

Ground squirrel coprolites preserve complex archives of ancient environmental DNA over 700,000 years

Corresponding Author: Dr Tyler Murchie

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Review of "Ground squirrel coprolites preserve complex archives of ancient environmental DNA over 700,000 years"

The authors investigate whether permafrost-preserved ground squirrel coprolites can function as long-term biological archives of past environments, and they evaluate the extent to which ancient environmental DNA from these deposits can inform ecological and evolutionary inferences across late Quaternary to Middle Pleistocene timescales. Specifically, the authors aim to (i) assess the potential of coprolites to preserve multi-taxon DNA signals informative of past biotic communities, (ii) evaluate how recovered signals vary across samples and temporal contexts, and (iii) use these data to refine reconstructions of past ecosystems and aspects of population history in Beringia, including insights enabled by the oldest material.

Overall, I find the data and many of the results convincing and potentially high impact. My main concern is the presentation: the manuscript reads more like a methods validation paper than a biology/ecology study, and the structure is at times difficult to follow. In my view, the manuscript would benefit substantially from refocusing the main text on the biological insights and moving much of the workflow validation and technical detail to the Methods and/or Supplementary Information.

Major comments

1. Framing and writing style: abstract/introduction are overly method-focused

The abstract and introduction place considerable emphasis on methods and pipeline validation. This is not the most effective format for a Nat Comm narrative, where it typically outlines the biological problem, the key discovery, and why it matters.

Suggestion: revise the abstract and introduction to focus primarily on (i) what coprolites can reveal about paleoecology and community composition, (ii) what is uniquely enabled by permafrost-preserved squirrel burrows, and (iii) the major biological/ecological conclusions. Methodological novelty should be mentioned only briefly and at a high level, with details reserved for Methods/SI.

2. Background section reads like a mini-review and distracts from the core story

Early in the manuscript, there is an extended overview of coprolite studies using ancient DNA. While some context is relevant, the length and stand-alone nature of this section takes focus away from the specific aims and storyline of the present study.

Suggestion: substantially condense this background and integrate only the most essential points into the introduction (i.e., what is known, what is missing, and why ground squirrel coprolites fill that gap).

3. Excessive emphasis on pipeline/extraction validation in the main text

The manuscript spends a large portion of the main text on extraction tests and stepwise descriptions of computational

processing. As written, the narrative feels methods-paper-ish and, in places, repeats workflows that are already published. There are also sentences that are repeated in both main text and methods.

Suggestions:

Move most of the extraction and pipeline validation detail to Methods/Supp, keeping only the minimum necessary to justify the reliability of the biological conclusions.

In the main text, describe only what is distinct from previously published approaches and why those differences matter for the downstream ecological inferences.

Consider a clearer Study design schematic early on, then streamline the workflow description in the Results.

4. Results / Discussion boundary unclear

Interpretation and justification (authenticity, false positives, damage patterns, simulation benchmarking) appear extensively before the Discussion section, and the Discussion itself is long and subdivided in a way that reads like a second Results section.

I suggest to structure by separating (i) results presentation (what was detected/observed) from (ii) authentication/limitations (what supports confidence, what remains uncertain) and from (iii) broader interpretation (ecological implications).

5. Low-count taxonomic calls: insufficient support without damage authentication

The manuscript reports taxa supported by very low read counts (sometimes <10 and <100). For many of these, the authors cannot validate authenticity via ancient DNA damage patterns and instead rely on pipeline conservatism, controls, and ecological plausibility. In my view, this is not sufficiently stringent for strong claims of taxon presence in deep-time samples.

Suggestions:

Apply and justify a more conservative minimum read threshold for reporting species-level presence, especially for key ecological inferences. Restrict low-count assignments (genus/family) unless damage/authentication is shown.

Minor comments / presentation

The Supplementary Information contains figures that feel overly detailed to the main message. For example, the map presentation in the Pacific Northwest Quaternary references (e.g., Fig. S3) seems unnecessary at the current level of detail.

Fig 9 main-text figure also feel more methodological than biological. Consider moving such figures to the Supplementary Information and prioritizing in the main text the figures that directly support paleoecological interpretation and key conclusions.

Recommendation

I appreciate the breadth of work and the extensive testing presented. However, the manuscript currently lacks focus and a clear narrative structure, which makes it more challenging to read than necessary. I encourage the authors to revise the manuscript with a stronger emphasis on the coprolite-derived biological results and paleoecological conclusions, and to streamline methodological validation by moving much of the technical detail to the Methods and/or Supplementary Information. This would better align the presentation with the expectations and readership of Nature Communications. With these changes, I believe the study will be of substantial interest to the journal's broad audience.

Lastly, I would like to thank the authors for the data availability statement and the commitment to make the raw sequencing data readily accessible. Providing unfiltered raw reads (rather than only reads that map or pass additional filtering) is essential for transparency and reuse, and sets a good standard for the field. On that note, since data from previous studies (e.g., Murchie et al.) are used for comparisons, I encourage the authors to ensure that the corresponding raw sequence datasets are clearly referenced with accession numbers and are publicly accessible. If applicable, this could include BioProjects such as PRJNA722670 and PRJNA752360 (and any additional accessions used).

