Peer Community In Zoology

A novel nematode species from the Siberian permafrost shares adaptive mechanisms for cryptobiotic survival with C. elegans dauer larva

Isa Schon *based on peer reviews by 3 anonymous reviewers*

Anastasia Shatilovich, Vamshidhar R. Gade, Martin Pippel, Tarja T. Hoffmeyer, Alexei V. Tchesunov, Lewis Stevens, Sylke Winkler, Graham M. Hughes, Sofia Traikov, Michael Hiller, Elizaveta Rivkina, Philipp H. Schiffer, Eugene W Myers, Teymuras V. Kurzchalia (2023) A novel nematode species from the Siberian permafrost shares adaptive mechanisms for cryptobiotic survival with C. elegans dauer larva. bioRxiv, ver. 6, peer-reviewed and recommended by Peer Community in Zoology.
https://doi.org/10.1101/2022.01.28.478251

Submitted: 20 May 2022, Recommended: 09 February 2023

Cite this recommendation as:
Schon, I. (2023) A novel nematode species from the Siberian permafrost shares adaptive mechanisms for cryptobiotic survival with C. elegans dauer larva. *Peer Community in Zoology*, 100130.
https://doi.org/10.24072/pci.zool.100130

Published: 09 February 2023
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This article [1] investigated two nematode genera, *Panagrolaimus* and *Plectus*, from the Siberian permafrost to unravel the adaptations allowing them to survive cryptobiosis; radio carbon dating showed that the individuals of *Panagrolaimus* had been in cryobiosis in Siberia for as long as 46,000 years!

I was impressed by the multidisciplinary approach of this study, including morphological as well as phylogenetic and -genomic analyses to describe a new species. In triploids as some of the species studied here, it is quite challenging to assemble a novel genome. The authors furthermore not only managed to successfully reanimate the Siberian specimens but could also expose them to repeated freezing and desiccation in the lab, not an easy task.

This study reports some amazing discoveries - comparing the molecular toolkits between *C. elegans* and *Panagrolaimus* and *Plectus* revealed that several components were orthologues. Likewise, some of the biochemical mechanisms for surviving freezing in the lab turned out to be similar for *C. elegans* and the Siberian nematodes. This study thus provides strong evidence that nematodes developed specific mechanisms allowing them to stay in cryobiosis over very long times.

A surprising additional experimental result concerns the well-studied *C. elegans* - dauer larvae of this species can stay viable much longer after periods of animated suspension than previously thought.

I highly recommend this article as it is an important contribution to the fields of evolution and molecular biology. This study greatly advanced our understanding of how nematodes could have adapted to cryobiosis. The applied techniques could also be useful for studying similar research questions in other organisms.

References:

[1] Shatilovich A, Gade VR, Pippel M, Hoffmeyer TT, Tchesunov AV, Stevens L, Winkler S, Hughes GM, Traikov S, Hiller M, Rivkina E, Schiffer PH, Myers EW, Kurzchalia TV (2023) A novel nematode species from the Siberian permafrost shares adaptive mechanisms for cryptobiotic survival with *C. elegans* dauer larva. bioRxiv, 2022.01.28.478251, ver. 6 peer-reviewed and recommended by Peer Community in Zoology. https://doi.org/10.1101/2022.01.28.478251

Reviews

Evaluation round #2

DOI or URL of the preprint: https://doi.org/10.1101/2022.01.28.478251
Version of the preprint: 5

Authors' reply, 25 January 2023

Dear Dr., Schon,

Thank you very much for your comments on the manuscript. We submit the revised manuscript and provide a detailed point- by- point reply to the comments. We hope that we addressed all the comments suggested above, and the manuscript is now acceptable. Once our manuscript qualifies/acceptable for publication, we kindly like to request a PCI recommendation from the editor for submission to PCI friendly journals.

With best wishes,

Philipp H. Schiffer (For all authors)

Download author's reply

Decision by Isa Schon, posted 10 January 2023, validated 11 January 2023

Minor revison

I would like to thank the authors for their extensive response to the reviewers' comments and the changes of the manuscript. Sorry that the evaluation of the revised version took some time – the xmas period is particularly difficult for the reviewing process.

Reviewer 3 found that your revised manuscript could be recommended now.

Reviewer 1 and 2 of the first round were unfortunately no longer available to reassess the revised version and I did not want to invite new reviewers for the second round of reviewing as this usually leads to problems and I consider as unfair. Instead, I have gone through the comments of reviewers 1 & 2 and your replies in the rebuttal myself.

