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A draft genome sequence of the elusive giant squid, Architeuthis dux

--Manuscript Draft--

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Abstract:

Background

The giant squid (Architeuthis dux; Steenstrup, 1857) is an enigmatic giant mollusk with a circumglobal distribution in the deep ocean, except in the high Arctic and Antarctic waters. The elusiveness of the species makes it difficult to study. Thus, having a genome assembled for this deep-sea dwelling species will allow unlocking several pending evolutionary questions.Findings

We present a draft genome assembly that includes 200 Gb of Illumina reads, 4 Gb of Moleculo synthetic long-reads and 108 Gb of Chicago libraries, with a final size matching the estimated genome size of 2.7 Gb, and a scaffold N50 of 4.8 Mb. We also present an alternative assembly including 27 Gb raw reads generated using the Pacific Biosciences platform. In addition, we sequenced the proteome of the same individual

	and RNA from three different tissue types from three other species of squid species (Onychoteuthis banksii, Dosidicus gigas, and Sthenoteuthis oualaniensis) to assist genome annotation. We annotated 33,406 protein coding genes supported by evidence and the genome completeness estimated by BUSCO reached 92%. Repetitive regions cover 49.17% of the genome.Conclusions This annotated draft genome of A. dux provides a critical resource to investigate the unique traits of this species, including its gigantism and key adaptations to deep-sea environments.
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Response to Reviewers:	Dear Editor, We herewith submit our revised manuscript ‘A draft genome sequence of the elusive giant squid, Architeuthis dux’. Regarding the points that you have highlighted, please find the answers below: 1) Please clarify the rationale for the unconventional assembly strategy in the revised manuscript. If this has "historic" rather than scientific reasons, the reviewer feels this may be fine, but I agree that the reasons should be discussed in the manuscript, for the benefit of readers who are looking for best practice examples. The reviewer is correct that there is some degree of history involved. We initially did the assembly without PacBio, and did the presented analyses on this. Later we were offered the chance to try and improve it with PacBio, which we did, but as you can see there was minimal improvement in the assembly statistics (Table 1 and Table S2), but i) an increase of the total genome size to 3.155 Gb, beyond the expected 2.7 Gb estimated in kmergenie, and ii) a slight decrease in the BUSCO completeness assessment. As such, we elected to retain the results based on the original assembly (based on Dovetail), but given that we assume others may wish to use the alternative assembly and explore the differences, we provide both. In the beginning of the “Data analyses” section, we now clearly state which assembly was used in the comparative genomics analyses (from Line 297) and provide an explanation for that choice. 2) Please expand on the methods for protein-coding gene modelling and have another look at your data whether 50K genes may be an overestimate. I also agree with the reviewer's recommendation to analyze gene models in BUSCO to give readers a better idea of their completeness. We now expanded the section detailing the filtering of the protein-coding gene set and

present a total of 33,406 gene annotations in the final set, as these have validation by matching to cephalopod transcripts and/or SwissProt/UniRef90 proteins. We also provide the results from BUSCO when using the gene models as input for comparison (added to Table 1).

Answers to the reviewer's comments:

Reviewer #1: In this study, de Fonseca et al. report the genome of the giant squid as a resource to investigate the unique traits of this fascinating organism. Two assemblies, which are of comparable contiguity to most other recently published molluscan genomes, as well as a set of over 51,000 gene models are reported. Analysis of the genome focuses on repetitive elements (e.g., TEs), non-coding RNAs, and gene families of interest to the authors (WNT genes, Protocadherins, Hox genes, and reflectins). Overall this is a straightforward study that provides a resource that will be broadly useful and I feel it should be published. However, I have a number of suggestions for improvement including a few important issues that need to be addressed.

Major points:

1.1. It is unclear why two different genome assemblies are presented instead of just one most optimal assembly. This is not the way I would have gone about assembling this combination of data but presumably Dovetail scaffolding and gene modelling were performed before PacBio sequencing and scaffolding? Re-doing the assembly would a more logical way would probably have relatively little improvement but a little more explanation of the rationale or 'historical' reasons for two different assemblies and/or this assembly strategy would be a helpful addition to readers looking in the literature for examples on best practices for genome assembly.

Thank you for this comment. The reviewer is correct that there is some degree of history involved. We initially did the assembly without PacBio, and did the presented analyses on this. Later we were offered the chance to try and improve it with PacBio, which we did, but as you can see there was minimal improvement in the assembly statistics (Table 1 and Table S2), but i) an increase of the total genome size to 3.155 Gb, beyond the expected 2.7 Gb estimated in kmergenie, and ii) a slight decrease in the BUSCO completeness assessment. As such, we elected to retain the results based on the Dovetail assembly, but given that we assume others may wish to use the alternative assembly and explore the differences, we provide both.

1.2. Related to this issue, there is little comparison of the two genome assemblies and it is unclear which assembly was used for what analyses and even Table 1 and Table S2's titles are a bit ambiguous with respect to which assembly statistics are presented. Please explicitly state which assembly was used for which analyses.

In the beginning of the "Data analyses" section, we now clearly state which assembly was used in the comparative genomics analyses (from Line 297) and provide an explanation for that choice. Additionally, we also mention the choice in the Methods section (Lines 183 to 185) before describing the strategies for annotation and comparative analyses.

1.3. The approach used for gene annotation is unconventional and the inferred number of protein-coding gene models is very high. This does not mean the gene model set is bad, but I feel that data needed for the reader to assess the quality of the gene models are lacking. Please run BUSCO on the gene models and report these data as well.

We now also provide the results from BUSCO when using the gene models as input for comparison.

1.4. Specimen collection data are not reported in the manuscript.

This information has now been added to Table S1.

Minor points:

1.5. Scientific names of species need to be italicized throughout.

Done.

1.6. Did all the giant squid DNA come from the same individual?

Yes, this is now clear in Line 172.

1.7. Lines 140-141: "currently increasing locally" is a bit awkward and vague.

Replaced by “in some regions”.

1.8. Line 176: Which reads? All Illumina reads? PE reads only?

This has now been clarified on Line 176.

1.9. Line 185: Again, this seems to me to be a strange assembly strategy and I think that it should be clearly stated that PacBio data became available 'late in the game' if that is the case. Otherwise, the logic behind this assembly strategy needs to be explained.

In the beginning of the “Data analyses” section, we now clearly state which assembly was used in the comparative genomics analyses (from Line 297) and provide an explanation for that choice.

1.10. Line 199: High-throughput is misspelled.

Done.

1.11. Line 203: Clarify what is meant by reference transcriptome. All reads from all tissues were pooled and assembled together?

This has now been clarified in Lines 203-204.

1.12. Line 205: "EvidencialGene" is a tyo.

Corrected.

1.13. Lines 261-262: Please provide details on exactly what was done in this study in the supplementary material. Description of how the final gene models were selected is vague.

We now further discuss the filters applied in lines 272-275. The total number of protein-coding genes passing all the filters is 33,406.

1.14. Line 277: What is meant by a "bespoke pipeline"? Custom scripts should be made available.

No custom analysis scripts were developed. We simply use ‘bespoke’ to mean 'tailored to our particular purpose'. Here this refers an analysis pipeline combining: a preliminary analysis using RepeatMasker, followed by a de novo analysis using RepeatModeler and a referenced and publicly available script by Platt et al, followed by a full annotation using RepeatMasker. These steps are fully outlined and referenced in the methods section. We have simplified the sentence which now reads:
“Repetitive elements were first identified using RepeatMasker v.4.0.8”

1.15. Line 450: Correct "Sampling was following"

Done.

1.16. BUSCO results are presented in the methods section (should be in the results by the way) for the pre-PacBio scaffolding genome but not the post-PacBio scaffolding genome.

The results of BUSCO for post-PacBio step are presented in Table S2 (as indicated in Line 186). We moved the description of the BUSCO results to the “Data analyses” section and added a clarification regarding the choice of the assembly for the overall comparative genomics analyses (from Line 297).

1.17. Table 1: BUSCO should be in all capital letters.

Done.

1.18. Figure 3: What does the note "Gene size only" mean?

This gene was reported to be fully isolated from other Hox genes in a different scaffold but was not alone in the scaffold. There were other non-Hox genes. Figure 3 aims to show both the organisation and the range occupied by Hox genes. Considering the organisation, the gene is isolated such as in O. bimaculoides. Regarding the size, the schematic representation indicates only the Hox “cluster” area. In O. bimaculoides, the scaffolds contain only the Hox genes. This means it could be possible for the cluster to be there but only when considering a very vast distance. In this scenario for C. teleta, the gene is found in the middle of the scaffold, surrounded by other genes. It is not part of the cluster. Indicating the full scaffold size could lead to a wrong interpretation of the gene size and of the Hox gene range. As such, only the gene size is indicated.

1.19. Table S1: Please provide total number of reads and somewhere it should be clarified how many different instrument runs were conducted and if different libraries were multiplexed on the Illumina platform.

This information has now been added to Table S1.

Reviewer #2: The authors present the genome of the giant squid Architeuthis dux. Several cephalopod genomes have been sequenced, but our genomic understanding of cephalopods living in the deep-sea environment is still poor. The authors sequenced a giant squid species A. dux together with several transcriptomes from the gonad, liver and brain tissues derived from three other squid species including Onychoteuthis banksii, Dosidicus gigas, and Sthenoteuthis oualaniensis.

Having a giant squid genome is an important contribution to the field of cephalopod genomics, especially for further meaningful comparative genomics. The authors provide a decent genome assembly. And the observation of a non-tightly physically linked Hox cluster is interesting. The manuscript is well written in general, however, there are a lot of editing errors throughout the whole manuscript, which distracts the reading. The authors need to carefully fix all these typos and errors during the revision. Further comments are provided below.

Major comments:

2.1. In the Abstract/Findings, there is a lot of information about "Methods" (e.g. how many raw reads, sequencing of proteome and RNA) instead of what the authors found from the genome itself. Also, the statement "RNA from three different tissue types from three other species of squid to assist genome annotation." is very vague. What tissue types from what species should be clearly described. The authors need to rewrite this section.

In the abstract we followed the format that is usual in a data note, providing detailed information on the data provided by this work. We have now added the names of the three species of squid to the abstract.

2.2. Line 153: Body patterning system? Usage of body patterning is confusing here since body patterning often refers to the developmental process during embryogenesis but not the skin color pattern.

