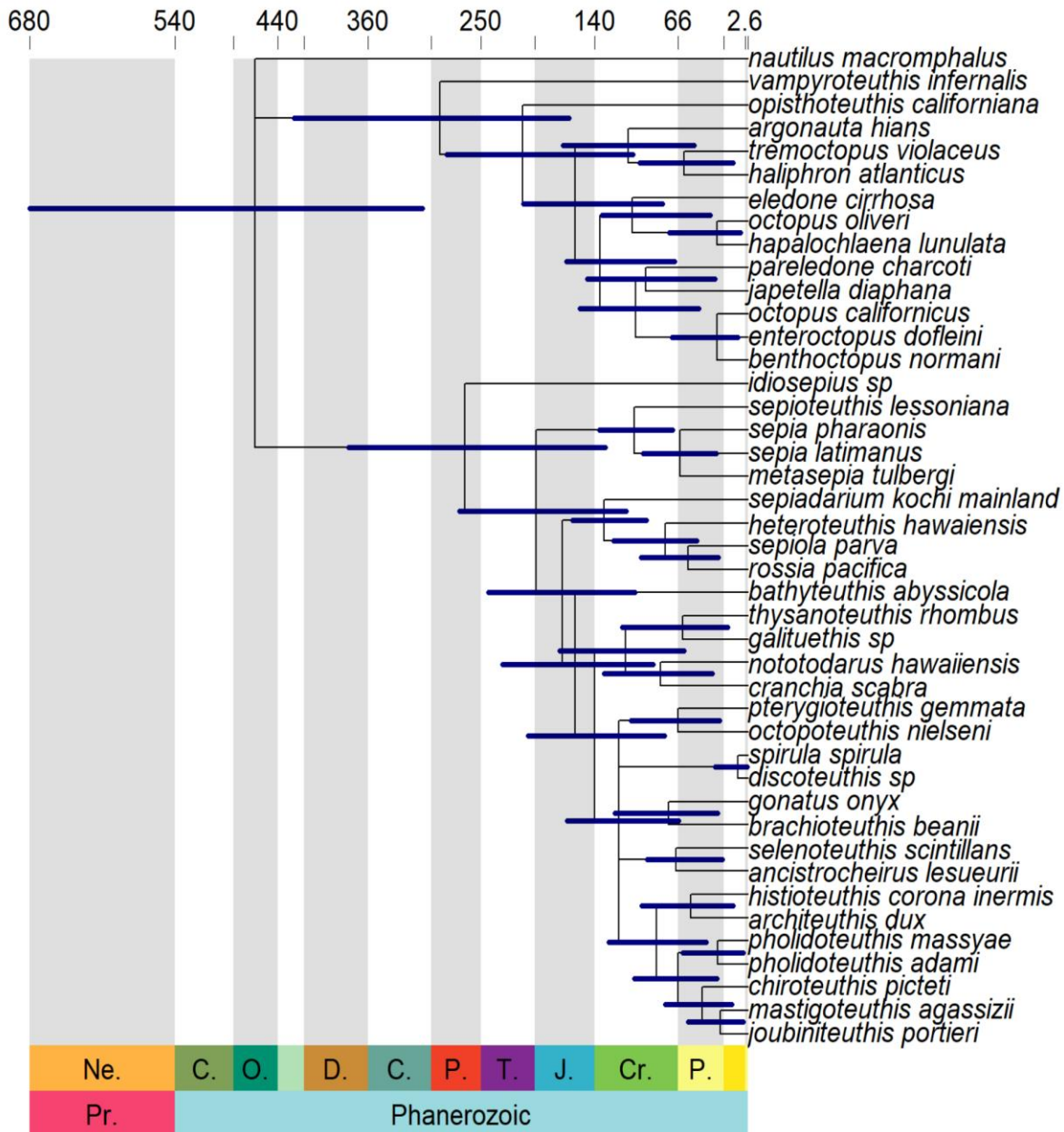


Figure 4: This visual was made using MCMCTreeR in R with the first of two output files generated by MrBayes software to estimate divergence times across Cephalopod species. The Coleoid Cephalopods appear to have diverged from their shelled ancestors and Nautiloid body design possibly during the middle of the Ordovician over 450 million years ago.

