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A comparison of eDNA to camera trapping for assessment of terrestrial mammal diversity

Kevin Leempoel, Trevor Hebert and Elizabeth A. Hadly

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Review timeline

Original submission:	26 June 2019
1st revised submission:	10 October 2019
2nd revised submission:	15 November 2019
3rd revised submission:	3 December 2019
Final acceptance:	4 December 2019

Note: Reports are unedited and appear as submitted by the referee. The review history appears in chronological order.

Review History

RSPB-2019-1509.R0 (Original submission)

Review form: Reviewer 1

Recommendation
Accept with minor revision (please list in comments)

Scientific importance: Is the manuscript an original and important contribution to its field?
Excellent

General interest: Is the paper of sufficient general interest?
Good

Quality of the paper: Is the overall quality of the paper suitable?
Excellent

Is the length of the paper justified?
Yes

Should the paper be seen by a specialist statistical reviewer?
No

Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.
No

It is a condition of publication that authors make their supporting data, code and materials available - either as supplementary material or hosted in an external repository. Please rate, if applicable, the supporting data on the following criteria.

Is it accessible?
Yes

Is it clear?
Yes

Is it adequate?
No

Do you have any ethical concerns with this paper?
No

Comments to the Author
Environmental DNA is an emerging tool for biodiversity monitoring. However, despite its importance, the application of the eDNA method to terrestrial mammal monitoring is much less than that of aquatic animals, and thus we still have very limited knowledge on terrestrial eDNA. Leempoel et al. evaluated the usefulness of environmental DNA (eDNA) approach as a tool for terrestrial (mammal) biodiversity monitoring by comparing its detection efficiency with that of a long-term camera-trap study. In addition, the authors examined the effects of soil sampling strategy, DNA extraction method and metabarcoding region on the detection efficiency. They collected 12 soil samples from seven/six sampling sites in Jasper Ridge Biological Preserve in California, USA and performed eDNA metabarcoding by using previously developed methods. One of the major findings of this study is that the eDNA approach detected almost all mammals regularly recorded with cameras, and that the eDNA approach detected many unrecorded small mammals that are usually difficult to be detected by cameras. While differences in soil sampling strategy and DNA extraction method did not produce significant differences, a long metabarcode was better than a short metabarcode in terms of taxonomic resolution. In addition, time-window based data analysis suggested that eDNA in soil could persist ca. 30-150 days.

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Review form: Reviewer 2

Recommendation
Major revision is needed (please make suggestions in comments)

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Good

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Protax uses a Bayesian framework and apart from the reference database it also accepts a reference taxonomy alongside. It accounts for an incomplete reference database and is able to place sequences with a certain probability to a genus even if the taxon is missing in the references. For more details and because they explain thing better than I please see the section on taxonomic assignment in the review of Piper et al 2019 doi: 10.1093/gigascience/giz092.

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Recommendation
Accept with minor revision (please list in comments)

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manuscript includes a small comparison of the efficacy of two different eDNA extraction methods; and the manuscript compares eDNA results with a long term inventory based on camera data. Comparisons of eDNA results and "traditional" inventory techniques are really important in this emerging field, and the system described here is ideal for such comparisons. Overall, I feel that the manuscript is well written and the science is sound, but I do have several suggestions that could improve the manuscript if addressed:

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- Lines 405-406 (or elsewhere in the discussion): I was surprised that the absence of raccoon from eDNA results was not discussed more thoroughly since it is was the only example where eDNA failed to find a camera result. What could explain this?
- Overall, another useful analysis to include that would help evaluate both eDNA and camera methodologies would be an evaluation of how many mammals were detected vs how many mammals are known to occur at the site overall (e.g., Line 443 indicates that there are 14 total bats known to occur here). Maybe a table with all known mammals at the site (based on any source) listed, then bold, underline, or both used to indicate which mammals were detected by which method.
- There is a pretty rich literature on metabarcoding mammal DNA from fecal samples that I was

surprised wasn't referenced in the introduction or discussion.

Decision letter (RSPB-2019-1509.R0)

13-Aug-2019

Dear Dr Leempoel:

I am writing to inform you that your manuscript RSPB-2019-1509 entitled "A comparison of eDNA to camera trapping for assessment of terrestrial mammal diversity" has, in its current form, been rejected for publication in Proceedings B.

This action has been taken on the advice of referees, who have recommended that substantial revisions are necessary. With this in mind we would be happy to consider a resubmission, provided the comments of the referees are fully addressed. However please note that this is not a provisional acceptance.

The resubmission will be treated as a new manuscript. However, we will approach the same reviewers if they are available and it is deemed appropriate to do so by the Editor. Please note that resubmissions must be submitted within six months of the date of this email. In exceptional circumstances, extensions may be possible if agreed with the Editorial Office. Manuscripts submitted after this date will be automatically rejected.

Please find below the comments made by the referees, not including confidential reports to the Editor, which I hope you will find useful. If you do choose to resubmit your manuscript, please upload the following:

1) A 'response to referees' document including details of how you have responded to the comments, and the adjustments you have made.
2) A clean copy of the manuscript and one with 'tracked changes' indicating your 'response to referees' comments document.
3) Line numbers in your main document.

To upload a resubmitted manuscript, log into http://mc.manuscriptcentral.com/prsb and enter your Author Centre, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions," click on "Create a Resubmission." Please be sure to indicate in your cover letter that it is a resubmission, and supply the previous reference number.

Sincerely,
Professor Hans Heesterbeek
mailto: proceedingsb@royalsociety.org

Reviewer(s)' Comments to Author:

Referee: 1

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• Overall, another useful analysis to include that would help evaluate both eDNA and camera methodologies would be an evaluation of how many mammals were detected vs how many mammals are known to occur at the site overall (e.g., Line 443 indicates that there are 14 total bats known to occur here). Maybe a table with all known mammals at the site (based on any source) listed, then bold, underline, or both used to indicate which mammals were detected by which method.