We have rephrased the sentence to: “Cephalopods can rapidly alter the texture, pattern, colour and brightness of their skin, and this both enables a complex communication system, as wells as provides exceptional camouflage and mimicry.”

2.3. The authors cited that there is a global proliferation of cephalopods (Lines 140 and 141) but later cited other studies saying that there is a regional extinction. It is a bit confusing whether cephalopods are undergoing proliferation or extinction. Given that the earlier citation is more recent (Doubleday et al., 2016) than others, it is wondering which condition is closer to the current situation.

We have removed the second statement to avoid confusion.

2.4. Although it is agreeable in general to have genome resources from unexplored species, the authors' argument in the last paragraph of Data description/Context is not convincing. The link between having a genome and aiding conservation efforts as well as ensuring continued existence is not clear.

Without a genome, population genomic studies that provide information regarding the genetic diversity and structure of populations becomes very challenging, with genome-wide data having to be produced from reduced-representation methods that have many biases. In this last paragraph, we state this specifically: “A genome is an important resource for future population genomics studies[…]”.

2.5. Do the authors have any idea why the genome contains so many protein-coding genes (51,225 genes predicted) in comparing to other cephalopod species usually having only 20,000-30,000 genes? For example, is it due to that A. dux has more lineage-specific genes or expansions of certain gene families?

We have revised our gene models and now further discuss the filters applied in lines 272-275. The total number of protein-coding genes passing all the filters is 33,406.

2.6. Given that genome size and polyploidy of the organisms are often correlated to increased body size (Session et al., 2016), have the authors checked if there is whole-genome duplication or polyploidy in the A. dux genome? Session et al. (2016) Genome evolution in the allotetraploid frog Xenopus laevis. Nature 538, 336-343.

We did confirm that the genome was not polyploid by testing for Hardy–Weinberg equilibrium using re-sequencing data from 32 giant squid individuals (Winkelman et al, unpublished results) and there is no evidence for an ancient duplication since we only found one intact Hox complement.

2.7. Figure 3: The authors should provide scaffold numbers for the Hox clusters from each species. Also, in most cases, Hox genes in the Hox cluster are adjacent to each other without the insertion of other non-Hox genes. If there is a special case in A. dux and E. scolopes, the authors should show the real gene arrangement on that scaffold, especially for the non-Hox genes (with brief annotation) that are in between Hox genes. This can be achieved by having an additional panel in the same figure. The authors are encouraged to show an illustration on the types of Hox gene organization in order to give the readers a better understanding of this context.

Figure 3 has received new panels. Scaffold information for A. dux was added in panel C (Figure 3-C). As the assemblies of the other species were retrieved from other studies, the readers are directed to the appropriate references for further detail. An extra panel depicting the Hox cluster organisation in more detail has been added. E. scolopes data is shown as reported in its published study. No non-Hox genes were indicated for the area covered in this representation. An additional panel with a simplified version of the various Hox "cluster" types was inserted in panel A (Figure 3-A).

Minor comments:

2.1.1. Line 149: ~2cm -> "~2 cm"
Done.

2.1.2. Line 150: 3 orders -> "three orders"
Done.

2.1.3. Line 150: Architeuthis dux -> "A. dux"
Done.

2.1.4. Lines 150 and 151: 10-12cm… 20m -> "10-12 cm… 20 m"
Done.

2.1.5. Line 152: 500kg -> "500 kg"
Done.

2.1.6. Line 171: a Architeuthis dux sample -> "an A. dux sample"
Done.

2.1.7. Line 172: What is CTAB?
CTAB = “cetyl trimethylammonium bromide”; this description has been included in the text (Line 172)

2.1.8. Line 184: For Eukaryota and Metazoa we identified… -> "For Eukaryota and Metazoa, we identified…"
Done.

2.1.9. Line 184: … 90.4 % and 92.1 %... -> "… 90.4% and 92.1%..."
Done.

2.1.10. Line 185: 23.38Gb -> "23.38 Gb"
Done.

2.1.11. Line 186: 14.79kb -> "14.79 kb"

Done.

2.1.12. "k-mer" (Line 204) or "kmer" (Line 176) to be consistent.
Chose to use “kmer”.

2.1.13. Line 216: 100,000 g -> "100,000×g"
Done.

2.1.14. Lines 219 and 222: SDS-PAGE -> "SDS-PAGE" (hyphen but not en dash)
Done.

2.1.15. Line 221: Tris - HCl -> Tris-HCl (single hyphen but not en dash with spaces)
Done.

2.1.16. Line 226: LC-MS/MS analyses -> "LC-MS/MS analyses" (hyphen but not en dash)
Done.

2.1.17. Line 254: Using italic for scientific names (i.e. Octopus bimaculatus, Crassostrea gigas, and Lottia gigantea)
Done.

2.1.18. Line 260: … 200kb (total length 199Mb)… -> "… 200 kb (total length 199 Mb)…"
Done.

2.1.19. Line 290: Transposable elements -> "transposable elements"
Done.

2.1.20. Line 300: Architeuthis dux -> "A. dux"
Done.

2.1.21. Line 323: ~5-8% -> "~5¬-8%" (en dash but not hyphen for a range)

	Done. 2.1.22. Line 381: Octopus bimaculoides -> "O. bimaculoides" Done. 2.1.23. Line 383: Euprymna scolopes -> "E. scolopes" (in italic) Done. 2.1.24. Line 395: Euprymna scolopes -> "E. scolopes" Done. 2.1.25. Lines 397 & 398: 500 - 10,000 kb -> "500-10,000 kb" (en dash but not hyphen for a range) Done. 2.1.26. Line 406: ... observed in Hox 1, Hox 4, ANTP, Lox 2, Lox 5, Post 1 and Post 2. Hox 1 did,... -> "... observed in Hox1, Hox4, ANTP, Lox2, Lox5, Post1 and Post2. Hox1 did,..." Done. 2.1.27. Line 407: Hox 1 -> "Hox1" Done. 2.1.28. Line 408: Hox 3 -> "Hox3" Done. 2.1.29. Line 409: Lox 4 -> "Lox4" Done. 2.1.30. Lines 410, 412 & 413: Hox 2 -> "Hox2" Done. 2.1.31. Line 421: ... contains 7 reflectin genes and 3 reflectin-like genes… -> "... contains seven reflectin genes and three reflectin-like genes…" Done. 2.1.32. Line 422: … exception of 1 reflectin gene, … -> "… exception of one reflectin gene, …" Done. 2.1.33. Line 436: … (tsa)… -> "… (TSA)…" Done. 2.1.34. Lines 647 & 657: Architeuthis dux -> "A. dux" Done. 2.1.35. Line 659: Hox 2 -> "Hox2" Done.
Additional Information:	
Question	**Response**
Are you submitting this manuscript to a special series or article collection?	No
Experimental design and statistics Full details of the experimental design and statistical methods used should be given in the Methods section, as detailed in our Minimum Standards Reporting Checklist. Information essential to interpreting the	Yes

data presented should be made available in the figure legends. Have you included all the information requested in your manuscript?	
Resources A description of all resources used, including antibodies, cell lines, animals and software tools, with enough information to allow them to be uniquely identified, should be included in the Methods section. Authors are strongly encouraged to cite Research Resource Identifiers (RRIDs) for antibodies, model organisms and tools, where possible. Have you included the information requested as detailed in our Minimum Standards Reporting Checklist?	Yes
Availability of data and materials All datasets and code on which the conclusions of the paper rely must be either included in your submission or deposited in publicly available repositories (where available and ethically appropriate), referencing such data using a unique identifier in the references and in the “Availability of Data and Materials” section of your manuscript. Have you have met the above requirement as detailed in our Minimum Standards Reporting Checklist?	Yes

A draft genome sequence of the elusive giant squid, *Architeuthis dux*

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Abstract

Background

The giant squid (*Architeuthis dux*; Steenstrup, 1857) is an enigmatic giant mollusk with a circumglobal distribution in the deep ocean, except in the high Arctic and Antarctic waters. The elusiveness of the species makes it difficult to study. Thus, having a genome assembled for this deep-sea dwelling species will allow unlocking several pending evolutionary questions.

Findings

We present a draft genome assembly that includes 200 Gb of Illumina reads, 4 Gb of Moleculo synthetic long-reads and 108 Gb of Chicago libraries, with a final size matching the estimated genome size of 2.7 Gb, and a scaffold N50 of 4.8 Mb. We also present an alternative assembly including 27 Gb raw reads generated using the Pacific Biosciences platform. In addition, we sequenced the proteome of the same individual and RNA from three different tissue types from three other species of squid species (*Onychoteuthis banksii*, *Dosidicus gigas*, and *Sthenoteuthis oualaniensis*) to assist genome annotation. We annotated 33,406 protein coding genes supported by evidence and the genome completeness estimated by BUSCO reached 92%. Repetitive regions cover 49.17% of the genome.

Conclusions

This annotated draft genome of *A. dux* provides a critical resource to investigate the unique traits of this species, including its gigantism and key adaptations to deep-sea environments.

Keywords

Cephalopod, invertebrate, genome assembly.

Data description

Context

Cephalopods are the most behaviourally complex of the invertebrate protostomes [1] . Their large, highly differentiated brains are comparable in relative size and complexity to those of vertebrates [2], as are their cognitive capabilities [1]. Cephalopods are distributed worldwide from tropical to polar marine habitats, from benthic to pelagic zones and from intertidal areas down to the abyssal parts of the deep sea, with the only exception being the Black Sea. Cephalopod populations are thought to be currently increasing in some regions for a variety of reasons [3], including potential predator release as a consequence of the depletion of fish stocks [4]. The class Cephalopoda contains approximately 800 species, with the vast majority belonging to the soft-bodied subclass Coleoidea (cuttlefishes, octopuses and squids), and a small handful belonging to the Nautiloidea (nautiluses) [5]. Cephalopods are ecologically important as a primary food source for marine mammals, birds and for many fish species. They are also increasingly important as a high-protein food source for humans and are a growing target for commercial fisheries and farming [6].

