GigaScience

Adaptive venom evolution and toxicity in octopods is driven by extensive novel gene formation, expansion and loss

--Manuscript Draft--

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Abstract:	Background Cephalopods represent a rich system for investigating the genetic basis underlying organismal novelties. This diverse group of specialized predators has evolved many adaptations including proteinaceous venom. Of particular interest is the blue-ringed-octopus genus (Hapalochlaena), which are the only octopods known to store large quantities of the potent neurotoxin, tetrodotoxin, within their tissues and venom gland. Findings To reveal genomic correlates of organismal novelties, we conducted a comparative study of three octopod genomes, including the Southern blue-ringed octopus (Hapalochlaena maculosa). We present the genome of this species and reveal highly dynamic evolutionary patterns at both non-coding and coding organizational levels. Gene family expansions previously reported in Octopus bimaculoides (e.g., zinc finger and cadherins, both associated with neural functions), as well as formation of novel gene families, dominate the genomic landscape in all octopods. Examination of tissue-specific genes in the posterior salivary gland (PSG) revealed that expression was dominated by serine proteases in non- tetrodotoxin bearing octopods, while this family was a minor component in H. maculosa . Moreover, voltage-gated sodium channels in H. maculosa contain a resistance mutation found in pufferfish and garter snakes, which is exclusive to the genus. Analysis of the PSG microbiome revealed a diverse array of bacterial species, including genera that can produce tetrodotoxin, suggestive of a possible production source. Conclusions We present the first tetrodotoxin-bearing octopod genome H. maculosa, which displays lineage-specific adaptations to tetrodotoxin acquisition. This genome, along with other recently published cephalopod genomes, represents a valuable resource from which future work could advance our understanding of the evolution of genomic novelty in this family.
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Response to Reviewers:

Dear Dr. Hongling Zhou

On behalf of my co-authors, I would like to thank you for the opportunity to revise and resubmit our research piece titled "Adaptive venom evolution and toxicity in octopods is driven
by extensive novel gene formation, expansion and loss" (GIGA-D-20-00135).

We would also like thank all three reviewers for their constructive criticism and advice on
how to improve our manuscript. All suggestions have been taken into account and incorporated resulting in a much-improved manuscript.

A response to reviewers has been provided in which we address each point providing the
original text followed by the revised text, including line numbers where changes have been
implemented. We also included a low resolution version of all corrected figures in the response.

Thank you again for your consideration of our revised manuscript

Yours sincerely,
Brooke L. Whitelaw

Reviewer reports:

Reviewer #1: The manuscript describes the genome of the southern blue-ringed octopus and provides comparisons between this genome and previously published octopus genomes, with a particular focus on venom. I did not see any major flaws in the paper, which I think will be a valuable contribution to the cephalopod genomics literature. The blue-ringed octopuses are of major interest due to their highly toxic venom, and I found the comparisons between this species and others in terms of venom production and resistance to be quite illuminating (though somewhat unsurprising, given what we already knew about the use of TTX by this species).

I found the paper to be fairly well written, though I do have several questions and comments that I hope will help clarify some issues. I will list those below, in the order I encountered them as I read the manuscript and supplementary materials. My only somewhat substantive concern first struck me as I read page 17 of the manuscript: "…suggesting a species-specific expansion of this cluster in C. minor". I think the authors should be a bit more careful with how they use the phrase "species-specific". They have included only three octopod species out of 300+ species in this study. Yes, any differences they detect between these species could be species specific, but I think it is more likely that the differences arose in ancestral lineages. For example, expansion of the serine protease cluster may have occurred only in C. minor, but it could also have occurred in the ancestor of Callistoctopus, or in some other ancestor. At present, the authors do not have sufficient sampling to know if any of the expansions, losses, shifts in expression, etc., they are seeing are truly species specific. Similarly, sentences like "Loss of serine protease genes can also be observed in H.

maculosa". The authors can certainly state that H. maculosa has fewer serine protease genes than O. bimaculoides and C. minor, but the *loss* of these genes may have occurred in H. maculosa *or* in any ancestral lineage after the divergence of Hapalochlaena from Octopus. I urge the authors to go through their manuscript carefully to find instances where they have evidence of differences among these species and to check that their descriptions of differences among these species are clear.

We agree with the reviewer and have carefully read through the manuscript and corrected sections where species-specific inferences were made to prevent miscommunication of the findings.

Original text:
“Loss of serine protease genes can also be observed in H. maculosa”

Revised text: (pg. 18, line: 315)
“Fewer serine protease genes can also be observed in H. maculosa”

Original text:
“The greatest proportion of genes in each species examined were not specific to octopods or an octopus species (ancient genes) (Fig 2a). Expression of these genes were enriched in neural tissues across all species, indicating the core conservation of neural development and function. However, we also find that genes specific to each octopod species also show this expression pattern”

Revised text: (pg. 13-14 , lines: 231-235)
“The greatest proportion of genes in each species examined were not specific to octopods or an octopus lineage (ancient genes) (Fig 2a). Expression of these genes were enriched in neural tissues across all species, indicating the core conservation of neural development and function. However, we also find that genes specific to each octopod lineage also show this expression pattern”

Original text:
“Absence of gene expression for genes whose orthologs have retained expression in one or more other species suggests a unique evolutionary trajectory from other octopods. It should be noted that differences in tissue sampling may in part influence these values.”

Revised text: (pg. 14, lines: 244-249)
“Absence of gene expression for genes whose orthologs have retained expression in one or more other species suggests a unique evolutionary trajectory from other octopods. It should be noted that differences in tissue sampling may in part influence these values and due to the limited sampling of species, loss of expression cannot be inferred at a species level and may have occurred at any point in the lineage.”

We have also removed the term ‘species-specific’ and replaced it with the more accurate term ‘lineage-specific’ to avoid confusion. : (pg. 2, line: 45) , (pg. 10, line: 178), (pg. 13, line:229, (pg. 13, line: 224), (pg. 14, line: 239), (pg. 15, line: 255), (pg. 18, line: 314), (pg. 34, line: 589) & (pg. 35, line: 601)

Figure 2 has also been corrected to replace ‘species specific’ with ‘lineage specific’. Low quality version shown here. The corrected version has been uploaded to replace the original

Minor points and suggestions

Check for subject-verb agreement in the abstract. For example, it should be "This diverse group of specilised (sic) predators has evolved…" (the subject is "group", not "predators").

We have corrected the spelling of ‘specialised’ and have replaced ‘have’ with ‘has’ (pg

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	1, lines: 24). Abstract: Last sentence might be better as "This genome, along with other recently published cephalopod genomes, represents a valuable resource from which future work could advance our understanding of the evolution of genomic novelty in this family" We agree that this sentence would fit better at the end of the abstract and have corrected this. Original sentence: “This genome along with other recently published cephalopod genomes represent a valuable resource from which future work could advance the evolution of genomic novelty within the family.“ Revised sentence (pg. 2, lines: 45-48): "This genome, along with other recently published cephalopod genomes, represents a valuable resource from which future work could advance our understanding of the evolution of genomic novelty in this family" Pg. 3: "underrepresented" - Underrepresented in what sense? In terms of genomic resources? When we used the term underrepresented, we were referring to the lack of published genomes for cephalopods. This has been slowly changing since the publication of the first cephalopod genome in 2015 by Albertin et al. The sentence has been modified to clarify this. Original sentence: “The recent publication of octopod genomes provides an opportunity to investigate the link between genomic and organismal evolution in this unique and underrepresented lineage” Revised sentence: (pg. 3 ,lines: 52-55). “The recent publication of octopod genomes provides an opportunity to investigate the link between genomic and organismal evolution in this unique lineage for which genomic resources have been lacking.1” Pg. 3: (FAO,) - Looks like a typo? Or incomplete switch to a different citation format? Corrected (pg. 3 ,line: 57) Pg. 3: "soft bodied" should be "soft-bodied" Corrected (pg. 3 ,line: 58) Pg. 3: Should be "that are well adapted" (no hyphen) Corrected (pg. 3 ,line: 58) Pg. 4: "How resistance to TTX" - This is not totally clear as written. Resistance in what? I think the authors are referring to how Hapalochlaena avoids being killed by its own TTX, but this could be rephrased to make it crystal clear. Also "remains a large unknown" is a little awkward…maybe "remains largely unknown" would be better? We agree that this sentence was unclear. Reviewer 3 also suggested that due the recent publication by Geffeney et al (2019) this sentence was no longer reflective of the current literature. In order to correct this we have updated this sentence to reflect the latest literature and taken care to ensure the sentences are clear. Original sentence: “How resistance to TTX has been acquired at the genetic level, remains a large unknown, with TTX resistance studied in only in a few select species (i.e. pufferfish13, newts14,15 and gastropods16).”

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Revised sentences (pg. 4, lines: 74-79):
"The mechanism of TTX resistance, which allows for safe sequestration of TTX, has been attributed to several substitutions in the p-loop regions of voltage-gated sodium channels(Nav) in H. lunulata24. However, these channels have yet to be examined in H. maculosa and H. fasciata. TTX resistance has also been studied in a range of other genera including, pufferfish25, newts26,27 arachnids28, snakes29 and gastropods30"

Pg. 4: "Primarily used for defense…" - Awkward sentence with a dangling modifier, which makes it read as though Hapalochlaena is primarily used for defense in other species.

We agree that this was a poor choice of wording and this sentence has been clarified as follows:

Original sentence:
"Primarily used for defense in other unrelated TTX-bearing species, Hapalochlaena is the only known taxa to utilise TTX in venom"

Revised sentence (pg. 4-5,lines: 82-84):
"While other unrelated TTX-bearing species primarily use TTX for defense, Hapalochlaena is the only known taxa to utilise TTX in venom23,35."

Pg. 5: "for example at the evolution of venoms" - Somewhat awkward, I think?

We agree and have modified the sentence to improve flow as follows:

Original sentence:
"By using a comparative genomic approach we are able to examine the emergence of octopod novelties, for example at the evolution of venoms, at a molecular level between H. maculosa and the two non-TTX bearing octopods: the California two-spot octopus (O. bimaculoides) and the long-armed octopus (Callistoctopus minor), while also addressing the species-specific evolution of tetrodotoxin acquisition and resistance in H. maculosa"

Revised sentence (pg. 5 ,lines: 93-98):
"By using a comparative genomic approach we are able to examine the emergence of octopod novelties, at a molecular level between H. maculosa and the two non-TTX bearing octopods: the California two-spot octopus (O. bimaculoides) and the long-armed octopus (Callistoctopus minor). We also address unique features of venom evolution in octopods while also addressing the species-specific evolution of tetrodotoxin acquisition and resistance in H. maculosa"

Pg. 6: Capitalize "bay"? (Port Phillip Bay)

Corrected (pg. 6, line: 108)

Pg. 7: Should be "shallow-water marine organisms".

Corrected (pg. 8, lines: 139)

Pg. 8: Should be "Southern Hemisphere".

Corrected (pg. 8, line: 142-143)

Pg. 8: Also here, how did they do their divergence time estimation?

Tanner et al. (2017) used a Bayesian approach to estimate divergence times, analyzing a concatenated alignment of 197 genes with Phylobayes. These details have been incorporated into the manuscript.

Original sentence:
"Previous phylogenies using a combination of a small number of mitochondrial and

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	nuclear genes46-48 and orthologs derived from transcriptomes49 support this topology. Likewise, divergence of the H. maculosa from Abdopus aculeatus has been previously estimated to be ~59 mya2" Modified sentence (pg. 9, lines: 151-155) : "Previous phylogenies using a combination of a small number of mitochondrial and nuclear genes49–51 and orthologs derived from transcriptomes52 support this topology. Likewise, estimates by Tanner et al.2, using a concatenated alignment of 197 genes with a Bayesian approach, placed divergence of H. maculosa from Abdopus aculeatus at ~59 mya2." Pg. 8: "maculosa from Abdopus" - "from" should not be italicized. Corrected (pg. 9, line: 154) Pg. 9: "sporadic occurrence" - I think the authors mean sporadic taxonomically here (i.e., some species have them, some do not), but this should be clarified (surely the authors don't mean that sometimes a given species has them and sometimes they don't!). In order to prevent confusion the sentence has been modified to include the term "taxonomically sporadic". Original sentence "Morphological variations of ocelli structure and colour, along with their sporadic occurrence within Octopus and Amphioctopus3, limits our interpretation as to the evolutionary history of this trait in octopods" Revised sentence: (pg. 9, lines: 160-163): "Morphological variations of ocelli structure and colour, in conjunction with the taxonomically sporadic occurrence of this trait across species within Octopus and Amphioctopus, limits our interpretation as to the evolutionary history of this trait in octopods." Pg. 11: Just a suggestion here: "splicing, embryonic and neural development" is clear, but it looks odd. How about "splicing and embryonic and neural development" or "splicing as well as embryonic and neural development". We have modified the sentence as suggested: Original sentence: "H. maculosa also shows expansions in the C2H2-type zinc finger family. Zinc fingers form an ancient family of transcription factors, which among other roles serve to regulate transposon splicing, embryonic and neural development 45,46." Revised sentence: (pg. 12 ,lines: 207-09) "H. maculosa also shows expansions in the C2H2-type zinc finger family. Zinc fingers form an ancient family of transcription factors, which among other roles serve to regulate transposon splicing as well as embryonic and neural development69,70." Pg. 11: Should be "this type of zinc finger in O. bimaculoides" Corrected (pg. 12, line: 210) Pg. 12: "High level examination" should be "High-level examination", "large scale expression patterns" should be "large-scale expression patterns", and "lineage specific loss" should be "lineage-specific loss". Corrected (pg. 13, lines: 227 & 230) Pg. 12: Unnecessary comma in "we also find that, genes specific to each octopod" Corrected (pg.13, line: 234)

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Pg. 13: "in tandem with overall reduction in genes number relative to the octopods" - This is unclear and poorly worded. I assume this is referring still to H. maculosa relative to other octopods?