• There is a pretty rich literature on metabarcoding mammal DNA from fecal samples that I was surprised wasn't referenced in the introduction or discussion.

Author's Response to Decision Letter for (RSPB-2019-1509.R0)

See Appendix A.

RSPB-2019-2353.R0

Review form: Reviewer 2

Recommendation
Accept with minor revision (please list in comments)

Scientific importance: Is the manuscript an original and important contribution to its field?
Excellent

General interest: Is the paper of sufficient general interest?
Excellent

Quality of the paper: Is the overall quality of the paper suitable?
Good

Is the length of the paper justified?
Yes

Should the paper be seen by a specialist statistical reviewer?
No

Do you have any concerns about statistical analyses in this paper? If so, please specify them explicitly in your report.
No

It is a condition of publication that authors make their supporting data, code and materials available - either as supplementary material or hosted in an external repository. Please rate, if applicable, the supporting data on the following criteria.

Is it accessible?
Yes

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
The resubmitted manuscript „a comparison of eDNA to camera trapping for assessment of terrestrial mammal diversity" by Leempoel et al. compares the results gained from camera

trapping studies with the results of DNA metabarcoding of soil samples. It tries to bridge a gap, to make the emerging field of eDNA a more applicable technique that can be used by ecologists and conservation biologists.
The methods, results and discussion are reasonable and the important issues are well addressed and I enjoyed reading it even a second time. However, I still see some options for optimization of the study design, especially when it comes to the lab processing and taxonomic assignment. But nevertheless I think the manuscript marks an important step in the transition from eDNA studies just being a proof of concept to a more applicable tool for standardized wildlife inventories. I have a few comments that Ill address in the following.

General comments:
• Lines given in the authors responses did not match to the manuscript (also not the one with tracked changes) that made it a bit hard to follow their changes.
• Reading the method section and looking at the supplement I wondered if the workflow chosen by the authors is applicable to larger scale studies because it seems to involve a lot of manual editing that might be feasible with 100 samples but would become infeasible for 1000 samples. Maybe the authors can comment on this, because it affects the usability for larger biodiversity screenings e.g. at the landscape level.
• If you use a fragment of a genetic marker as barcode it would be good to use the more conventional gene name like 12S and 16S (in italics) instead of M12s and M16s.

Specific comments:
• Line 217; you compared MOTUs reported by both barcodes. To me this makes only sense if you bring them on the same taxonomic level otherwise it's a bit like comparing apples and oranges.
• Line 218, you did not evaluate technical replicates you evaluated different sampling strategies.
• Line 222-223; you used accumulation curves to compare assignment results of barcodes, sampling design and extraction methods. The latter two I would agree that it makes sense but for the assignment results I think it makes no sense if they are on different taxonomic levels (same as for line 217). If one is just not able to split the feline species into bobcat and puma it will never be as good as the other (another example see also line 259-261).
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• Line 269; Species names should be in italics.
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Decision letter (RSPB-2019-2353.R0)

30-Oct-2019

Dear Dr Leempoel:

Your manuscript has now been peer reviewed and the reviews have been assessed by an Associate Editor. The reviewers' comments (not including confidential comments to the Editor) and the comments from the Associate Editor are included at the end of this email for your reference. As you will see, the reviewer and the Associate Editor are positive about the manuscript but the reviewer has raised some (minor) issues that need to be addressed.

We do not allow multiple rounds of revision so we urge you to make every effort to fully address all of the comments at this stage. If deemed necessary by the Associate Editor, your manuscript will be sent back to one or more of the original reviewers for assessment. If the original reviewers are not available we may invite new reviewers. Please note that we cannot guarantee eventual acceptance of your manuscript at this stage.

To submit your revision please log into http://mc.manuscriptcentral.com/prsb and enter your Author Centre, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions", click on "Create a Revision". Your manuscript number has been appended to denote a revision.

When submitting your revision please upload a file under "Response to Referees" in the "File Upload" section. This should document, point by point, how you have responded to the reviewers' and Editors' comments, and the adjustments you have made to the manuscript. We require a copy of the manuscript with revisions made since the previous version marked as 'tracked changes' to be included in the 'response to referees' document.

Your main manuscript should be submitted as a text file (doc, txt, rtf or tex), not a PDF. Your figures should be submitted as separate files and not included within the main manuscript file.

When revising your manuscript you should also ensure that it adheres to our editorial policies (https://royalsociety.org/journals/ethics-policies/). You should pay particular attention to the following:

Research ethics:
If your study contains research on humans please ensure that you detail in the methods section whether you obtained ethical approval from your local research ethics committee and gained informed consent to participate from each of the participants.

Use of animals and field studies:
If your study uses animals please include details in the methods section of any approval and licences given to carry out the study and include full details of how animal welfare standards were ensured. Field studies should be conducted in accordance with local legislation; please include details of the appropriate permission and licences that you obtained to carry out the field work.

Data accessibility and data citation:
It is a condition of publication that you make available the data and research materials supporting the results in the article. Datasets should be deposited in an appropriate publicly available repository and details of the associated accession number, link or DOI to the datasets must be included in the Data Accessibility section of the article (https://royalsociety.org/journals/ethics-policies/data-sharing-mining/). Reference(s) to datasets should also be included in the reference list of the article with DOIs (where available).

In order to ensure effective and robust dissemination and appropriate credit to authors the dataset(s) used should also be fully cited and listed in the references.