Cephalopods show a wide variety of morphologies, lifestyles and behaviours [7], but with the exception of the nautiluses they are characterised by having rapid growth and short lifespans, despite a considerable investment in costly sensory adaptations [2]. They range in size from the tiny pygmy squids (~2 cm) to animals that are nearly three orders of magnitude larger, such as the giant squid, *A. du*x (average length 10–12 m, and reported up to 20 m total length) [6,8,9], to the colossal squid, *Mesonychoteuthis hamiltoni* (maximum length remains unclear, but a recorded weight of 500 kg makes it the largest known invertebrate [10]). Cephalopods can rapidly alter the texture, pattern, colour and brightness of their skin, and this both enables a complex communication system, as wells as provides exceptional camouflage and mimicry [11]. Together these allow cephalopods to both avoid predators, and hunt prey highly efficiently, making them some of the top predators in the ocean. The remarkable adaptations of cephalopods also extend to their genome, with recent work demonstrating increased levels of RNA editing to diversify proteins involved in neural functions [12].

Over recent years, oceanic warming and acidification, pollution, expanding hypoxia and fishing [13–15] have been shown to affect cephalopod populations. Mercury has been found in high concentrations in the tissue of giant squid specimens [16], and accumulation of flame retardant chemicals has also been detected in the tissue of deep-sea cephalopods [17]. Consequently, there is an urgent need for greater biological understanding of these important, but rarely encountered animals, in order to aid conservation efforts and ensure their continued existence. A genome is an important resource for future population genomics studies aiming at characterizing the diversity of the legendary giant squid, the species which has inspired generations to tell tales of the fabled Kraken.

Methods

DNA extraction, library building, and de novo genome assembly

High-molecular-weight genomic DNA was extracted from a single *A. du*x individual (NCBI taxon id: 256136) using a cetyl trimethylammonium bromide (CTAB) based buffer followed by organic solvent purification, following Winkelmann et al [18] (details in the Supplementary Information). We generated 116 Gb of raw reads from Illumina short-insert libraries, 76 Gb of paired-end reads from libraries ranging from 500 bp to 800 bp in insert size, and 5.4 Gb of mate-pair with a 5 kb insert (Table S1). Furthermore, we generated 3.7 Gb of paired-end reads using Moleculo libraries (3 High-Throughput libraries and 4 High-Fidelity libraries). The kmer distribution of the reads under a diploid model in kmergenie [19] predicted the genome size to be 2.7 Gb.

An initial assembly generated with Meraculous [20] using Illumina and Moleculo data (N50 of 32 Kb, assembly statistics in Table S2) was used as input for Dovetail Genomic’s HiRise scaffolding software together with the Hi-C data generated from two Chicago libraries corresponding to a physical coverage of the genome of 52.1X. This “Meraculous + Dovetail” assembly (statistics in Table 1) was the one used for the genome annotation (non-coding RNAs, protein-coding genes and repeats) and comparative genomics analyses presented in this paper. Further scaffolding was done using 23.38 Gb of PacBio reads (19 SMRT cells, average read length is 14.79 kb) using the default parameters in PBJelly [21] (see assembly statistics in Table S2). The genome gene content completeness was evaluated through the Benchmarking Universal Single-Copy Orthologs (BUSCO v.3.0.2, datasets: Eukaryota, Metazoan) [22].

Transcriptome sequencing and de novo assembly

Given the extreme rarity of live giant squid sightings, we were unable to collect fresh organ samples (following the recommendations in [23]) containing intact RNA from the species to assist with the genome annotation. As an alternative, we extracted total RNA from gonad, liver and brain tissue from live caught specimens of three other oegopsid squid species (*Onychoteuthis banksii*, *Dosidicus gigas*, and *Sthenoteuthis oualaniensis*; NCBI taxon ids 392296, 346249 and 34553, respectively; Supplementary Figure S1), using the Qiagen RNeasy extraction kit (Qiagen,CA, USA). The RNA integrity and quantity was measured on a Qubit fluorometer (Invitrogen, OR, USA) and on the Agilent Bioanalyzer 2100 (Agilent, CA, USA). The Illumina TruSeq Kit v.2.0 was used to isolate the mRNA and prepare cDNA libraries for sequencing, following the recommended protocol. Compatible index sequences were assigned to individual libraries to allow for multiplexing on four lanes of 100bp paired-end technology on an Illumina HiSeq 2000 flow cell. Sequencing of the cDNA libraries was done at the National High-Throughput Sequencing Center at the University of Copenhagen in Denmark. We assessed the quality of the raw reads using FastQC v0.10.0 [24]. After removing indexes and adaptors with CutAdapt [25], we trimmed the reads with the FASTX-toolkit (http://hannonlab.cshl.edu/fastx_toolkit) removing bases with a Phred-scale quality score lower than 25. Reference transcriptomes for each species were built after pooling the reads from all tissues and using these as input in Trinity [26]. This software was used with the default settings including a fixed kmer size of 25 as suggested by the authors. Annotation of coding regions was done with the EvidentialGene pipeline [27].

Protein extraction, separation by 1D SDS–PAGE, MALDI-TOF/TOF and Protein Identification

Given the practical impossibility of obtaining RNA from a giant squid specimen, we produced a library of giant squid peptide sequences to guide the gene annotation process.

Proteins were solubilised from a giant squid mantle tissue sample according to the procedure described by Kleffmann et al. [28] and employing the following buffers: (1) 40 mM Tris–HCl, 5 mM MgCl2 and 1 mM DTT, pH 8.5; (2) 8 M urea, 20 mM Tris, 5 mM $MgCl_2$ and 20 mM DTT; (3) 7 M urea, 2 M thiourea, 20 mM Tris, 40 mM DTT, 2% CHAPS (w ⁄ v) and 1% Triton X-100 (v ⁄ v) and (4) 40 mM Tris, 4% SDS (w ⁄ v) and 40 mm DTT. All buffers were augmented with protease inhibitors (Halt™ Protease Inhibitor Cocktail, EDTA-Free, Thermo Scientific). Tissue samples were ground in liquid nitrogen before homogenization, or homogenized directly with ultrasound (probe sonication at 60 Hz, for 3 min) in buffer 1. Solubilised proteins were collected by ultracentrifugation at 100,000xg and 4 ºC. Each extraction was performed in duplicate for each specific buffer and extracts were pooled. Protein extracts were subsequently stored at -20 ºC. Total protein content was estimated according to the Bradford (1976) method [29].

Protein separation by 1D SDS-PAGE electrophoresis was carried out as described in Santos et al. [30]. 53 µL of sample (39 µg protein) was diluted in 72 µL of Loading Buffer (0.01% bromophenol blue, 2% SDS (Sodium-DodecylSulfate), 20% glycerol, 5% β-mercaptoethanol (w/v/v) in 62.5 mM Tris-HCl, pH 6.8). The resulting solution was heated for 3 min at 99°C. Proteins were separated by SDS–PAGE with 12% (w/v) polyacrylamide gels. Electrophoresis was carried out using the mini Protean Cell (BioRad) at a constant voltage of 150 V. The separated proteins were visualized by staining with Colloidal Coomassie Brilliant Blue (CCB) [31], and lanes were cut into 15 gel sections for subsequent LC-MS/MS analysis.

LC-MS/MS analyses

All samples were analysed with the Easy-nLC system (Thermo Fisher Scientific), connected online to a Q Exactive mass spectrometer (Thermo Fisher Scientific) equipped with a nanoelectrospray ion source

(Thermo Fisher Scientific). Tryptic peptides were loaded in a fused silica column (75 µm inner diameter) packed with C18 resin (3-µm beads, Reprosil, Dr. Maisch), with solvent A (0.5% acetic acid). They were then eluted with a 120 minute gradient of solvent B (80% ACN, 0.5% acetic acid) with a constant flow of 250 nL/min. The Q exactive was operated in positive mode with a capillary temperature of 250 °C, using the data dependent acquisition method, which switches from full MS scans to MS/MS scans for the 12 most intense ions. Fragmentation was achieved by higher-energy collisional dissociation (HCD) with a normalized collisional energy (NCE) of 25. Full MS ranged from 300 to 1750 m/z at a resolution of 70,000, an Automatic Gain Control (AGC) of 1e6 and a maximum injection time of 120 ms, whereas MS/MS events were scanned at a resolution of 35,000, an AGC of 1e5, maximum injection time of 124 ms, isolation windows of 2 m/z and an exclusion window of 45 seconds.

de novo peptide prediction

Raw LC-MS/MS data were read using Thermo Fisher MSRawFileReader 2.2 library and imported into PEAKS Studio 7.0 and subsequently preprocessed for precursor mass and charge correction, MS/MS de-isotoping, and deconvolution. PEAKS de novo sequencing [31] was performed on each refined MS/MS spectrum with a precursor and fragment ion error tolerance of 7 ppm and 0.02 da respectively. Carbamidomethylation (Cys) was set as a fixed modification and oxidation (Met) and N-terminal Acetylation as variable modifications. At most, five variable modifications per peptide were allowed. For each tandem spectrum, five *de novo* candidates were reported along with their Local Confidence Scores (the likelihood of each amino acid assignment in a de novo candidate peptide). This score was used to determine the accuracy of the de novo peptide sequences. The top de novo peptide for each spectrum was determined by the highest Average Local Confidence score (ALC) among the candidates for that spectrum.

Genome annotation

Protein-coding genes were predicted by ExonHunter [32] , which combines probabilistic models of sequence features with external evidence from alignments. As external evidence, we have used known proteins from *Octopus bimaculatus*, *Crassostrea gigas* (Pacific oyster) and *Lottia gigantea* (Giant owl limpet) and the predicted proteins encoded by the transcriptomes of the three other oegopsid species analysed in this paper (*O. banksii*, *D. gigas*, and *S. oualaniensis)*. These proteins were aligned to the genome by BLASTX. De-novo identified MS/MS-based peptides were initially also considered as external evidence, but were later omitted due to low coverage. Evidence from predicted repeat locations was used to discourage the model to predict genes overlapping repeats. Since no sufficiently close annotated genome was available for training gene finding parameters, ExonHunter was first run using Drosophila melanogaster parameters on a randomly chosen subset of 118 scaffolds longer than 200kb (total length 199 Mb). Out of 12,912 exons predicted in this run, 5,716 were supported by protein alignment data and selected to train the parameters of the gene finding model for *A. dux*, using the methods described in [32]. Rerunning ExonHunter with the resulting A. dux model parameters on the entire genome yielded 51,225 gene predictions genes. Gene prediction in A. dux is challenging due to the fragmentary nature of the genome assembly (60% of predictions span a sequencing gap). This results in a significant number of artifacts, for example short genes with long introns spanning gaps in the assembly. 18,054 predictions yield protein product shorter than 100 amino acids, yet the median span of these predictions is more than 4kb and only 32% of them are supported by transcript or protein alignments. In contrast, 83% of genes with product longer than 100aa are supported. In most of the analyses below, we consider only 33,406 genes that were found to have transcript evidence (blastp match to a sequence from a cephalopod transcriptome, with at least 50% of the giant squid coding region covered) and/or matches in Swissprot or UniRef90 databases (Table 1). This supported set contains much fewer extremely short genes (Figure S4).