In order to improve the clarity and wording of this sentence it has been rephrased as follows:

Original sentence:
“In order to understand the implications of gene expression loss, in tandem with overall reduction in genes number relative to the octopods, further investigation is required.”

Revised sentence: (pg. 14, lines: 249-251)
“In order to fully understand the implications of the gene family contractions and loss of expression in H. maculosa, relative to other octopods, further investigation is required.”

Pg. 15: Unnecessary comma in "More notable, were differences"

Corrected (pg. 17, line: 288)

Pg. 16: Should be "primary venom-producing gland".

Corrected (pg. 17, line: 296)

Pg. 16: Haplochlaena should be italicized in "hypothesized that the Hapalochlaena PSF…" Also, who has hypothesized that the Hapalochlaena PSF will exhibit a loss of redundant proteinaceous toxins? This seems to call for a citation.

This statement was poorly worded and has been corrected to reflect that we proposed the hypothesis. Hapalochlaena has also been italicized.

Original sentence:
“It has been hypothesized that the Hapalochlaena PSG will exhibit a loss of redundant proteinaceous toxins due to the presence of TTX.”

Revised sentence: (pg. 17, lines: 299-300)
“We hypothesize that the Hapalochlaena PSG will exhibit a loss of redundant proteinaceous toxins due to the presence of TTX.”

Pg. 16: "A total of 623 genes were exclusive to H. maculosa PSF…exclusive to O. bimaculoides and C. minor, respectively". Should this be "exclusive to the O. bimaculoides and C. minor PSGs, respectively"?

Yes the reviewer is correct. This sentence has been corrected as suggested.

Original sentence:
“A total of 623 genes were exclusive to H. maculosa PSG compared to only 230 and 164 exclusive to O. bimaculoides and C. minor, respectively”

Revised sentence (pg.17-18, lines 302-304)
“A total of 623 genes were exclusive to H. maculosa PSG compared to only 230 and 164 exclusive to O. bimaculoides and C. minor PSGs, respectively.”

Pg. 16: "Additionally, H. maculosa PSG is predicted to be" - Predicted by whom? The authors? I think so, so they should make that clear, e.g., "we predict that the H. maculosa PSG is functionally more diverse…"

This sentence has been corrected as suggested:

Revised sentence (pg. 18, lines: 304-306)
“Additionally, we predict that the H. maculosa PSG is functionally more diverse based on the number of Pfam families detected, 532 in total.”

Pg. 17: The sentence about reprolysin doesn't make sense to me. The authors

describe shifting expression in this species (see my comment above) but then note that there is a complete loss
of orthologs from the genome. This latter comment suggests that reprolysin doesn't even exist in the H. maculosa genome, but it must, if it is showing different expression patterns than the other species. Can the authors clarify this?

The sentence that “there is a complete loss of orthologs from the genome” was incorrect. It should have read “there is a complete loss of paralogs from the genome”. This has been corrected in the sentence below.

Revised sentence (pg. 18, lines: 316-318)
“Similarly, reprolysin (M12B) exhibits shifting expression in H. maculosa, presumably from the PSG to the branchial heart, and a complete loss of paralogs from the genome.”

Pg. 18: "the cephalopod specific clade" should be "cephalopod-specific clade".

Corrected (pg. 19 ,line: 324)

Pg. 18: "…hyaluronidase, which often serve as dispersal factors" seems odd. Should this be "hyaluronidase, which often serves as a dispersal factor"?

Yes and we have modified the sentence as suggested.

Original sentence:
“Previous proteomic analysis of the H. maculosa PSG revealed high expression of hyaluronidase, which often serve as dispersal factors within snake venom, facilitating the spread of toxin while not being directly toxic to their prey9,72”

Revised sentence: (pg. 19, lines: 330-333)
“Previous proteomic analysis of the H. maculosa PSG revealed high expression of hyaluronidase, which often serves as a dispersal factor within snake venom, facilitating the spread of toxin while not being directly toxic to their prey9,72.”

Pg. 19: "Two Nav genes"…should the "v" be a subscript here?

Corrected (pg. 20, line: 352)

Pg. 19: "latter regions in DIII and DIV" - Is "latter" the best adjective here?

“Latter” is not an ideal word choice and the sentence has been modified.

Original sentence:
“The latter regions in DIII and DIV”

Revised sentence: (pg. 21, line: 356)
“The regions DIII and DIV closer to the C-terminal end of the protein”

Pg. 20: "In previous studies, when examined individually, the Met- Thr substitution in a TTX sensitive Nav1.4 rat channel decreased binding affinity in pufferfish by 15-fold" - This could be more clear. A "rat channel"? As in, a channel in rats? But the sentence says "in pufferfish". Please clarify this.

Jost et al 2008 found a Met-Thr substitution in the third p-loop region of a pufferfish sodium channel. This substitution was induced in a TTX sensitive rat channel through site-directed mutagenesis and the rat channel was then expressed in the oocytes of the African clawed frog (Xenopus). In our original sentence some of these taxonomical/methodological details obscured the ultimate finding which is the introduction of a Met-Thr substitution, in an otherwise TTX-sensitive channel, inhibits TTX binding by 15-fold. Our revised sentence has removed reference to taxonomy to convey the main finding more clearly.

Original sentence:
"In previous studies, when examined individually, the Met- Thr substitution in a TTX

	sensitive Nav1.4 rat channel decreased binding affinity in pufferfish by 15-fold" Revised sentence (pg. 21,lines: 365-366): “In a previous study a Met to Thr substitution into a TTX sensitive Nav1.4 channel decreased binding affinity to TTX by 15-fold87.” Pg. 21: "It has yet to be established if these mutations are derived from a shared ancestor or have occurred independently" - Excellent. This is exactly the clarity I think the authors can bring to their other statements about gains, losses, etc. that I pointed out in a previous comment. As detailed in the first response to reviewers we have clarified these statements throughout. Pg. 21: I would write "While Hapalochlaena remains" here. Corrected (pg. 22 ,line: 379) Pg. 21: Should be "STX-contaminated bivalves" (and "STX-contaminated fish" and "STX-contaminated food sources" below…and "TTX-producing bacteria" and "TTX-producing strains" on pg. 23). Corrected (pg. 22-24, lines: 383, 391-392, 394, 416, 422) Pg. 21: "Humboldt" should be capitalized, as it is a proper name. Corrected (pg. 18, line: 389, 391) Pg. 23: "Sequestration of TTX is not exclusive to the blue-ringed octopus among molluscs. Gastropods such as Pleurobranchaea maculata and Niotha clathrata, as well as some bivalves, are capable of sequestering the similar toxin STX " - TTX and STX are similar, but not the same. Are there other examples of actual TTX sequestration in molluscs, outside of Hapalochlaena? If not, this should be rephrased. Yes, sequestration of TTX does occur in molluscs aside from Hapalochlaena, including but not necessarily limited to the gastropods Pleurobranchaea maculata and Niotha clathrata. In the original sentence we mistakenly said these species were capable of sequestering STX as opposed to TTX. This has been corrected in the revised sentence. Original sentence: “Gastropods such as Pleurobranchaea maculata and Niotha clathrata, as well as some bivalves, are capable of sequestering the similar toxin STX” Revised sentence: (pg. 24 ,lines: 409-420) “Gastropods such as Pleurobranchaea maculata and Niotha clathrata, as well as some bivalves, are also capable of sequestering TTX” Pg. 23: "highly diverse composition of genera" may be better as "highly diverse composition of bacterial genera" Corrected (pg. 24, lines: 413) Pg. 23: "Diversity of bacterial genera much like the H. maculosa in this study was high" - This seems awkward and unclear." This sentence was unclear and has been modified to: Original sentence: “Diversity of bacterial genera much like the H. maculosa in this study was high and this may complicate identification of species responsible for TTX production” Revised sentence (pg. 25 , lines: 423-425): “Congruent with our findings the diversity of bacterial genera was high and this may

complicate identification of species responsible for TTX production"

Pg. 24: "TTX bearing mollusk genome" - Hmm…does the genome bear TTX? The mollusk does, I suppose, but it's produced by bacteria as described above. This could be rephrased.

The sentence has been rephrased to improve clarity to:

Original sentence:
"This work describes the genome of a unique TTX bearing mollusc genome, the southern blue-ringed octopus (Hapalochlaena maculosa)."

Revised sentence (pg. 25 ,lines: 434-435):
"This work describes the genome of a unique TTX bearing mollusc, the southern blue-ringed octopus (Hapalochlaena maculosa)."

Pg. 28: What kit or method was used to construct the cDNA libraries for transcriptome sequencing?

Construction of cDNA libraries was outsourced to AGRF (Australian Genome Research Facility), Melbourne, and conducted using their TruSeq mRNA Library Prep with polyA selection and unique dual indexing method. This information has been included in the methods section "Transcriptome sequencing"

Revised text: (pg. 29, lines: 499-504)
"Construction of cDNA libraries was outsourced to AGRF (Australian Genome Research Facility), Melbourne and conducted using their TruSeq mRNA Library Prep with polyA selection and unique dual indexing method. Libraries were constructed using 3 µg of RNA at a concentration of >100 ng/µ L. Each tissue was sequenced on 1/12th of an Illumina HiSeq2000 lane with one lane used in total."

Pg. 30: Which assemblies of the molluscan genomes (Crassostrea, etc.) were used? Where did the transcriptomes for Sepia and Idiosepius come from? Were reads downloaded from the NCBI SRA and assembled in Trinity? If so, what were the BioProejct numbers for the transcriptome data?

We have modified the methods section "Calibration of sequence divergence with respect to time" to include details as to the origin of both the transcriptomes and the genomes used in this study.

Revised text: (pg. 31-32, lines: 539-546)
Bioprojects for each genome used are as follows:Crassostera gigas (PRJNA629593 & PRJEB3535), Lottia gigantea (PRJNA259762 & PRJNA175706), Aplysia californica (PRJNA629593 & PRJNA13635) and (Euprymna scolopes PRJNA47095). Octopus bimaculoides was obtained from http://octopus.unit.oist.jp/OCTDATA/BASIC/Metazome/Obimaculoides_280.fa.gz. The , Idiosepius notoides (BioProject: PRJNA302677) transcriptome was sequenced and assembled using the same method previously described for the H. maculosa transcriptome.

Pg. 32: I think when the authors write "H. maculosa is a single generation species", they mean that it is semelparous, but I don't see why that is relevant for mutation rate calculations.

We completely agree that this has little impact on mutation rate (measured per generation). This was included in an early draft (that discussed the coalescent demographic analyses in more detail) and was kept in by mistake. The text has been revised to remove "H. maculosa is a single generation species"

Original sentence:
"Per base neutral substitution between lineages was determined using the mean dS value divided by divergence time (refer to Calibration of sequence divergence with respect to time) usually over number of generations, however H. maculosa is a single generation species"

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Revised sentence: (pg. 34, lines: 575-577)
“Per base neutral substitution between lineages was determined using the mean dS value divided by divergence time (refer to Calibration of sequence divergence with respect to time) over the number of generations.”

Pg. 33: "genes with expression within one or more tissues was determined" should be "…were determined".

Corrected (pg. 35, line: 592).

Pg. 34: Something is strange in this sentence - "A loss of expression requires a gene to be present in all three octopods with and expressed in one or more species while having no detectable expression in at least one species" (an extra "with"?)

Corrected (pg. 35, lines: 602-604).

Pg. 34: Individual mutation with potential" should be "mutations"

Corrected (pg. 36, line: 613-614).

Figure 5: Loligo pealei and Doryteuthis pealeii are the same species. The latter name is the correct one.

Figure 5 has been corrected by removing the duplicate sequence and retaining the correct name Doryteuthis pealeii

Low quality version shown here. The corrected version has been uploaded to replace the original

Supplementary Material

4.1: a)What models and settings were used in RAxML and PhyloBayes?
b)How were those models chosen?
c)What calibrations were used for the divergence time analysis?
d)How was convergence inferred for the PhyloBayes run?
e)Also, Supplementary Figure 3 is a a "QITREE" tree…do the authors mean IQ-TREE? If so, why is IQ-TREE not mentioned in the text? This tree also differs slightly from the tree presented in Figure 1a, though I doubt the difference is of any consequence for this paper.

In order to clearly address the points raised by Reviewer1, we have answered each question separately.

a)The models chosen for RaxML and Phylobayes were GTR+G+I and strict clock with a mixture model of F81 + G respectively.
b) These models were selected based on results from JmodelTest.
c) Calibrations were used on two nodes for the Phylobayes run : divergence between H. maculosa and E. scolopes 275mya & divergence between C. gigas and E. scolopes 500mya
d) Convergence was ascertained using tracecomp from the Phylobayes package
e) Two trees were run using the same alignment, the first was run with RAXML and the resulting tree used to inform Phylobayes, the second was run with IQTREE and included as a supplementary as it does not differ from the previous tree in any significant capacity. We have corrected the typo QITREE in the text.

We have added the details mentioned above to the supplementary materials section “4.1 Multi-gene cephalopod phylogeny and dating”.