While all comments have been addressed in the rebuttal letter, not all comments also led to changes in the revised manuscript. I would like to ask the authors to do so for the points specified below. This is especially relevant for the concerns on homology of mechanisms, which was raised by both reviewer 1 and reviewer 2. Likewise, for certain points, especially reviewer 1 asked for a more balanced discussion or interpretation, which I feel has not been addressed in the revised manuscript. In view of this, my editorial assessment is minor

revision.
I outline the reviewers' comments below, which I feel need still to be addressed: Abstract, line 35:
I agree with reviewer 1 that the expression "genetic and biochemical programs" is not a good choice of words; please use instead the appropriate expression which you mention in the rebuttal letter ("combination of genetic and biochemical pathways that are upregulated upon preconditioning"). Point 2) Phylogenetic placement and species description of reviewer 1:
1. Nowhere in the manuscript is explained which species concept is applied to justify that the novel parthenogenetic Panagrolaimus strain is a new species. Please add this important information.
2. I still feel that the possibility that the basal position of the Siberian strain in the phylogeny could indicate that this strain is a hybrid parent is not sufficiently addressed, neither in the rebuttal nor in the revised manuscript. The newly added lines (178-182) do still not discuss this possibility . I would like to ask the author to provide the possibility of a hybrid parent at least as alternative explanation in the manuscript, even more so as the bootstrap support of the outside grouping is really low in Figure S3.
3. Please provide more information on the abbreviations now used in the new version – for example - what is GRAMPA (line 178)? Point 4) C. elegans dauer of reviewer 1:
While the new version of the text does now partly address this comment, the fact that larvae are not metabolically active is still not specifically mentioned. Not all readers will understand what "hypometabolic" means. Point 5) Panagrolaimus developmental stage of reviewer 1:
I still find the term "mixed population" confusing is this is normally not used to describe populations with different larval stages. The authors corrected this only in one place in the manuscript and I recommend to include the same corrections also everywhere else where this is mentioned. Point 6) Homology of mechanisms of reviewer 1 and point 2. of reviewer 2:
While indeed some suggestions for additional research have been added in the revised version of the manuscript in lines 301-303, these still do not include RNAi or inhibitor-based experiments nor is it clear from the text that this kind of experiment is planned for the future. I would like to recommend to the authors that they should add these specifics not only in the rebuttal but also in the manuscript.
I also cannot see how the "correlation is not causality" comment has been addressed. This is not the case in the mentioned lines (190, 200-201). Comment on line 132 of reviewer 1:
While the authors explained in the rebuttal that the strains were grown for multiple generations in several labs and that the strain was no longer frozen when received, none of this information made it into the manuscript although this is absolutely vital information and absolutely needs to be added. Suggestion of reviewer 1 to remove lines 241-243 and 244-251:
I agree with the reviewer that these lines would better fit the introduction and found the reply by the authors not convincing. Sorry for asking this again but I would recommend another careful language check editing of the new parts of the manuscript as lines 178-182. Also for example in line 270, "fine" is not a suitable word.

Evaluation round #1

DOI or URL of the preprint: https://doi.org/10.1101/2022.01.28.478251
Version of the preprint: 2

Authors' reply, 02 December 2022

Dear Dr., Schön,
Thank you very much for giving us the opportunity to reply to the reviewer's comments and to improve our manuscript. We uploaded the revised manuscript on bioRxiv and provide a detailed point- by- point reply to the comments. We hope that we addressed all the comments of the reviewers, and the manuscript is now acceptable for a recommendation from PCI.

With best wishes,
Philipp H. Schiffer (For all authors)
Download author's reply

Decision by Isa Schon, posted 19 September 2022

Dear Dr Shatilovich and co-authors,

your manuscript has been evaluated by three different reviewers. All three were very enthusiastic about the manuscript and found your results exciting. Especially the multidisciplinary approach was very much appreciated and the relevance of your manuscript for the evolutionary community as well as the Celegans community is clear. However, all three reviewers made suggestions on various aspects of the manuscript which could be improved. This includes the suggstion of a formal species description, which is in my opinion relevant. I would furthermore recommend that the comments dealing with gene and functional homology and the question of genome assembly in light of hybrid origin and triploidy are given full attention. At least two reviewers commented on data accessibility and I agree that open access data should be obvious and clearly structured; please improve them. Several additional references were also suggested which should be added. Reviewer 3 made several useful suggestions on the used terminology which should be rethought; this reviewer also pointed out a list of typo's - please check these carefully.