If you wish to submit your data to Dryad (http://datadryad.org/) and have not already done so you can submit your data via this link
http://datadryad.org/submit?journalID=RSPB&manu=(Document not available), which will take you to your unique entry in the Dryad repository.

If you have already submitted your data to dryad you can make any necessary revisions to your dataset by following the above link.

For more information please see our open data policy http://royalsocietypublishing.org/data-sharing.

Electronic supplementary material:
All supplementary materials accompanying an accepted article will be treated as in their final form. They will be published alongside the paper on the journal website and posted on the online figshare repository. Files on figshare will be made available approximately one week before the accompanying article so that the supplementary material can be attributed a unique DOI. Please try to submit all supplementary material as a single file.

Online supplementary material will also carry the title and description provided during submission, so please ensure these are accurate and informative. Note that the Royal Society will not edit or typeset supplementary material and it will be hosted as provided. Please ensure that

the supplementary material includes the paper details (authors, title, journal name, article DOI). Your article DOI will be 10.1098/rspb.[paper ID in form xxxx.xxxx e.g. 10.1098/rspb.2016.0049].

Please submit a copy of your revised paper within three weeks. If we do not hear from you within this time your manuscript will be rejected. If you are unable to meet this deadline please let us know as soon as possible, as we may be able to grant a short extension.

Thank you for submitting your manuscript to Proceedings B; we look forward to receiving your revision. If you have any questions at all, please do not hesitate to get in touch.

Best wishes,
Professor Hans Heesterbeek
mailto: proceedingsb@royalsociety.org

Associate Editor Board Member
Comments to Author:
The authors have comprehensively addressed the comments from three reviewers of the original submission. One of the original reviewers is very positive about the resubmission, but has provided a very helpful set of constructive criticisms and recommendations which the authors should address.

Reviewer(s)' Comments to Author:

Referee: 2

Comments to the Author(s).
The resubmitted manuscript „a comparison of eDNA to camera trapping for assessment of terrestrial mammal diversity" by Leempoel et al. compares the results gained from camera trapping studies with the results of DNA metabarcoding of soil samples. It tries to bridge a gap, to make the emerging field of eDNA a more applicable technique that can be used by ecologists and conservation biologists.
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Author's Response to Decision Letter for (RSPB-2019-2353.R0)

See Appendix B.

Decision letter (RSPB-2019-2353.R1)

25-Nov-2019

Dear Dr Leempoel

I am pleased to inform you that your manuscript RSPB-2019-2353.R1 entitled "A comparison of eDNA to camera trapping for assessment of terrestrial mammal diversity" has been accepted for publication in Proceedings B.

The Associate Editor has recommended publication, but also suggests some final minor revision to your manuscript. Therefore, I invite you to respond to the comments and revise your manuscript. Because the schedule for publication is very tight, it is a condition of publication that you submit the revised version of your manuscript within 7 days. If you do not think you will be able to meet this date please let us know.

To revise your manuscript, log into https://mc.manuscriptcentral.com/prsb and enter your Author Centre, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions," click on "Create a Revision." Your manuscript number has been appended to denote a revision. You will be unable to make your revisions on the originally submitted version of the manuscript. Instead, revise your manuscript and upload a new version through your Author Centre.

When submitting your revised manuscript, you will be able to respond to the comments made by the referee(s) and upload a file "Response to Referees". You can use this to document any changes you make to the original manuscript. We require a copy of the manuscript with revisions made since the previous version marked as ‘tracked changes’ to be included in the ‘response to referees’ document.

Before uploading your revised files please make sure that you have:

1) A text file of the manuscript (doc, txt, rtf or tex), including the references, tables (including captions) and figure captions. Please remove any tracked changes from the text before submission. PDF files are not an accepted format for the "Main Document".

2) A separate electronic file of each figure (tiff, EPS or print-quality PDF preferred). The format should be produced directly from original creation package, or original software format. PowerPoint files are not accepted.

3) Electronic supplementary material: this should be contained in a separate file and where possible, all ESM should be combined into a single file. All supplementary materials accompanying an accepted article will be treated as in their final form. They will be published alongside the paper on the journal website and posted on the online figshare repository. Files on figshare will be made available approximately one week before the accompanying article so that the supplementary material can be attributed a unique DOI.

Online supplementary material will also carry the title and description provided during submission, so please ensure these are accurate and informative. Note that the Royal Society will not edit or typeset supplementary material and it will be hosted as provided. Please ensure that the supplementary material includes the paper details (authors, title, journal name, article DOI). Your article DOI will be 10.1098/rspb.[paper ID in form xxxx.xxxx e.g. 10.1098/rspb.2016.0049].

4) A media summary: a short non-technical summary (up to 100 words) of the key findings/importance of your manuscript.

5) Data accessibility section and data citation
It is a condition of publication that data supporting your paper are made available either in the electronic supplementary material or through an appropriate repository.

In order to ensure effective and robust dissemination and appropriate credit to authors the dataset(s) used should be fully cited. To ensure archived data are available to readers, authors should include a 'data accessibility' section immediately after the acknowledgements section. This should list the database and accession number for all data from the article that has been made publicly available, for instance:
• DNA sequences: Genbank accessions F234391-F234402
• Phylogenetic data: TreeBASE accession number S9123
• Final DNA sequence assembly uploaded as online supplemental material
• Climate data and MaxEnt input files: Dryad doi:10.5521/dryad.12311
NB. From April 1 2013, peer reviewed articles based on research funded wholly or partly by RCUK must include, if applicable, a statement on how the underlying research materials – such as data, samples or models – can be accessed. This statement should be included in the data accessibility section.