The function of the protein-coding genes was inferred with Annocript 0.2 [33], which is based on the results from blastp [34] runs against the SwissProt (SP) and UniRef90 (Uf). In addition, we performed a rpsblast search using matrices from the conserved domain database (CDD) to annotate specific domains present on the protein queries.

Non-coding RNAs were annotated using the cmsearch program from INFERNAL 1.1 and the covariance models (CMs) from the Rfam database v12.0 [35,36]. All matches above the curated GA threshold were included. INFERNAL was selected because it implements the CMs that provide the most accurate bioinformatic annotation tool for ncRNAs available [37]. tRNA-scan v.1.3.1 was subsequently used to refine the annotation of tRNA genes (Table S3). The method uses a number of heuristics to increase the search-speed, annotates the Isoacceptor Type of each prediction, infers if predictions are likely to be functional or tRNA-derived pseudogenes [38,39]. This method uses CMs to identify tRNAs. Rfam matches and the tRNA-scan results for families belonging to the same clan were then “competed”, so that only the best match was retained for any genomic region [36].

Transposable element annotation

Repetitive elements were first identified using RepeatMasker v.4.0.8 [40] with the eukaryota RepBase [41] repeat library. Low-complexity repeats were ignored (-nolow) and a sensitive (-s) search was performed. Following this, a de novo repeat library was constructed using RepeatModeler v.1.0.11 [42] , including RECON v.1.08 [43] and RepeatScout v.1.0.5 [44]. Novel repeats identified by RepeatModeler were analyzed with a ‘BLAST, Extract, Extend’ process to characterise elements along their entire length [45]. Consensus sequences and classification information for each repeat family were generated. The resulting de novo repeat library was utilized to identify repetitive elements using RepeatMasker.

Data analyses

We present a main draft genome assembly produced using 200 Gb of Illumina reads, 4 Gb of Moleculo synthetic long-reads and 108 Gb of Chicago libraries, with a final size matching the estimated genome size of 2.7 Gb, and a scaffold N50 of 4.8 Mb (assembly and annotation statistics in Table 1). Genome completeness estimated by BUSCO reached 90.4% (Eukaryota) and 92.1% (Metazoa), and the completeness for the 33,406 protein-coding genes was 91.2% (Eukaryota) and 84.0 (Metazoa).

We also produced an alternative assembly including 27 Gb raw reads generated using the Pacific Biosciences platform, but this showed minimal improvement in assembly statistics, genome size larger than the predicted and lower BUSCO completeness (Table S2).

Comparative analyses of transposable elements

We estimated the total repeat content of the giant squid genome to be approximately half its total size (~49.1%) (Figure 1, Supplementary Table S4). Out of all the repeats present in the giant squid genome, only a few were predicted to be small RNAs, satellites, simple or low complexity repeats (~0.89% of the total genome), with the vast majority (~48.21%) instead consisting of transposable elements (TEs; i.e. SINEs, LINEs, LTR retrotransposons, and DNA transposons; Figure 1, Supplementary Table S4). Of the TE portion of the giant squid genome, the main contribution from annotated TEs is from DNA elements (11.06%) and LINEs (6.96%), with only a small contribution from SINEs (1.99%) and LTR elements (0.72%). TEs are a nearly universal feature of eukaryotic genomes, often comprising a large proportion of the total genomic DNA (e.g. the maize genome is ~85% TEs [46], stick insect genome is ~52% TEs [47], and the human genome is >45% TEs [48]), consequently these account for the majority of observed genome size variation among animals.

In Figure 1, we summarise the recently reported TE analyses performed on assembled cephalopod genomes, as follows: California two-spot octopus (*Octopus bimaculatus*) [11] and long-arm octopus (*O. minor*) [49], Hawaiian bobtail squid (*Euprymna scolopes*) [50], and giant squid (*A. dux*). The varying sequencing strategies employed to generate currently available cephalopod genomes (and accompanying variation in assembly quality) complicates the comparative analysis of TE content for this group. However, notwithstanding this caveat, it does seem clear that TEs make up a large fraction of the total genomic content across all cephalopod genomes published to date (Figure 1). DNA transposons and LINEs dominate in available cephalopod genomes, while LTR elements and SINEs generally represent a minor portion of cephalopod TEs (Figure 1). Within decapod cephalopods (i.e. squid and cuttlefish), patterns in TE content are generally similar, however, the giant squid has a notably larger proportion of DNA transposons (1,626,482 elements, 11.06% of the total genome) than the Hawaiian bobtail squid (855,308 elements, 4.05% of the total genome), with the bobtail squid in turn having a similar proportion of LINEs (752,629 elements, 6.83% of the total genome) than the giant squid (766,382 elements, 6.96% of the total genome; Figure 1).

The defining ability of TEs to mobilise, in other words, to transfer copies of themselves into other parts of the genome, can result in harmful mutations. However, TEs can also facilitate the generation of genomic novelty, and there is increasing evidence of their importance for the evolution of host-adaptive processes [51]. In the giant squid genome, all classes of TEs were more frequent (~38.23) in intergenic regions (here defined as regions >2kb upstream or downstream of an annotated gene), than in genic regions versus % of the genome in intergenic regions (~16.6%; Figure 2A). These findings are broadly similar to those reported for other cephalopods, although a larger proportion of the giant squid genome is composed of repeats located within genic regions (percentage of the genome represented by TEs for *O. bimaculoides*: ~6% genic versus ~30% intergenic, and for *O. minor* ~6% genic versus ~40% intergenic [49]).

A Kimura distance-based copy divergence analysis revealed that the most frequent TE sequence divergence relative to the TE consensus sequence in the giant squid genome was ~5–8% across all repeat classes, suggesting a relatively recent transposition burst across all major TE types (Figure 2B). Divergence peaks were most pronounced in LINE RTE elements, Tc/Mar and hAT DNA transposons, and unclassified TEs, with smaller divergence peaks in SINE tRNA elements and Penelope LINE elements (Figure 2B). Divergence peaks were most pronounced in LINE RTE elements, Tc/Mar and hAT DNA transposons, and unclassified TEs, with smaller divergence peaks in SINE tRNA elements and Penelope LINE elements (Figure 2B). In comparison to observations from other cephalopods, these results suggest a shorter and more intense burst of recent TE activity in the giant squid genome. Overall, further genomic sampling within each of the cephalopod clades will be needed to understand TE evolution, as closely related species can show significant differences (*e.g.*, *O. bimaculoides* to *O. vulgaris*) [52].

Non-coding RNAs

We identified 50,598 ncRNA associated loci in the squid sequencing data, using curated homology-based probabilistic models from the Rfam database[53] and the specialized tRNAscan-SE transfer RNA annotation tool [38]. The essential and well conserved Metazoan ncRNAs: tRNAs, rRNAs (5S, 5.8S, SSU and LSU), RNase P, RNase MRP, SRP and the major spliceosomal snRNAs (U1, U2, U4, U5, U6), as well as the minor spliceosomal snRNAs (U11, U12, U4atac & U6atac), are all found in the *A. dux* genome. Some of the copy numbers associated with the core ncRNAs are extreme. For example, we identified: i) approximately 24,000 loci that appear to derive from 5S rRNA; ii) approximately 17,000 loci that are predicted to be tRNA derived; iii) approximately 3,200 Valine tRNAs isotypes and approximately 1,300 U2 spliceosomal RNAs. The microRNA mir-598 also exhibits high copy-numbers at 172. Many of these are likely to be SINEs derived by transposition. All 20 tRNA isotypes were identified in *A. dux* genome. Again, many of these had relatively large copy numbers (summarised in Table 1). These ranged from 46 (Cys) up to 2,541 (Val). We identified 174 loci that share homology with 34 known snoRNA families, these included 15 scaRNA, 41 H/ACA box and 118 C/D box snoRNA associated loci [10]. The snoRNAs are predominantly involved in rRNA maturation. We identified 7,049 loci that share homology with 283 families of microRNA. Some of these may be of limited reliability, as CMs for simple hairpin structures can also match other, non-homologous, hairpin-like structures in the genome e.g. inverted repeats. A number of cis-regulatory elements were also identified. These included 235 hammerhead 1 ribozymes, 133 Histone 30 UTR stem-loops, and 14 Potassium channel RNA editing signal sequences. There are very few matches to obvious non-metazoan RNA families in the current assemblies. The only notable exceptions are bablM, IMES-2, PhotoRC-II and rspL. Each of these families are also found in marine metagenomic datasets, possibly explaining their presence as "contamination" from the environment.

Analyses of specific gene families

Several gene families involved in development, such as transcription factors or signaling ligands, are highly conserved across metazoans and may therefore reveal signatures of genomic events, such as a whole genome duplication.

WNT is a family of secreted lipid-modified signaling glycoproteins with a key role during development [54]. Comparative analysis of molluscan genomes indicates that the ancestral state was 12 *WNT* genes, as *Wnt3* is absent in all protostomes examined thus far [55]. The giant squid has the typical 12 lophotrochozoan WNTs (1, 2, 4, A, 5, 6, 7, 8, 9, 10, 11 and 16; Supplementary Figure S2), and therefore has retained the ancestral molluscan complement, including *Wnt8*, which is absent, for instance, in the genome of the slipper snail *Lottia gigantea* [56].

Protocadherins are a family of cell adhesion molecules that appear to play an important role in vertebrate brain development [57]. It is thought that they act as multimers at the cell surface in a manner akin to DSCAM in flies, which lack protocadherins [58]. Cephalopods have massively expanded this family, with 168 identified in the *O. bimaculoides* genome, whereas only 17-25 protocadherins have been identified in the genomes of annelids and non-cephalopod molluscs [11]. We identified approximately 135 protocadherin genes in *A. dux*, many of which are located in clusters in the genome. The possibility that this gene family plays a developmental role parallel to that of protocadherins in vertebrate neurodevelopment thus remains a compelling hypothesis.