Original text:
“A total of 2,108 clusters were obtained. Phylogenies were constructed using RAxML31 and Phylobayes32. Divergence times were calculated using Phylobayes, calibrations, setting and model used”

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Revised text: (pg. 15-16, lines: 222-232)
"A total of 2,108 clusters were obtained. Phylogenies were constructed using RAxML v8.031 and divergence times estimated by Phylobayes v4.132. RAxML v8.031 was run using the GTR+G+I model ascertained from JmodelTest v2.1.10. using the cAIC criterion for 100 bootstraps. Phylobayes estimated divergence times under a strict clock with a mixture model of F81+G with a burn-in of 10%. Calibrations were used as follows : divergence between H. maculosa and E. scolopes 275mya & divergence between C. gigas and E. scolopes 500mya. Two runs were performed and convergence verified using bpcomp, which confirmed a maximum difference of < 0.1 and tracecomp, which also indicated convergence with an effective sample size(EES) of > 200 for all parameters. Both programs used were from the Phylobayes package."

Reviewer #2: Review
Manuscript Number: GIGA-D-20-00135
Title: Adaptive venom evolution and toxicity in octopods is driven by extensive novel gene formation, expansion and loss
submitted to: GigaScience

This manuscript presents interesting data sets of both the genomic sequence of the TTX-bearing octopus Hapalochlaena maculosa as well as transcriptomes from twelve different tissues. The methods used were appropriate for the aims of the study including the use of two different methods to prepare (Illumina and Chicago), sequence (Illumina HiSeq 2000 and Dovetail) and assemble the genome (Illumina and HiRise). The authors do a good job reporting the statistical analysis of their assembly and comparing their statistics to two other octopus genomes, Callistoctopus minor and Octopus bimaculoides. Their methods of transcriptome sequencing, analysis and assembly were appropriate. Finally, their analysis of the completeness of their genome was appropriate and indicate that their genome is well constructed.
Their further analysis of the assembled genome and transcriptome are interesting and appropriate including the examination of the expansion of the zinc finger and cadherin/protocadherin gene families that have previously been identified in octopuses. Their analysis of expression differences in genes expressed in the posterior salivary gland between non-TTX bearing octopuses and the TTX-bearing H. maculosa is informative and suggests that the expression of serine protease venoms found in non TTX-bearing octopuses is reduced in H. maculosa. Finally, the authors confirm that H. maculosa has the same set of amino acid substitutions that are found in the voltage-gated sodium channel NaV1 of Hapalochlaena lunalata. In both species, these changes in channel structure are likely to impart TTX resistance and explain the genetic mechanism underlying TTX resistance in the genus.
The authors appear to have met the minimum standard of reporting for the journal. However, the authors have not done an adequate job of reviewing the scientific literature that would contextualize their work and this has led to inaccurate statements in the manuscript. The manuscript requires editing for clarity. I will highlight several of the problem sections below.

1) In the abstract/background the authors state "Cephalopods represent a rich system for investigating the genetic basis underlying organismal novelties. This diverse group of specilised predators have evolved many unique adaptations including proteinaceous venom." Proteinaceous venoms are not unique to cephalopods. Snakes have evolved the use of proteinaceous venoms that function as enzymes including serine proteases that the authors suggest are unique to cephalopods. For an example, see a review in Toxicon from 2013 by Solange and Serrano. The authors could strengthen this manuscript by discussing their work in the context of the independent evolution in vertebrates and invertebrate lineages of the use of this enzyme class. The author's interesting report that serine protease expression is reduced in a tetrodotoxin (TTX) bearing cephalopod compared to non-TTX bearing cephalopods is overshadowed by this mischaracterization of the uniqueness of this character in cephalopods.

It was not our intention to convey that cephalopods are the only taxa to have evolved proteinaceous venom and to rectify this we have modified the sentence in the abstract to more accurately represent the literature. Additionally, we clarified the independent evolution between invertebrates and vertebrates in the introduction.

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Abstract:
Original text:
"Cephalopods represent a rich system for investigating the genetic basis underlying organismal novelties. This diverse group of specilised predators have evolved many unique adaptations including proteinaceous venom."

Revised text: (pg.1, lines:23-25)
“Cephalopods represent a rich system for investigating the genetic basis underlying organismal novelties. This diverse group of specialized predators has evolved many adaptations including proteinaceous venom.”

Background:
Original text:
“Furthermore, proteinaceous venom is produced and stored within a specialised gland in cephalopods known as the posterior salivary gland (PSG)”

Revised text: (pg.3-4, lines:63-71)
“Furthermore, the cephalopods have independently evolved proteinaceous venom, which is produced and stored within a specialised gland in known as the posterior salivary gland (PSG). All octopods are believed to possess a form of proteinaceous venom used to subdue prey8–10. Serine proteases are a common component of cephalopod venoms and have been observed in the PSG of squids, cuttlefish and octopods10–13. Convergent recruitment of serine proteases has been observed between many vertebrate (Squamata14–16 and Monotremata17) and invertebrate (Hymenoptera18, Arachnida19, Gastropoda20, Remipedia21 and Cnidarian22) venomous lineages.”

2)In the abstract/findings description the authors state "…voltage-gated sodium channels in H. maculosa contain a resistance mutation found in pufferfish and garter snakes, which is absent in other octopods." Hapalochlaena maculosa has the same amino acid sequences encoded in the voltage-gated sodium channel genes NaV1 and NaV2 as previously reported for the Greater Blue-ringed octopus Hapalochlaena lunulata, in Toxicon from 2019 by Geffeney and colleagues.

This sentence has been corrected to reflect the recent finding of the resistance mutations by Gefferny et al 2019 in H. lunulata.

Original sentence:
“Moreover, voltage-gated sodium channels in H. maculosa contain a resistance mutation found in pufferfish and garter snakes, which is absent in other octopods”

Modified sentence: (pg.2, lines:38-40)
“Moreover, voltage-gated sodium channels in H. maculosa contain a resistance mutation found in pufferfish and garter snakes, which is exclusive to the genus.”

3) In the background section of the main body the authors state "How resistance to TTX has been acquired at the genetic level, remains a large unknown, with TTX resistance studied in only in a few select species (i.e. pufferfish13, newts14,15 and gastropods16)." The changes in voltage-gated sodium channel genes that lead to TTX-resistance are well understood. Genetic changes that lead to TTX resistance have been examined in groups not included in the authors list including other invertebrates (e.g. insects and blue-ringed octopuses) as well as snakes. The authors statement mischaracterizes the body of literature examining the evolution of TTX resistance.

This section has been modified to more accurately depict the current literature by providing more examples of taxa which exhibit resistance to TTX. We now included an example of an arachnid whose channels have been examined for TTX resistance, however we were unable to find an example of an insect as suggested above by reviewer 2. We would be happy to include an example of an insect if the reference could be provided.

Original text:

"How resistance to TTX has been acquired at the genetic level, remains a large unknown, with TTX resistance studied in only in a few select species (i.e. pufferfish13, newts14,15 and gastropods16)."

Revised text: (pg. 4, lines: 74-89)
“The mechanism of TTX resistance, which allows for safe sequestration of TTX, has been attributed to several substitutions in the p-loop regions of voltage-gated sodium channels(Nav) in H. lunulata24. However, these channels have yet to be examined in H. maculosa and H. fasciata. TTX resistance has also been studied in a range of other genera including, pufferfish25, newts26,27 arachnids28, snakes29 and gastropods30.”

4)In the data description the authors discuss their work to identify the expansion of genes in the cadherin/protocadherin gene family. This section requires citations as well as correction of existing citations.

a.The authors state "H. maculosa and C. minor exhibit expansions in the cadherin gene family, characteristic of other octopod genomes, including O. bimaculoides (Fig1b)." without including a reference. This statement requires a reference and the discussion of their data would be improved by comparing their findings to other articles that have examined the expansion of the cadherin gene family and specifically protocadherins, for example Styfhals et al. (2019) in Frontiers in Physiology.

We agree with the reviewer that further discussion was required for this section and have restructured the paragraph to the following:

Original text:
“Organismal impact of novel genes and gene family expansions
Gene family expansions between octopods (O. bimaculoides, C. minor and H. maculosa) and three other molluscan genomes (Aplysia californica, Lottia gigantea and Crassostrea gigas) were examined using Pfam annotations. A total of 5565 Pfam domains were identified among six molluscan genomes. H. maculosa and C. minor exhibit expansions in the cadherin gene family, characteristic of other octopod genomes, including O. bimaculoides (Fig1b). C. minor, in particular, shows the greatest expansion of this family within octopods. Expansions of protocadherins, a subset of the cadherin family, have also occurred independently in squid 20, with the octopod expansions occuring post divergence ~135 mya 20. Cadherins, specifically protocadherins, are essential mediators of short-range neuronal connections in mammals42 43. Due to the absence of a myelin sheath in octopods, short-range connections are integral to maintaining signal fidelity over distance44.”

Revised text: (pg. 10-12, lines: 183-206)
“Organismal impact of novel genes and gene family expansions
Gene family expansions between octopods (O. bimaculoides, C. minor and H. maculosa) and three other molluscan genomes (Aplysia californica, Lottia gigantea and Crassostrea gigas) were examined using Pfam annotations. A total of 5565 Pfam domains were identified among six molluscan genomes. H. maculosa and C. minor exhibit expansions in the cadherin gene family, characteristic of other octopod genomes, including O. bimaculoides (Fig1b)42,64. C. minor, in particular, shows the greatest expansion of this family within octopods. Expansions of protocadherins, a subset of the cadherin family, have also occurred independently in squid42, with the octopod expansions occurring post divergence ~135 mya42. The shared ancestry of octopod cadherins was also documented by Styfhals et al64 using phylogenetic inference between O. bimaculoides and O. vulgaris.Cadherins, specifically protocadherins, play crucial roles in synapse formation, elimination and axon targeting within mammals and are essential mediators of short-range neuronal connections65–68. It should be noted that octopods lack a myelin sheath, as a result short-range connections are integral to maintaining signal fidelity over distance6. The independent expansions of protocadherins within chordate and cephalopod lineages are believed to be associated with increased neuronal complexity42,64. Elevated expression of protocadherins within neural tissues have been observed in O. vulgaris and O. bimaculoides by both Styfhals et al64 and Albertin et al42 respectively. In particular Styfhals et al64 noted differential expression across neural tissues including supra-esophageal mass, sub-esophageal mass, optic lobe and the stellate ganglion64.

	However, functional implications of observed expression patterns remain speculative without further study.” b.The next sentence ("Expansions of protocadherins, a subset of the cadherin family, have also occurred independently in squid 20, with the octopod expansions occuring post divergence ~135 mya 20.") incorrectly references Williams et al. (2012, reference 20) but should reference Albertin et al. (2015, reference 29). This reference was corrected as suggested: (pg. 11, line:190-192) “Expansions of protocadherins, a subset of the cadherin family, have also occurred independently in squid42, with the octopod expansions occurring post divergence ~135 mya42” c.The authors state "Cadherins, specifically protocadherins, are essential mediators of short-range neuronal connections in mammals42 43. Due to the absence of a myelin sheath in octopods, short-range connections are integral to maintaining signal fidelity over distance44." None of the citations in these two sentences are correct and no correct references can be found in the list of citations. These final statements should include the fact that expansion in the number of protocadherin genes also occurs in chordates (for example, Hulpiau & van Roy, 2010 from Molecular Biology and Evolution). There is good evidence that protocadherins have roles in multiple aspects of proper synapse formation in mammals including synapse generation, synapse elimination and axon targeting (for example see reviews by de Wit and Ghosh from 2016 in Nature Reviews Neuroscience as well as Peek et al. from 2017 in Cellular and Molecular Life Sciences). Though synapses are "short-range connections", proper synapse formation is important for vertebrates and invertebrates with complex nervous systems whether or not that have myelinated axons. The expansion of protocadherin genes in both cephalopods and chordates independently is thought to be linked to increased neuronal circuit complexity. We have corrected the miscitations and restructured the paragraph to incorporate the reviewers suggestions and more accurately describe the evolution and role of protocadherins in vertebrates and invertebrates. Original text: “Cadherins, specifically protocadherins, are essential mediators of short-range neuronal connections in mammals42 43. Due to the absence of a myelin sheath in octopods, short-range connections are integral to maintaining signal fidelity over distance44.” Revised text: (pg. 11-12, lines: 194-206) “Cadherins, specifically protocadherins, play crucial roles in synapse formation, elimination and axon targeting within mammals and are essential mediators of short-range neuronal connections65–68. It should be noted that octopods lack a myelin sheath, as a result short-range connections are integral to maintaining signal fidelity over distance6. The independent expansions of protocadherins within chordate and cephalopod lineages are believed to be associated with increased neuronal complexity42,64. Elevated expression of protocadherins within neural tissues have been observed in O. vulgaris and O. bimaculoides by both Styfhals et al64 and Albertin et al42 respectively. In particular Styfhals et al64 noted differential expression across neural tissues including supra-esophageal mass, sub-esophageal mass, optic lobe and the stellate ganglion64. However, functional implications of observed expression patterns remain speculative without further study.” 5)In the data description the authors discuss their work to identify the expansion of genes in the zinc finger gene family. The author state "H. maculosa also shows expansions in the C2H2-type zinc finger family. Zinc fingers form an ancient family of transcription factors, which among other roles serve to regulate transposon splicing, embryonic and neural development 45,46." These references are not correct for this statement. The manuscript would be strengthened by proper citations in this section, for example Fedotova and colleagues (2017) have a review in Acta Naturae. Additionally, there is evidence that these proteins have roles in both transposon suppression and alternative splicing.

Citations were corrected as suggested: (pg. 12, lines: 207-209)

"H. maculosa also shows expansions in the C2H2-type zinc finger family. Zinc fingers form an ancient family of transcription factors, which among other roles serve to regulate transposon splicing as well as embryonic and neural development69,70."

