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

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Submitted by Philipp Schiffer 20 May 2022 14:32

Abstract

Some organisms in nature have developed the ability to enter a state of suspended metabolism called cryptobiosis1 when environmental conditions are unfavorable. This state-transition requires the execution of complex genetic and biochemical programs1,2,3, that enables the organism to survive for prolonged periods. Recently, nematode individuals have been reanimated from Siberian permafrost after remaining in cryptobiosis. Preliminary analysis indicates that these nematodes belong to the genera Panagrolaimus and Plectus4. Here, we present precise radiocarbon dating indicating that the Panagrolaimus individuals have remained in cryptobiosis since the late Pleistocene (~46,000 years). Phylogenetic inference based on our genome assembly and a detailed morphological analysis demonstrate that they belong to an undescribed species, which we named Panagrolaimus n. sp. Comparative genome analysis revealed that the molecular toolkit for cryptobiosis in Panagrolaimus n. sp. and in C. elegans is partly orthologous. We show that biochemical mechanisms employed by these two species to survive desiccation and freezing under laboratory conditions are similar. Our experimental evidence also reveals that C. elegans dauer larvae can remain viable for longer periods in suspended animation than previously reported. Altogether, our findings demonstrate that nematodes evolved mechanisms potentially allowing them to suspend life over geological time scales.

Keywords: Cryptobiosis, Pleistocene, C. elegans, Permafrost, Suspended animation
Round #1

by Isa Schon, 19 Sep 2022 15:57
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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 suggestion 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

Dear Dr., Schon,

Thank you very much for giving us the opportunity to reply to the reviewer's comments and to improve our 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 of the reviewers, and the manuscript is now acceptable for a recommendation from PCI.

With best wishes,

Philipp H. Schiffer
(For all authors)

Response to reviewers:

Reviews

Reviewer 1

Reviewed by anonymous reviewer, 01 Sep 2022 15:37
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 shown 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).

We thank the reviewer for prodding us on citating the appropriate literature. We would like to kindly remind this reference(*1*) was cited several times in our manuscript, lines 85, 86, 91, 262, 285. We further included the references mentioned above in the line 198.

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.

To address this issue we have now implemented the MUL tree approach with GRAMPA as used in (*2*) and describe it in detail in the supplementary information (Fig.S6). The MUL tree supports the same topology as the originally obtained phylogeny (Lines 178-182). We thus assume that the homeologs do not affect topology of the tree.

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.

As described above we have used a MUL tree approach to settle the “homeolog issue”. It still appears that the strain is an outgroup to other *Panagrolaimus*. It remains an issue that the Biological Species Concept cannot be applied to parthenogens. We have included few more

statements (Lines 178-182) in this regard in the text and include a reference to a population genomic analysis of *Panagrolaimus* strains.

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.

In 1959, David Keilin, in his van Leeuwenhoek Lecture has given a fundamental definition of cryptobiosis(*3*). This state is defined as "the state of an organism when it shows no visible signs of life and when its metabolic activity becomes hardly measurable or comes reversibly to a standstill". We would like to stress that only very few organisms have ability entering this state to withstand adverse environmental conditions. As we and others have shown, that this ability depends on elaborated mechanisms of preconditioning(*4*)(*5*)(*6*)(*7*).

Cryptobiosis is different from cryoprotection or being frozen without damage. The former is an intrinsic property of an organism, whereas cryopreservation is aided by exogenous chemicals (e.g., glycerol, DMSO). In our experiments, we expose dauer larvae to two adverse conditions: desiccation and freezing (-80°C) and do not use any cryoprotectant (Like glycerol, trehalose or DMSO). Therefore, we think it is appropriate to use "cryptobiotic" than "frozen."

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.

We took the reviewer's suggestion and clarified the distinction in the text. The dauer larvae is in hypometabolic state in comparison to larval stages in reproductive life cycle. We now mentioned the hypometabolic state of the dauer larvae in the text (Lines 206- 208).

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.

This point is clarified in the reply to the comment 3 above.
Again, dauer larvae are cryptobiotic because they have intrinsic ability to survive desiccation and freezing(*8*)(*4*)(*5*). Mammalian oocytes are not cryptobiotic, they can be frozen only in the presence of an added cryoprotectant. The reviewer notices that dauer larvae can be frozen after dessication, thus once they are in suspended animation and in this way survive in cryptobiotic state much longer. This remarkable observation might be one of the most interesting points of our study.
We would like to kindly remind the reviewer that we haven't made a comparative statement about freezing ability of dauer larvae to non-dauer larval stages in our manuscript. Non-dauer stages (Specifically L1 larva) survives freezing more easily, however they need a cryoprotectant (15% Glycerol) to survive freezing whereas dauer larvae that are desiccated survive to freezing without any cryoprotectant.