Development organisation of the highly diverse body plans found in the Metazoa is controlled by a conserved cluster of homeotic genes, which includes, among others, the Hox genes. These are characterized by a DNA sequence referred to as the homeobox, comprising 180 nucleotides that encode the homeodomain [59]. Hox genes are usually found in tight physical clusters in the genome and are sequentially expressed in the same chronological order as they are physically located in the DNA (temporal and spatial collinearity) [60]. Different combinations of Hox gene expression in the same tissue type can lead to a wide variety of different structures [61]. This makes the Hox genes a key subject for understanding the origins of the multitude of forms found in the cephalopods. In *O. bimaculoides* genome assembly no scaffold contained more than a single Hox gene, meaning that they are fully atomised [11]. However, in *E. scolopes*, the Hox cluster was found spanning two scaffolds [50]. In the giant squid, we recovered a full Hox gene cluster in a single scaffold (Figure 3-B). The Hox gene organization found in the giant squid genome suggests either the presence of a disorganised cluster, so-called type D, or atomised clusters, type A [61], or possibly a combination of the two (the genes are still organized, but physically distant from each other). The existence of a "true" cluster seems unlikely, given the presence of other unrelated genes in between and the relatively large distances (Figure 3-C). The classification as type A (atomised) might seem most obvious, despite the co-presence of the genes in a single scaffold, due to these large distances. However, the definition of type D (disorganised) does allow for the presence of non-Hox genes in between members of the cluster (Figure 3-A). Thus, it is difficult to clearly categorise the recovered "cluster", but it does remain clear that these genes are not as tightly bundled as they are in other Bilateria lineages. The *A. dux* Hox “cluster” is spread across 11 Mb of a 38 Mb scaffold, and this suggests a far larger size range in the cephalopods than in other described animals, as recently suggested based on the genome of *E. scolopes* [50]. It is possible that this is the reason for the apparent atomisation of Hox genes in the more fragmented *O. bimaculoides* assembly. Hox clusters are usually found in contigs of around 100 kb length in vertebrates [6, 7] and between 500 – 10,000 kb in invertebrates [8] An assembled contig easily containing the complete cluster for these smaller cluster sizes, would manage to cover only one member of the Hox gene cluster in the studied coleoids. As such, our results suggest that the Hox cluster may not be fully atomised in *O. bimaculoides* as previously hypothesised. Further improvements of genome assemblies in cephalopods will be required to address this question. The biological reason for this dramatic increase in the distance between the genes in the Hox cluster presents an intriguing avenue of future research. The homeodomain of all the obtained Hox genes in cephalopods were compared with those of other mollusks. Few differences were found relative to a previous study [62], as no significant modifications were observed in Hox1, Hox4, ANTP, Lox2, Lox5, Post1 and Post2. Hox1 did, however, show reduced conservation in residues 22 to 25 in the *A. dux* sequence. This observation for Hox1 in *A. dux* is visible only in the Pacbio assembly. Additionally, the Hox3 homeodomain analysis supports a basal placement of the nautiloids within cephalopods. The Lox4 gene was the most variable among all groups. As of to date, Hox2 still remains undetected in the coleoid cephalopods [63]. Assembly errors notwithstanding, gain and loss of Hox genes has been attributed to fundamental changes in animal body plans, and the apparent loss of Hox2 may therefore be significant. For example, Hox gene loss has been associated with the reduced body-plan segmentation of spider mites [42]. The circumstance that Hox2 has been readily found in *Nautilus*, but remains undetected in all coleoids sequenced thus far, might signify an important developmental split within the Cephalopoda. Alternatively, and equally intriguing, this Hox gene may have undergone such drastic evolutionary modifications that it is presently undetectable by conventional means.

On a final note, we analyzed genes encoding reflectins, a class of cephalopod-specific proteins first described in *E. scolopes* [64]. Reflectins form flat structures that reflect ambient light (other marine animals use purine-based platelets), thus modulating iridescence for communication or camouflage purposes [65]. The giant squid genome contains seven reflectin genes and three reflectin-like genes (Supplementary Figure S3). All of these genes, with the exception of one reflectin gene, appear on the same scaffold, which corresponds very well with the distribution pattern of octopus reflectin genes [11]).

Conclusions

Not only because of its astonishing proportions, but also for the lack of knowledge of the key facets of its deep-sea lifestyle, the giant squid has long captured the imagination of scientists and the general public alike. With the release of this annotated giant squid genome, we set the stage for future research into the enigmas that enshroud this truly awe-inspiring creature. Further, given the paucity of available cephalopod genomes, we provide a valuable contribution to the genomic description of cephalopods, and more widely to the growing number of fields that are recognizing the potential, which this group of behaviourally advanced invertebrates holds for improving our understanding of the diversity of life on Earth in general.

Availability of supporting data

The data sets supporting the results of this article are available in the NCBI database a Bioproject PRJNA534469. The three transcriptome data sets (TSA) have ids GHKK01000000, GHKL01000000 and GHKH01000000 and the sequence data used for the genome assemblies has id VCCN01000000.

Additional files

Supplement.txt. Supplementary methods, tables and figures.

Declarations

Abbreviations

Gb: gigabase pairs; Mb: megabase pairs; BUSCO: Benchmarking Universal Single-copy Orthologs; bp: base pair; NCBI: National Center for Biotechnology Information; LC-MS/MS: liquid chromatography (LC) tandem mass spectrometry (MS); CCB: Colloidal Coomassie Brilliant Blue; HCD: higher-energy collisional dissociation; NCE: normalized collisional energy; AGC: Automatic Gain Control; ALC: Average Local Confidence; SP: SwissProt; Uf: UniRef90; CDD: conserved domain database; CM: covariance model; TE: transposable element; LINE: Long interspersed nuclear element; SINE: Short interspersed nuclear element; LRT: long terminal repeat.

Ethics statement

Sampling followed the recommendations from Moltschaniwskyj et al., 2007 [23].

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors contributions

R.D.F. and M.T.P.G. designed the study. J.S., H-J.H. AND R.T. carried out the sampling. Alex.C., A.R., B.F., G.C.A.Jr, H.O. and I.W. performed the laboratory work. R.D.F., Alv.C., A.M., C.B.A., F.S., P.G., T.B., A.H., I.B.H., C.C., B.P., F.P., M.P., F.M., O.S., S.R., M.Z.R. and D.P. analyzed the data. E.J., G.Z., J.V., O.F. and Q.L. contributed with genomic resources. R.D.F., L.F.C.C., A.A., Y.W., B.M., R.S., T.V., B.B., T.S-P., M.T.P.G. contributed with supervision and computational resources. R.R.F., T.S-P., R.N., M.T.P.G paid for sequencing. R.D.F. wrote the manuscript with contributions from all authors. All authors have read and approved the manuscript.

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Tables

Table 1. Statistics of the giant squid genome assembly (Meraculous + Dovetail) and corresponding gene prediction and functional annotation. The transcript evidence was confirmed by blastp hits with e-value $< 10E^{-6}$ using the transcriptomes of three other species of squid (see the "Transcriptome sequencing" section).

Global Statistics		
Genome assembly*	**Genome**	**Gene models with evidence**
Input assembly	Meraculous	
Contig N50 length (Mb)	0.005	
Longest contig (Mb)	0.120	
Scaffold N50 length (Mb)	4.852	
Longest scaffold (Mb)	32.889	
Total length (Gb)	2.693	
BUSCO statistics (^{1}Euk / ^{2}Met)		
Complete BUSCOs, (%)	86.1 / 88.5	81.6 / 78.3
Complete and single-copy, (%)	85.1 / 87.6	79.9 / 77.7
Complete and duplicated, (%)	1.0 / 0.9	1.7 / 0.6
Partial, (%)	4.3 / 3.6	9.6 / 5.7
Missing, (%)	9.6 / 7.9	8.8 / 16.0
Total Buscos found, (%)	90.4 / 92.1	91.2 / 84.0
Genome annotation / Gene Prediction		
Protein-coding gene number	33,406	
Transcript evidence	30,472	
Average Protein length, (aa)	339	
Longest Protein, (aa)	17,047	
Average CDS length, (bp)	1,015	
Longest CDS, (bp)	51,138	

Average exon length, (bp)	199
Average exons per gene	5
Functional annotation (Number of Hits)	
Swissprot	15,749
Uniref90	29,553
GO Terms	4,712
Conserved Domains Database (CDD)	15,280

*****The presented statistics are to contigs/scaffolds with length >= 500 bp.

[1] Euk: Database of Eukaryota orthologs genes, containing a total of 303 BUSCO groups.

[2] Met: Database of Metazoa orthologs genes, containing a total of 978 BUSCO groups.

Figure legends

Figure 1. Comparison of genome repeat content among available cephalopod genomes with assembled genomes (repeat data for *O. minor* and *O. bimaculoides* from [49] and for *E. scolopes* from [50]). The tree indicates evolutionary relationships among the two available octopod cephalopods and the two available decapod cephalopods. Pie charts are scaled according to genome size (*O. bimaculoides*: 2.7Gb, *O. minor*: 5.09Gb, *E. scolopes*: 5.1Gb, *A. dux*: 2.7Gb), with different repeat types indicated by the colours presented in the key.

Figure 2. A) Stacked bar chart illustrating the proportions (expressed as percentage of the total genome) of repeats found in genic (≤2kb from an annotated gene) and intergenic regions (>2kb from an annotated gene) for the giant squid genome. **B)** Transposable element (TE) accumulation history in the giant squid genome, based on a Kimura distance-based copy divergence analysis of TEs, with Kimura substitution level (CpG adjusted) illustrated on the x-axis, and percentage of the genome represented by each repeat type on the y-axis. Repeat type is indicated by the colour chart below the x-axis.