I believe that all changes are minor and easy to address and I hope that I can recommend the revised version of this preprint.

With kind regards,
Isa Schön

Reviewed by anonymous reviewer 1, 01 September 2022

This manuscript follows on the discovery of a parthenogenetic *Panagrolaimus* nematode in the Siberian permafrost, as previously reported by Shatilovich et al. (ref. 4). The present data concern:

1. the radiocarbon dating of the permafrost sample to ca. 46,000 years;
2. a genome assembly showing that, like previously studied parthenogenetic *Panagrolaimus*, this strain is triploid; that the strain is an outgroup to all Panagrolaimus species that have genome assemblies at this day, and that parthenogenesis is not monophyletic in the genus;
3. the formal morphological description and naming of the permafrost strain as a new species, the justification being genome divergence;
4. studies of the strain's ability to withstand dessication and freezing, as previously shown by others for other *Panagrolaimus*, including biochemical data showing upregulation of the trehalose content;
5. data showing that *C. elegans* dauer larvae can be frozen after dessication.

This manuscript is an assemblage of an impressively diverse array of methodologies to characterize the *Panagrolaimus* strain from permafrost. The finding of nematodes in permafrost is exciting. The genomic data showing triploidy are convincing.

However, the manuscript suffers from several issues, which I would urge the authors to consider. The conclusions and terminology the authors use are often not substantiated by the data.

The C. elegans dauer data may not be particularly relevant here.

1) Citation of previous work:

It was known that some *Panagrolaimus* species can withstand dessication and freezing. One parthenogenetic *Panagrolaimus* was found living in ice in Antarctica. Especially the parthenogenetic strain complex was hown to be particularly amenable to dessication and to freezing (Mc Gill et al. 2015). This literature is poorly reported.

Previous articles by others on trehalose synthesis and dessication in *Panagrolaimus* should be mentioned and discussed: for example doi.org/10.1242/jeb.0162 or doi:10.1242/bio.023341.

See also doi:10.1590/1678-4685-GMB-2017-0030 and articles on gene silencing in *Panagrolaimus* strains.

line 196; a *tps-2* and a *gob-1* homolog was previously studied in a dessication-resistant *Panagrolaimus* (doi:10.1242/bio.023341).

2) Phylogenetic placement and species description:

The phylogenetic placement is unclear given the potentially hybrid origin of the triploid strain. Schiffer et al. 2019 previously assembled genomes of parthenogenetic *Panagrolaimus* strains and found them to be triploid and likely hybrids between quite distant species in this genus. In this previous article, the different homeologs were distinguished, with estimates of the divergence date between them in millions of years, and thus a distinct phylogenetic placement of the homeologs. In the present manuscript, it is unclear how the different homeologs were treated and thus how they would each map on a phylogenetic reconstruction (as that in Schiffer et al. 2019, Fig 1B). It is therefore uncertain whether the basal position of the Siberian strain represents that of one of the hybrid parents in Schiffer et al. If this were the case, it may not be distinct from the other triploid parthenogenetic strains.

As long as the homeolog issue is not settled, species description based on genome divergence may not be recommended: it is unclear whether the strain should be in a distinct branch compared to the triploid parthenogenetic complex including the described *Panagrolaimus davidi*.

[If the strain turns out from homeolog distinction to be part of the monophyletic parthenogenetic complex, whether to treat it as a new species is a question of species definition. Further studies of the complex would be welcome.]

3) Terminology:

In the title, abstract, and throughout, the authors use the term 'cryptobiosis/tic' to refer to the metabolically suspended frozen nematodes in the permafrost or in the laboratory. Many nematode species can be frozen in the laboratory and *C. elegans* is routinely frozen in mixed-stage populations. They are not called cryptobiotic for this ability. Why not just say 'frozen' instead of 'cryptobiotic', which is both clearer, more precise and correct? This particularly applies for the *C. elegans* dauer (next point), but not only.

4) *C. elegans* dauer:

C. elegans can adopt a developmentally arrested juvenile stage called dauer, which is metabolically active and not cryptobiotic per se. The metabolism of dauer larvae differs from that of the feeding larvae or adults, in particular through the use of the glyoxylate shunt of the TCA cycle. As previously shown by the authors, the dauer larva can be dessicated, and then enters a metabolically inactive state. The distinction between the metabolically active dauer stage and the dauer larvae that have been dessicated in the laboratory should be clear in the text.