Reviewer #2

(Remarks to the Author)

Murchie et al. have produced a thorough ancient environmental DNA study of ground squirrel coprolites spanning 700,000 years with some impressive results (e.g. mitogenomes of not just ground squirrels but also mammoths, bison, and horses). The amount of data, analyses, and information gleaned from these coprolites is a lot to say the least. The figures are all of high quality. Whenever I had a question or query about the methods or results, they were mostly answered in the main manuscript and supplementary information. This study is probably one of the most thorough I have seen on ancient environmental DNA from coprolites, showing when done well, they can be as powerful results wise as ancient DNA from individual bones. I hope the following comments are helpful in revising the manuscript.

Major comments:

I felt that the introduction needed to be reworked as it left me somewhat confused and was not as polished as other parts of the manuscript. The opening paragraph (Lines 54-65) was repeated a lot in the first paragraph of the "ancient DNA from coprolites" section (Lines 80-88). I also felt that the second paragraph of the introduction (Lines 66-78) should be at the end of the introduction. These changes, and the ones detailed below (Line 157), would give you the space to have a big picture opening to the introduction built around coprolites as time capsules for reconstructing ecosystems (if only the associated problems could be solved).

Line 157: I would rename this section "Geological Setting" and have it separate to the introduction or at least make it clear that this is what this section is about. This change would make it clear why results (Line 160-165) are in this section.

Lines 194-220: Given the impressive amount of research and results that are presented in this study, I really liked the summary section you have at the start of the results. I would have liked to see another of these summaries about the big picture implications and discussion at the start of the discussion (similar to lines 924-942) before the in-depth discussion is undertaken. This would ensure the reader is not lost in the large amounts of data and analyses this study has undertaken.

Lines 782-784: If I'm not mistaken (but happy to be proven wrong), ancient environmental DNA analysis was not undertaken on sediment surrounding the coprolites. Why was this not done as I thought this would have been a good way to test for DNA leaching and where the ancient environmental DNA signal is coming from (more so than you talk about in the manuscript, especially for samples that might not all be coprolite as you discuss)?

Minor comments:

Lines 114-117: This sentence is confusing and needs rewriting. Remove the parentheses around "Sciuridae", and make it clear the family contains the species in question, rather than ground squirrels contain the species in question.

Line 121 and elsewhere: Scientific names (genus and species) should be in italics (e.g. *Urocyon*).

Line 122 and elsewhere: Geographic names should be in capitals (e.g. Bering Strait and Arctic ground squirrel).

Line 126: Remove the extra ")" from this line.

Line 144 and elsewhere: Can you please include scientific names for species when you first introduce them with common names...this is done inconsistently in the manuscript (e.g. snowshoe hares).

Lines 237: In general, numbers ten or less need to be spelled out, while 11 and above can be numbers.

Lines 337-338 and elsewhere: Please keep use of capitals in common names consistent throughout the manuscript (e.g. golden-mantled ground squirrel vs Thirteen-lined ground squirrel).

Line 545: Remove the ")" from this line.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

I think the manuscript now reads a lot better, I thank the authors for this rework.

While I think this manuscript and data will become valuable resource to the community I still have a challenge with the way the authors handled my criticism on the low read taxa reporting. Besides from this last critique point, I think the authors answered my other concerns.

Major

I do appreciate the authors' explanation and agree that low-count signals can be useful for hypothesis generation and targeted follow-up. But I do not think it is sufficient to address this issue only through general wording in the figure captions. The manuscript still needs a clearer distinction between exploratory low-count detections and taxa that are sufficiently supported for inference in the present study.

I would therefore suggest either (i) moving unauthenticated low-read assignments to the Supplementary Information as tentative detections, or (ii) retaining them in the figure but marking the relevant taxonomic names with an asterisk or similar symbol, with the caption explicitly stating that these taxa had insufficient reads across all samples for authentication and should therefore be regarded as tentative.

While I agree with the authors that there is no universally accepted minimum read threshold in the field, I do not think that this in itself justifies continuing to report weak signals on the same footing as more strongly supported detections. The key issue is not the absence of a universal cutoff, but the need to communicate clearly which taxa are sufficiently supported for interpretation and which should remain exploratory. In my view, an important broader point here is that studies such as this one help set the standard for future work, and the authors therefore have an opportunity to guide the field toward a clearer and more transparent treatment of weak taxonomic signals.

Take for example, the total of 5 reads assigned to the genus *Puma*, 5 reads to *Felinae*, and 14 reads to *Felidae*, all

recovered in only a single sample, that makes this taxonomic signal highly uncertain. This seems particularly so given that the broader ecological signal, including the recovered plant taxa, that obviously does not support such an interpretation. I therefore think such cases, and not just the Puma signal, should be presented much more cautiously and not placed on the same footing as better-supported detections.