Figure 3. Schematic representation of the Hox gene clusters. Different scaffolds are separated by two slashes. **A)** Simplified classification of the Hox clusters genomic organisation. Type A identifies the lack of a "typical" Hox cluster configuration, i.e. genes are scattered through the genome (not closely placed); Type S indicates a Hox cluster that is separated by a chromosomal breakpoint; Type D clusters comprehend all the genes in the same location but encompassing a larger region than in organised clusters and may display non-Hox genes and repeats in between; Type O indicates a very compact cluster embracing a short region with only Hox genes. Non-coding RNA and miRNA can be found. **B)** Simplified scheme of the chromosomal organisation in various invertebrates. Scaffold length is shown underneath. Unlike in other coleoids, for *Architeuthis dux* all Hox genes were found in the same scaffold. However, the distance between the genes was larger than expected for invertebrate organisms, and

non-homeobox genes were also present within the cluster. Hox2 remains undetected in coleoids. *A. dux* cluster can be found in scaffold25. *E. scolopes*, *O. bimaculoides*, *L. gigantea*, *C. teleta* and *D. melanogaster* assemblies and Hox cluster details can be found in [11,50,56,66]. (*) This gene was reported in a different scaffold, adjacent to non-Hox genes (the length corresponds to the size of the gene). **C)** Complete representation of the Hox cluster found in *A. dux* including the non-Hox genes. PO – Predicted open reading frame; TATDN2 – Putative deoxyribonuclease TATDN2; ZMYM1 – Zinc finger MYM-type protein 1; POGK – Pogo transposable element with KRAB; Zinc finger – Zinc finger protein; MYB-like – Putative Myb-like DNA-binding domain protein; MAPRE1 – Microtubule-associated protein RP/EB family member 1; MGC12965 – Similar to Cytochrome c, somatic.

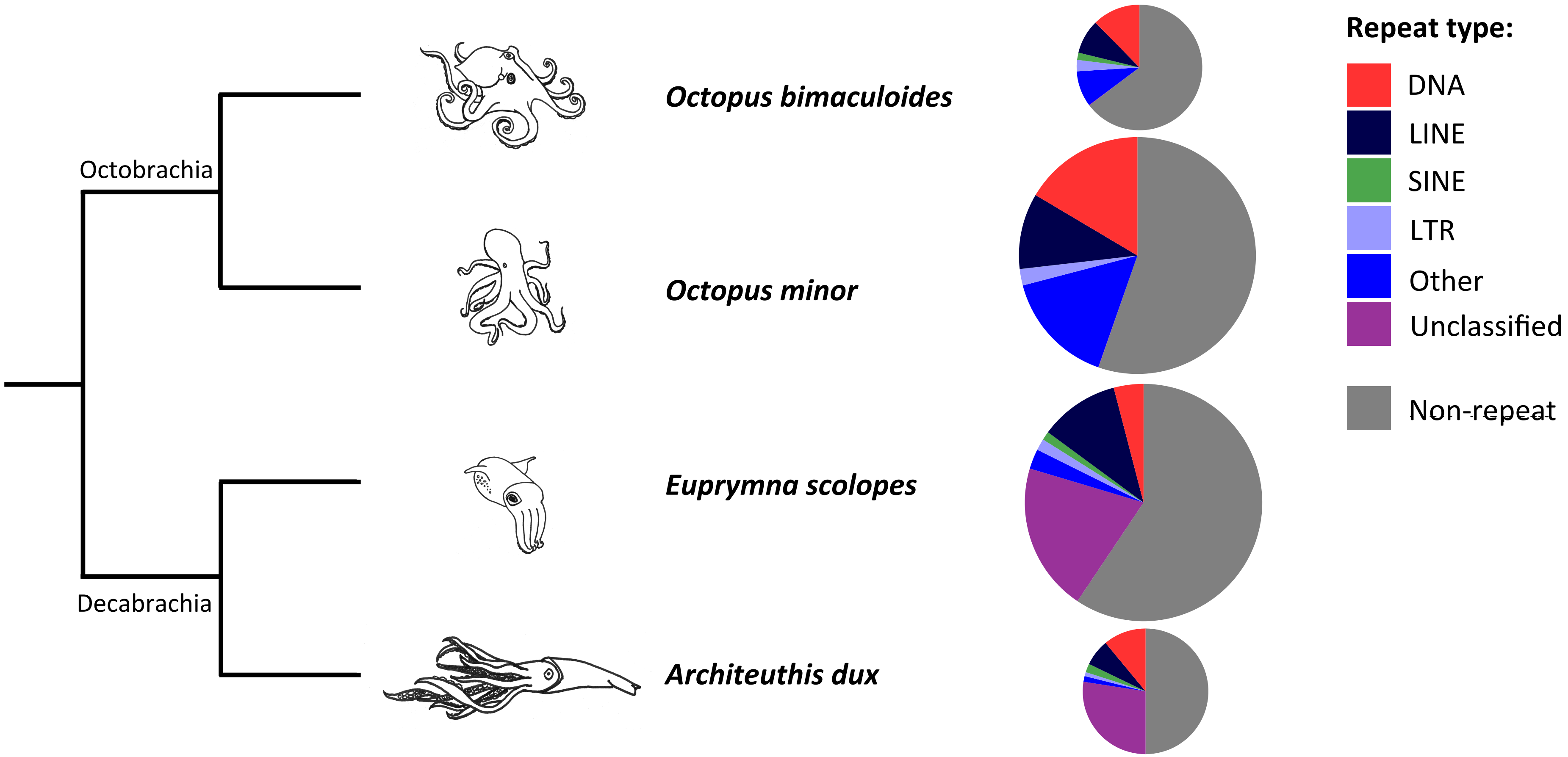

Octobrachia
Decabrachia
Octopus bimaculoides
Octopus minor
Euprymna scolopes
Architeuthis dux
Repeat type:
DNA
LINE
SINE
LTR
Other
Unclassified
Non-repeat

Figure 2

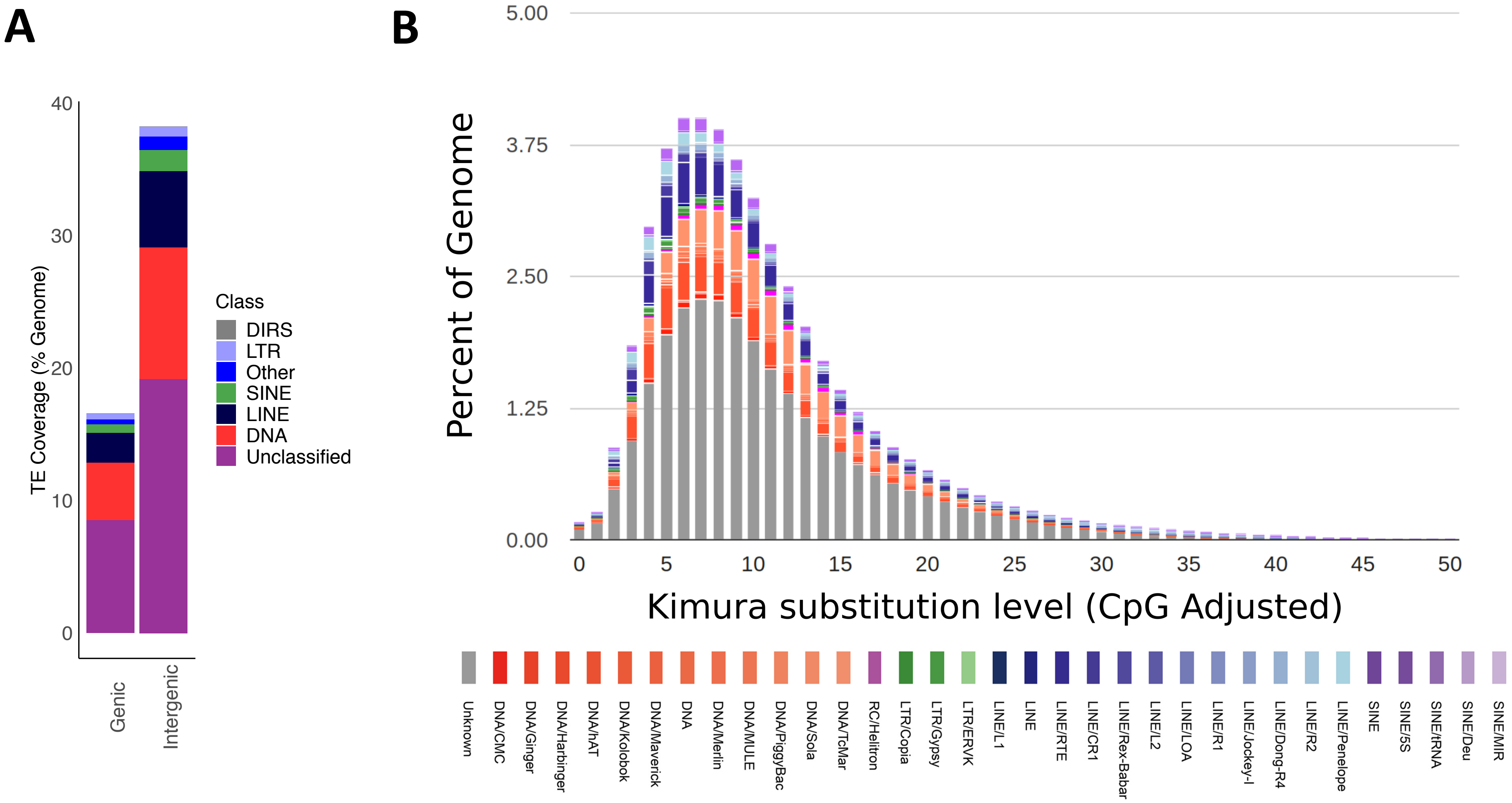
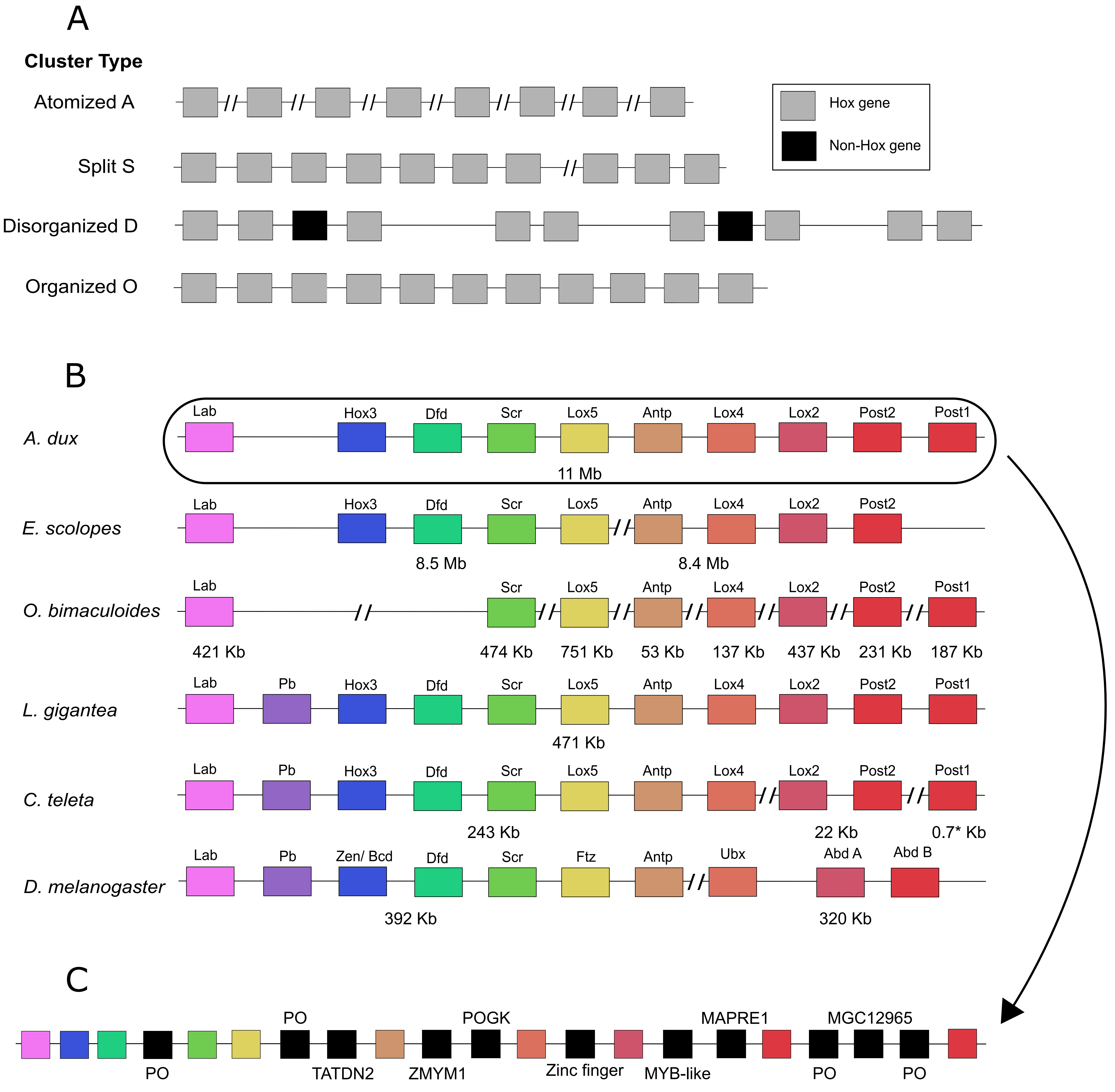

