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Mitonuclear interactions and introgression genomics of macaque monkeys (*Macaca*) highlight the influence of behaviour on genome evolution

Ben J. Evans, Benjamin M. Peter, Don J. Melnick, Noviar Andayani, Jatna Supriatna and Anthony J. Tosi

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Original submission:	17 May 2021
1st revised submission:	5 August 2021
2nd revised submission:	2 September 2021
Final acceptance:	14 September 2021

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

Review History

RSPB-2021-1107.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?
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
This study investigates challenging questions related to the effects of male-biased dispersal and mitonuclear interactions on genome-wide patterns of population structure and introgression. In my view, the authors made a compelling argument that this complex of southeast Asian macaque species is a particularly interesting and valuable one to test predictions about the evolution of mitonuclear incompatibilities. The resulting evidence for differential evolutionary pressures on "N-interacting" genes seemed less compelling, as there were a number of conflicting signals and alternative interpretations. But I thought the authors did a good job in providing a balanced discussion of this nuanced dataset. The analysis is extensive, and I found that the manuscript was well written and clearly presented. Overall, I feel that it makes a valuable contribution, and I only have a few minor comments.

1. To what extent could the finding of higher population structure for the X-chromosome than for autosomes be attributable to lower effective population size for the X? I believe humans also have more structure for the X than autosomes even though the discussed mechanisms of the Large X Effect and male-biased dispersal may be less relevant.

Ramachandran S, Rosenberg NA, Zhivotovsky LA, Feldman MW. 2004 Robustness of the inference of human population structure: a comparison of X-chromosomal and autosomal microsatellites. Hum. Genomics 1, 87 – 97.

Li JZ et al. 2008 Worldwide human relationships inferred from genome-wide patterns of variation. Science 319, 1100 – 1104.

2. Line 306. I understand the point about NDUFAF3 not co-precipitating with ND1, but this also raises the point that the wealth of structural data for mitonuclear enzyme complexes in mammals is not used in this study. One common discussion point in the literature is that mitonuclear incompatibilities might come down to a small number of positions, leading to signal to noise problems in this type of genome-wide scan. In that sense, taking structural data into account could produce a more targeted set of potential incompatibilities. For example, a resource such as MitImpact 3 (Castellana et al. 2021 NAR) could be useful in this respect. Given the already extensive analysis, I do not think it is necessary to incorporate this type of structural

analysis, but the authors could point to this area as an important one for future work.

3. Typos:

Line 60. ARP2 should be ARS2.
Line 267. support gene flow [in] the hybrid zone

Review form: Reviewer 2

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

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

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

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

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

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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
This manuscript reports the sequencing of 29 individuals from eight macaque species, and the interspecies differentiation and metrics of positive selection, low intraspecies polymorphism, and atypically long runs of homozygosity associated with nuclear- encoded genes that interact with mitochondria-encoded genes. This study represents a valuable attempt providing novel insights

into the evolutionary genomics of macaques. Detailed comments are below:

1. Line 88-91: These two sentences are also reviewed in reference 17?
2. Line 100: seven or eight species? It is not necessary to discuss the species clarification here. It is better to follow the current description.
3. Line 118: Table S1 and Fig 1 just showed the sample list and sampling locations. It is necessary to show the divergence of the mt-genomes of herein studied samples.
4. Fig 1 and Table S1: there are serial numbers in Fig 1 and it is also necessary to add in Table S1 for each samples. There are 29 samples in Table S1 and only 27 samples in Fig 1. I know maybe two samples are not with geographical information. But in this case it will confuse the reviewers and readers.
5. Fig 1: Why is the sample 22 in a different color comparing to other Tonkean macaques. Might be another species? It will confuse the readers.
6. Fig 1 and Table S1: M. brunnescens or M. o. brunnescens? It looks like that M. o. brunnescens is one the two subspecies of Macaca ochreata?
7. Line 402: eight or nine macaque species? Line 280 "eight macaque species"?
8. Does the N-interact genes show the similar pattern in other macaques or primates with highly-diverged mt-genomes? Or an opposite pattern in other macaques or primates with slightly-diverged mt-genomes? I think it should be an important comparison about this issue.
9. Line 380: even N-interact genes are frequently subjected to positive selection, and frequently embedded in atypically long ROHs, I am afraid that they are not the direct evidence for the links between behaviour and genome evolution. Maybe these genes could be affected by other factors.
10. I also suggest the authors to describe the non-synonymous and synonymous mutations in N-interact genes.