6)The authors state "It has been hypothesized that the Hapalochlaena PSG will exhibit a loss of redundant proteinaceous toxins due to the presence of TTX." This sentence should have a citation or the authors should explain that this statement is their hypothesis.

This statement is a hypothesis by the authors and the sentence has been modified to reflect this.

Original sentence:
"It has been hypothesized that the Hapalochlaena PSG will exhibit a loss of redundant proteinaceous toxins due to the presence of TTX."

Revised sentence: (pg.17, lines: 299-300)
"We hypothesize that the Hapalochlaena PSG will exhibit a loss of redundant proteinaceous toxins due to the presence of TTX."

7)There are minor errors in the sequences presented in Figure 5. In multiple invertebrate species, phenylalanine (F) replaces tyrosine (Y) in the D1 pore. In pufferfish cysteine (C) replaces tyrosine (Y). The figure is constructed in a way that suggests that these amino acids replace a neighboring aspartic acid (D).

Figure 5 has been modified so all sequences align correctly.
Low quality version shown here. The corrected version has been uploaded to replace the original

Reviewer #3
A truly excellent paper that was a pleasure to read. My comments are very minor:
- TTX resistance in Thamnophis species of snakes should be referenced in the sentence "How resistance to TTX has been acquired at the genetic level, remains a large unknown, with TTX resistance studied in only in a few select species (i.e. pufferfish13, newts14,15 and gastropods16)". This is cited later (ref 58) but it would be appropriate for inclusion in this sentence too.
This citation has been added as suggested to a modified version of this sentence as suggested by reviewer 1:

Original text:
"How resistance to TTX has been acquired at the genetic level, remains a large unknown, with TTX resistance studied in only in a few select species (i.e. pufferfish13, newts14,15 and gastropods16)"
Revised text: (pg. 4, lines: 74-79)
"The mechanism of TTX resistance, which allows for safe sequestration of TTX, has been attributed to several substitutions in the p-loop regions of voltage-gated sodium channels(Nav) in H. lunulata13. However, these channels have yet to be examined in H. maculosa and H. fasciata. TTX resistance has also been studied in a range of other genera including, pufferfish14, newts15,16 arachnids17, snakes18 and gastropods19.

- For the PSG specific genes, calculations of the relative rates of evolution would be informative as this would be suggestive of adaptive evolution eg are the abundant serine proteases C. minor showing signs of accelerated evolution seen in other venomous lineages such as snakes? Previous work has shown that the sites on the molecular surface are undergoing episodic diversification when compared across a wide range of lineages. In this case, it would be interesting to see what the evolutionary patterns are for C. minor, in that is the extensive duplication accompanied by signs of diversification?
We investigated the potential of positive selection within serine proteases with a focus

on genes specifically expressed in the posterior salivary gland (venom gland). Unfortunately, we did not find strong evidence of accelerated evolution in these genes with the method described below. In the future we look forward to conducting a more in-depth analysis of this interesting family with a more comprehensive sampling across coleoid cephalopods.
This section below, describing our additional analyses, has been added to the supplementary materials: (pg. 24-25, lines: 355-382)
6.4 Examination of selection and evolutionary rates in octopod serine proteases
Gene models (aa) from the three octopod genomes (H. maculosa, O. bimaculoides and C. minor) were annotated with Interproscan and serine proteases with the Pfam PF00089 extracted for examination. Gene models and their corresponding CDS sequences were imported into Geneious v10.2.6 and selected for a single trypsin (PF00089) domain greater than 200aa/600bp long. The region containing the trypsin domain was then extracted from the nucleic acid sequences and MAFFT v7.407 was used to align sequences using Translation align in Geneious v10.2.6, which interpreted the first codon as the start of the codon region and used the first translation frame. The resulting alignment was tested for an appropriate substitution model in jModelTest v2.2.10 and a tree was generated with RAxML v8.0 using the GTR +G+I model and 100 bootstraps. The resulting tree and alignment were examined using codeml via EasyCodeml v1.21 from the PAML package to examine non-synonymous to synonymous substitution rates for evidence of positive selection. We first used a site-based model which allows for ω values to vary between sites along the protein. Comparison of the nested models (M1a-M2a) and (M7-M8) did not reveal any sites under positive selection ($p > 0.05$). In order to access the potential for different rates of evolution within specific lineages we used a branch site model which allows for ω values to vary between sites and branches. For the foreground a large clade of genes, majority of which were specifically expressed in the posterior salivary gland (PSG) was selected and compared to all other non-PSG specific genes. No sites among the foreground branches were significantly accelerated relative to the background. The last method implemented is similar to the branch site model, however, the rate along sites is constant and the rate between the background and foreground can differ. This also found no evidence of positive selection between the background and foreground lineages. It should be noted that serine proteases are a large and complex family and are due a more in-depth analysis in coleoid cephalopods, which could form a complete stand-alone study.
- The M12B metalloprotease type in snake venom has a wide range of demonstrated activities, both anticoagulant (fibrinogenolytic) but also procoagulant (Factor X activating [Atractaspis and Daboia venoms] and prothrombin activating (Bothrops, Echis, and Dispholidus/Thelatornis venoms]) prothrombin activating metalloproteases from Dispholidus typus (boomslang) and Thelotornis mossambicanus (twig snake).
" Comp Biochem Physiol C Toxicol Pharmacol: 108625. Oulion, B., J. S. Dobson, C. N. Zdenek, K. Arbuckle, C. Lister, F. C. P. Coimbra, B. Op den Brouw, J. Debono, A. Rogalski, A. Violette, R. Fourmy, N. Frank and B. G. Fry (2018). "Factor X activating Atractaspis snake venoms and the relative coagulotoxicity neutralising efficacy of African antivenoms." Toxicol Lett 288: 119-128.
Rogalski, A., C. Soerensen, B. Op den Brouw, C. Lister, D. Dashevsky, K. Arbuckle, A. Gloria, C. N. Zdenek, N. R. Casewell, J. M. Gutierrez, W. Wuster, S. A. Ali, P. Masci, P. Rowley, N. Frank and B. G. Fry (2017). "Differential procoagulant effects of saw-scaled viper (Serpentes: Viperidae: Echis) snake venoms on human plasma and the narrow taxonomic ranges of antivenom efficacies." Toxicol Lett 280: 159-170.
Sousa, L. F., C. N. Zdenek, J. S. Dobson, B. Op den Brouw, F. Coimbra, A. Gillett, T. H. M. Del-Rei, H. M. Chalkidis, S. Sant'Anna, M. M. Teixeira-da-Rocha, K. Grego, S. R. Travaglia Cardoso, A. M. Moura da Silva and B. G. Fry (2018). "Coagulotoxicity of Bothrops (Lancehead Pit-Vipers) Venoms from Brazil: Differential Biochemistry and Antivenom Efficacy Resulting from Prey-Driven Venom Variation." Toxins (Basel) 10(10): 411.
We agree that these references should be included in the manuscript and have added them as appropriate. (pg. 18, lines:316-319)

“Similarly, reprolysin (M12B) exhibits shifting expression in H. maculosa, presumably from the PSG to the branchial heart, and a complete loss of paralogs from the genome. While the function of this protein has not been assessed in octopus, members of this protein family exhibit anticoagulant properties in snake venom75–78.

Additional Information:

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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 data presented should be made available in the figure legends. Have you included all the information requested in your manuscript?	Yes
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.	Yes

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1 # Adaptive venom evolution and toxicity in octopods is driven by
2 # extensive novel gene formation, expansion and loss

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21 ## *Abstract*

22 ***Background***

23 Cephalopods represent a rich system for investigating the genetic basis underlying

24 organismal novelties. This diverse group of specialized predators has evolved many

25 adaptations including proteinaceous venom. Of particular interest is the blue-ringed-

26 octopus genus (*Hapalochlaena*), which are the only octopods known to store large

27 quantities of the potent neurotoxin, tetrodotoxin, within their tissues and venom gland.

28 ***Findings***

29 To reveal genomic correlates of organismal novelties, we conducted a comparative

30 study of three octopod genomes, including the Southern blue-ringed octopus

31 (*Hapalochlaena maculosa*). We present the genome of this species and reveal highly

32 dynamic evolutionary patterns at both non-coding and coding organizational levels.

Gene family expansions previously reported in *Octopus bimaculoides* (e.g., zinc finger and cadherins, both associated with neural functions), as well as formation of novel gene families, dominate the genomic landscape in all octopods. Examination of tissue-specific genes in the posterior salivary gland (PSG) revealed that expression was dominated by serine proteases in non- tetrodotoxin bearing octopods, while this family was a minor component in *H. maculosa*. Moreover, voltage-gated sodium channels in *H. maculosa* contain a resistance mutation found in pufferfish and garter snakes, which is exclusive to the genus. Analysis of the PSG microbiome revealed a diverse array of bacterial species, including genera that can produce tetrodotoxin, suggestive of a possible production source.

Conclusions

We present the first tetrodotoxin-bearing octopod genome *H. maculosa,* which displays lineage-specific adaptations to tetrodotoxin acquisition. This genome, along with other recently published cephalopod genomes, represents a valuable resource from which future work could advance our understanding of the evolution of genomic novelty in this family.

50 ***Background***

51 Reconstructing the evolution of novelties at the genomic level is becoming an
52 increasingly viable approach to understand their origin. The recent publication of
53 octopod genomes provides an opportunity to investigate the link between genomic and
54 organismal evolution in this unique lineage for which genomic resources have been
55 lacking[1]. From their emergence 275 mya[2], octopods have diversified into > 300
56 species, inhabiting tropical to polar regions, from the deep sea to shallow intertidal
57 zones[3]. As a highly diverse group, octopods show remarkable variation in body form
58 and function. They are specialised soft-bodied predators that are well adapted to their
59 environment with prehensile limbs lined with chemosensory suckers[4], the ability to
60 manipulate skin texture and colour using specialised chromatophores[5], the largest
61 invertebrate nervous systems (excluding those of other cephalopods)[6], and a relatively
62 large circumesophageal brain allowing for complex problem solving and retention of
63 information[7]. Furthermore, the cephalopods have independently evolved proteinaceous
64 venom, which is produced and stored within a specialised gland in known as the
65 posterior salivary gland (PSG). All octopods are believed to possess a form of

66 proteinaceous venom used to subdue prey[8–10]. Serine proteases are a common

67 component of cephalopod venoms and have been observed in the PSG of squids,

68 cuttlefish and octopods[10–13]. Convergent recruitment of serine proteases has been

69 observed between many vertebrate (Squamata[14–16] and Monotremata[17]) and

70 invertebrate (Hymenoptera[18], Arachnida[19], Gastropoda[20], Remipedia[21] and Cnidarian[22])

71 venomous lineages.

72 In addition to these proteinaceous venoms, the blue-ringed octopus (genus

73 *Hapalochlaena*) is the only group that also contains the potent non-proteinaceous

74 neurotoxin, tetrodotoxin (TTX)[12,23]. The mechanism of TTX resistance, which allows for

75 safe sequestration of TTX, has been attributed to several substitutions in the p-loop

76 regions of voltage-gated sodium channels(Na_v) in *H. lunulata*[24]. However, these

77 channels have yet to be examined in *H. maculosa* and *H. fasciata*. TTX resistance has

78 also been studied in a range of other genera including, pufferfish[25], newts[26,27]

79 arachnids[28], snakes[29] and gastropods[30].

80 The blue-ringed octopus is easily identified by iridescent blue rings, which

81 advertise its toxicity in an aposematic display[31–33]. Sequestration of the TTX within

82 bodily tissues is unique to this genus among cephalopods[32,34]. While other unrelated

83 TTX-bearing species primarily use TTX for defense, *Hapalochlaena* is the only known
84 taxa to utilise TTX in venom[23,35]. The impact of TTX inclusion on venom composition
85 and function has been previously investigated in the southern blue-ringed octopus (*H.*
86 *maculosa*)[9]. Relative to the non-TTX bearing species *Octopus kaurna*, *H. maculosa*
87 exhibited greater expression of putative dispersal factors such as hyaluronidase, which
88 serve to aid in the dispersal of toxic venom components[9]. Conversely, tachykinins-
89 neurotoxins known from other octopods[36,37] were absent from the *H. maculosa* PSG[9].
90 Further investigation into the broader impact of TTX on the evolutionary trajectory of
91 the species has yet to be addressed due to the absence of a genome.
92 This study presents the genome of the southern blue-ringed octopus (*H.*
93 *maculosa*), the first from the genus *Hapalochlaena.* By using a comparative genomic
94 approach we are able to examine the emergence of octopod novelties, at a molecular
95 level between *H. maculosa* and the two non-TTX bearing octopods: the California two-
96 spot octopus (*O. bimaculoides*) and the long-armed octopus (*Callistoctopus minor*). We
97 also address unique features of venom evolution in octopods while also addressing the
98 species-specific evolution of tetrodotoxin acquisition and resistance in *H. maculosa*.
99

100 *Keywords:* cephalopod genome, comparative genomics, gene family expansions,

101 transposable elements, venom evolution

102

103

104 ## *Data Description*

105 ### ***Genome assembly and annotation***

106 The southern blue-ringed octopus genome was sequenced using Illumina paired

107 end and Dovetail sequencing from a single female collected at Beaumaris Sea Scout

108 Boat Shed, Beaumaris, Port Phillip Bay, Victoria, Australia. The assembly was

109 composed of 48,285 scaffolds with an N50 of 0.93 Mb and total size of 4.08 GB. A total

110 of 29,328 inferred protein coding genes were predicted using a PASA[38] and an

111 Augustus[39] pipeline and supplemented with zinc finger and cadherin genes obtained

112 from aligning *H. maculosa* transcripts to *O. bimaculoides* gene models(Supplementary

113 notes 1.1-1.4). Completeness of the genome was estimated using BUSCO[40], which

114 identified 87.7% complete and 7.5% fragmented genes against the metazoan database

115 of 978 groups (Supplementary notes 3.2).