A
40
30
20
10
0
TE Coverage (% Genome)
Genic
Intergenic
Class
DIRS
LTR
Other
SINE
LINE
DNA
Unclassified
B
5.00
3.75
2.50
1.25
0.00
Percent of Genome
0
5
10
15
20
25
30
35
40
45
50
Kimura substitution level (CpG Adjusted)
Unknown
DNA/CMC
DNA/Ginger
DNA/Harbinger
DNA/hAT
DNA/Kolobok
DNA/Maverick
DNA
DNA/Merlin
DNA/MULE
DNA/PiggyBac
DNA/Sola
DNA/TcMar
RC/Helitron
LTR/Copia
LTR/Gypsy
LTR/ERVK
LINE/L1
LINE
LINE/RTE
LINE/CR1
LINE/Rex-Babar
LINE/L2
LINE/LOA
LINE/R1
LINE/Jockey-I
LINE/Dong-R4
LINE/R2
LINE/Penelope
SINE
SINE/5S
SINE/tRNA
SINE/Deu
SINE/MIR

Figure 3


A
Cluster Type
Atomized A
Split S
Disorganized D
Organized O
Hox gene
Non-Hox gene
B
A. dux
Lab
Hox3
Dfd
Scr
Lox5
Antp
Lox4
Lox2
Post2
Post1
11 Mb
E. scolopes
Lab
Hox3
Dfd
Scr
Lox5
Antp
Lox4
Lox2
Post2
8.5 Mb
8.4 Mb
O. bimaculoides
Lab
Scr
Lox5
Antp
Lox4
Lox2
Post2
Post1
421 Kb
474 Kb
751 Kb
53 Kb
137 Kb
437 Kb
231 Kb
187 Kb
L. gigantea
Lab
Pb
Hox3
Dfd
Scr
Lox5
Antp
Lox4
Lox2
Post2
Post1
471 Kb
C. teleta
Lab
Pb
Hox3
Dfd
Scr
Lox5
Antp
Lox4
Lox2
Post2
Post1
243 Kb
22 Kb
0.7* Kb
D. melanogaster
Lab
Pb
Zen/ Bcd
Dfd
Scr
Ftz
Antp
Ubx
Abd A
Abd B
392 Kb
320 Kb
C
PO
POGK
MAPRE1
MGC12965
PO
TATDN2
ZMYM1
Zinc finger
MYB-like
PO
PO

Supplementary Material

Dear Editor,

We herewith submit our revised manuscript ‘A draft genome sequence of the elusive giant squid, *Architeuthis dux*’.

Regarding the points that you have highlighted, please find the answers below:

1) Please clarify the rationale for the unconventional assembly strategy in the revised manuscript. If this has "historic" rather than scientific reasons, the reviewer feels this may be fine, but I agree that the reasons should be discussed in the manuscript, for the benefit of readers who are looking for best practice examples.

The reviewer is correct that there is some degree of history involved. We initially did the assembly without PacBio, and did the presented analyses on this. Later we were offered the chance to try and improve it with PacBio, which we did, but as you can see there was minimal improvement in the assembly statistics (Table 1 and Table S2), but i) an increase of the total genome size to 3.155 Gb, beyond the expected 2.7 Gb estimated in kmergenie, and ii) a slight decrease in the BUSCO completeness assessment. As such, we elected to retain the results based on the original assembly (based on Dovetail), but given that we assume others may wish to use the alternative assembly and explore the differences, we provide both.
In the beginning of the “Data analyses” section, we now clearly state which assembly was used in the comparative genomics analyses (from Line 297) and provide an explanation for that choice.

2) Please expand on the methods for protein-coding gene modelling and have another look at your data whether 50K genes may be an overestimate. I also agree with the reviewer's recommendation to analyze gene models in BUSCO to give readers a better idea of their completeness.

We now expanded the section detailing the filtering of the protein-coding gene set and present a total of 33,406 gene annotations in the final set, as these have validation by matching to cephalopod transcripts and/or SwissProt/UniRef90 proteins. We also provide the results from BUSCO when using the gene models as input for comparison (added to Table 1).

Answers to the reviewer’s comments:

Reviewer #1: In this study, de Fonseca et al. report the genome of the giant squid as a resource to investigate the unique traits of this fascinating organism. Two assemblies, which are of comparable contiguity to most other recently published molluscan genomes, as well as a set of over 51,000 gene models are reported. Analysis of the genome focuses on repetitive elements (e.g., TEs), non-coding RNAs, and gene families of interest to the authors (WNT genes, Protocadherins, Hox genes, and reflectins). Overall this is a straightforward study that provides a resource that will be broadly useful and I feel it should be published. However, I have a number of suggestions for improvement including a few important issues that need to be addressed.

Major points:

1.1. It is unclear why two different genome assemblies are presented instead of just one most optimal assembly. This is not the way I would have gone about assembling this combination of data but presumably Dovetail scaffolding and gene modelling were performed before PacBio sequencing and scaffolding? Re-doing the assembly would a more logical way would probably have relatively little improvement but a little more explanation of the rationale or 'historical' reasons for two different assemblies and/or this assembly strategy would be a helpful addition to readers looking in the literature for examples on best practices for genome assembly.

Thank you for this comment. The reviewer is correct that there is some degree of history involved. We initially did the assembly without PacBio, and did the presented analyses on this. Later we were offered the chance to try and improve it with PacBio, which we did, but as you can see there was minimal improvement in the assembly statistics (Table 1 and Table S2), but i) an increase of the total genome size to 3.155 Gb, beyond the expected 2.7 Gb estimated in kmergenie, and ii) a slight decrease in the BUSCO completeness assessment. As such, we elected to retain the results based on the Dovetail assembly, but given that we assume others may wish to use the alternative assembly and explore the differences, we provide both.

1.2. Related to this issue, there is little comparison of the two genome assemblies and it is unclear which assembly was used for what analyses and even Table 1 and Table S2's titles are a bit ambiguous with respect to which assembly statistics are presented. Please explicitly state which assembly was used for which analyses.

In the beginning of the “Data analyses” section, we now clearly state which assembly was used in the comparative genomics analyses (from Line 297) and provide an explanation for that choice. Additionally, we also mention the choice in the Methods section (Lines 183 to 185) before describing the strategies for annotation and comparative analyses.

1.3. The approach used for gene annotation is unconventional and the inferred number of protein-coding gene models is very high. This does not mean the gene model set is bad, but I feel that data needed for the reader to assess the quality of the gene models are lacking. Please run BUSCO on the gene models and report these data as well.

We now also provide the results from BUSCO when using the gene models as input for comparison.

1.4. Specimen collection data are not reported in the manuscript.

This information has now been added to Table S1.

Minor points:

1.5. Scientific names of species need to be italicized throughout.

Done.

1.6. Did all the giant squid DNA come from the same individual?

Yes, this is now clear in Line 172.

1.7. Lines 140-141: "currently increasing locally" is a bit awkward and vague.

Replace by “in some regions”.

1.8. Line 176: Which reads? All Illumina reads? PE reads only?

This has now been clarified on Line 176.

1.9. Line 185: Again, this seems to me to be a strange assembly strategy and I think that it should be clearly stated that PacBio data became available 'late in the game' if that is the case. Otherwise, the logic behind this assembly strategy needs to be explained.

In the beginning of the “Data analyses” section, we now clearly state which assembly was used in the comparative genomics analyses (from Line 297) and provide an explanation for that choice.

1.10. Line 199: High-throughput is misspelled.

Done.

1.11. Line 203: Clarify what is meant by reference transcriptome. All reads from all tissues were pooled and assembled together?

This has now been clarified in Lines 203-204.

1.12. Line 205: "EvidencialGene" is a tyo.

Corrected.