Above all, I would like to suggest a "major revision" for this manuscript. However, its final acceptance depends on the revision of the manuscript that satisfactorily responds to the comments from reviewers. The data is valuable for publication, but I have to say the authors have not fully demonstrate their results. I also hope the authors will re-organize the languages throughout the text.

Review form: Reviewer 3

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

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

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

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

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No

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No

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Yes

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No

Comments to the Author
The authors present a study on how behavior may influence genome evolution, using genomic data from eight species of macaque monkeys. Most macaque monkeys have sex-biased dispersal, with females staying within their natal range while males migrate. This means that mitochondria lineages will evolve relatively independently between populations, which is also manifested as high differentiation between species. The authors hypothesize that the high level of diversity of mitochondrial DNA may create positive selection for compensatory mutations on genes which interact with mitochondrial genes and mitochondrial DNA. Since females carry two X chromosomes but males only one, the authors also hypothesize that the social system of these monkeys will lead to higher intraspecific population structure across hybrid zones for the X chromosome, compared to autosomes.

To test these hypotheses, the authors first identify 211 nuclear-encoded genes which interact with mitochondria-encoded genes ("$N_{interact}$"). They use different population genetics metrics (Fst, Tajima's D, π, Fay and Wu's H, ROH) to test if genome windows (100 kb) containing these genes differ from other genome windows. They also study mitochondria introgression and effects of sex-specific gene flow on autosomes relative to the X chromosome. They find that Fst outliers are more common for $N_{interact}$ windows than windows containing other genes, and that ROH is larger for $N_{interact}$ windows. They find stronger population differentiation on the X chromosome than autosomes and found evidence of introgression across hybrid zones.

The bioinformatic methodology in this paper is very good and the questions that the authors are addressing are interesting. The manuscript is very well written. However, I would suggest incorporating more of the Supplementary results into the Main text, to make it more independent. While I think the authors have done an overall good job with this paper, I do have some concerns and suggestions which I believe would make it stronger and the results more convincing.

General comments:

Firstly, I suggest including a genome-wide plot showing the different population genomic statistics (e.g. Fst, Tajima's D) and gene density, while highlighting the $N_{interact}$ windows. That would show whether the $N_{interact}$ genes are clustered or evenly dispersed throughout the genome, and if some of the results may be explained by chromosome position (e.g. many $N_{interact}$ windows close to centromeres).

Secondly, I was wondering how you reached the decision of analysing the data in 100 kb windows. 100 kb sounds like quite a large window size as genes in mammals (to my knowledge) very rarely exceed 30 kb. Since gene density seem to be an important covariate in these analyses, I am wondering whether using a smaller window size may lead to more evenly distributed number of genes (among the windows which will still contain genes), and also ensure that the genome statistics are actually driven by the $N_{interact}$ genes rather than neighboring ones. Showing consistent results with the current ones, while using another window size might also be a good argument to back up the claims in this paper.

Thirdly, did you consider constructing gene trees from the $N_{interact}$ genes and use e.g. the PAML package to test directly for positive selection? To me, tests for positive selection on a gene level would be more convincing than the current approach of quite large genome windows. Calculating total branch length within gene trees may also give an estimate of gene divergence, which could be compared to a subset of non-$N_{interact}$ genes.

Below you will find some line-specific comments:

Lines 38-39: I would rephrase the way the term "genome sequence" is being used throughout this manuscript. The phrasing "Using 29 new genome sequences from eight species" sounds odd to me. I suggest changing to something like: "Using genomic data from 29 individuals from eight species".

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Line 226-233: The X chromosome (or Z chromosome) has been shown to have higher intraspecies divergence than autosomes across a wide range of species, which do not have the social system of these monkeys. Given this knowledge, I would be careful in phrasing this as a test of "expectations associated with the social system of these monkeys". Is this not more likely to be an effect of for example genetic drift, which is stronger on sex chromosomes than autosomes?

Line 259-263: Should this not be mentioned only in the discussion?

Figure 2: Consider changing the format of this plot. As it is now, it is very hard to get an idea of the number of data points in each group, and the differential distribution of Fst values among $N_{interact}$ and non-$N_{interact}$ windows. Perhaps a grouped boxplot?