116 *H. maculosa* has a highly heterozygous genome (0.95%), similar to *O. vulgaris*
117 (1.1%)[41] but far higher than *O. bimaculoides* (0.08%)[42]. While the low heterozygosity of
118 *O. bimaculoides* is surprising, other molluscs also have highly heterozygous genomes in
119 accordance with *H. maculosa*, including the gastropods (1-3.66%)[43,44] and bivalves
120 (0.51-3%)[45–51](Supplementary table 5).
121

122 ***PSMC (Pairwise Sequentially Markovian Coalescent) and mutation rate***

123 The mutation rate for *H. maculosa* was estimated to be 2.4 x 10^{-9} per site per
124 generation based on analysis of synonymous differences with *O. bimaculoides*
125 (Supplementary note 1.5). The mutation rate is comparable to the average mammalian
126 mutation rate of 2.2 x 10^{-9} per site per generation, and *Drosophila*, 2.8 x 10^{-9}[52,53]. Due to
127 the unavailability of a suitable closely related and comprehensive genome until the
128 publication of *O. bimaculoides* in 2015[42], this is the first genome-wide mutation rate
129 estimated for any cephalopod genome.
130 The historic effective population size (Ne) of *H. maculosa* was estimated using
131 the pairwise sequentially Markovian coalescent (PSMC) model (Supplementary Fig 2).
132 Population size was found to initially increase during the early Pleistocene, followed by

133 a steady decline which slows slightly around 100kya. Note that PSMC estimates are not

134 reliable at very recent times due to a scarcity of genomic blocks that share a recent

135 common ancestor in this highly heterozygous genome. A decline in population size

136 started during the mid-Pleistocene approximately 1mya, a time of unstable

137 environmental conditions with fluctuations in both temperature and glaciation events[54–

138 56]. Corals in the genus *Acropora* show a similar pattern of expansion and contraction

139 attributed to niche availability post mass extinction of shallow-water marine organisms

140 2-3 mya, followed by the unstable mid-Pleistocene climate[57,58]. A similar pattern of

141 expansion and decline in effective population size has also been observed in the

142 Antarctic ice fish among other marine organisms distributed in the Southern

143 Hemisphere[59].

144

145 ***Phylogenomics***

146 A total of 2,108 (single copy/ 1-to-1) orthologous clusters were identified

147 between the molluscan genomes and transcriptomes of 11 species and used to construct

148 a time-calibrated maximum likelihood tree(Fig 1a). The phylogenetic reconstruction

149 estimated the divergence time between *H. maculosa* and its nearest relative, *O.*

150 *bimaculoides*, to be ~59 mya. *C. minor* diverged from this clade much earlier ~183

151 mya. Previous phylogenies using a combination of a small number of mitochondrial

152 and nuclear genes[60–62] and orthologs derived from transcriptomes[63] support this

153 topology. Likewise, estimates by Tanner et al.[2], using a concatenated alignment of 197

154 genes with a Bayesian approach, placed divergence of *H. maculosa* from *Abdopus*

155 *aculeatus* at ~59 mya[2].

156 Inference of "shared" phenotypic traits can be difficult to resolve with the

157 current literature. For example, false eye spots/ocelli observed in both *O. bimaculoides*

158 and *H. maculosa* are structurally very different. Each ocellus in *H. maculosa* is composed

159 of a continuous single blue ring[33], while *O. bimaculoides* has a blue ring composed of

160 multiple small rings. Morphological variations of ocelli structure and colour, in

161 conjunction with the taxonomically sporadic occurrence of this trait across species

162 within *Octopus* and *Amphioctopus,* limits our interpretation as to the evolutionary

163 history of this trait in octopods[3] . Large gaps remain in the literature between

164 phenotypic traits in cephalopods and their genomic source[1]. This study aims to provide

165 a genomic framework to enable resolution of these features by profiling changes in

166 several genomic characters: (i) gene duplications, (ii) novel gene formation, and (iii)

167 non-coding element evolution.

168

169 **Fig 1. Comparisons of molluscan genomes and gene families a)** Time-calibrated maximum likelihood

170 phylogeny of seven molluscan genomes (*Aplysia californica, Lottia gigantea, Crassostrea gigas, Euprymna*

171 *scolopes, Octopus bimaculoides, Callistoctopus minor and Hapalochlaena maculosa*) and four transcriptomes

172 (*Octopus kaurna, Octopus vulgaris, Sepia officinalis* and *Idiosepius notoides*) using 2,108 single copy

173 orthologous sequence clusters. Node labels show divergence times in millions of years (mya), blue

174 (divergence to octopods) and orange bars (decopods) represent standard error within a 95% confidence

175 interval. Octopodiformes lineages are highlighted in blue and decapod orange. Scale bar represents

176 millions of year (mya). **b)** Expansions of octopod gene families relative to molluscan genomes *Aplysia*

177 *californica* (A. cali), *Biomphalaria glabrata* (B. glab), *Crassostrea gigas* (C. gig), *Lottia gigantea* (L. gig),

178 *Euprymna scolopes* (E. scol) **c)** Lineage-specific gene expansions in the octopod genomes *Callistoctopus*

179 *minor* (C. min), *Octopus bimaculoides* (O. bim) and *Hapalochlaena maculosa* (H. mac). Domains

180 abbreviated: Chondroitin N-acetylgalactosaminyltransferase (CHGN), C2H2(Cys2-His2) zinc finger and

181 Cornifin SPRR(small proline-rich proteins).

182

183 ***Organismal impact of novel genes and gene family expansions***

184 Gene family expansions between octopods (*O. bimaculoides*, *C. minor* and *H.*
185 *maculosa)* and three other molluscan genomes (*Aplysia californica*, *Lottia gigantea* and
186 *Crassostrea gigas)* were examined using Pfam annotations. A total of 5565 Pfam
187 domains were identified among six molluscan genomes. *H. maculosa* and *C. minor*
188 exhibit expansions in the cadherin gene family, characteristic of other octopod
189 genomes, including *O. bimaculoides* (Fig1b)[42,64]. *C. minor,* in particular, shows the
190 greatest expansion of this family within octopods. Expansions of protocadherins, a
191 subset of the cadherin family, have also occurred independently in squid[42], with the
192 octopod expansions occurring post divergence ~135 mya[42]. The shared ancestry of
193 octopod cadherins was also documented by Styfhals et al[64] using phylogenetic
194 inference between *O. bimaculoides* and *O. vulgaris*.Cadherins, specifically
195 protocadherins, play crucial roles in synapse formation, elimination and axon targeting
196 within mammals and are essential mediators of short-range neuronal connections[65–68]. It
197 should be noted that octopods lack a myelin sheath, as a result short-range connections
198 are integral to maintaining signal fidelity over distance[6]. The independent expansions
199 of protocadherins within chordate and cephalopod lineages are believed to be
200 associated with increased neuronal complexity[42,64]. Elevated expression of

201 protocadherins within neural tissues have been observed in *O. vulgaris* and *O.*

202 *bimaculoides* by both Styfhals et al[64] and Albertin et al[42] respectively. In particular

203 Styfhals et al[64] noted differential expression across neural tissues including supra-

204 esophageal mass, sub-esophageal mass, optic lobe and the stellate ganglion[64]. However,

205 functional implications of observed expression patterns remain speculative without

206 further study.

207 *H. maculosa* also shows expansions in the C2H2-type zinc finger family. Zinc

208 fingers form an ancient family of transcription factors, which among other roles serve

209 to regulate transposon splicing as well as embryonic and neural development[69,70].

210 Expansion of this type of zinc finger in *O. bimaculoides* has been associated with neural

211 tissues. It should be noted that due to the inherent difficulty in fully annotating the

212 zinc finger family, alternative methods were used to examine the number of exons in *C.*

213 *minor* with high similarity to annotated zinc finger genes in *O. bimaculoides*

214 (Supplementary notes 5.1). A total of 609 exons (not captured by published gene

215 models) from *C. minor* were found with high similarity to accepted zinc finger genes in

216 *O. bimaculoides,* suggesting this family is larger than that which the genome annotation

217 infers.

218 Examination of genes specifically expressed within neural tissues found that

219 cadherins were among the most highly expressed gene families of all octopod species.

220 Particularly in *C. minor*, relative to the other octopods, such a trend reflects the gene

221 family expansions found in this species (Fig2c). Zinc fingers were less pronounced,

222 representing 1.1% of overall expression in *C. minor* compared to cadherins at 11.3%.

223 Overall, neural tissues express a large diversity of Pfams with each species, exhibiting a

224 similar profile and proportion of orthologous to lineage-specific genes.

225

226 ***Novel patterns of gene expression***

227 High-level examination of gene dynamics (expression, loss of expression and

228 absence of expression) between octopods across different levels of orthology provides

229 insight into large-scale expression patterns and highlights lineage-specific loss of

230 expression.

231 The greatest proportion of genes in each species examined were not specific to

232 octopods or an octopus lineage (ancient genes) (Fig 2a). Expression of these genes were

233 enriched in neural tissues across all species, indicating the core conservation of neural

234 development and function. However, we also find that genes specific to each octopod

235 species also show this expression pattern. The overall elevated expression of genes

236 within neural tissues could be reflective of the extensive neural network present in

237 cephalopods, which comprises around 520 million nerve cells[71], rivalling

238 vertebrates/mammals in size[6]. Expression of many novel genes in the nervous system

239 may also indicate contribution of those genes to lineage-specific neural network

240 evolution. In contrast, genes that date back to the shared octopod ancestor show

241 highest expression in male reproductive tissues in all species.

242 Loss of expression between octopod genomes is exhibited most clearly in *H. maculosa*

243 with 11% (1993 genes) of all ancient genes having no expression, compared to 1% in

244 both *O. bimaculoides* and *C. minor*. Absence of gene expression for genes whose

245 orthologs have retained expression in one or more other species suggests a unique

246 evolutionary trajectory from other octopods. It should be noted that differences in

247 tissue sampling may in part influence these values and due to the limited sampling of

248 species, loss of expression cannot be inferred at a species level and may have occurred

249 at any point in the lineage. In order to fully understand the implications of the gene

250 family contractions and loss of expression in *H. maculosa*, relative to other octopods,

251 further investigation is required.

252

253 **Fig2. Dynamics of gene expression in octopod genomes**. Proportion of gene expression across levels

254 of specificity from not specific to octopods or an octopus species (left) to octopod-specific (middle) and

255 lineage-specific (right). Donut plots show gene expression as some expression in any tissue (purple), no

256 expression (blue) or expression that has been lost (dark blue). Loss of expression requires an ortholog of

257 the gene to be expressed in one or more species and not expressed in the other species. Heatmaps at each

258 specificity level show average expression of genes within their respective tissues, low expression (cream)

259 to high expression (dark red).

260

261 **Fig3. Dynamics of gene expression in neural and venom producing tissues of octopods.** a) Tissue

262 specific expression of genes within the brain of *H. maculosa, O. bimaculoides* and *C. minor* (red). Venn

263 diagram shows numbers of shared and exclusive genes between species (Left). Bar chart of the top 5

264 Pfams and their contribution to overall expression in the brain (right). b) Tissue specific expression of

265 genes within the posterior salivary gland (PSG) of *H. maculosa, O. bimaculoides* and *C. minor* (Blue). Venn

266 diagram shows numbers of shared and exclusive genes between species (left). Bar chart of the top 5

267 Pfams and their contribution to overall expression in the PSG (right).

268

269 ***Evolution of the octopod non-coding genome***

270 Similar to other cephalopod genomes, the *H. maculosa* genome has a high repeat
271 content of 37.09% (bases masked). *O bimaculoides* and *C. minor* are also highly
272 repetitive with 46.39% and 44% of their genomes composed of transposable elements
273 (TE) respectively. Of the repetitive elements, LINEs dominate the decapodiform
274 *Euprymna scolopes* genome accounting for its larger genome size[72], while SINEs are
275 expanded in all four octopod genomes. SINEs have been previously documented in *O.*
276 *bimaculoides* (7.86%)[42], comparable with *H. maculosa* (7.53%), while fewer SINEs were
277 previously reported for *C. minor* (4.7%)[73]. SINE elements also dominate the *O. vulgaris*
278 genome with an expansion occurring post divergence from *O. bimaculoides*[41]. Rolling
279 circle (RC) elements are a prominent minor component in octopods, particularly in *H.*
280 *maculosa*. RC transposons have been isolated from plant (*Zea mays*) and mammalian
281 genomes. They depend greatly on proteins used in host DNA replication and are the
282 only known class of eukaryotic mobile element (transposon) to have this dependence[74].
283 TE elements in cephalopod lineages show differing expansions between most of the
284 genomes currently available, suggesting they are highly active and play a strong role in
285 cephalopod evolution.