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).

Indeed, *Panagrolaimus* genus do not have a dauer larva stage(*9*), therefore we performed experiments with mixed population of worms (Lines 211-12). Moreover, we exclusively went for mixed population for the survival and biochemistry experiments to reduce stage specific bias on our results.
We agree with the reviewer in ambiguity in the line 99. Therefore, we now modified the sentence.

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.

Indeed, the presence of homologous genes in two species does not necessarily demonstrate their functionality in both. However, we would like to kindly remind that our conclusion is based on the biochemical evidence of accumulation of trehalose and depletion of triacylglycerol which ensures the functionality of trehalose biosynthesis pathway and utilization of the glyoxylate shunt during desiccation in *Panagrolaimus*. Without the activity of the enzyme TPS-2 and glyoxylate shunt, it is not possible to synthesize trehalose in nematodes, especially to upregulate trehalose levels upon preconditioning. We do not eliminate the possibility of other biochemical features that might contribute to desiccation survival ability of *Panagrolaimus,* but with regards to trehalose biosynthesis and the glyoxylate shunt, our data suggest that molecular tool kit is partially orthologous. In our near future, we intend to perform the RNAi or inhibitor-based experiments to infer the concrete mechanisms. Indeed, we emphasized this point in our discussion lines (301-303).
We agree with your suggestion of 'correlation is not causation' hence we now modified line 190, 200-1, figure 4 legend title line.

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.

We thank the reviewer for this question. We have mentioned this in our manuscript in lines 115-117 that the burrow in which our species was isolated had been frozen and thawed only in the laboratory after isolation.
As shown in our previous studies, unfrozen water in permafrost deposits estimated as 3-8% by weight and occurs most often in the form of films covering soil particles or, occasionally, as brine pockets. The thickness of these films depends on the permafrost temperature and is about 5 nm at -10°C(*10*)(*11*, *12*). It is shown that this amount of unfrozen water is enough to keep the metabolism of bacterial cells at a very low level. We are convinced that the metabolism of *Panagrolaimus* in frozen sediments under such conditions is not possible in contrast to *P. davidi,* which receives enough melt water and food during the Antarctic summer.

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.

We thank the reviewer for pointing this out. Indeed, the isolation was previously reported in (*13*). We now changed the author contributions for AS.

- Please give a strain name to the culture. Is it an isofemale line?

Thank you for the comment, the name and a more detailed description of the culture is given in the Supplementary information. All investigations were carried out using Pn2-1 strain obtained from single female.

- 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?

In our previous paper (*8*) we have reported that non-dauer larvae stages are dead even upon mild desiccation. We mentioned this in the text lines 205-6.

- Please indicate which strain are parthenogenetic on the representation of inferred phylogenetic relationships.

As reviewer suggested, we now indicate the parthenogenetic strains on the representation of inferred phylogenetic relationship in our Figure S6.

- line 219: please explain why labeling of acetate makes you reach this conclusion of origin from TAGs.

Radioactive acetate labeling is an old and well-established technique to label metabolites of citric acid cycle and lipid biosynthesis. We used this method in our previous reports(*8*)(*14*)(*15*)(*16*)(*5*) . We now included an explanation in the manuscript lines (Lines 223-26).

- line 223: explain how you identify spot 7's chemical nature.

The chemical nature of the spot 7 is identified by fragmentation pattern of the molecule on the mass spectrometer. Furthermore, we have used a standard (Trehalose-6-phosphate) to overlap the fragmentation pattern. We now mentioned this in the text lines (229-30).

- line 224: the presence of a chemical does not prove that it is used 'to resist harsh dessication'.

We would like to mention that trehalose accumulation in response to desiccation has been observed in several nematodes. Using a combination of genetic and biochemical approach, we have shown that the accumulated trehalose in *C. elegans* dauer larvae is essential for anhydrobiosis(*8*). We are not claiming that the presence of the direct precursor of trehalose (trehalose-6-phosphate) is a proof of its role in desiccation resistance. We think it is a good indicator on trehalose pathway intensity in *Panagrolaimus*. The enzyme activity of GOB-

1(trehalose phosphatase) might be lower than activity of TPS-2, therefore we observe accumulation of trehalose-6-phosphate.