If you wish to submit your data to Dryad (http://datadryad.org/) and have not already done so you can submit your data via this link
http://datadryad.org/submit?journalID=RSPB&manu=(Document not available) which will take you to your unique entry in the Dryad repository. If you have already submitted your data to dryad you can make any necessary revisions to your dataset by following the above link. Please see https://royalsociety.org/journals/ethics-policies/data-sharing-mining/ for more details.

6) For more information on our Licence to Publish, Open Access, Cover images and Media summaries, please visit https://royalsociety.org/journals/authors/author-guidelines/.

Once again, thank you for submitting your manuscript to Proceedings B and I look forward to receiving your revision. If you have any questions at all, please do not hesitate to get in touch.

Sincerely,
Professor Hans Heesterbeek
Editor, Proceedings B
mailto:proceedingsb@royalsociety.org

Associate Editor:
Comments to Author:
The authors have now addressed all remaining referee comments and made appropriate changes. I encourage the authors to ensure that Table 1 is of publication quality, with a fully informative caption explaining the taxonomic levels and abbreviations, and consistent row headings. I would encourage one row or set of rows explicitly for each of the named 15 families listed in the

response to referees - perhaps with the species, genus or sub-family in parenthesis after the family name, if there was only one possible representative of the lowest taxonomic level. This would be consistent with the focus at the family level. Please also consider whether the three repeated columns of 16S, 12S and Camera Trap could be simplified in some way to aid the presentation.

Decision letter (RSPB-2019-2353.R2)

04-Dec-2019

Dear Dr Leempoel

I am pleased to inform you that your manuscript entitled "A comparison of eDNA to camera trapping for assessment of terrestrial mammal diversity" has been accepted for publication in Proceedings B.

You can expect to receive a proof of your article from our Production office in due course, please check your spam filter if you do not receive it. PLEASE NOTE: you will be given the exact page length of your paper which may be different from the estimation from Editorial and you may be asked to reduce your paper if it goes over the 10 page limit.

If you are likely to be away from e-mail contact please let us know. Due to rapid publication and an extremely tight schedule, if comments are not received, we may publish the paper as it stands.

If you have any queries regarding the production of your final article or the publication date please contact procb_proofs@royalsociety.org

Your article has been estimated as being 10 pages long. Our Production Office will be able to confirm the exact length at proof stage.

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Electronic supplementary material:
All supplementary materials accompanying an accepted article will be treated as in their final form. They will be published alongside the paper on the journal website and posted on the online figshare repository. Files on figshare will be made available approximately one week before the accompanying article so that the supplementary material can be attributed a unique DOI.

You are allowed to post any version of your manuscript on a personal website, repository or preprint server. However, the work remains under media embargo and you should not discuss it

with the press until the date of publication. Please visit https://royalsociety.org/journals/ethics-policies/media-embargo for more information.

Thank you for your fine contribution. On behalf of the Editors of the Proceedings B, we look forward to your continued contributions to the Journal.

Sincerely,
Proceedings B
mailto: proceedingsb@royalsociety.org

Appendix A

Referee: 1

Environmental DNA is an emerging tool for biodiversity monitoring. However, despite its importance, the application of the eDNA method to terrestrial mammal monitoring is much less than that of aquatic animals, and thus we still have very limited knowledge on terrestrial eDNA. Leempoel et al. evaluated the usefulness of environmental DNA (eDNA) approach as a tool for terrestrial (mammal) biodiversity monitoring by comparing its detection efficiency with that of a long-term camera-trap study. In addition, the authors examined the effects of soil sampling strategy, DNA extraction method and metabarcoding region on the detection efficiency. They collected 12 soil samples from seven/six sampling sites in Jasper Ridge Biological Preserve in California, USA and performed eDNA metabarcoding by using previously developed methods. One of the major findings of this study is that the eDNA approach detected almost all mammals regularly recorded with cameras, and that the eDNA approach detected many unrecorded small mammals that are usually difficult to be detected by cameras. While differences in soil sampling strategy and DNA extraction method did not produce significant differences, a long metabarcode was better than a short metabarcode in terms of taxonomic resolution. In addition, time-window based data analysis suggested that eDNA in soil could persist ca. 30-150 days.

This is an interesting and well-written manuscript that provides important information on eDNA-based mammal biodiversity monitoring. The methodology, results and discussion are all reasonable and they are mostly properly described in the main text and supplementary information. The main result that the eDNA-based survey detected all regularly recorded mammals is good news for ecologists. The non-significant differences in the soil sampling strategy and DNA extraction method are practically important. In addition, although the authors did not state in the Abstract, the finding that soil mammalian eDNA could persist ca. 30-150 days is very interesting.

Overall, I think that this study is timely, important and concise enough, and thus is worth publishing. I appreciate the authors' effort to perform the study. I really enjoyed reading the manuscript.

Based on my reading, there is no serious flaw in the method (i.e., soil sampling, DNA extraction, library preparation and sequence processing). Therefore, I have only a few comments on the manuscript.

- Abstract: The finding that soil eDNA could persist ca. 30-150 days may be included.

We modified the concluding paragraph to highlight this finding.

- L61-63: Ref. 19 performed their study in a tropical lowland forest in Borneo. Therefore, you should mention that ref. 19 is not done in enclosed environment.

We expanded this paragraph by adding more references. L60-67

- L89: I do not recommend to use the term "parameters" here. This is because "parameter" is often used in the context of statistics and thus the use of "parameter" here might be confusing. Another word such as "protocols" or "procedures" would be better.

We modified the term to "experimental design" instead, to reflect the diverse sampling, extraction and primers chosen. L92

- L118 (first 2 cm): Please indicate that this is a depth.

Modified. L121

- L150-152: Did you include replicates in the first PCR (e.g., duplicate first PCR for each DNA extract)? If yes, please provide the number of replicates. If no, please clearly state that the first PCR does not include replicates.