The authors show here that these *C. elegans* dauer stages (at least those induced by a temperature-sensitive mutation in the insulin receptor gene *daf-2*) can be frozen after dessication, thus once they already are in suspended animation. This is to my knowledge new. Mammalian embryos can be frozen but arguably cannot be called 'cryptobiotic' nor adapted to it. I would urge the authors to remove the term cryptobiotic at the least for this *C. elegans* case, if not everywhere. 'dessicated' and 'frozen' appear better descriptions.

Non-dauer stages freeze more easily, without preconditioning, so in terms of lab freezing method, they will remain a preferred stage. This may be worth noting.

5) *Panagrolaimus* developmental stage:

The manuscript is confusing and ambiguous relative to the dauer diapause stage in *Panagrolaimus*. Please explicit whether this species was seen to undergo dauer diapause. To my knowledge, *Panagrolaimus* species do not have a dauer larval stage. Throughout the manuscript is written with emphasis on the similarity between *Panagrolaimus* (as far as I can tell, non-dauer) and *C. elegans* (dauer).For example, on line 99 "we

demonstrate that *Panagrolaimus* and *C. elegans* dauer larvae utilize similar adaptive mechanisms to survive extreme dessication and freezing': The sentence is ambiguous because it is easy to read '*Panagrolaimus* dauer larvae'.

Methods are silent as to the *Panagrolaimus* stage that was studied in the dessication protocol ('mixed populations', line 437).

6) Homology of mechanisms:

The presence of homologs of genes necessary for the glyoxylate shunt, trehalose synthesis or the insulin receptor does not make them demonstrated functional components for dessication nor freezing (nor - indeed- dauer formation for the latter) in *Panagrolaimus*.

Abstract line 44: the sentence with "the molecular toolkit for cryptobiosis in *Panagrolaimus*... is partially orthologous" is thus inappropriate. Furthermore, it could well be that *Panagrolaimus* have other biochemical features that allow them to dessicate; and maybe that non-freezable *Panagrolaimus* species have a glyoxylate shunt?

line 276: 'homology of molecular and biochemical mechanisms'. Again, metabolic genes may be homologous, but talking about homology of mechanisms is more problematic. Idem for Fig 4 title, line 188, line 192, lines 200-1: what is a 'molecular toolkit for cryptobiosis' of an organism where no functional data are reported (or cited)? Experimental gene silencing by RNA interference has been reported in dessication-tolerant strains so this could be used to infer 'mechanisms'. Correlation is not causation.

7) Dating:

Is it clear that the *Panagrolaimus* was frozen in the permafrost sample? Are there any unfrozen films of water? Given the data with *P. davidi* in Antarctic ice, living in permafrost with freezing avoidance appears a possibility. It may be difficult to determine its state on the sampling site, but please discuss this point. This is not about sterility and the statement on lines 122-126 do not suffice to address the point for a new group of organisms.

ADDITIONAL COMMENTS

- From the 'Contributions of authors' section, I do not understand why the first author's contribution is listed as 'performed isolation and cultivation of nematodes'. The isolation was previously reported. A previously reported culture should be freely available. Please clarify.
- Please give a strain name to the culture. Is it an isofemale line?
- line 440: preconditioning of *Panagrolaimus* at 98% relative humidity for 4 d (ref. 24). What happens to non-dauer larvae in this environment in terms of development?
- Please indicate which strain are parthenogenetic on the representation of inferred phylogenetic relationships.
- line 219: please explain why labeling of acetate makes you reach this conclusion of origin from TAGs.
- line 223: explain how you identify spot 7's chemical nature.
- line 224: the presence of a chemical does not prove that it is used 'to resist harsh dessication'.
- Please provide some details on the survival test.
- The sentences on lines 238-9 and 242-243 need to be removed or rephrased.
- Fig S4: what is meant by the absence of a *daf-28* ortholog in *Panagrolaimus*? Was the whole insulin gene family studied?
- Fig S5: a quantification of biological replicates (not a technical replicate with n=2) would be better to reach a conclusion (and perform a t-test). The panels are misnamed in the legend.