## Molecular Clock Analysis Tree 2:

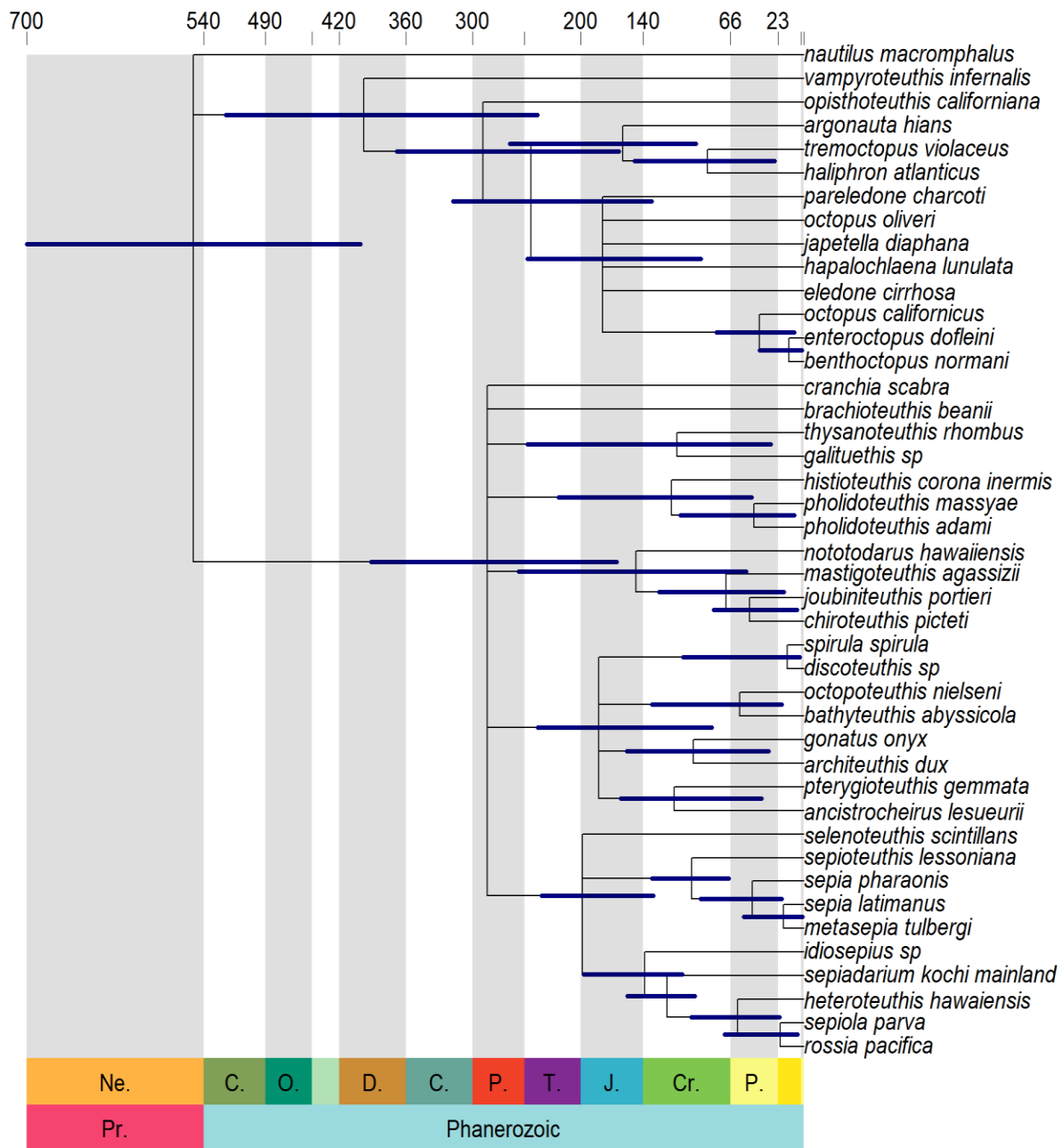


Figure 5: This visual displays the results of the second of the two MrBayes outputs and shows a similar hypothesis but with several important distinctions, most notably the overall age of this tree being different by approximately 75 million years and the emergence of *vampyroteuthis infernalis* (vampire squid) to be much earlier as well in the mid Devonian.

## Discussion

Both trees estimate the rapid diversification of both Octopodiformes and Decapodiformes in the Permian predating the most recent estimates slightly which credit these events to later periods of the Mesozoic. In addition to the discrepancy between the two trees in overall age and the estimated emergence range of vampyroteuthids, other distinctions between the two hypotheses are equally worthy of note and further investigation. The timeline of the emergence of the pygmy squid *Idiosepius*, a relative of other bottle and bobtail squid, differs by about 100 million years and the ages of true squids show clade inconsistencies between the two as well, though by much smaller margins. One consistent yet surprising finding is in the shared lineage of *Spirula spirula* and *Discoteuthis* sp extending all the way up to approximately 10 million years ago before diverging to become the only known member of the family *Spirulidae*.

The newly resolved phylogeny, and particularly the nodes relating to the Coleoid Cephalopods support and add larger context to recent studies into the relatedness of species within the class Cephalopoda. While the molecular clock figures present interesting hypotheses in resolving the divergence times of these species, more data will need to be incorporated into this analysis to resolve discrepancies and add confidence to the resulting estimate. Additionally, seeking out improved genomic data for several of the species and incorporating it into future analyses could also yield additional clarity and context. Once accomplished, taken together, these references could enhance and support continued efforts to investigate the enigmatic genome underlying the fascinating biology of these uniquely advanced marine invertebrates and establish cephalopods as a model species from which to study the genetic basis for a wide range of topics, most notably gene editing events and advanced cognition.

I intend to use them as a foundational reference from which to conduct research investigating changes in cephalopod gene expression and regulatory networks associated with changes in behavior. Studies of this nature will uncover insights into the molecular basis of behavioral responses. Such work is currently being conducted on model organisms to explore a host of topics from how species will cope with climate change to understanding disorders affecting the human species. In the long-term I hope to develop cephalopods into a study model to further these aims and to begin to develop a more fundamental understanding of the genetic basis of advanced cognition found several times over across life forms on our planet.

## References

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