We did not perform PCR replicates but have multiple extractions per soil sample. We summarized the number of PCRs, samples etc for clarity (L169-172). We have also included

results from combined 12s and 16s data (L277-288), and further discussed replication issues (L384-393).

- L165: If computer scripts to perform the pipeline described here is available (e.g., in Github), please describe it.

We published the scripts on Github.
https://github.com/kleempoel/Soil_eDNA_Leempoeletal2019.git

- Figure 1: Please indicate what the gray regions indicate. (95% confidence intervals?)

Legend modified. Gray areas correspond to the standard deviation, estimated from random permutations.

- L368-371: "types of habitat" was not well explained in the Method. If you discuss this, you need to explain either in "Study area and camera trapping" or in Supplementary Information (e.g., as caption in Figure S1).

Habitats were added to Figure S1 and mentioned line 110

- L394-408: I think this paragraph provides important information on terrestrial eDNA behavior (just a comment).

We thought so too, thank you.

- Figure S1: It was a bit difficult to understand what "the 7 sampling sites" in Figure S1 means. According to the Method, you collected soil samples from six sites, so why "the spring" is mentioned in the caption? Where is the spring (E0)? You need to add some more explanations on the sampling sites.

We initially collected water at the spring but decided not to include that site in the paper. We modified the legend accordingly.

Referee: 2

Thanks for sharing this nice and interesting work with us; I enjoyed reading it. The study intents to validate eDNA matabarcoding in comparison to camera trapping and the overall study design is well elaborated and scientifically sound. The presentation of the results could be a bit more straightforward and clearer, but the discussion is very well written, comprehensive and thoroughly. The study is of interest because despite the numerous eDNA publications popping up everywhere there are only a few studies so far comparing eDNA metabarcding with other conventional methods and evaluating it and the authors address some important questions in their manuscript. Thus, the manuscript is interesting for any researcher assessing or monitoring terrestrial biodiversity. Although I am aware that the authors worked very thoroughly I have one criticism; they addressed so many different issues (different sampling methods, different extractions) that they lack of technical replicates for some comparisons and (with all their precautions) positive hits were accepted based on single positive PCRs. Given that you work with degraded DNA which increases the stochasticity of PCR a lot this is not something I would recommend. For good reason there is a lot of discussion on the acceptance of false-positives and/or false-negatives and why replication is so important in eDNA studies (please see Ficetola et al 2014 doi: 10.1111/1755-0998.12338, Ficetola et al 2016 doi: 10.1111/1755-0998.12508, Taberlet et al 2018 doi:10.1093/oso/9780198767220.001.0001, Zepeda-Mendoza et al 2016 doi: 10.1186/s13104-016-2064-9).

Nevertheless I still consider this manuscript as to valuable than to reject it because of that and the authors have replicates at higher levels (12S/16S, different sampling strategies). I would just like to see that they are a bit more aware of this issue and I would like to combine 12S and 16S replicates in table 1 in a way that the reader can see at one glance in how many replicates of a site an taxonomic assignment (or MOTU) was positive. I would suggest to order the MOTUs in rows taxonomically (first family, then indented species) and for each row you can then say yes or no (or ss20/ss80/both) for the regarding replicate.

Yes, it's true we should have covered PCR replicates more extensively. We therefore changed Table 1 to have the number of positive PCRs at each site, modified the results accordingly (L277-286), and included a paragraph in the discussion (L384-393).

To be honest I got a bit lost with the total number of replicates per site and PM and PB (apart from "one is cheaper than the other" I miss a comparison of their results). With 2 sampling strategies (SS20/SS80), 2 extraction methods (PM/PB) and 2 genetic markers (12S/16S) I would end up with 2x2x2=8 replicates per site (that'd be 12S/SS20/PM, 12S/SS80/PM, 12S/SS20/PB, 12S/SS80/PM, 16S/SS20/PM, 16S/SS80/PM, 16S/SS20/PB, 16S/SS80/PM) but the authors have 6 per site(?). If they have 6 per site in total, how can they have 36 PCRs for 12S and 36 PCRs for 16S as shown in figure 1? Maybe the authors can make it a bit more easy and clear for stupid people like me. How many replicates were there per site?

We summarized the number of PCRs per soil sample at the end of the methods (L169-172). We had 6 sites, from which we collected 2 soil samples each. From each soil sample, we performed 3 extraction (1 PM and 2 PB) and for each extraction, we performed 2 PCRs (12s and 16s) . So 6x2x3x2=72 PCRs.

Lines 10-12: The first sentence is so not correct because there are already a few studies comparing metabarcoding results with camera trapping data; e.g. please see Weiskopf et al 2018 doi: 10.1111/1365-2664.13111, Abrams et al 2019 doi: 10.1111/1365-2664.13411 or Drinkwater et al 2018 doi: 10.1111/1755-0998.12943 who at least compare their results a bit with CT data.

Thank you for these references, we updated the introduction (L51-67).

Lines 60-64: Not knowing all papers, that might be true for soil derived eDNA put there are several other studies on different eDNA sources like water holes, salt licks, vegetation swaps, snow or invertebrate derived DNA (iDNA), which is just a sub-discipline of eDNA, that focused on terrestrial mammals.

Same as previous comment.

Lines 261-263: That is exactly why you should rely on replicates. In Fig 1a you see the noise that is caused by PCR stochasticity. In Fig 1b you look at the replicates and then 12S and 16S are more comparable and represent reality most likely much better.

To get a clearer picture, we added accumulation curves where we compared extraction methods and soil sampling by grouping 12s and 16s (L283-289). We expanded these results and discussion and think the results are clearer.