Decision letter (RSPB-2021-1107.R0)

19-Jul-2021

Dear Dr Evans:

I am writing to inform you that your manuscript RSPB-2021-1107 entitled "Mitonuclear interactions and introgression genomics of macaque monkeys (*Macaca*) highlight the influence of behaviour on genome evolution" 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.
4) Data - please see our policies on data sharing to ensure that you are complying (https://royalsociety.org/journals/authors/author-guidelines/#data).

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Sincerely,
Dr Locke Rowe
mailto: proceedingsb@royalsociety.org

Associate Editor
Comments to Author:
In this paper, the authors analyse close to 30 genome sequences from 8 different species of macaque, to reveal some interesting signatures of selection associated with selection on joint mitochondrial-nuclear genotype, which seem to be shaped by the social system of these primates (extreme female philopatry, and male dispersal). In particular, the authors present several analyses that suggest coadaptation between mitochondrial and nuclear genomes -- "mitonuclear compatibility" to preserve functional metabolic capacity. The authors also find population structure on the X chromosome is higher than on the autosomes.

This is an interesting paper, and was thus sent out to peer review. Three expert referees agree the paper contains high quality analyses providing new insights into the evolutionary genomics of macaques. The referees have provided some very insightful and constructive comments / queries -- suggesting a number of areas in which analyses can be improved and extended on, and alternative analyses conducted to strengthen the evidence for the key results; and also pointed out alternative explanations for results (e.g. regarding the population structure of the X chromosome) that the authors need to carefully consider. I ask that the authors to pay careful attention to these suggestions to further probe their data and test the validity of conclusions.

Reviewer(s)' Comments to Author:
Referee: 1
Comments to the Author(s)
This study investigates challenging questions related to the effects of male-biased dispersal and mitonuclear interactions on genome-wide patterns of population structure and introgression. In my view, the authors made a compelling argument that this complex of southeast Asian macaque species is a particularly interesting and valuable one to test predictions about the evolution of mitonuclear incompatibilities. The resulting evidence for differential evolutionary pressures on

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Author's Response to Decision Letter for (RSPB-2021-1107.R0)

See Appendix A.

RSPB-2021-1756.R0

Review form: Reviewer 3

Recommendation
Accept as is

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

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

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

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Is it accessible?
No

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
The authors have addressed all my comments in a satisfactory way and adjusted the manuscript according to most suggestions. I am happy to see that the results are consistent also when using a smaller window size (30kb), and that the results from the dN/dS analysis are in line with expectations. I believe this manuscript is fit for publication and congratulate the authors on an interesting study.

I found one typo in the manuscript (line 168 in the version without track changes): "2111Ninteract" instead of "211 Ninteract".

Decision letter (RSPB-2021-1756.R0)

01-Sep-2021

Dear Dr Evans

I am pleased to inform you that your Review manuscript RSPB-2021-1756 entitled "Mitonuclear interactions and introgression genomics of macaque monkeys (Macaca) highlight the influence of behaviour on genome evolution" has been accepted for publication in Proceedings B.

The referee does not recommend any further changes. Therefore, please proof-read your manuscript carefully and upload your final files for publication. 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 me know immediately.

To upload your 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 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, upload a new version through your Author Centre.

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

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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. Please note that PowerPoint files are not accepted.

3) Electronic supplementary material: this should be contained in a separate file from the main text and the file name should contain the author's name and journal name, e.g authorname_procb_ESM_figures.pdf
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 see: https://royalsociety.org/journals/authors/author-guidelines/

4) Data-Sharing and data citation
It is a condition of publication that data supporting your paper are made available. Data should be made available either in the electronic supplementary material or through an appropriate repository. Details of how to access data should be included in your paper. Please see https://royalsociety.org/journals/ethics-policies/data-sharing-mining/ for more details.

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=RSPB-2021-1756 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.

5) 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 final version. If you have any questions at all, please do not hesitate to get in touch.

Sincerely,
Dr Locke Rowe

mailto:proceedingsb@royalsociety.org

Associate Editor
Comments to Author:
The authors have done an excellent job of accounting for the referee comments and revising their manuscript, and have provided a very well reasoned and comprehensive response to the referee comments. The paper will make an excellent contribution to the literature on the evolutionary significance of mitochondrial-nuclear interactions.

Reviewer(s)' Comments to Author:
Referee: 3
Comments to the Author(s).
The authors have addressed all my comments in a satisfactory way and adjusted the manuscript according to most suggestions. I am happy to see that the results are consistent also when using a smaller window size (30kb), and that the results from the dN/dS analysis are in line with expectations. I believe this manuscript is fit for publication and congratulate the authors on an interesting study.