Minor

In main Figures 1, 3, 4, and 5, there is a general challenge in balancing overall figure size and font size. However, in several cases there also appears to be room to increase the font size, which I think is necessary for readers to be able to view and interpret the figures properly where possible.

The resolution in the submitted manus also appears to be low. I assume this may be an artefact of the submission system, but if so, this should be corrected before any eventual publication.

Reviewer #2

(Remarks to the Author)

Murchie et al. have done an in-depth and thorough revision and provided thoughtful explanations for their revisions. All my comments (and I believe the other reviewers) have been thoroughly addressed, especially the setting up of the study and why it's important in the introduction. I look forward to seeing this paper in print. I hope the following very minor comments are helpful.

Line 77: You have already defined ancient environmental DNA, so just use aeDNA.

Line 78: Change "those" to "the resulting"

Line 265: Can you please give the common name (snowshoe hare) for *Lepus americanus*.

Figure 3 and 4: "10" should be "ten"

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Thank you for the opportunity to revise our manuscript submission. The reviewer comments have significantly improved the quality of this work. What follows are our responses to the reviewer comments in blue. The original reviewer comments are in black text. In addition to the changes described below, we changed the citation style to *Nature Communications*.

RESPONSE TO REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

Review of "Ground squirrel coprolites preserve complex archives of ancient environmental DNA over 700,000 years"

The authors investigate whether permafrost-preserved ground squirrel coprolites can function as long-term biological archives of past environments, and they evaluate the extent to which ancient environmental DNA from these deposits can inform ecological and evolutionary inferences across late Quaternary to Middle Pleistocene timescales. Specifically, the authors aim to (i) assess the potential of coprolites to preserve multi-taxon DNA signals informative of past biotic communities, (ii) evaluate how recovered signals vary across samples and temporal contexts, and (iii) use these data to refine reconstructions of past ecosystems and aspects of population history in Beringia, including insights enabled by the oldest material.

Overall, I find the data and many of the results convincing and potentially high impact. My main concern is the presentation: the manuscript reads more like a methods validation paper than a biology/ecology study, and the structure is at times difficult to follow. In my view, the manuscript would benefit substantially from refocusing the main text on the biological insights and moving much of the workflow validation and technical detail to the Methods and/or Supplementary Information.

Thank you for your comments. Upon re-reading the manuscript with fresh eyes, we can see how the framing of the article itself became lost in all of the technical details. To address this limitation, we significantly reframed the abstract and introduction for the article and moved around the many elements as suggested to achieve a more streamlined structure.

Major comments

1. Framing and writing style: abstract/introduction are overly method-focused

The abstract and introduction place considerable emphasis on methods and pipeline validation. This is not the most effective format for a Nat Comm narrative, where it typically outlines the biological problem, the key discovery, and why it matters.

Suggestion: revise the abstract and introduction to focus primarily on (i) what coprolites can reveal about paleoecology and community composition, (ii) what is uniquely enabled by permafrost-preserved squirrel burrows, and (iii) the major biological/ecological conclusions. Methodological novelty should be mentioned only briefly and at a high level, with details reserved for Methods/SI.

Great suggestion. The originally submitted manuscript had a clunky abstract, introduction, and background section and were too methods focused given the journal. The abstract and introduction have both been rephrased substantially (L31-L45 and L52-L131). We removed the 'background' sections and integrated the important information from those into the introduction and results. The biological context has been placed at the forefront. We moved other material to the methods/SI. We believe the flow of the article is now significantly improved with clear framing, rationale, and the early repetition has been removed.

2. Background section reads like a mini-review and distracts from the core story

Early in the manuscript, there is an extended overview of coprolite studies using ancient DNA. While some context is relevant, the length and stand-alone nature of this section takes focus away from the specific aims and storyline of the present study.

Suggestion: substantially condense this background and integrate only the most essential points into the introduction (i.e., what is known, what is missing, and why ground squirrel coprolites fill that gap).

Following from comment 1, we have removed the 'background' sections entirely, moved the important material from that into the introduction to provide a proper setup for the rest of the article, and moved other extraneous details to the results, methods, and supplement. There was also a fair bit of repetition before. That repetition has now been removed.

3. Excessive emphasis on pipeline/extraction validation in the main text

The manuscript spends a large portion of the main text on extraction tests and stepwise descriptions of computational processing. As written, the narrative feels methods-paper-ish and, in places, repeats workflows that are already published. There are also sentences that are repeated in both main text and methods.

Suggestions:

Move most of the extraction and pipeline validation detail to Methods/SI, keeping only the minimum necessary to justify the reliability of the biological conclusions.

In the main text, describe only what is distinct from previously published approaches and why those differences matter for the downstream ecological inferences.

Consider a clearer Study design schematic early on, then streamline the workflow description in the Results.