286 Enrichment of transposable elements associated with genes (flanking regions
287 10kb up- and downstream) was not observed compared to the whole genome for any
288 species examined. More notable were differences between species, in particular *C.*
289 *minor* shows a greater proportion of LINE to SINE elements relative to both *O.*
290 *bimaculoides* and *H. maculosa*.
291 Together, this highlights a very dynamic evolutionary composition of repeats in
292 cephalopods, that requires further study to test for any potential association with
293 changes in gene expression or genome evolution.
294
295 ***Dynamics of gene expression in the posterior salivary gland (PSG)***
296 The posterior salivary gland is the primary venom-producing gland in octopods.
297 Venom composition in the majority of octopods is primarily composed of proteinaceous
298 toxins. *Hapalochlaena* is an exception containing an additional non-proteinaceous
299 neurotoxin, TTX, within their venom. We hypothesize that the *Hapalochlaena* PSG will
300 exhibit a loss of redundant proteinaceous toxins due to the presence of TTX.
301 Examination of all PSG-specific genes from the three octopods revealed a
302 disproportionate number of genes exclusive to *H. maculosa* (Fig 3a). A total of 623

303 genes were exclusive to *H. maculosa* PSG compared to only 230 and 164 exclusive to *O.*

304 *bimaculoides* and *C. minor* PSGs, respectively. Additionally, we predict that the *H.*

305 *maculosa* PSG is functionally more diverse based on the number of Pfam families

306 detected, 532 in total. Comparatively, the PSG genes in *O. bimaculoides* and *C. minor*

307 are fewer and more specialised. Gene family expansions of serine proteases dominate

308 expression comprising over 30% of total PSG-specific expression in *C. minor* and 17-

309 20% in *O. bimaculoides* (Fig 3b). Serine proteases were also among genes whose

310 expression appears to have shifted between octopod species. Several serine proteases

311 show specific expression to the PSG of *O. bimaculoides* and *C. minor* while being

312 expressed in a non-specific pattern among brain, skin, muscle and anterior salivary

313 gland tissues in *H. maculosa* (Fig 4b). Most notable is the absence of many paralogs in

314 both *H. maculosa* and *O. bimaculoides* suggesting a lineage-specific expansion of this

315 cluster in *C. minor*. Fewer serine protease genes can also be observed in *H. maculosa*

316 (Fig 4c). Similarly, reprolysin (M12B) exhibits shifting expression in *H. maculosa*,

317 presumably from the PSG to the branchial heart, and a complete loss of paralogs from

318 the genome. While the function of this protein has not been assessed in octopus,

319 members of this protein family exhibit anticoagulant properties in snake venom[75–78].

320 Serine proteases have been previously documented in cephalopod venom and
321 are prime candidates for conserved toxins in octopods. Cephalopod-specific expansions
322 have been identified with strong association to the PSG in 11 cephalopods (seven
323 octopus, two squid and two cuttlefish)[8,13]. All serine proteases identified from the PSG
324 of these species were found to belong to the cephalopod-specific clade. Functionally,
325 cephalopod venom serine proteases have yet to be assessed. However, octopod venom
326 has been observed to have strong digestive and hemolytic properties, which may be in
327 part due to this crucial protein family[79–81]. The reduced number and expression of
328 serine proteases in *H. maculosa* suggests a change in function of the PSG for this
329 species. These results support the hypothesis of toxin redundancy in the *H. maculosa*
330 PSG due to the incorporation of tetrodotoxin. Previous proteomic analysis of the *H.*
331 *maculosa* PSG revealed high expression of hyaluronidase, which often serves as a
332 dispersal factor within snake venom, facilitating the spread of toxin while not being
333 directly toxic to their prey[9,82]. While further investigation is required, the incorporation
334 of TTX within *H. maculosa* venom may have contributed to a shift in function, with
335 proteins present acting to support the spread of venom and digestion of tissues.
336

337 **Fig 4. Examination of posterior salivary gland (PSG) gene expression between three octopod**

338 **genomes. a)** Heatmap of genes expressed specifically in the PSG of *H. maculosa* (tau > 0.8) and their

339 orthologs in *O. bimaculoides* and *C. minor* lacking specific expression to the PSG (tau < 0.8). Genes with

340 an ortholog lacking expression are coloured in grey while the absence of an ortholog is white. **b)**

341 Heatmap of genes expressed specifically in the (PSG) of both *O. bimaculoides* and *C. minor* (tau >0.8)

342 and their orthologs in *H. maculosa* lacking specific expression to the PSG.

343

344 ***TTX resistance of the Na_v channels***

345 To identify the mechanism of TTX resistance in *H. maculosa,* the voltage gated

346 sodium channel (Na_v) sequences were compared between susceptible (human) and

347 resistant (pufferfish, salamanders and garter snakes) species. TTX binds to the p-loop

348 regions of sodium channels, inhibiting the flow of sodium ions in neurons, resulting in

349 paralysis[83,84]. Inhibition of TTX binding has been observed in species which either

350 ingest TTX via prey, such as garter snakes[85], and in those which retain TTX within their

351 tissues like pufferfish[86].

352 Two Na_v genes were identified in the *H. maculosa* genome (Na_v1 and Na_v2), this

353 is congruent with the recent identification of two Na_v isoforms in *H.*

354 *lunulata*[24](Supplementary Fig 8 & 9). Among cephalopods with sequenced Na_v1

355 channels, p-loop regions are highly conserved with both DI and DII shared between all

356 species. The regions DIII and DIV closer to the C-terminal end of the protein in

357 *Hapalochlaena* sp. contain mutations, which may impact TTX binding and differ

358 between families and species as follows. Similar to the pufferfish (*Arothron,*

359 *Canthigaster, Takifugu* and *Tetraodon*)[87]and garter snake *Thamnophis couchii*[88], *H.*

360 *maculosa* Nav1 has a mutation within the third p-loop at site (DIII) from M1406T,

361 while all other cephalopods have an Ile(I) at this position (Fig 5a). The dumbo octopus

362 (*Grimpoteuthis*) is the only exception retaining the susceptible M at this site similar to

363 humans and other non-resistant mammals[83]. Additionally, the fourth p-loop (DIV) in *H.*

364 *maculosa* exhibits two substitutions at known TTX binding sites: D1669H and H1670S.

365 In a previous study a Met to Thr substitution into a TTX sensitive Nav1.4 channel

366 decreased binding affinity to TTX by 15-fold[87]. Likewise, a 10-fold increase in

367 sensitivity was observed from a T1674M substitution in a mite (*Varroa destructor*)

368 channel VdNav1[28]. However, resistance is often a result of multiple substitutions and

369 when I1674T/D1967S occur together in VdNav1, resistance is multiplicative resulting

370 in “super resistant” channels with binding inhibition of 1000-fold. The combination of

371 M1406T/ D1669H in *H. maculosa* also occurs in the turbellarian flatworm *Bdelloura*

372 *candida*(BcNav1)[87,89]. While it has yet to be assessed for TTX resistance, the replacement

373 of Asp in *B. candida* with a neutral amino acid has been predicted to disrupt TTX

374 binding by preventing formation of a hydrogen bond[90]. These three substitutions

375 (M1406T, D1669H and H1670S) in *H. maculosa*, with the potential to inhibit TTX

376 binding, have also been identified by Geffeney et al[24] in *H. lunulata*. It has yet to be

377 established if these mutations are derived from a shared ancestor or have occurred

378 independently.

379 While *Hapalochlaena* remains the best documented example of TTX resistance

380 among cephalopods, other species may contain some level of TTX resistance (e.g.

381 *Octopus vulgaris*)[91,92]. Saxitoxin (STX) is a similar toxin in structure and function, and

382 mutations resistant to TTX are often also STX inhibiting[93] *O. vulgaris* has been observed

383 consuming STX-contaminated bivalves with no negative impacts and as such is

384 believed to be resistant[92]. However, no mutations known to reduce TTX/STX binding

385 affinity occur in its Nav1[92,94]. The selective pressure facilitating the evolution of

386 STX/TTX resistance in these shallow water benthic octopods may be toxic prey, similar

387 to garter snakes. STX is also known as a paralytic shellfish poison (PSP). Produced by

388 photosynthetic dinoflagellates and bioaccumulated in bivalves[95], this toxin

389 contaminates a common octopus food source. Pelagic squids such as the Humboldt (*D.*

390 *gigas*) and longfin inshore squid (*D. pealeii*) do not appear to be TTX/STX resistant;

391 mass strandings of Humboldt squid have been associated with ingestion of STX-

392 contaminated fish[96]. Likewise, no evidence of resistance was found in the sodium

393 channel of the dumbo octopus (*Grimpoteuthis*). This species typically inhabits depths of

394 2000-5000m and is unlikely to encounter STX-contaminated food sources[97].

395

396 **Fig 5. Mechanism of tetrodotoxin resistance within the posterior salivary gland of *H. maculosa***

397 **(PSG) a)** Alignment of voltage gated sodium channel alpha subunits (DI, DII,DIII & DIV) p-loop regions.

398 Mutations conferring resistance are coloured in green (pufferfish), orange (salamander), purple (clam)

399 and blue (octopus). Susceptible mutations at the same site are Black and bolded. Sites which may be

400 involved with resistance are in bold. **b)** Schematic of voltage-gated sodium channel (Na_v) alpha subunits

401 (DI, DII, DIII and DIV). Each unit is composed of six subunits 1-4 (blue) and 5-6 (yellow). Alternating

402 extra and intercellular loops are shown in black with the p-loops between subunits 5 and 6 highlighted

403 in red. Mutations conferring resistance are shown within black circles on p-loops.

404

405 ***Microbiome of the PSG***

406 TTX is produced through a wide variety of bacteria, which are common in
407 marine sediments and have been isolated from organisms such as pufferfish[25,98,99].
408 Sequestration of TTX is not exclusive to the blue-ringed octopus among molluscs.
409 Gastropods such as *Pleurobranchaea maculata*[100] and *Niotha clathrata*[30], as well as some
410 bivalves, are also capable of sequestering TTX[95]. The commonly held hypothesis for
411 TTX acquisition within *Hapalochlaena* is that it is bacterial in origin, and is either
412 ingested or endosymbiotic[100,101]. Analysis of a ribo-depleted RNA sample from the PSG
413 of *H. maculosa* revealed a highly diverse composition of bacterial genera with Simpson's
414 and Shannon’s diversity indices of 4.77 and 0.94, respectively. The dominant phyla
415 were Proteobacteria and Firmicutes, composing respectively 41% and 22% of overall
416 bacterial species detected (Fig 5a-b). To date, 151 strains of TTX-producing bacteria
417 have been identified from 31 genera. Of these, 104 are members of Proteobacteria[102].
418 The genera *Pseudomonas* and *Bacillus* belonging to the phyla Proteobacteria and
419 Firmicutes, respectively, have been previously identified in the PSG of *Hapalochlaena* sp
420 *(Octopus maculosus)*[101]. Examination of these bacterial strains revealed TTX production,
421 and extracts injected into mice proved to be lethal[101]. A more recent study on the
422 bacterial composition of *H. maculosa* PSG did not identify TTX-producing strains[100].

423 However, only a small subset of the many strains identified were tested. Congruent
424 with our findings the diversity of bacterial genera was high and this may complicate
425 identification of species responsible for TTX production. The biosynthetic pathway of
426 TTX has yet to be elucidated, and as a result, only culturable bacterial species can be
427 tested for TTX production.

428

429 **Fig 6. Assessment of bacteria within the posterior salivary gland of *H. maculosa* (PSG)**. **a)** Bacterial composition at
430 the phylum level of a *H. maculosa* posterior salivary/venom gland. **b)** Composition of the largest Phylum,
431 Protobacteria of a *H. maculosa* posterior salivary/venom gland.

432

433 ***Conclusions***

434 This work describes the genome of a unique TTX bearing mollusc, the southern
435 blue-ringed octopus (*Hapalochlaena maculosa*). Much of cephalopod evolution is barely
436 understood due to sparseness of genomic data. Our analysis provides the first glimpse
437 into genomic changes underlying genome evolution of closely related octopod species.
438 While the size, heterozygosity and repetitiveness of the blue ring genome is congruent
439 with previously published octopod genomes, we find similar yet independent
440 expansions of key neuronal gene families across all three species and show evidence for

441 the involvement of gene novelty in the evolution of key neuronal, reproductive, and
442 sensory tissues. The evolution of venom in octopods also differs between species, with
443 *H. maculos*a showing a reduction in the number and expression of serine proteases in
444 their venom gland relative to the other octopods in this study. Inclusion of TTX in *H.*
445 *maculosa* distinguishes this species from related octopods and is believed to impact
446 toxin recruitment and retention, as the highly potent TTX is sufficient to subdue
447 common octopod prey without additional toxins.
448

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455

456 **Ethics declaration**

457 Animal Ethics Approval

458 Field collection of fishes, cephalopods (nautiluses, squids, cuttlefishes and octopuses}

459 and decapod crustaceans (crabs, lobsters, crayfishes and their allies) in for Museum

460 Victoria" (Animal Ethics Committee: Museums Victoria; AEC Approval Number: 10006)

461

462 **Competing interests**

463 Authors have no conflicts/competing interests to declare.

464

465 **Data availability statement:**

466 Genomic and transcriptomic data produced and used in this paper have been made

467 available in the NCBI BioProject: PRJNA602771 under the following accession

468 numbers: raw transcriptome(SAMN13930963 - SAMN13930975), genome assembly(to

469 be provided). Voucher specimen for the transcriptome is stored at Melbourne museum

470 (number to be provided). (Data is not yet available om NCBI as we would like to

471 release it on publication).

472

473 **Code availability statement:**

474 Custom scripts and pipelines used in all of the analyses are published in

475 GitHub(https://github.com/blwhitelaw/BRO_annotation).

476

477 **Abbreviations**

478 TTX: Tetrodotoxin, STX: Saxitoxin, PSG: Posterior Salivary Gland, CHGN:Chondroitin

479 N-acetylgalactosaminyltransferase, C2H2(Cys2-His2) zinc finger, Cornifin SPRR:Small

480 Proline-Rich Proteins, LINE: Long Interspersed Nuclear Element, SINE: Short

481 Interspersed Nuclear Element, Mya: Million Years Ago, BUSCO: Benchmarking

482 Universal Single-Copy Orthologs, PSMC: *Pairwise Sequentially Markovian Coalescent* and

483 MSMC:multiple sequentially Markovian coalescent

484

485

486 ***Methods***

487 ***Genome sequencing and assembly***

488 DNA was extracted from a single *H. maculosa* female collected at Port Phillip Bay,

489 Victoria, Australia. Two types of Illumina libraries were constructed, standard paired

490 end and Illumina mate pairs (Supplementary data 2). Dovetail sequencing, Chicago

491 libraries improved upon original sequencing resulting in an overall coverage of 71X.