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Decision letter (RSPB-2021-1756.R1)

03-Sep-2021

Dear Dr Evans

I am pleased to inform you that your manuscript entitled "Mitonuclear interactions and introgression genomics of macaque monkeys (*Macaca*) highlight the influence of behaviour on genome evolution" 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

Associate Editor
Comments to Author:
In this paper, the authors analyse close to 30 genome sequences from 8 different species of macaque, to reveal some interesting signatures of selection associated with selection on joint mitochondrial-nuclear genotype, which seem to be shaped by the social system of these primates (extreme female philopatry, and male dispersal). In particular, the authors present several analyses that suggest coadaptation between mitochondrial and nuclear genomes -- "mitonuclear compatibility" to preserve functional metabolic capacity. The authors also find population structure on the X chromosome is higher than on the autosomes.

This is an interesting paper, and was thus sent out to peer review. Three expert referees agree the paper contains high quality analyses providing new insights into the evolutionary genomics of macaques. The referees have provided some very insightful and constructive comments / queries -- suggesting a number of areas in which analyses can be improved and extended on, and alternative analyses conducted to strengthen the evidence for the key results; and also pointed out alternative explanations for results (e.g. regarding the population structure of the X chromosome) that the authors need to carefully consider. I ask that the authors to pay careful attention to these suggestions to further probe their data and test the validity of conclusions.

> **Response:** Thank you for this balanced assessment of our manuscript. Below we itemize how we have addressed all of these comments.

Reviewer(s)' Comments to Author:

Referee: 1

Comments to the Author(s)
This study investigates challenging questions related to the effects of male-biased dispersal and mitonuclear interactions on genome-wide patterns of population structure and introgression. In my view, the authors made a compelling argument that this complex of southeast Asian macaque species is a particularly interesting and valuable one to test predictions about the evolution of mitonuclear incompatibilities. The resulting evidence for differential evolutionary pressures on “N-interacting” genes seemed less compelling, as there were a number of conflicting signals and alternative interpretations. But I thought the authors did a good job in providing a balanced discussion of this nuanced dataset. The analysis is extensive, and I found that the manuscript was well written and clearly presented. Overall, I feel that it makes a valuable contribution, and I only have a few minor comments.

> **Response:** Thank you for this positive feedback.

1. To what extent could the finding of higher population structure for the X-chromosome than for autosomes be attributable to lower effective population size for the X? I believe

humans also have more structure for the X than autosomes even though the discussed mechanisms of the Large X Effect and male-biased dispersal may be less relevant.

Ramachandran S, Rosenberg NA, Zhivotovsky LA, Feldman MW. 2004 Robustness of the inference of human population structure: a comparison of X-chromosomal and autosomal microsatellites. Hum. Genomics 1, 87 – 97.

Li JZ et al. 2008 Worldwide human relationships inferred from genome-wide patterns of variation. Science 319, 1100 – 1104.

> **Response:** Thank you for this valuable comment, which was also expressed by another reviewer. We completely agree. We have revised our manuscript to no longer have this observation as an explicit expectation because (as you and the other reviewer point out) we do not attempt to disentangle the independent effects of social system and genetic drift. We have also moved the Fst figure from the main text to the supplement and mentioned the higher population structure of the X versus the autosome as an observation rather than an expectation.

2. Line 306. I understand the point about NDUFAF3 not co-precipitating with ND1, but this also raises the point that the wealth of structural data for mitonuclear enzyme complexes in mammals is not used in this study. One common discussion point in the literature is that mitonuclear incompatibilities might come down to a small number of positions, leading to signal to noise problems in this type of genome-wide scan. In that sense, taking structural data into account could produce a more targeted set of potential incompatibilities. For example, a resource such as MitImpact 3 (Castellana et al. 2021 NAR) could be useful in this respect. Given the already extensive analysis, I do not think it is necessary to incorporate this type of structural analysis, but the authors could point to this area as an important one for future work.

> **Response:** We agree and have added this to the concluding parahraph: “One way to distinguish effects of mitonuclear interactions from other correlated phenomena would be to test for correlated evolution evolution of mitochondrial and nuclear-encoded protein motifs that interact directly based on structural data for mitonuclear enzyme complexes; this is an exciting direction for future work.”

3. Typos:

Line 60. ARP2 should be ARS2.

> **Response:** Corrected; thank you!