We moved the following methods heavy result sections to the supplementary text and left behind minimal necessary summaries relevant to the discussion to point interested readers there: extraction tests (and the former Fig. 3, now part of Fig. S1), macrofossil details, metagenomic simulation validations, fungal metagenomics, *Lepus* phylogenetics, and thermal age). Because the supplement now has many Supplementary Text sections, we added a proper

legend to the SOM with page numbers for those main extended results sections. This shift in focus improves both the flow of the main text and the utility of the supplementary materials. Thanks for this excellent suggestion.

To clarify, the reason for the methodological focus in the submission, and for what remains in this revision, is two-fold. First, this project essentially began between TJM and SC as a methodological curiosity, and our attempts to pull useful aeDNA from the coprolites was a multi year side project (to emphasize, essentially none of these samples worked at first and would have been forgotten had we not tried to optimize the methods for these samples). It was only when we realized how good the data was when we finally sequenced it that the project shifted in a fundamental sense.

Building off of that, secondly, we believe that the methodological focus of the aDNA work we present (here and elsewhere) is inextricable from the palaeoecological discussion. We intentionally wish to resist the growing trend in the aDNA field to sideline methodological caveats for simpler ecological stories that hide the detail needed to interpret ecological insights at varying levels of confidence. We do agree that the methods here were too front and center. And to aid this limitation, we have moved much of that material you mention in your review to the supplement. However, one of the most important take-aways from this work is almost none of these samples worked initially. But by caring about the iterative wet-lab details and not giving up on these failed samples, we were able to recover a pretty remarkable dataset of aeDNA from samples we didn't expect much of anything from. This context we believe is important to this work and future work that might make the most of these sorts of coprolites to create large, holistic datasets of Quaternary ecosystems. We have added further emphasis to this point on L810 in the final paragraph of the discussion. Future research efforts will likely struggle with the inherent per-sample methodological troubleshooting needed to get viable aeDNA from coprolite samples such as these. We want to make sure that a record of our troubleshooting efforts is available to help fast track future efforts since these information dense records are thawing at alarming rates. Sites visited one year where abundant sample sets could be collected are entirely gone the next year. We want folks doing this work to not overlook coprolites (especially ground squirrel coprolites) as they're incredibly information rich, but also to not give up on them when they present troubles during processing.

4. Results / Discussion boundary unclear

Interpretation and justification (authenticity, false positives, damage patterns, simulation benchmarking) appear extensively before the Discussion section, and the Discussion itself is long and subdivided in a way that reads like a second Results section.

I suggest to structure by separating (i) results presentation (what was detected/observed) from (ii) authentication/limitations (what supports confidence, what remains uncertain) and from (iii) broader interpretation (ecological implications).

Authenticating aDNA inherently is, we believe, a blurred boundary case that doesn't easily fit into a strict results/discussion partition. We included a listing of authentication criteria together in the results so that it's easy for readers to see all of that related information together (which is very important for aDNA literature), but so as not to take away from the far more interesting discussion of what the data means. In our view of the results -> discussion spectrum, whether

aDNA is likely to be ancient is a result in itself (admittedly, a very low-level interpretation, but so are most of the processing results of aDNA, arguably). What that means ecologically with our data being ancient, and from a specific place, is the discussion. In that framing, we have all of core results together (with authenticity at the end since it's pushing that boundary), and we save the integration with the rest of the literature to the discussion. We saved the "Authenticity and Caveats" section for the discussion so that anyone who skips over the results "Authentication" for the discussion will be more likely to see the caveats and use those to tune their cautious interpretations (as we agree that caveating aDNA research for what we can believe with confidence and what is simply an interesting signal worthy of further study, is very important).

Our discussion is focused on evolutionary implications, diet vs. exogenous midden DNA, novel palaeoecological insights, caveats, and how we might move forward with ground squirrel coprolite aeDNA to make the most of these incredible remains and the critters that created them. We believe the types of interpretation/discussion in the manuscript here are quite far away on that spectrum from the "interpreting" of human DNA as being contamination or a deamination proportion of ~20% as suggesting DNA is old. Those results are so tied to their meaning that it seems disingenuous to suggest that they're interpretations in the manner that we have utilized our discussion section here. We'll hope you agree that by dedicating space in each figure for deamination plots, and the many figures in the supplement to the metaDMG authentication plots, helps shows we think authenticating aDNA is critical this field. But that what we can say once we think there are sufficient lines of evidence that the DNA is real is on an entirely separate level, ergo our split of "results" and "discussion" here.

To some degree, this differing of opinion is partly the result of having so many interesting elements to discuss far beyond whether the DNA is old or not. But at the core here, whether there is a sufficient abundance of authenticity lines of evidence is, we believe, just a later stage of "results". Actual interpretations in the sense you have emphasized in your review (the biological conclusions), are what we think all of this aeDNA means given our result indicating that it's likely old and real.