492 Assembly-stats (https://github.com/sanger-pathogens/assembly-stats) was used to

493 ascertain the quality of the assembly and relevant metrics (Supplementary notes 1).

494

495 ***Transcriptome sequencing***

496 The *H. maculosa* transcriptome was generated using 12 tissues (brain, anterior salivary

497 gland, digestive gland, renal, brachial heart, male reproductive tract, systemic heart,

498 eyeballs, gills, posterior salivary gland, dorsal mantle and ventral mantle tissue). RNA

499 was extracted using the Qiagen RNeasy kit. Construction of cDNA libraries was

500 outsourced to AGRF (Australian Genome Research Facility), Melbourne and conducted

501 using their TruSeq mRNA Library Prep with polyA selection and unique dual indexing

502 method. Libraries were constructed using 3 μg of RNA at a concentration of >100

503 ng/μL. Each tissue was sequenced on 1/12th of an Illumina HiSeq2000 lane with one

504 lane used in total.

505

506 ***De novo transcriptome assembly***

507 *De novo* assembly of the *H. maculosa* transcriptome was conducted using sequencing
508 data from 11 tissues (as listed above) and Trinity (v10.11.201). Default parameters
509 were used aside from kmer coverage, which was set to three to account for the large
510 data volume. Protein coding sequences were identified using Trinotate[103] and domains
511 assigned by Interpro[104].
512
513 ***Genome annotation***
514 Genes were annotated using a *de novo* predictor supplemented with transcriptomic
515 evidence. Training models were produced by PASA[38] using a transcriptome composed
516 of 12 tissues (as listed above) and supplied to the *de novo* predictor Augustus[39] along
517 with intron, exon and repeat hints (generated by repeatmasker). Alternative splicing of
518 gene models was also predicted using PASA. Methods used for annotation have been
519 documented in the git https://github.com/blwhitelaw/BRO_annotation. Additional
520 genes were predicted by mapping raw expressed reads against the genome. Functional
521 annotation of gene models was achieved using InterPro v72.0[104]. Completeness of genes
522 was assessed using BUSCO v3 Metazoan database[40].
523

524 ***Heterozygosity***

525 JELLYFISH v2.2.1 was used in conjunction with GenomeScope[105] to calculate

526 heterozygosity in *H. maculosa* using a kmer frequency of 21 (Supplementary table 5).

527

528 ***Repetitive and transposable elements***

529 Repetitive and transposable elements were annotated using RepeatModeler v1.0.9

530 (RepeatScout) and masking performed with RepeatMasker v4.0.8[106](Supplementary

531 notes 3.3). Analysis of gene associated TE was conducted by extracting TE within

532 flanking regions 10K upstream and downstream of genes using Bedtools v2.27.1.

533

534 ***Calibration of sequence divergence with respect to time***

535 Divergence times between the molluscan genomes (*Crassostrea gigas, Lottia gigantea,*

536 *Aplysia californica, Euprymna scolopes, Octopus bimaculoides, Callistoctopus minor* and

537 *Hapalochlaena maculosa*) and transcriptomes (*Sepia officinalis, idiosepius notoides,*

538 *Octopus kaurna* and *Octopus vulgaris*) was obtained using a mutual best hit (MBH)

539 approach. Bioprojects for each genome used are as follows: *Crassostera gigas*

540 (PRJNA629593 & PRJEB3535), *Lottia gigantea* (PRJNA259762 & PRJNA175706),

541 *Aplysia californica* (PRJNA629593 & PRJNA13635) and (*Euprymna scolopes*

542 PRJNA47095). *Octopus bimaculoides* was obtained from

543 http://octopus.unit.oist.jp/OCTDATA/BASIC/Metazome/Obimaculoides_280.fa.gz. The

544 *, Idiosepius notoides (*BioProject: PRJNA302677) transcriptome was sequenced and

545 assembled using the same method previously described for the *H. maculosa*

546 transcriptome. Whole genomes and transcriptomes were BLASTed against *Octopus*

547 *bimaculoides*. The resulting hits were filtered, and alignments shared between all

548 species extracted. A maximum likelihood phylogeny was generated using RAxML

549 v8.0[107]. Phylobayes v3.3[108] was used to calculate divergence times (Supplementary 4.1).

550

551 ***Effective population size (PSMC)***

552 Historical changes in effective population size were estimated using PSMC

553 implemented in the software MSMC[109,110]. To generate inputs for MSMC we selected a

554 subset of the reads used for genome assembly corresponding to 38x coverage of reads

555 from libraries with short (500bp) insert sizes. These were pre-processed according to

556 GATK best practices; briefly, adapters were marked with Picard 2.2.1, reads were

557 mapped to the *H. maculosa* genome using bwa mem v 0.7.17 and PCR duplicates

558 identified using Picard v2.2.1. In order to avoid inaccuracies due to poor coverage or
559 ambiguous read mapping we masked regions where short reads would be unable to
560 find unique matches using SNPable (http://lh3lh3.users.sourceforge.net/snpable.shtml)
561 and where coverage was more than double or less than half the genome wide average
562 of 38x. Variant sites were called within unmasked regions and results converted to
563 MSMC input format using msmc-tools https://github.com/stschiff/msmc-tools. All data
564 for *H. maculosa* scaffolds of length greater than 1Mb was then used to generate 100
565 bootstrap replicates by dividing data into 500kb chunks and assembling them into 20
566 chromosomes with 100 chunks each. We then ran msmc2 on each bootstrap replicate
567 and assembled imported the resulting data into R for plotting. A mutation rate of 2.4e-
568 9 per base per year and a generation time of 1 year were assumed in order to set a
569 timescale in years and convert coalescence rates to effective population size.
570

571 ***Mutation rate***

572 Mutation rate was calculated by extracting orthologous genes from *O. bimaculoides* and
573 *H. maculosa.* Neutrality was assumed for genes with very low expression (>10 TMP
574 across all tissues). Neutral genes were aligned using MAFFT v7.407[111] and codeml[112]

575 was used to calculate substitution metrics (dS). Per base neutral substitution between

576 lineages was determined using the mean dS value divided by divergence time (refer to

577 *Calibration of sequence divergence with respect to time*) over the number of generations.

578 As octopus are diploid the rate was divided by two. Divergence between species was

579 calculated using Phylobayes v3.3[108].

580

581 ***Quantifying gene expression/ specificity***

582 Gene expression within individual tissues was calculated using Kallisto[113] for the

583 transcriptomic data sets of *H. maculosa*, *O. bimaculoides* and *C. minor*. Defaults were

584 used and counts were calculated as TPM. Gene specificity was defined as any gene with

585 a tau value > 0.80.

586

587 ***Gene model expression dynamics***

588 Patterns of gene expression and loss were assessed across octopod genomes at differing

589 taxonomic/organismal levels. Gene models were classified as lineage-specific, octopod

590 specific or non-specific (orthologous to a gene outside of octopods). Expression at each

591 level was determined using whole transcriptomes from all tissues of each species. Genes

592 with expression within one or more tissues were determined to be expressed, loss of
593 expression was classified as a gene with a single ortholog in each species, which is
594 expressed in one or more species and not expressed in the remaining species.
595

596 ***Dynamics of PSG gene expression***

597 In order to identify patterns of PSG specific gene expression (losses and shifts) between
598 the three available octopod genomes, genes with expression specific to the PSG of each
599 species were examined separately. Specific gene expression was defined as a tau value
600 > 0.8. Orthologous groups were identified between species using Orthovenn2[114] and
601 sequences which were identified as lineage-specific were confirmed using BLAST. Types
602 of expressions were categorized as follows: A loss of expression requires a gene to be
603 present in all three octopods and expressed in one or more species while having no
604 detectable expression in at least one species. A shift in expression occurs when an
605 ortholog present in all species is expressed in different tissues.
606

607 ***The role of the Nav in TTX resistance***

608 Sodium channels for the three octopus genomes along with all available in-house

609 cephalopod transcriptomes were extracted manually using a series of BLAST searches

610 against the nr database. Annotation was achieved using Interpro v72.0[104] and

611 identification and extraction of p-loop regions of the sodium channel alpha subunit

612 were manually performed. Where sodium channels were incomplete alignment against

613 related complete channels were used to extract the p-loop regions. Individual

614 mutations with potential to confer resistance were identified manually in Geneious

615 v10.1 (https://www.geneious.com).

616

617

618 ***Microbiome of PSG***

619 A single ribo-depleted RNA sample of *H. maculosa* PSG was examined using the

620 SAMSA2 pipeline[115] to identify the bacterial composition and corresponding molecular

621 functions. Two databases were used Subsys and NCBI RefBac. The Krona package[116]

622 was used to produce visualizations of each dataset.

623

624 **Supplementary Information**

625 Supplementary Notes 1-8, Supplementary Tables 1-8, Supplementary Figs 1-10

626 Supplementary Data 2: Table of genomic Illumina library insert sizes

627

628

629 **References**

630

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927

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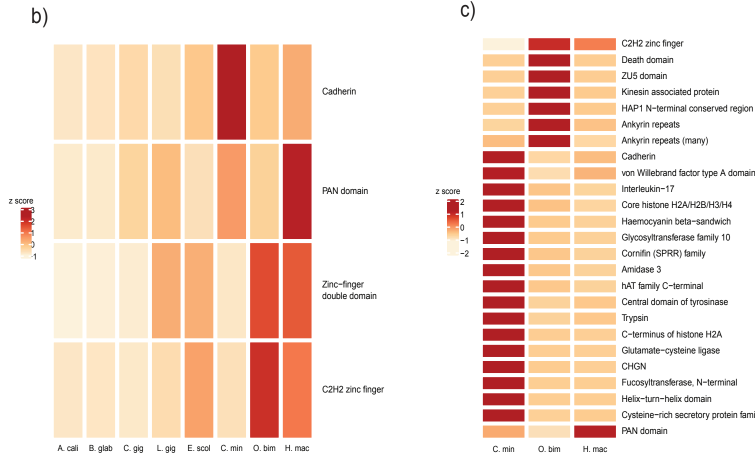
Figure1

a)

Octopus kaurna
Octopus vulgaris
Octopus bimaculoides
Hapalochlaena maculosa
Sepia officinalis
Idiosepius notoides
Euprymna scolopes
Crassostrea gigas
Lottia gigantea
Aplysia californica
Octopods
Decapods
90.91
183.56
29.88
59.45
261.53
102.75
201.7
549.5
533.89
259.6
60.0



Revised Figure 2

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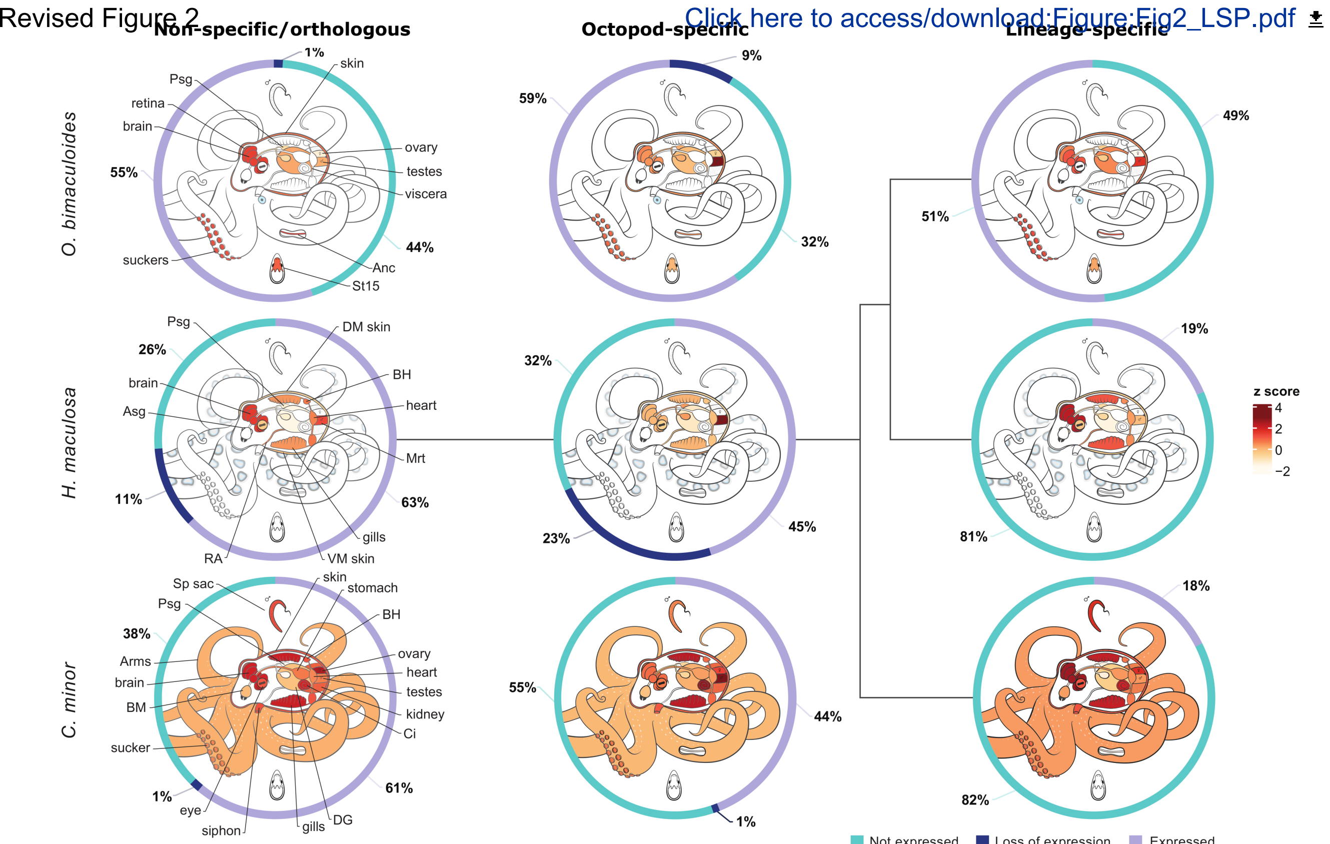