Line 328 "…could offer a meaningful alternative…" I don't think it can be an alternative. Both methods have their pros and cons and it depends on the research question. I would say it's a good addition.

Changed to "complement"

Line 363: "..ideal for extraction in the field…" A major risk in metabarcoding studies are contaminations and the do occur even under lab conditions. Thus I would not recommend extraction of nucleic acids in the field. Not everything that's possible must be right.

We haven't tried extractions in the field and thus cannot compare with the results in this paper. However, it is difficult to get exportation permits for samples in certain countries and, therefore, being able to perform the extraction, PCR and sequencing in the field would be advantageous.

Line 364-367: First the authors state that their results are in line others and that it is etter to have many small samples, then in the next sentence they say that their results show it's good to have large amounts of soil per site…

Are there pros and cons for both methods and you can't say which one is better? I don't know, but this contradicts each other a bit.

Yes, we understand it is a bit misleading. But our point is to say that, for one soil sample, it is better to collect multiple sub-samples and pool them together, rather than collecting it all in one soil pit, in part because the surface of the soil is where DNA from animals is most likely to collect. We feel that collecting a large volume is necessary given low deposition rate of shed DNA from animals that are walking across the ground surface. We modified the text accordingly and also added a recommendation for more PCR replicates (L384-393).

Lines 368-371: Could it be due to the UV radiation degrading the DNA?

Yes, that is our interpretation and we added it to the text (L391).

Lines 373-375: Please see Abrams et al 2019 doi: 10.1111/1365-2664.13411

Added, L394-398

Lines 378-379: Good point! Don't want to bring up just negative points. This discussion is bringing up several good points and gives good arguments. There were also some other sections before, very nice.

Thanks!

Lines 411-419: Regarding incomplete reference databases maybe you should read this paper Axtner et al 2019 doi: 10.1093/gigascience/giz029

Thank you for the reference. Our understanding of this paper is that they base their analysis on curated databases such as BOL or Silva. Combined with taxonomic assignment approaches based on phylogeny, this seem to be an ideal solution. But the issue we brought up as more to do with the lack of reference sequences for many species, which we believe is a major obstacle for a universal use of eDNA.

Lines 425-428: Good point, very true

Lines 438-442: Instead of using simple similarity scores the authors might consider doing their taxonomic assignment using PROTAX from Somervuo et al 2016 doi: 10.1093/bioinformatics/btw346. Protax uses a Bayesian framework and apart from the reference database it also accepts a reference taxonomy alongside. It accounts for an incomplete reference database and is able to place sequences with a certain probability to a genus even if the taxon is missing in the references. For more details and because they explain thing better than I please see the section on taxonomic assignment in the review of Piper et al 2019 doi: 10.1093/gigascience/giz092.

Thank you for the suggestion, we mentioned this method in the discussion (L449).

Fig 1 In the manuscript the authors are talking of Fig 1a and Fig 1b, I'm missing that in the figure, should be consistent.

Figure updated with a and b.

Fig 2 The vole in the figure is obviously not a vole (long tail, big ears), maybe the authors can find a better image.

We changed the drawing of the vole.

Referee: 3

I have reviewed the manuscript "A comparison of eDNA to camera trapping for assessment of terrestrial mammal diversity by Leempoel et al. The manuscript describes the application of eDNA metabarcoding using two different assays designed to amplify across mammals; the manuscript includes a small comparison of the efficacy of two different eDNA extraction methods; and the manuscript compares eDNA results with a long term inventory based on camera data. Comparisons of eDNA results and "traditional" inventory techniques are really important in this emerging field, and the system described here is ideal for such comparisons. Overall, I feel that the manuscript is well written and the science is sound, but I do have several suggestions that could improve the manuscript if addressed:

- *Lines 10, 328: In several instances, eDNA is described as an "alternative" to traditional sampling methods, which I think is a mischaracterization that is dismissive to practitioners of such traditional methods and, as the data in this manuscript illustrate, not scientifically sound. I think it would be better to describe eDNA as an additional tool in the toolbox or complement to existing methods rather than a method that will replace others.*

We modified the instances of "alternative" and modified our concluding paragraph (L480-493).

- *Lines 76-79: this sentence feels incomplete; WHY is extracellular eDNA adsorption inconvenient? Long-term preservation of eDNA leading to spurious positive detections? Resistance to extraction methods?*

Yes, the issue is related to the resistance of soil particles to releasing DNA. By saturating the mixture with a phosphate buffer, DNA fragments are more likely to be liberated from the soil matrix. We modified this sentence and refer to 2 references for further detail (L80-82)

- *Line 119: "Falcon" is a brand name and should be capitalized. OR just refer to the tube as a generic centrifuge tube.*

Changed.

- *Line 159: "each primer pairs" should be "each primer pair"*

Updated

- *Line 216: "using a 1000 permutations" should be "using 1000 permutations"*

Updated

- *Lines 248-249: Why was the gray fox considered a contaminant species? It is listed in Figure 2 as being detected by camera traps, so it is present at the site.*

The gray fox was considered a contaminant in M12s analyses because of the high relative read abundance in the negative controls, but yes it was present at many sites. We still decided to keep it in some of our results.

- *Table 1: It would be helpful to state that each column represents a site. Also, the site names are confusing- do the two B-sites have something in common (and the two E-sites)? I suspect these site names are carryovers from the cited work, but it would be useful to explain site ID in a little more detail (or rename sites for this study) to avoid unnecessary confusion.*

We renamed sites to ABCDEF. The initial names had to do with the network of camera traps (cameras are connected wirelessly, and the letter is related the installation time).