5. Low-count taxonomic calls: insufficient support without damage authentication

The manuscript reports taxa supported by very low read counts (sometimes <10 and <100). For many of these, the authors cannot validate authenticity via ancient DNA damage patterns and instead rely on pipeline conservatism, controls, and ecological plausibility. In my view, this is not sufficiently stringent for strong claims of taxon presence in deep-time samples.

Suggestions:

Apply and justify a more conservative minimum read threshold for reporting species-level presence, especially for key ecological inferences. Restrict low-count assignments (genus/family) unless damage/authentication is shown.

We completely agree that it would be wrong to make strong claims (key ecological inferences) about taxon presence in instances where our read counts are low. Which is why we have carefully avoided such and only discussed ecological inferences based on taxa with strong

support (either damage indications or high counts). To make that clearer, we have added new captions to what are now figures 3 and 4 emphasizing to readers to be cautious of low read count taxa. We have also added a new figure to the supplement (Fig. S102) that has deamination profiles for low count animal taxa.

We disagree however that the best solution for low count taxa is to utilize significantly higher minimal read cut-offs, especially 100 reads which we would argue is by no means a norm in the field of aeDNA/sedaDNA to have such a high minimal read cutoff. The reason for this disagreement is twofold.

First, our metagenomic classifications are already highly stringent (beyond the norm in many comparable manuscripts in the field). Reads here were first map-filtered to a curated organelle database, processed through several additional filters, then again aligned against all of NCBI-NT, and then LCA assigned through a third primary layer of filtering, which together only brings through reads which have sufficient reference database availability to classify well. This alone is already an excellent strategy for removing most of the spurious false positives that might come from investigating nuclear data that has poor reference sequence coverage. Had we just been metagenomically classifying without first-map filtering, I'd agree that a higher cut-off and high skepticism is warranted because those datasets are far more vulnerable to false positives. But, our approach consistently produces very low rates of false positives as demonstrated in our supplementary simulation tests, and so seeing faint signals of low count taxa can then become a tool (importantly here, a cautious one) for diving in deeper with capture enrichment to pull out low signals from those specific organisms.

The second point here is an example related to our mammoth positive samples. After the initial batch 3 run, the metagenomic classifications called several taxa with low read counts. For instance, 9 reads to Elephantidae for SC-14, and 20 reads for SC-9 (both, which in context essentially mean, *Mammuthus primigenius*). Those low counts of course couldn't be map/damage authenticated, but we were interested if we could go back to those samples with a Proboscidean bait-set to pull out those faint signals (this was the case for all of the mammoth positive samples where we re-enriched to pull more of those signals out). Following that, we were able to re-assemble mammoth mitochondrial genomes from 6 squirrel faecal samples, including the 700 kya sample. The implications of those genomes are fascinating (an entire second paper is dedicated to the mammoth evolutionary history we were able to reconstruct from these samples, forthcoming soon). But had we simply called our read counts at >10, or went very high and called them at >100, we would have entirely missed that faint signal. A similar signal for mammoth exists in sample SC-6 (7 reads to Elephantidae), as it does for many other organisms in our dataset. We want to retain those faint signals so that collaborators who are interested in specific species have the option to ask us for those libraries to do a deeper enrichment, or ask us to collaborate to do that work so that they can investigate what those genomic datasets might say. For these reasons we believe that it is necessary to keep our minimal read counts for our LCA in the manuscript.

We do however, very much agree that we don't want anyone mis-interpreting low read count taxa as anything definitive. This is why we propose an in-between approach. Changing the captions on Figures 3 and 4 to make it clear to readers that low read taxa must be interpreted with caution. We have also generated a new figure for low count animal taxa (Fig. S102) where we have indications of ancient authenticity.

Minor comments / presentation

The Supplementary Information contains figures that feel overly detailed to the main message. For example, the map presentation in the Pacific Northwest Quaternary references (e.g., Fig. S3) seems unnecessary at the current level of detail.

Figure S3 is a supplemental figure that establishes the geographic scope for a large comprehensive reference panel for map-filtering our metagenomic data that we will refer back to in several forthcoming publications where we've built in-house RNA baits based on this reference set that we will make publicly available (including with a in-house bait production method). This figure is an important touchstone for those future publications using this panel and sets the data scope used for our map-filtering panel (a critical part of our map-filtered metagenomic classifications). This is an important figure in the supplement. This goes for other supplementary figures that together all support the main message of the manuscript and help facilitate future research efforts.

Fig 9 main-text figure also feel more methodological than biological. Consider moving such figures to the Supplementary Information and prioritizing in the main text the figures that directly support paleoecological interpretation and key conclusions.

As previously discussed, we do not believe that the methodology of recovering aeDNA from coprolites can be separated from the biological insights. A key piece of this paper is setting the methodological picture that detailed palaeoecological records can be recovered from coprolites and that we need large scale efforts processing these materials that have been found, as well as dedicated field efforts to find and save these samples before they're all gone from rapid thaw in the arctic. Bones, sediments, and coprolites will likely form much of the picture of Quaternary aeDNA to be reconstructed in coming years. And this figure we feel is critical to the message that it would be a mistake to overlook coprolites (as is so often done) because of their immense potential for ecological and evolutionary insights.