- Discussion regarding species age: There are several problems here. One is the definition of a species across geological time: the age of a species depends on this definition, so there is no way to 'anticipate' (line 308) a species age without narrowing down what is meant. A second problem is the effective generation time: there may be outlier individuals with a long generation time, but they may not participate much to the gene pool.

The language needs editing throughout. For example (but not only):

- Abstract line 35: remove the comma in 'programs that enable'. What is a 'complex genetic and biochemical program'?
- lines 93-94: remove this vague sentence.
- lines 95-96: the sentence structure is ambiguous and its meaning is unclear. What is a 'detailed morphological, phylogenetic analysis'?
- line 98: remove 'powerful'.
- line 132: why 100 generations of culture before studying it? Did you not maintain it frozen?
- line 153: 'links...show' not 'shows'
- Box 1 line 3: typo at 'within'
- line 180: add the word 'a' before monophyletic trait
- l202-204 the sentence needs rephrasing. As it is, it may seem that only the dauer larva can be frozen, which is wrong. It is best to avoid ambiguous language.
- l 210: survive 'better' not 'higher', or 'in a higher proportion'.
- l. 212 is ambiguous: which developmental stages of C. elegans? Fig. 4B shows daf-2 dauer larvae.
- 'preconditioning' is unclear. Why not just provide a word describing the treatment, for example 'partial dessication'? Or is there a specific freezing preconditioning? It is not always clear whether freezing was tested after full or only partial dessication.
- l 225, 293: 'the' glyoxylate shunt
- l 227: add suggests 'that' the flux. What does 'the latter' refer to?
- l 231: remove comma.
- line 239: 'extremely long periods of time'?! C. elegans has been kept frozen by others for half a century, not 480 days.
- remove lines 241-3. l. 244-251 belong to the introduction.
- l. 253: what is an undescribed strain?
- l. 255 'davidi' not 'davidii'
- l. 255-7: justify that the genus Panagroliamus is exceptional.
- line 259: remove 'makes'. The sentence structure and vocabulary are awkward.
- l 266: add 'this' species and remove 'of' at the end of the line.
- l. 273: 'species identification' is incorrect. You do not identify it.
- l. 276-8: remove.
- l 284 'in' detail
- l 288 rephrase to something like 'renders them dessication tolerant'?
- l 290 'upregulates' implies some change of condition, which is not specified; change to: 'than in C. elegans'
- Remove lines 291-2. If you are to talk about these genes, cite previous work monitoring and silencing these genes in Panagrolaimus.
- l 299: 'to survive'.
- l 301: 'survive the ...'
- l 315: make a sentence
- l 321 and throughout: coli with a small 'c'
- l 348: space missing between words
- l 363, 394: italics missing

- l 365: has 'a' length. The section below is in grey font.

- l 436: reference missing.

- l 440: add 'C. elegans' dauer larvae. Which developmental stage are the Panagrolaimus animals? The conditions were not described before for this species.

- line 446: which condition of recovery?

- l 482: 'left shaking' or 'on the shaker'.

- lines 485-6: The sentence needs rewriting.

- l. 823 'n. sp sp. n.' seems redundant.

- Fig. S3: C. sp. 34 is now described as C. inopinata (Kanzaki et al. 2018). Its phylogenetic relationship is odd here, as it is believed to be a sister to C. elegans (Kanzaki et al.). Is it due to the too small gene set? Any conclusion?

- line 997: why a plural?

- Software and databases need referencing.

- It would be appropriate to acknowledge CGC: https://cgc.umn.edu/acknowledging-the-cgc

- Be sure to explain all abbreviations., for example 'TG' line 496 or

- Check all references. ref. 11 and 12 are incomplete.

Reviewed by anonymous reviewer 3, 10 August 2022

Shatilovich et al. describe a new nematode species reanimated from permafrost. Plant material from the same burrow is radiodated to 46K years ago. Its genome is sequenced, revealing a triploid structure. Phylogenetic analysis places the species at the base of the *Panagrolaimus* clade. They show that the genome contains similar genes used in *C. elegans* for cryptobiosis. This is a very pithy, well-written and organized paper that reports an exciting discovery and good in-depth analysis.