- *Table 1: I expected more rigorous discussion of differences between markers at specific sites (although some of this does appear briefly in the discussion Lines 385-388). For example, Felidae is detected by M16s at site A2 but not B2; Puma concolor is detected by M12s at site B2 but not A2. Why?*

We attempted an explanation and extended the discussion at line 407-416.

- *Table 1: I am not sure what the purpose of the "%Both" row is. Additional discussion of this point would be helpful.*

We changed Table 1 but kept and improved the comparison of primers in the text (L267-280)

- *Lines 284-286 and Lines 364-365: Although it is intuitively appealing, I disagree that this study demonstrates the benefit of 80 samples over 20 samples. By my count, SS80 "outperformed" SS20 in M12s by a "Score" of 24 to 22 (SS80 working exclusively vs SS20 working exclusively). Lines 284-286 suggests that this is a "win" for SS80, but in M16s, SS20 "won" by a similar margin 14-12, which would lead to the exact conclusion. Why is one "win" more conclusive than the other?*

Sorry if this was confusing. We meant to say that with the 12s, SS80 slightly outperformed SS20, while with the 16s, the curves are similar. Not a clearcut result and, with the combination of 12s and 16s, this advantage disappears. We modified the results and discussion accordingly (L287-295).

- *Line 308 and Figure 3: If the relationship between # sites and # eDNA detections were correlated significantly only at 30 days, then what is Figure 3 (right) showing? It appears that significant results ($p<0.0028$) occur at many time points.*

Figure 3 right shows that the relationship was significant at 30 days (black point), whereas all the periods are not significant (represented by a red point). More generally, we see the regression coefficient decreasing with time, suggesting the relationship between camera trapping and eDNA is less accurate as we go further back in time.

- *Line 349: An additional explanation for bovid and suid detection may be reagents- many laboratory reagents (e.g., the obvious bovine serum albumin BSA) are potential sources of contamination in metabarcoding studies and may be worth evaluating.*

Yes, that is true and we added it to the list; indeed in other projects we discovered agricultural contaminants in our reagents and tubes! We did not use BSA in this project, but several colleagues are using it (L365).

- *Line 399: "This suggest" should be "This suggests"*

Corrected

- *Lines 405-406 (or elsewhere in the discussion): I was surprised that the absence of raccoon from eDNA results was not discussed more thoroughly since it is was the only example where eDNA failed to find a camera result. What could explain this?*

We explain why at line 422-427. Raccoons were rarely seen on cameras, even in 2009. After the return of puma in 2013, they were spotted even less. We believe these two factors explain why we did not detect them.

- *Overall, another useful analysis to include that would help evaluate both eDNA and camera methodologies would be an evaluation of how many mammals were detected vs how many mammals are known to occur at the site overall (e.g., Line 443 indicates that there are 14 total bats known to occur here). Maybe a table with all known mammals at the site (based on any source) listed, then bold, underline, or both used to indicate which mammals were detected by which method.*

Yes it's a good idea. We added that table in supplementary tables. However, the list of mammals in the preserve is not based on very recent surveys, except for the species recorded by cameras. Our study points to the weakness of using historic distribution maps for summaries of species occurrence data even in protected areas such as JRBP.

- *There is a pretty rich literature on metabarcoding mammal DNA from fecal samples that I was surprised wasn't referenced in the introduction or discussion.*

Yes, we mentioned that field of eDNA studies in the introduction (L54-56).

Appendix B

Response to Referees
The authors have comprehensively addressed the comments from three reviewers of the original submission. One of the original reviewers is very positive about the resubmission, but has provided a very helpful set of constructive criticisms and recommendations which the authors should address.

Reviewer(s)' Comments to Author:

Referee: 2

Comments to the Author(s).
The resubmitted manuscript „a comparison of eDNA to camera trapping for assessment of terrestrial mammal diversity" by Leempoel et al. compares the results gained from camera trapping studies with the results of DNA metabarcoding of soil samples. It tries to bridge a gap, to make the emerging field of eDNA a more applicable technique that can be used by ecologists and conservation biologists.
The methods, results and discussion are reasonable and the important issues are well addressed and I enjoyed reading it even a second time. However, I still see some options for optimization of the study design, especially when it comes to the lab processing and taxonomic assignment. But nevertheless I think the manuscript marks an important step in the transition from eDNA studies just being a proof of concept to a more applicable tool for standardized wildlife inventories. I have a few comments that Ill address in the following.

We first want to thank all reviewers for their comments and suggestions. Our paper was significantly improved thanks to their positive and meaningful inputs, we are very grateful for that.

General comments:
- Lines given in the authors responses did not match to the manuscript (also not the one with tracked changes) that made it a bit hard to follow their changes.

We made sure to check line numbers twice this time. Line numbers are those of the non-track change version

- Reading the method section and looking at the supplement I wondered if the workflow chosen by the authors is applicable to larger scale studies because it seems to involve a lot of manual editing that might be feasible with 100 samples but would become infeasible for 1000 samples. Maybe the authors can comment on this, because it affects the usability for larger biodiversity screenings e.g. at the landscape level.

Yes it's a good point, this filtering is at the moment only applicable to relatively small datasets. More complete and curated reference databases, as well as standardized protocol are needed before eDNA biodiversity monitoring can be scaled up. We modified the conclusion to reflect these drawbacks (L465-468)

- If you use a fragment of a genetic marker as barcode it would be good to use the more conventional gene name like 12S and 16S (in italics) instead of M12s and M16s.

We updated all names throughout the manuscript.

Specific comments:
- Line 217; you compared MOTUs reported by both barcodes. To me this makes only sense if you bring them on the same taxonomic level otherwise it's a bit like comparing apples and oranges.