Line 267. support gene flow [in] the hybrid zone

> **Response:** Corrected.

Referee: 2

Comments to the Author(s)
This manuscript reports the sequencing of 29 individuals from eight macaque species, and the interspecies differentiation and metrics of positive selection, low intraspecies polymorphism, and atypically long runs of homozygosity associated with nuclear-encoded genes that interact with mitochondria-encoded genes. This study represents a valuable attempt providing novel insights into the evolutionary genomics of macaques. Detailed comments are below:

1. Line 88-91: These two sentences are also reviewed in reference 17?

> **Response:** We have added this citation to follow this sentence as well.

2. Line 100: seven or eight species? It is not necessary to discuss the species clarification here. It is better to follow the current description.

> **Response:** We agree and changed this to read: **"**We set out to test these expectations using 29 genomes from pigtail macaques (*M. nemestrina*; six individuals) from Sumatra and Borneo and eight Sulawesi macaque species [18] (23 individuals)."

3. Line 118: Table S1 and Fig 1 just showed the sample list and sampling locations. It is necessary to show the divergence of the mt-genomes of herein studied samples.

> **Response:** We have added a table with pairwise nucleotide and protein divergences to the supplement (Table S2). We also discuss a summary of these divergences in the first section of the Supplement Results.

4. Fig 1 and Table S1: there are serial numbers in Fig 1 and it is also necessary to add in Table S1 for each samples. There are 29 samples in Table S1 and only 27 samples in Fig 1. I know maybe two samples are not with geographical information. But in this case it will confuse the reviewers and readers.

> **Response:** Thank you for this helpful suggestion. We have added a column to Table S1 that enumerates for each sample the geographical origins in Fig. 1. This information is also presented in the legend of Fig. 1.

5. Fig 1: Why is the sample 22 in a different color comparing to other Tonkean macaques. Might be another species? It will confuse the readers.

> **Response:** This reviewer may be aware that some aspects of macaque taxonomy are still a work in progress. In this revision, we now consistently use

the species name *M. togeanus* throughout the manuscript following Froehlich and Supriatna 1996. This information now is updated in Table S1 as well.

6. Fig 1 and Table S1: M. brunnescens or M. o. brunnescens? It looks like that M. o. brunnescens is one the two subspecies of Macaca ochreata?

Response: This reviewer correctly points out that some researchers consider the macaques on Buton Island to be a subspecies of *M. ochreata* (*M. o. brunnescens* and those on the Southeast peninsula of Sulawesi to be another subspecies (*M. o.* ochreata; e.g., Roos et al. 2014 Asian Primates Journal 4(1):2-38). We certainly do not object to this nomenclature. However, here we have chosen to follow the nomenclature of Fooden 1969, which recognizes *M. brunnescens* as a species separate from *M. ochreata*. This nomenclature (along with the recognition of *M. togeanus*) is in line with several of our previous papers (e.g., Evans et al. 2020, J. Hum. Evol. 146: 102852) and is not intended to support or refute other taxonomies. We have added citations and clarifying edits to the "Samples and nomenclature" section in the supplement to explain and justify the nomenclature used in this manuscript.

7. Line 402: eight or nine macaque species? Line 280 "eight macaque species"?

Response: We have clarified this to be nine species, which includes recognition of *M. togeanus* as discussed above, and justified with relevant citations in the supplement.

8. Does the N-interact genes show the similar pattern in other macaques or primates with highly-diverged mt-genomes? Or an opposite pattern in other macaques or primates with slightly-diverged mt-genomes? I think it should be an important comparison about this issue.

Response: We completely agree and highlight this point in the concluding paragraph: "Our findings in Southeast Asian macaques establish clear predictions for $N_{interact}$ genes in other species with similar social systems, including biomedically important species such as rhesus and longtail macaques.".

The current study is the first we are aware of to explore this issue in depth in eight of the ~20 macaque species (~1/3 of the species diversity in the genus). Scrutiny of other macaque species is certainly interesting, but well beyond the scope of the current study which we view to be a very comprehensive contribution. We are currently investigating this question in several other macaque species; please stay tuned!

9. Line 380: even [if?] N-interact genes are frequently subjected to positive selection, and frequently embedded in atypically long ROHs, I am afraid that they are not the

direct evidence for the links between behaviour and genome evolution. Maybe these genes could be affected by other factors.