Recommendation

I appreciate the breadth of work and the extensive testing presented. However, the manuscript currently lacks focus and a clear narrative structure, which makes it more challenging to read than necessary. I encourage the authors to revise the manuscript with a stronger emphasis on the coprolite-derived biological results and paleoecological conclusions, and to streamline methodological validation by moving much of the technical detail to the Methods and/or Supplementary Information. This would better align the presentation with the expectations and readership of Nature Communications. With these changes, I believe the study will be of substantial interest to the journal's broad audience.

Lastly, I would like to thank the authors for the data availability statement and the commitment to make the raw sequencing data readily accessible. Providing unfiltered raw reads (rather than only reads that map or pass additional filtering) is essential for transparency and reuse, and sets a good standard for the field. On that note, since data from previous studies (e.g., Murchie et al.) are used for comparisons, I encourage the authors to ensure that the corresponding raw

sequence datasets are clearly referenced with accession numbers and are publicly accessible. If applicable, this could include BioProjects such as PRJNA722670 and PRJNA752360 (and any additional accessions used).

Thanks, we agree it sets a good standard for the field. We have added to the data availability statement for all BioProject raw data used here. This includes the bone mammoth raw data, sediments, and coprolites. Here's the revised statement:

"All raw sequence data for our coprolite samples have been uploaded to the NCBI SRA (BioProject: PRJNA1180709) and will be released at the time of publication. Processed files derived from these raw sequence data will be stored on servers at the Hakai Institute and McMaster University and be available upon request. Sedimentary raw sequence data used here for analysis from Murchie et al. (2021/2022/2023) are also available on the NCBI SRA under BioProjects: PRJNA1004851, PRJNA752360 and PRJNA722670. The mammoth bone raw sequence data from Rowe et al. (2024) are available under BioProject PRJNA1405728. All other sample data are available in the main text or supplementary materials. The PNW Quaternary References are located here <https://doi.org/10.5281/zenodo.16621723> and the PalaeoChip Arctic v1.0 bait sequences used for capture enrichment are located at <https://doi.org/10.5281/zenodo.5643845>."

Reviewer #2 (Remarks to the Author):

Murchie et al. have produced a thorough ancient environmental DNA study of ground squirrel coprolites spanning 700,000 years with some impressive results (e.g. mitogenomes of not just ground squirrels but also mammoths, bison, and horses). The amount of data, analyses, and information gleaned from these coprolites is a lot to say the least. The figures are all of high quality. Whenever I had a question or query about the methods or results, they were mostly answered in the main manuscript and supplementary information. This study is probably one of the most thorough I have seen on ancient environmental DNA from coprolites, showing when done well, they can be as powerful results wise as ancient DNA from individual bones. I hope the following comments are helpful in revising the manuscript.

Thank you for your comments, together with review 1, they have helped make this manuscript much clearer!

Major comments:

I felt that the introduction needed to be reworked as it left me somewhat confused and was not as polished as other parts of the manuscript. The opening paragraph (Lines 54-65) was repeated a lot in the first paragraph of the "ancient DNA from coprolites" section (Lines 80-88). I also felt that the second paragraph of the introduction (Lines 66-78) should be at the end of the introduction. These changes, and the ones detailed below (Line 157), would give you the space to have a big picture opening to the introduction built around coprolites as time capsules for reconstructing ecosystems (if only the associated problems could be solved).

We totally agree, we reworked this completely. The introduction/background area was a problem. See L31-45 and L52-131 for the revised intro.

Line 157: I would rename this section "Geological Setting" and have it separate to the introduction or at least make it clear that this is what this section is about. This change would make it clear why results (Line 160-165) are in this section.

Great point. See above about the background sections that are integrated now with the background. Because there were 'results' in this section, we moved it to the results.

Lines 194-220: Given the impressive amount of research and results that are presented in this study, I really liked the summary section you have at the start of the results. I would have liked to see another of these summaries about the big picture implications and discussion at the start of the discussion (similar to lines 924-942) before the in-depth discussion is undertaken. This would ensure the reader is not lost in the large amounts of data and analyses this study has undertaken.

Excellent point, we added this at the start of the discussion, see L514-526.

Lines 782-784: If I'm not mistaken (but happy to be proven wrong), ancient environmental DNA analysis was not undertaken on sediment surrounding the coprolites. Why was this not done as I thought this would have been a good way to test for DNA leaching and where the ancient environmental DNA signal is coming from (more so than you talk about in the manuscript, especially for samples that might not all be coprolite as you discuss)?