L217-222, L263-264 & Fig1. Yes, that is very true. We brought all MOTUs to family level and modified the graphs as well as the description of those results accordingly.

- Line 218, you did not evaluate technical replicates you evaluated different sampling strategies.

It is indeed an incorrect way of describing it. We removed the sentence.

- Line 222-223; you used accumulation curves to compare assignment results of barcodes, sampling design and extraction methods. The latter two I would agree that it makes sense but for the assignment results I think it makes no sense if they are on different taxonomic levels (same as for line 217). If one is just not able to split the feline species into bobcat and puma it will never be as good as the other (another example see also line 259-261).

L263-264, Fig1 and FigS2. Given the lack of PCR replications, and to be coherent with the comparison of metabarcodes, we decided to bring all these comparisons at family level. We thus report families that were detected by one or the other subsampling/extraction kit, and updated accumulation curves accordingly.

- Lines 246-248; "…detected one additional contaminant, the gray fox…" I think you should add that you detected it in the negative controls; it makes it clearer why you discarded it.

L245-247. Sentence was clarified accordingly

- Line 252; are you sure it's 16 families? I counted only 15, but asking taxonomist is like asking lawyers, two have three different opinions.

L254 & Table 1. We recounted and indeed found 15:

Canidae, Cricetidae, Cervidae, Felidae, Geomyidae, Muridae, Leporidae, Talpidae, Mephitidae, Didelphidae, Equidae, Sciuridae, Suidae, Bovidae, Vespertilionidae, .

- Line 261; you use "Tamias spp." but list only the genus only once in the table 1. The abbreviation is used if you had several species of the genus Tamias in you samples (like for Myotis), was that the case? Then lease correct table 1 otherwise use Tamias sp.

L262. Corrected

- Line 269; Species names should be in italics.

L270. Corrected

- Line 271; if this is the final table it should replace table 1! Why are you hiding this wonderful piece of information? Although I appreciate that you added the number positive replicates to the table 1, but still I find it hard to compare the results of both marker with each other because you use so many different taxonomic levels. Actually I was about to asked you for a similar layout as in the supplement table 14 (Please see attached example table). Also I would like to see the camera trapping (CT) results for comparison. I think such a format including the CT results would be really, really helpful for the readers.

Table 1. The format you proposed is indeed an improvement to our supplementary table and we therefore decided to replace table 1 with this one.

- Lines 291-304; just to get this right, you got a correlation with the animals recorded in the camera traps using an occasion length of 30 days but you did not find consistency in site occurrences of species between soil and camera results. I think this is quite a discrepancy in the results that makes me feel a bit

unconfident on the conclusion. I don't see how DNA of a species at site A should be related to the presence of a species at site D documented by an image. The time corridor of 30 days seems reasonable to me if I expect to detect DNA at site A originating from the same animal that I observed with an camera at site A. In the discussion you discuss first this discrepancy and then the temporal aspect. I would switch this, and suggest to talk first about the temporal relation (30 days window), and then display the contrasting result that there was no spatial relation. I think you should point out to this discrepancy a bit more and very careful with your conclusion in this direction.

We agree with this suggestion and have separated the discussion on spatial and temporal accuracy in two sections. We start with the temporal accuracy and frequency of sightings (L370-386), while the following paragraph focuses on the lack of spatial consistency (L387-405).

- Figure 3; maybe you can make it unique with figure 1 using A and B instead of left and right.

Fig 3. Modified

- Line 317-318; blastn should be BLASTN and maybe give reference.

L327. Modified and added reference

- Line 336-338; low quality DNA is one of the reasons why you should focus a bit more on PCR stochasticity, the use of replicates and the discussion on false-positive / false-negative detections. I still think you don't address these points enough throughout the discussion (also line 347-349) and I think they are really essential for producing reliable results in DNA metabarcoding, especially if you are dealing with low amounts and/or bad quality of target DNA. There is a lot of papers already out there and I am missing them somehow, especially as you observe large standard deviations in your accumulation curves.

We understand the concern and have switched these two paragraphs in the discussion. We thus discuss first the high standard deviation, lack of PCR replicates and existing literature (L350-361). Then address experimental design in the following paragraph, in light of that stochasticity (L362-369). While we fully agree that our accumulation curves are limiting our analyses, we still find that our comparison of experimental designs valuable when merging metabarcodes at family level and combining all sites.

- Line 389 & figure 2; What is the time window of detections that resulted in the figure 2? Asking, because the badger and the mustelid are not in there.

Fig2, Table S11 and L380-386. Yes, thank you for pointing that out. We decided to add all mammals recorded by cameras during the year before sampling. Domestic cat and weasel were thus added to the figure. These species were not initially in the figure because of the low number of recordings. Note that badger have not been seen at these sites since 2013. We interpret why they were not detected in the discussion.

- Line 403-410; Already last time I pointed out to Axtner et al. in 2019 in Gigascience because what you mention here is exactly what they observed. If you look at their figure 6 they show a comparison on how many tetrapode species listed in the IT IS database could be identified to the species level (or higher taxonomic level) based on curated GenBank entries. For 12S and 16S it was 25-30% for all mammals. Furthermore, working in a biodiversity hotspot they found only half of their focus-species represented with a single haplotype in only one of the four markers they used. That is why I pointed this study out to you, they analyzed exactly what you are talking of in line 395-415.

L421-426. Thank you for reminding us of that study, it is indeed of great support to our argument and we expanded this section with their findings as follows. If I read their paper correctly, they found that each marker covers more than half of the target species, but none achieve >85% coverage. And by combining all markers for all known tetrapod, not more that 50% of species per class are covered at species level. We decided not to include their finding on the lack of haplotype diversity as we are unfortunately running out of space and find the point less relevant to our paper.