- Please provide some details on the survival test.

Thank you for this comment. The details of desiccation survival assay protocol are published in our previous report(*8*). We provide the reference to this work in Methods part.

- The sentences on lines 238-9 and 242-243 need to be removed or rephrased.

As the reviewer suggested these lines are now rephrased accordingly.

- Fig S4: what is meant by the absence of a daf-28 ortholog in Panagrolaimus? Was the whole insulin gene family studied?

Phylogenetic analysis was performed on the entire ortholog cluster containing the *C. elegans* sequence of daf-28. However, for the context of this manuscript we only mentioned *daf-28* as an example.

Furthermore, this cluster contained other *C. elegans* insulin genes (*ins-29, ins-25, ins-27, ins-9, ins-8, ins-7, ins-4, ins-6, ins-5, ins-2*). The cluster did not contain sequences of any other species, suggesting that these genes might only occur in *C. elegans*. Because even in complete genome assemblies some genes might be missing or so divergent that they do not cluster together in the OrthoFinder analysis, the phylogenies are not sufficient as a proof that a certain gene is not encoded in the *Panagrolaimus n. sp.* genome. We can only say with certainty that there is strong evidence for detected genes to be indeed orthologs to the respective *C. elegans* genes.

- 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.

Thank you for pointing this out. We would like to clarify that we performed the quantification from two biological replicates with two technical replicates performed on two independent days. We now clarified this in the figure legend as well. We did perform a t-test.

The panels are now properly named 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.

This is indeed an important point raised by the reviewer and we have tried to clarify this now by amending our statement (Lines 314-17). We like to note that in the case of parthenogens every individual “is its own gene pool”, concepts as in biological species do not apply.

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

The language editing and grammatical mistakes mentioned below are now rectified. We got our manuscript proofread by a native speaker.

- Abstract line 35: remove the comma in 'programs that enable'. What is a 'complex genetic and biochemical program'?

We removed the comma in “programs that enable”. A complex genetic and biochemical program is a combination of genetic and biochemical pathways that are upregulated upon preconditioning.

- lines 93-94: remove this vague sentence.

We removed this sentence now.

- lines 95-96: the sentence structure is ambiguous and its meaning is unclear. What is a 'detailed morphological, phylogenetic analysis'?

We modified the structure of this sentence now.

- line 98: remove 'powerful'.

We removed powerful

- line 132: why 100 generations of culture before studying it? Did you not maintain it frozen?

To make sure the strain adapts to laboratory environments we grew them for multiple generations in several labs (Please kindly notice the affiliations of the authors). By the time we received the strain from our co-author, it was grown for several generations. We now maintain it frozen but while we were performing the experiments, we grew them in culture for many generations.

- line 153: 'links...show' not 'shows'

We modified to links show

- Box 1 line 3: typo at 'within'

We corrected the typo.

- line 180: add the word 'a' before monophyletic trait

We modified to “a 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.

We rephrased the sentence to reduce the ambiguity (Lines 204-207).

- l 210: survive 'better' not 'higher', or 'in a higher proportion'.

We modified to higher proportion

- l. 212 is ambiguous: which developmental stages of C. elegans? Fig. 4B shows daf-2 dauer larvae.

We now changed this to *C. elegans* 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.

The notion of preconditioning was introduced several decades ago(*17*)(*18*)(*7*)(*6*). We used the nomenclature of 'preconditioning' for *C. elegans* in our previous reports(*5*)(*8*)(*14*)(*4*). Moreover, in our previous report(*5*) we have shown that *C. elegans* dauer larvae have a general program (preconditioning) to survive different kinds of abiotic stress. We have also observed the same in our pilot experiment (Data not shown) with *Panagrolaimus sp.n.* Therefore, we only showed the freezing survival ability after the nematodes are fully desiccated.

- l 225, 293: 'the' glyoxylate shunt

This is changed to 'the glyoxylate shunt'

- l 227: add suggests 'that' the flux. What does 'the latter' refer to?

We added "suggests that the flux now". We rephrased the 'latter' in the sentence.

- l 231: remove comma.

The comma is removed

- line 239: 'extremely long periods of time'?! C. elegans has been kept frozen by others for half a century, not 480 days.