This is a great idea, and had the project started with the foreknowledge of the excellent metagenomics results we'd get from the samples, we would have done this. As we mention at L774 and L649, we agree that studying the sediments from around these coprolites is an excellent next step in coprolite aeDNA research. Our sediment samples from the same region help build support here in our metagenomic comparisons, but not nearly as strong as a direct on site 1:1 comparison would, of course. We intend to process sediments from around ground squirrel middens in several new research efforts in cases where we have both available (often the sites are thawing at the rate now that you can't go back to resample next session because the site has entirely slumped). At this time, this suggestion is beyond the scope of this research effort, but we hope to investigate that more fully to resolve this question in the near future.

Minor comments:

Lines 114-117: This sentence is confusing and needs rewriting. Remove the parentheses around "Sciuridae", and make it clear the family contains the species in question, rather than ground squirrels contain the species in question.

This sentence as been re-written now (L81)

Line 121 and elsewhere: Scientific names (genus and species) should be in italics (e.g. *Urocitellus*).

Thanks for finding this one (fixed now L87), we went through carefully to fix other mis-italicised instances where we found them.

Line 122 and elsewhere: Geographic names should be in capitals (e.g. Bering Strait and Arctic ground squirrel).

Fixed, L88, and all instances of Arctic/Subarctic have been properly capitalized now.

Line 126: Remove the extra ")" from this line.

Removed, L92 (and switched to Nat. Comms. style superscript citations).

Line 144 and elsewhere: Can you please include scientific names for species when you first introduce them with common names...this is done inconsistently in the manuscript (e.g. snowshoe hares).

We fixed this (now L107) and went through to make sure Latin names are included in the first instance with organisms discussed here.

Lines 237: In general, numbers ten or less need to be spelled out, while 11 and above can be numbers.

This paragraph (L224-233) has now been fixed. We have tried to make sure the rest of the article has this fixed as well.

Lines 337-338 and elsewhere: Please keep use of capitals in common names consistent throughout the manuscript (e.g. golden-mantled ground squirrel vs Thirteen-lined ground squirrel).

Fixed, L255

Line 545: Remove the ")" from this line.

Fixed, L452.

Thank you for the opportunity to revise and resubmit and thanks to both reviewers for their thoughtful comments. Our responses to the reviewer comments are provided below in blue.

RESPONSE TO REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

I think the manuscript now reads a lot better, I thank the authors for this rework. While I think this manuscript and data will become valuable resource to the community I still have a challenge with the way the authors handled my criticism on the low read taxa reporting. Besides from this last critique point, I think the authors answered my other concerns.

Thanks!

Major

I do appreciate the authors' explanation and agree that low-count signals can be useful for hypothesis generation and targeted follow-up. But I do not think it is sufficient to address this issue only through general wording in the figure captions. The manuscript still needs a clearer distinction between exploratory low-count detections and taxa that are sufficiently supported for inference in the present study.

I would therefore suggest either (i) moving unauthenticated low-read assignments to the Supplementary Information as tentative detections, or (ii) retaining them in the figure but marking the relevant taxonomic names with an asterisk or similar symbol, with the caption explicitly stating that these taxa had insufficient reads across all samples for authentication and should therefore be regarded as tentative.

While I agree with the authors that there is no universally accepted minimum read threshold in the field, I do not think that this in itself justifies continuing to report weak signals on the same footing as more strongly supported detections. The key issue is not the absence of a universal cutoff, but the need to communicate clearly which taxa are sufficiently supported for interpretation and which should remain exploratory. In my view, an important broader point here is that studies such as this one help set the standard for future work, and the authors therefore have an opportunity to guide the field toward a clearer and more transparent treatment of weak taxonomic signals.

Take for example, the total of 5 reads assigned to the genus Puma, 5 reads to Felinae, and 14 reads to Felidae, all recovered in only a single sample, that makes this taxonomic signal highly uncertain. This seems particularly so given that the broader ecological signal, including the recovered plant taxa, that obviously does not support such an interpretation. I therefore think such cases, and not just the Puma signal, should be presented much more cautiously and not placed on the same footing as better-supported detections.

Response: We thank the reviewer for this important comment. We agree that the previous revision did not sufficiently distinguish stronger detections from exploratory or weakly supported assignments. We have revised the analysis and presentation accordingly. Taxa are no longer treated on the same footing based on read counts alone; instead, support is assessed using an integrated framework combining the highly conservative count-matrix support from our map-filtered BLASTn to MEGAN workflow, library-specific Holi/metaDMG damage and significance, simple mapping/fit QC, negative-control screening, independent corroboration from macrofossils where available, and ecological/biogeographic plausibility. Thankfully, all of this data was already available but underutilized, we just needed to combine these workflows together into an ensemble summary table.