1.13. Lines 261-262: Please provide details on exactly what was done in this study in the supplementary material. Description of how the final gene models were selected is vague.

We now further discuss the filters applied in lines 272-275. The total number of protein-coding genes passing all the filters is 33,406.

1.14. Line 277: What is meant by a "bespoke pipeline"? Custom scripts should be made available.

No custom analysis scripts were developed. We simply use ‘bespoke’ to mean 'tailored to our particular purpose'. Here this refers an analysis pipeline combining: a preliminary analysis using RepeatMasker, followed by a *de novo* analysis using RepeatModeler and a referenced and publicly available script by Platt et al, followed by a full annotation using RepeatMasker. These steps are fully outlined and referenced in the methods section. We have simplified the sentence which now reads:

“Repetitive elements were first identified using RepeatMasker v.4.0.8”

1.15. Line 450: Correct "Sampling was following"
Done.

1.16. BUSCO results are presented in the methods section (should be in the results by the way) for the pre-PacBio scaffolding genome but not the post-PacBio scaffolding genome.

The results of BUSCO for post-PacBio step are presented in Table S2 (as indicated in Line 186). We moved the description of the BUSCO results to the “Data analyses” section and added a clarification regarding the choice of the assembly for the overall comparative genomics analyses (from Line 297).

1.17. Table 1: BUSCO should be in all capital letters.

Done.

1.18. Figure 3: What does the note "Gene size only" mean?

This gene was reported to be fully isolated from other Hox genes in a different scaffold but was not alone in the scaffold. There were other non-Hox genes. Figure 3 aims to show both the organisation and the range occupied by Hox genes. Considering the organisation, the gene is isolated such as in *O. bimaculoides*. Regarding the size, the schematic representation indicates only the Hox “cluster” area. In *O. bimaculoides*, the scaffolds contain only the Hox genes. This means it could be possible for the cluster to be there but only when considering a very vast distance. In this scenario for *C. teleta*, the gene is found in the middle of the scaffold, surrounded by other genes. It is not part of the cluster. Indicating the full scaffold size could lead to a wrong interpretation of the gene size and of the Hox gene range. As such, only the gene size is indicated.

1.19. Table S1: Please provide total number of reads and somewhere it should be clarified how many different instrument runs were conducted and if different libraries were multiplexed on the Illumina platform.

This information has now been added to Table S1.

Reviewer #2: The authors present the genome of the giant squid Architeuthis dux. Several cephalopod genomes have been sequenced, but our genomic understanding of cephalopods living in the deep-sea environment is still poor. The authors sequenced a giant squid species A. dux together with several transcriptomes from the gonad, liver and brain tissues derived from three other squid species including Onychoteuthis banksii, Dosidicus gigas, and Sthenoteuthis oualaniensis.

Having a giant squid genome is an important contribution to the field of cephalopod genomics, especially for further meaningful comparative genomics. The authors provide a decent genome assembly. And the observation of a non-tightly physically linked Hox cluster is interesting. The manuscript is well written in general, however, there are a lot of editing errors throughout the whole manuscript, which distracts the reading. The authors need to carefully fix all these typos and errors during the revision. Further comments are provided below.

Major comments:

2.1. In the Abstract/Findings, there is a lot of information about "Methods" (e.g. how many raw reads, sequencing of proteome and RNA) instead of what the authors found from the genome itself. Also, the statement "RNA from three different tissue types from three other species of squid to assist genome annotation." is very vague. What tissue types from what species should be clearly described. The authors need to rewrite this section.

In the abstract we followed the format that is usual in a data note, providing detailed information on the data provided by this work. We have now added the names of the three species of squid to the abstract.

2.2. Line 153: Body patterning system? Usage of body patterning is confusing here since body patterning often refers to the developmental process during embryogenesis but not the skin color pattern.

We have rephrased the sentence to: "Cephalopods can rapidly alter the texture, pattern, colour and brightness of their skin, and this both enables a complex communication system, as wells as provides exceptional camouflage and mimicry."

2.3. The authors cited that there is a global proliferation of cephalopods (Lines 140 and 141) but later cited other studies saying that there is a regional extinction. It is a bit confusing whether cephalopods are undergoing proliferation or extinction. Given that the earlier citation is more recent (Doubleday et al., 2016) than others, it is wondering which condition is closer to the current situation.

We have removed the second statement to avoid confusion.

2.4. Although it is agreeable in general to have genome resources from unexplored species, the authors' argument in the last paragraph of Data description/Context is not convincing. The link between having a genome and aiding conservation efforts as well as ensuring continued existence is not clear.

Without a genome, population genomic studies that provide information regarding the genetic diversity and structure of populations becomes very challenging, with genome-wide data having to be produced from reduced-representation methods that have many biases. In this last paragraph, we state this specifically: "A genome is an important resource for future population genomics studies[…]".

2.5. Do the authors have any idea why the genome contains so many protein-coding genes (51,225 genes predicted) in comparing to other cephalopod species usually having only 20,000-30,000 genes? For example, is it due to that A. dux has more lineage-specific genes or expansions of certain gene families?

We have revised our gene models and now further discuss the filters applied in lines 272-275. The total number of protein-coding genes passing all the filters is 33,406.

2.6. Given that genome size and polyploidy of the organisms are often correlated to increased body size (Session et al., 2016), have the authors checked if there is whole-genome duplication or polyploidy in the A. dux genome? Session et al. (2016) Genome evolution in the allotetraploid frog Xenopus laevis. Nature 538, 336-343.

We did confirm that the genome was not polyploid by testing for Hardy–Weinberg equilibrium using re-sequencing data from 32 giant squid individuals (Winkelman et al, unpublished results) and there is no evidence for an ancient duplication since we only found one intact Hox complement.

2.7. Figure 3: The authors should provide scaffold numbers for the Hox clusters from each species. Also, in most cases, Hox genes in the Hox cluster are adjacent to each other without the insertion of other non-Hox genes. If there is a special case in A. dux and E. scolopes, the authors should show the real gene arrangement on that scaffold, especially for the non-Hox genes (with brief annotation) that are in between Hox genes. This can be achieved by having an additional panel in the same figure. The authors are encouraged to show an illustration on the types of Hox gene organization in order to give the readers a better understanding of this context.

Figure 3 has received new panels. Scaffold information for *A. dux* was added in panel C (Figure 3-C). As the assemblies of the other species were retrieved from other studies, the readers are directed to the appropriate references for further detail. An extra panel depicting the Hox cluster organisation in more detail has been added. *E. scolopes* data is shown as reported in its published study. No non-Hox genes were indicated for the area covered in this representation. An additional panel with a simplified version of the various Hox "cluster" types was inserted in panel A (Figure 3-A).

Minor comments:

2.1.1. Line 149: ~2cm -> "~2 cm"

Done.

2.1.2. Line 150: 3 orders -> "three orders"

Done.

2.1.3. Line 150: Architeuthis dux -> "A. dux"

Done.

2.1.4. Lines 150 and 151: 10-12cm… 20m -> "10-12 cm… 20 m"

Done.

2.1.5. Line 152: 500kg -> "500 kg"

Done.

2.1.6. Line 171: a Architeuthis dux sample -> "an A. dux sample"

Done.

2.1.7. Line 172: What is CTAB?

CTAB = “cetyl trimethylammonium bromide”; this description has been included in the text (Line 172)

2.1.8. Line 184: For Eukaryota and Metazoa we identified… -> "For Eukaryota and Metazoa, we identified…"

Done.

2.1.9. Line 184: … 90.4 % and 92.1 %... -> "… 90.4% and 92.1%..."

Done.

2.1.10. Line 185: 23.38Gb -> "23.38 Gb"

Done.

2.1.11. Line 186: 14.79kb -> "14.79 kb"

Done.

2.1.12. "k-mer" (Line 204) or "kmer" (Line 176) to be consistent.

Chose to use “kmer”.

2.1.13. Line 216: 100,000 g -> "100,000×g"

Done.

2.1.14. Lines 219 and 222: SDS-PAGE -> "SDS-PAGE" (hyphen but not en dash)

Done.

2.1.15. Line 221: Tris - HCl -> Tris-HCl (single hyphen but not en dash with spaces)

Done.

2.1.16. Line 226: LC-MS/MS analyses -> "LC-MS/MS analyses" (hyphen but not en dash)

Done.

2.1.17. Line 254: Using italic for scientific names (i.e. Octopus bimaculatus, Crassostrea gigas, and Lottia gigantea)

Done.

2.1.18. Line 260: … 200kb (total length 199Mb)… -> "… 200 kb (total length 199 Mb)…"

Done.

2.1.19. Line 290: Transposable elements -> "transposable elements"

Done.

2.1.20. Line 300: Architeuthis dux -> "A. dux"

Done.

2.1.21. Line 323: ~5-8% -> "~5¬-8%" (en dash but not hyphen for a range)

Done.

2.1.22. Line 381: Octopus bimaculoides -> "O. bimaculoides"

Done.

2.1.23. Line 383: Euprymna scolopes -> "E. scolopes" (in italic)

Done.

2.1.24. Line 395: Euprymna scolopes -> "E. scolopes"

Done.

2.1.25. Lines 397 & 398: 500 - 10,000 kb -> "500-10,000 kb" (en dash but not hyphen for a range)

Done.

2.1.26. Line 406: ... observed in Hox 1, Hox 4, ANTP, Lox 2, Lox 5, Post 1 and Post 2. Hox 1 did,... -> "... observed in Hox1, Hox4, ANTP, Lox2, Lox5, Post1 and Post2. Hox1 did,..."

Done.

2.1.27. Line 407: Hox 1 -> "Hox1"

Done.

2.1.28. Line 408: Hox 3 -> "Hox3"

Done.

2.1.29. Line 409: Lox 4 -> "Lox4"

Done.

2.1.30. Lines 410, 412 & 413: Hox 2 -> "Hox2"

Done.

2.1.31. Line 421: ... contains 7 reflectin genes and 3 reflectin-like genes… -> "... contains seven reflectin genes and three reflectin-like genes…"

Done.

2.1.32. Line 422: … exception of 1 reflectin gene, … -> "… exception of one reflectin gene, …"

Done.

2.1.33. Line 436: … (tsa)… -> "… (TSA)…"
Done.

2.1.34. Lines 647 & 657: Architeuthis dux -> "A. dux"

Done.

2.1.35. Line 659: Hox 2 -> "Hox2"

Done.