We kindly refer to our previous reply in the major comments section. The freezing of *C. elegans* using cryoprotectants and their inherent ability to survive freezing after being desiccated are two different experiments. We do not make a comparative claim between these two experiments anywhere in our manuscript. As we have mentioned in our manuscript lines (236-38), even in its desiccated state *C. elegans* dauer larvae do not survive more than 10 days, on that comparative scale we think it is extremely long periods of time.

- remove lines 241-3. l. 244-251 belong to the introduction

We would like to kindly disagree with the reviewer on removing or moving them to introduction. We are summarizing our results, so our sentences are within the context of our discussion. For the lines 244-51, our intention here is to discuss the lack of attention to the findings made on organisms isolated from Siberian permafrost. Moreover, in the introduction

we do not plan to discuss these historical findings, as it does not fit with the succinct flow of the text we have there.

- l. 253: what is an undescribed strain?

We removed the word ‘undescribed’ in the sentence.

- l. 255 'davidi' not 'davidii'

We modified it to ‘davidi’.

- l. 255-7: justify that the genus Panagroliamus is exceptional.

We justified our phrase by mentioning the exceptional nature, because of its morphological uniformity of the nematodes. For instance, in the genus *Caenorhabditis* the morphological uniformity is little.

- line 259: remove 'makes'. The sentence structure and vocabulary are awkward.

We removed ‘makes’. This was a mistake while editing.

- l 266: add 'this' species and remove 'of' at the end of the line.

We added ‘this’ species and removed ‘of’

- l. 273: 'species identification' is incorrect. You do not identify it.

We removed these sentences based on your suggestion above.

- l. 276-8: remove.

It is not clear to us, what to remove here. We explained our reasons for these sentences in the comments above.

- l 284 'in' detail

We added ‘in’ detail

- l 288 rephrase to something like 'renders them dessication tolerant'?

We rephrased it to ‘renders them desiccation tolerant’

- l 290 'upregulates' implies some change of condition, which is not specified; change to: 'than in C. elegans'

We changed to ‘elevated’

- Remove lines 291-2. If you are to talk about these genes, cite previous work monitoring and silencing these genes in Panagrolaimus.

Thank you for this suggestion. We now cited the previous work that performed silencing these genes in *Panagrolaimus*

- l 299: 'to survive'.

'to survive'

- l 301: 'survive the ...'

'survive the'

- l 315: make a sentence

We made a sentence here.

- l 321 and throughout: coli with a small 'c'

Changed to 'E. coli'

- l 348: space missing between words

We corrected the spacing

- l 363, 394: italics missing

We added the missing italics now.

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

The font is changed to black now

- l 436: reference missing.

Thanks for pointing this missing reference. We now added this.

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

We added *C. elegans* dauer larvae. We also included that we performed our experiments with mixed populations of *Panagrolaimus*.

- line 446: which condition of recovery?

We removed 'recovery' and added "overnight incubation at 15°C".

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

We changed the sentence to "left on the shaker".

- lines 485-6: The sentence needs rewriting.

'We rephrased these lines (509-511).

“The dried samples were reconstituted in a volume of 300 µl of 4:2:1 (Isopropanol:Methanol:Chloroform). Volume corresponding to 1 µg was used for injection”.

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

We rectified our typo and removed the redundancy.

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?

We corrected the name of C. sp. 34. It is indeed possible that this is an artefact. We like to note that we only include *Caenorhabditis* species here for reference. The tree is much more stable in clade IV, containing more species and our target organisms. The position of individual species in *Caenorhabditis* might not be congruent with phylogenies centering on that taxon.

- line 997: why a plural?

We removed ‘a’ from the plural.

- Software and databases need referencing.

All the software and databases are now referred in the manuscript.

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

Thank you for making us aware of this link. We now included appropriate acknowledgement for CGC

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

We checked all the abbreviations in the manuscript and made sure they are consistent in the text.

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

Thank you for this comment. We checked all the references, rectified, included, and completed the references including 11 and 12.

Reviewed by anonymous reviewer, 10 Aug 2022 19:26

Reviewer 2

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.

1. 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.

We thank the reviewer for this question.

In our earlier versions on bioRxiv we did mention the species name in the main text. As we are obliged by the rules of PCI not to use the species name in the preprints, we had to modify our manuscript accordingly. Please find the concern expressed by PCI editor below:

“Preprints should indeed never name new species to avoid nomenclature confusion. Additionally, the description of a new species should always be part of the main text of an article, and should not be limited to the supplementary material. Therefore, your article has to be corrected, so that "Panagrolaimus kolymaensis" is replaced by "Panagrolaimus n. sp.", and so that the description of the species is moved to the main text.”