Figure3

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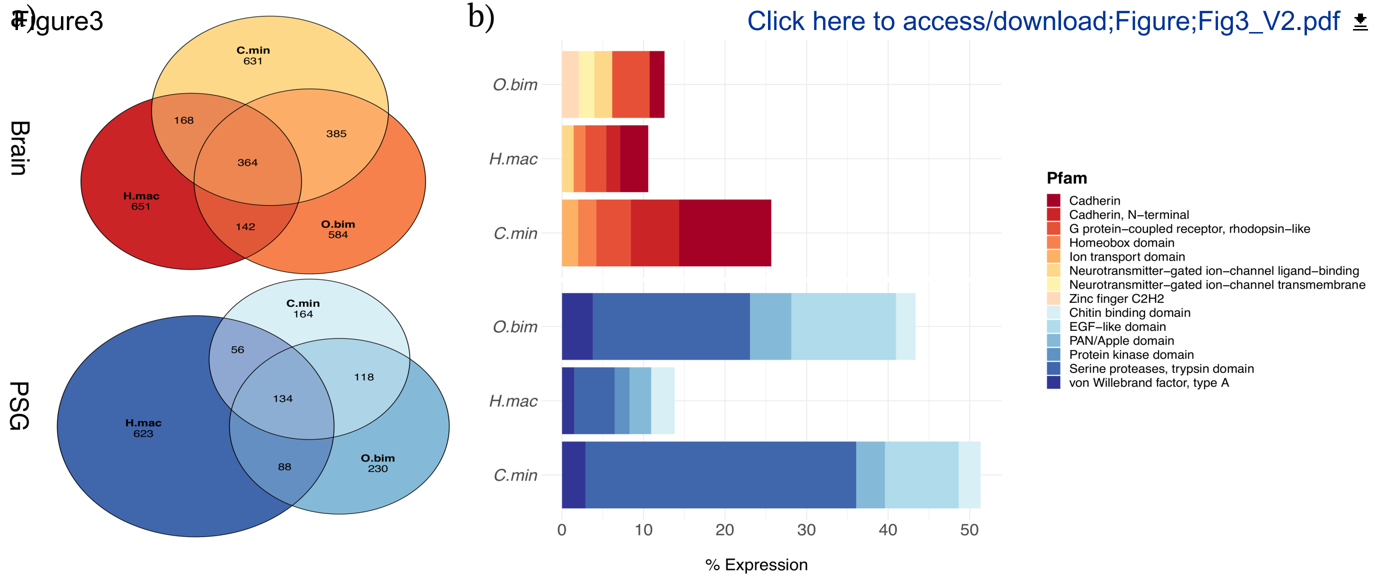

Figure4

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a)

H. maculosa *O. bimaculoides* *C. minor*

Chitin binding
Chymotrypsin S1
Core-2/I-Branching enzyme
Cysteine-rich secretory protein(CAP)
Domain of unknown function (DUF4371)
EGF-like domain, chitin binding
DNA-binding domain
Homeobox domain
Hsp20/alpha crystallin family
Laminin G domain
Profilin
Scavenger receptor cysteine-rich domain
Unknown
VWF factor, type A

H. maculosa: Posterior salivary gland, Ventral mantle muscle skin, Anterior salivary gland, Dorsal mantle skin, Gills, Brain, Digestive gland, Eye, Male reproductive tract, Renal appendage, Systemic heart, Branchial heart

O. bimaculoides: Posterior salivary gland, Sucker, Skin, Brain, Anc, St15, Retina, Testes, Ovary, Viscera

C. minor: Stomach, Buccal mass, Posterior salivary gland, Cecum intestine, Brain, Ovary, Spermatophore sac, Sucker, Skin, Siphon, Arms, Eye, Kidney, Digestive gland, Gills, Branchial heart, Testes, Systemic heart

z score
3
2
1
0
−1

b)

H. maculosa *O. bimaculoides* *C. minor*

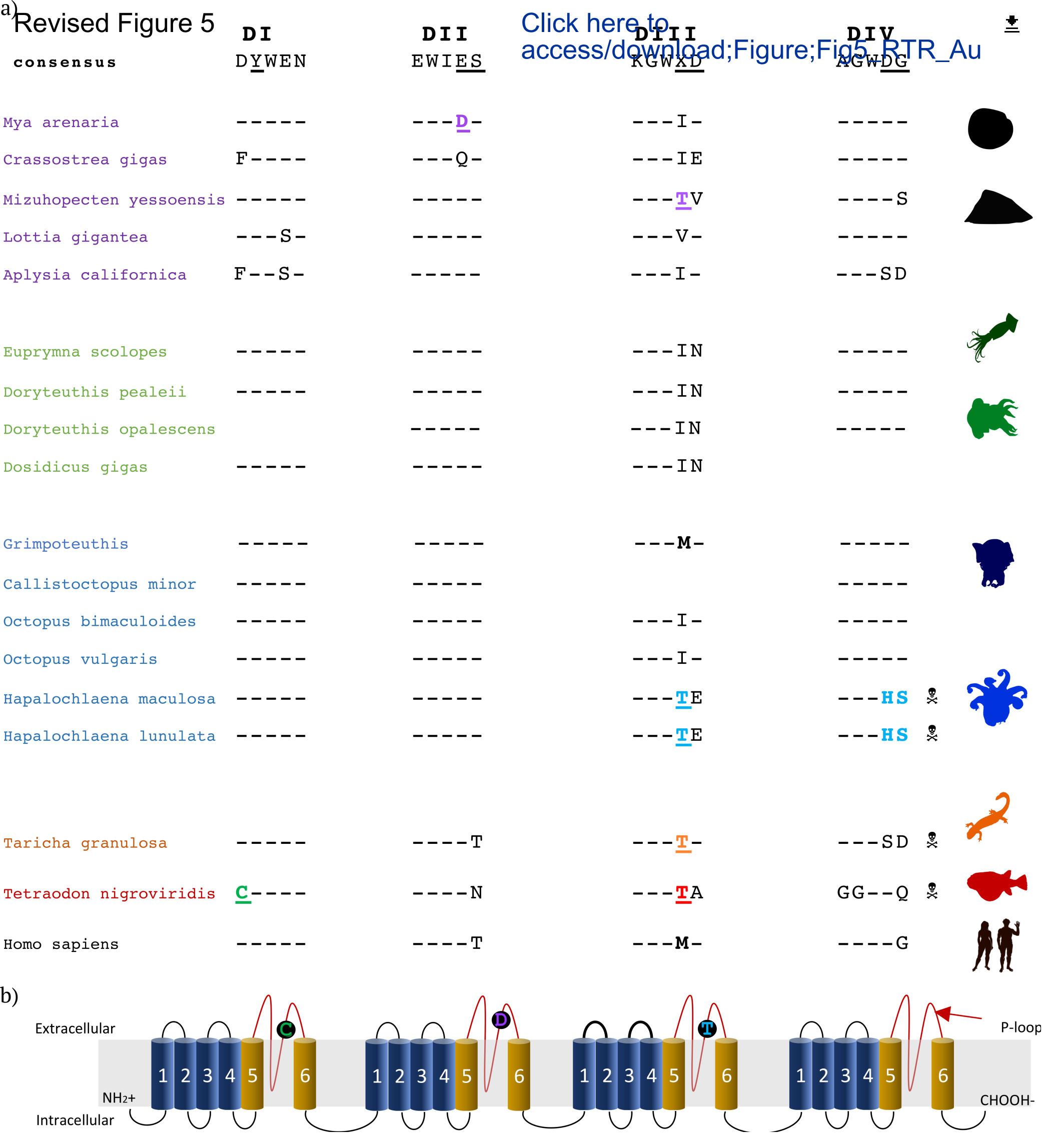
7TM GPCR
Cadherin
Peptidase M12B
VWF factor, type A
Serine Protease
Unknown

H. maculosa: Posterior salivary gland, Ventral mantle muscle skin, Barin, Anterior salivary gland, Systemic heart, Digestive gland, Eye, Gills, Dorsal mantle skin, Renal appendage, Male reproductive tract, Branchial heart

O. bimaculoides: Posterior salivary gland, Anc, St15, OL, Sub, Supra, Viscera, Ovary, Testes, Retina, Skin, Sucker

C. minor: Posterior salivary gland, Branchial heart, Siphon, Gills, Systemic heart, Buccal mass, Sucker, Brain, Kidney, Stomach, Skin, Testes, Ovary, Digestive gland, Cecum intestine, Arm, Spermatophore sac, Eye

z score
3
2
1
0
−1
−2



Revised Figure 5
Click here to access/download;Figure;Fig5_RTR_Au
a)
consensus
DI DYWEN
DII EWIES
DIII KGWXD
DIV AGWDG
Mya arenaria ----- ---D- ---I- -----
Crassostrea gigas F---- ---Q- ---IE -----
Mizuhopecten yessoensis ----- ----- ---TV ----S
Lottia gigantea ---S- ----- ---V- -----
Aplysia californica F--S- ----- ---I- ---SD
Euprymna scolopes ----- ----- ---IN -----
Doryteuthis pealeii ----- ----- ---IN -----
Doryteuthis opalescens ----- ---IN -----
Dosidicus gigas ----- ----- ---IN
Grimpoteuthis ----- ----- ---M- -----
Callistoctopus minor ----- ----- -----
Octopus bimaculoides ----- ----- ---I- -----
Octopus vulgaris ----- ----- ---I- -----
Hapalochlaena maculosa ----- ----- ---TE ---HS
Hapalochlaena lunulata ----- ----- ---TE ---HS
Taricha granulosa ----- ----T ---T- ---SD
Tetraodon nigroviridis C---- ----N ---TA GG--Q
Homo sapiens ----- ----T ---M- ----G
b)
Extracellular
Intracellular
NH2+
CHOOH-
P-loop
C D T
1 2 3 4 5 6

Figure6

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a)

Other 12%

Bacteroidetes 10%

Proteobacteria 39%

Actinobacteria 17%

Firicutes 22%

b)

other 9%

Betaproteobacteria 15%

Alphaproteobacteria 31%

Gammaproteobacteria 45%

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Supplementary Material
BRO Sequencing Insert Sizes.xlsx

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Cairns
Singapore
Townsville

Dear Dr. Goodman

I am pleased to submit an original research piece titled "**Adaptive venom evolution and toxicity in octopods is driven by extensive novel gene formation, expansion and loss** " for consideration to be published in *GigaScience*.

Much of cephalopod evolution remains unknown due to sparseness of their genomic sampling. Cephalopod genomes are some of the largest and most repetitive animal genomes and exhibit drastically different evolutionary trajectories relative to other better documented lineages. A more focused genomic study to reveal how individual genomic changes are associated with the evolution of novel organs, tissues, or adaptations, within a single group of cephalopods has been missing so far. We present such a study, focussing on adaptations in the toxic blue-ringed octopus the *Hapalochlaena maculosa*, for which we provide a high quality genome assembly based on multiple technologies. Members of the genus *Hapalochlaena* are the only octopods to contain the lethal neurotoxin, tetrodotoxin (TTX), within their venom and tissues and are a prime example of the origin of evolutionary novelties within octopods.

Using global comparative genomics approaches and focused study on TTX evolution, we report key findings:

- Gene family expansions crucial for the development of complex neural networks are present in cephalopods, yet are differentially expanding in all three octopod species
- Novel gene formation at different phylogenetic levels can be associated with evolution in a specific set of cephalopod tissues
- Changes in Posterior Salivary Gland composition (PSG) between TTX bearing and non-TTX bearing species
- Convergently evolved mutations consistent with TTX resistance detected in *H. maculosa*

We firmly believe that our manuscript is suited for publication by *GigaScience* as one of the first to explore the evolutionary genomic basis for novelties within octopods and cephalopods in general. Our whole genomic comparisons provide insight into the defining structure/features of octopod genomes at the species-specific level. Additionally, we

examine the impact of TTX on the evolution of venom in *H. maculosa* relative to non-TTX bearing octopods.

Yours sincerely,

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Cover letter for revisions

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Dear Dr. Hongling Zhou

On behalf of my co-authors, I would like to thank you for the opportunity to revise and resubmit our research piece titled "*Adaptive venom evolution and toxicity in octopods is driven by extensive novel gene formation, expansion and loss*" (GIGA-D-20-00135).

We would also like thank all three reviewers for their constructive criticism and advice on how to improve our manuscript. All suggestions have been taken into account and incorporated resulting in a much-improved manuscript.

A response to reviewers has been provided in which we address each point providing the original text followed by the revised text, including line numbers where changes have been implemented. We also included a low resolution version of all corrected figures in the response.

Thank you again for your consideration of our revised manuscript

Yours sincerely,

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