These support classes (detailed at the end of this response) and Holi/metaDMG data (previously only used in the supplementary significance/damage figures) are now reported explicitly in the revised supplementary source-data workbooks (Data S3), source data files, and represented in the updated figure symbology. This revision also clarifies why a universal read cut-off is not appropriate across classifiers. The workflows used here differ significantly in stringency, such that the same taxon can accumulate many more reads in broader mapping or damage-model contexts than in our conservative PNW map-filtered BLASTn to MEGAN workflow. *Puma* is a useful example: in Holi/metaDMG, the best library (SC-10) shows 414 reads, with damage = 0.124, significance = 2.33, alignments/read = 3.62, rho_Ac = 0.126, and MAP_valid = TRUE, whereas the conservative PNW map-filtered BLASTn to MEGAN-LCA workflow recovered only 5 reads for this taxon. We suspect that this organism is likely not *Puma* actually but its close relative *Miracinonyx* or another felid (driven by database incompleteness), but this still highlights that a read cut-off suitable for Holi/metaDMG is not transferable to a different classification workflow. Taken together, these results indicate an ancient-looking felid signal, which we discuss in the text as being of uncertain species origin (but potentially *Miracinonyx* given the relatedness to *Puma* and the biogeographic context). We therefore classify this taxon in the figure as tentative because of the ecological and biogeographic uncertainty (despite all of the metrics from Holi/metaDMG being sufficient to call this taxon as 'supported' based on damage and counts). The counts in other classifiers far exceed those in our conservative map->blast->megan workflow, highlighting that read counts across classifiers are not directly comparable. Our revised ensemble-support approach provides a more transparent and appropriately cautious treatment of weak, tentative, and higher-confidence signals. These new categories/data have been applied to figures 3/4/5 and this system was applied to the entire map->blast->megan and megablast->megan classified dataset (Supplementary_Data_3.xlsx).

Under this revised framework, taxa with exact damage support plus independent conservative-workflow support are distinguished from taxa that are only lineage-supported, ecologically plausible, weakly supported, or blank-associated. This allows low-support or biogeographically uncertain detections to be retained for completeness without placing them on the same footing as more strongly supported assignments. We thank the reviewer for raising this concern, because we think this ensemble revision has significantly improved the manuscript, and we expect to implement a version of this approach from the outset in future projects.

Below is a summary of the classification logic for the categories, which we have included in a new paragraph in the methods (L1259-1350, with mentions now at L170-173, captions for figures 3/4/5, and detailed in both the Source Data File and Supplementary_Data_3.xlsx).

aDNA Support

Very high confidence

- Exact Holi/metaDMG support (damage > 0.01, significance > 2, N_reads >= 100)
- strong conservative count-matrix support from BLASTn (>= 50 reads in one ancient library; present in >= 2)
- not blank-associated
- no strong QC concern (multi-taxon mapping impacting damage assessments)

High confidence

- Exact Holi/metaDMG support (damage > 0.01, significance > 2)
- strong conservative count-matrix support
- not blank-associated
- but below the extra N_reads >= 100 threshold and/or with QC caution

Supported

- Strong count support and/or exact damage support that falls short of "high confidence", such as Holi QC suggesting mixed mapping caution (to be expected with aeDNA and is observed even among organisms where we reconstructed high coverage mitogenomes such as mammoth, horse, and bison)
- not blank-associated
- and/or low-rank lineage-consistent damage support

Tentative

- Weaker DNA support, but ecologically/biogeographically plausible and/or corroborated by macrofossils or other evidence
- not blank-associated
- Also includes ecologically suspect organisms (far outside of known range), but that have damage and count support sufficient for "Supported" (e.g. Puma)

Weak support

- Weak DNA support (damage/count/coverage)
- not blank-associated
- no corroboration and uncertain ecological fit

Blank-associated / likely artefactual

- Substantial overlap with negative controls and weak ancient-sample support

Minor

In main Figures 1, 3, 4, and 5, there is a general challenge in balancing overall figure size and font size. However, in several cases there also appears to be room to increase the font size, which I think is necessary for readers to be able to view and interpret the figures properly where possible.

The resolution in the submitted manus also appears to be low. I assume this may be an artefact of the submission system, but if so, this should be corrected before any eventual publication.

We have gone through to improve font sizes with minor stylistic tweaks throughout for all of the figures. There are some limitations here because we have so much data to present, but the text size in all cases exceeds the sizes provided by Nat. Comms. in their formatting documentation. We can modified further if needed by the journal, but hopefully this should suffice. The low resolution I think did come from the submission system because the submitted files all have 300 dpi figures. We'll be submitting vector editable PDFs of all the main-text figures.

Reviewer #2 (Remarks to the Author):

Murchie et al. have done an in-depth and thorough revision and provided thoughtful explanations for their revisions. All my comments (and I believe the other reviewers) have been thoroughly addressed, especially the setting up of the study and why it's important in the introduction. I look forward to seeing this paper in print. I hope the following very minor comments are helpful.

Thanks!

Line 77: You have already defined ancient environmental DNA, so just use aeDNA.

Changed

Line 78: Change "those" to "the resulting"

Changed

Line 265: Can you please give the common name (snowshoe hare) for *Lepus americanus*.

Changed

Figure 3 and 4: "10" should be "ten"

Changed