However, we would gladly take your kind suggestion and include the species name in the final submission to a journal.

2.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.

We agree with the reviewer’s comment that indeed the presence of homologous genes in two species does not necessarily demonstrate their functionality in both. However, we would like

to kindly remind that our conclusion is based on the biochemical evidence of accumulation of trehalose and depletion of triacylglycerol which ensures the functionality of trehalose biosynthesis pathway and utilization of the glyoxylate shunt during desiccation in *Panagrolaimus*. Without the activity of the enzyme tps-2 and glyoxylate shunt, it is not possible to synthesize trehalose in nematodes, especially to upregulate trehalose levels upon preconditioning. We do not eliminate the possibility of other biochemical features that might contribute to desiccation survival ability of *Panagrolaimus,* but with regards to trehalose biosynthesis and the glyoxylate shunt, our data suggest that molecular tool kit is partially orthologous. We should admit that the present results need further comprehensive investigation to provide a mechanistic insight. In our near future, we intend to perform RNAi or perturbation experiments to infer the concrete mechanisms. Indeed, we emphasized this point in our discussion lines (301-303).

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

We are elated to see a positive comment on our *C. elegans* data. Indeed, we hope our cryopreservation methods will be appreciated and used by *C. elegans* community.

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

Single species are routinely called “outgroup” in phylogenetic analyses. We thus suggest to stick with this technical term here.

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.

We would like to kindly mention to the reviewer that we did provide a supplementary table (Supplementary table 2 in the SI) listing the accession number for the 18S and 28S sequences used in the analysis. We now mentioned this in the methods (lines 431-435).

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!

This is a very good point. We certainly resonate with the idea of the reviewer, and it would have been a great addition to our manuscript. Unfortunately, we did not make an isolation from the non-permafrost soil in the same area.

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

Certainly, we will soon submit our strain to CGC.

Reviewed by anonymous reviewer, 23 Aug 2022 18:57

Reviewer 3

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.
1.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.

We thank the reviewer for this suggestion, and we completely agree about the user-unfriendly folder set up we provided in the zip file. We now made subfolders in our supplementary data to make it is more user friendly and accessible. We also included a readme file in the folder.

2.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?

We agree with the reviewer that we did not provide enough detail in the figure legend to interpret the Circos plot. We have therefore added additional detail to the figure legend (including the number of homeologs and how they were inferred). We opted not to mention the reasons for the gaps (which is likely due to repeat expansion and/or gene loss in one of the three haplotypes) because that would require a substantial additional analysis that is unrelated to the aim of the figure (which is to show that the genome is triploid). We have also opted not to remove the yellow lines because that would detract from the aim of showing the three-way relationship between the haplotypes. The author is correct that it is possible to scaffold the contigs where they are broken at different places (that is in fact what we attempted to do, starting with this region). However, one quickly run in into regions that are either broken at the same place or that are more fragmented than what is shown in Figure 3B. As a result, scaffolding the entire genome using this approach (or even extending further from what is shown in 3B) would not be possible.

3. 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.

We thank the reviewer for raising this intriguing point. Even in the complete genome assemblies, some genes might be missing, or are so divergent that they do not cluster together in the OrthoFinder analysis. As spurious sequences were removed for the phylogenetic analysis, the phylogenies cannot sufficiently describe for instance the number of duplicates in *Panagrolaimus n. sp.* for a particular gene. Additionally, if a gene is not detected, it does not mean that it is not there. However, our data provides strong evidence for the existence for detected orthologs.

For DAF-16 in particular, the alignment of all sequences in the cluster (providedinthesupplementarydataunderSurvey/alignment_and_phylogeny_files/OG0002030_ DAF16.fa.aln) shows that there are 2 sequences that are long towards one end, two sequences long towards the other end, and one sequence that spans both, so it is indeed probable that there are actually only the three usual homeologs here, even though there are tiny differences in the different sequences. For the above-mentioned reasons this cannot be entirely solved here though. Thus, our analysis aimed at detecting the presence of homologs to certain genes, rather than focussing on paralogs or the absence of genes. It surely will be necessary to scaffold the genome using, for example Hi-C derived data, in the future to completely resolve the questions of what are homeologs, what are paralogs, and which genes are missing.

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}; “C. elegans dauer larvae desiccation assays were performed as described in.”

Thank you for mentioning this, we rectified our spelling mistakes, corrected, and included the missing references in the manuscript.

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