I do not understand why the authors only mention the new species' name once, and do not provide a complete taxonomic declaration. The species description fits within "Box 1", which is okay (an alternative is an appendix?), but the name should be declared as a genus-species binomen with the associated author name(s) and year (e.g. *Panagrolaimus kolymaensis* Shatilovich and Kurzchalia, 2022, in Shatilovich et al., 2022. It would also be great to have this in the paper title: e.g. "*Panagrolaimus kolymaensis* n. sp. from the Siberian permafrost..." The combination of morphometrics and phylogenetic analysis clearly establish the nematode as a new "species" (one could use the phylogenetic species concept, I guess?). Anyway, giving a name to an OTU is useful.

The statement that Panagrolaimus n. sp. and C. elegans "utilize similar mechanisms to enter and remain in cryptobiotic state..." is premature, despite the work showing orthologies in the "cryptobiosis toolkit". As the authors themselves acknowledge, "while further functional analyses are needed...our results hint at convergence or parallelism..." In the absence of functional studies in the new species (e.g. RNAi knockdowns?) it could also be the case that a new mechanism of cryptobiosis has evolved for the new species while the "toolkit" persists. Most of these genes have pleiotropic functions and would be maintained anyway by selection. Their existence alone is insufficient evidence for establishing that these two species use the same molecular pathway for cryptobiotic functions.

The *C. elegans* community will love learning how to make cryopreservation more efficient and effective, so the *C. elegans* experiments will be appreciated.

Picayune point: in line 266, "outgroup" should be "outgroup representative", since a single species cannot be the entire outgroup.

Data accessibility. Please provide a table (e.g. in supplement) listing all the GenBank accession numbers for the 18S and 28S sequences used (even if not sequenced for the first time here), along with the species names. Also, is please provide the genome sequence project ID.

One thing I got curious about and would make an amazing addition to the paper (optional): Are there (possibly descendent/related) populations of parthenogenic Panagrolaimus in the non-permafrost soil in the

same area as the revived isolate? Perhaps a molecular clock could be calibrated!

Send a live culture to the Caenorhabditis Genetics Center to keep in cryptobiosis for other researchers!

Reviewed by anonymous reviewer 2, 23 August 2022

Shatilovich et al. provide a fascinating molecular insight into a nematode species that has remained in the permafrost for tens of thousands of years. They take an exemplary transdisciplinary approach, combining a broad range of techniques including systematics, genomics, analytical chemistry, and biochemistry. Their findings are original and will be of interest to a broad audience.

My sole concerns relate to the genome assembly and its analysis.

First, on a practical level, having a supplementary data repository that is a single zip folder containing 138,315 files is hardly user-friendly. A division of the repository into different zip folders in broad categories would be appreciated. Additionally, the lack of an explanatory catalogue of contents (e.g. for "OrthoFinder") renders the data next-to unusable. I apologise if it should have been obvious, but I was unable to find the assembly and gene predictions, either in the supplementary data or referred to in the text. A search at Genbank was also fruitless. This obviously limits the possibility of evaluating the quality of the assembly.

The authors conclude that the nematode genome is triploid. Fig 3B shows the triploid structure of the Panagrolaimus kolymensis genome using a Circos plot. These can only be interpreted properly if the parameters used in the analysis are given. The authors should consider removing the yellow lines as their inverted orientation does not allow the synteny to be visualised simply. They could productively mention the reason for areas where there is a gap (e.g. before 2M on tig00000955). More importantly, given that the contig breaks are in different positions for each pseudohaplotype assembly, why can the 3 pseudohaplotype sequences not be used to assemble better the individual pseudohaplotype contigs, even if the joins are of undetermined sequence?

In the text reporting their analyses of orthologues, I could find no mention of the variable copy number of certain C. elegans single copy genes. This is a particular importance for the key stress resistance regulators such as DAF-16. In this case, they report 5 orthologues, with tandem duplications on 2 of the 3 pseudohaplotype sequences (HLNpanKol1|jg25880.t1/ HLNpanKol1|jg25881.t1 and HLNpanKol1|jg48128.t1/HLNpanKol1|jg48129.t1). Especially as the supposed copies are neighbouring, this is quite likely to be the result of a consensus alignment issue and so an in silico artefact, rather than a real tandem duplication that has not affected one pseudohaplotype. The authors need to provide figures of the reads mapping to these regions to allay such doubts.

Generally it is clearly and well written, with only occasional spelling mistakes (e.g. homeolog), and problems with references in the Methods section (e.g. \Anaconda Software Distribution; \Dainat, https://www.doi.org/10.5281/zenodo.3552717\TU\textbackslash; "C. elegans dauer larvae desiccation assays were performed as described in."