> **Response:** We agree with this point and appreciate the balanced skepticism. We have added text to the conclusion section that reflects this: “We acknowledge that other explanations are possible, such variation in local environmental conditions being associated with positive selection on pleiotropic functions of $N_{interact}$ genes that are not directly related to mitonuclear interactions.”.

10. I also suggest the authors to describe the non-synonymous and synonymous mutations in N-interact genes.

> **Response:** This constructive suggestion was echoed as well by Reviewer 3. As detailed below, we have performed a transcriptome-wide analysis of nonsynonymous and synonymous substitutions, and the results out consistent with our other analyses.

Above all, I would like to suggest a “major revision” for this manuscript. However, its final acceptance depends on the revision of the manuscript that satisfactorily responds to the comments from reviewers. The data is valuable for publication, but I have to say the authors have not fully demonstrate their results. I also hope the authors will re-organize the languages throughout the text.

> **Response:** We have addressed all concerns, including new analyses (e.g. dN/dS), new information (e.g. genetic distances), a new supplemental figure (grouped box plots of Fst), and extensive revisions and clarifications (e.g., pertaining to Ne of the X and autosomes, movement of the Fst figure to the Suppl). We feel these changes have improved and streamlined our manuscript, which constitutes a compelling study.

Referee: 3

Comments to the Author(s)
The authors present a study on how behavior may influence genome evolution, using genomic data from eight species of macaque monkeys. Most macaque monkeys have sex-biased dispersal, with females staying within their natal range while males migrate. This means that mitochondria lineages will evolve relatively independently between populations, which is also manifested as high differentiation between species. The authors hypothesize that the high level of diversity of mitochondrial DNA may create positive selection for compensatory mutations on genes which interact with mitochondrial genes and mitochondrial DNA. Since females carry two X chromosomes but males only one, the authors also hypothesize that the social system of these monkeys will lead to higher intraspecific population structure across hybrid zones for the X chromosome, compared to autosomes.

To test these hypotheses, the authors first identify 211 nuclear-encoded genes which interact with mitochondria-encoded genes ("$N_{interact}$"). They use different population genetics metrics (Fst, Tajima's D, π, Fay and Wu's H, ROH) to test if genome windows (100 kb) containing these genes differ from other genome windows. They also study mitochondria introgression and effects of sex-specific gene flow on autosomes relative to the X chromosome. They find that Fst outliers are more common for $N_{interact}$ windows than windows containing other genes, and that ROH is larger for $N_{interact}$ windows. They find stronger population differentiation on the X chromosome than autosomes and found evidence of introgression across hybrid zones.

The bioinformatic methodology in this paper is very good and the questions that the authors are addressing are interesting. The manuscript is very well written. However, I would suggest incorporating more of the Supplementary results into the Main text, to make it more independent. While I think the authors have done an overall good job with this paper, I do have some concerns and suggestions which I believe would make it stronger and the results more convincing.

> **Response:** Thank you for these positive comments. We agree that some material in the Supplement would also be well placed in the main text. However, Proc. Roy. Soc. B. has a strict 10-page limit for the main text, which limits our capacity to transfer content from the supplement to the main text. However, we were able to address all of the concerns of the three reviews while still staying within this limit, which included expanded discussion on several issues including a new analysis of dN/dS ratios and 20kb windows, a new supplemental table and figure, and expanded interpretation of nomenclature, future directions, and population structure on the X.

General comments:

Firstly, I suggest including a genome-wide plot showing the different population genomic statistics (e.g. Fst, Tajima's D) and gene density, while highlighting the $N_{interact}$ windows. That would show whether the $N_{interact}$ genes are clustered or evenly dispersed throughout the genome, and if some of the results may be explained by chromosome position (e.g. many $N_{interact}$ windows close to centromeres).

> **Response:** This is a helpful suggestion that we had considered in depth and revisited for consideration during the preparation of this revision. During the preparation of our initial submission, we generated circle plots with these statistics (and also Patterson's D) to accompany the circle plots in Fig. 4, and Figs. S10-S13. We also generated linear plots with genome-wide data for each of the 20 chromosomes. However, we opted to *not* include these plots for several reasons. Most importantly, the resolution of the patterns is not clearly evident on a genome-wide scale when rendered on a single page. As well the nature of the

data would necessitate many figures, panels, or overlay lines (e.g., for each of 20 chromosomes for each of 10 pairwise comparisons for Fst or for each of 20 chromosomes for each of 5 species for pi, TajD and F&WH) which we found to be noisy and of limited value.

As an alternative, we opted instead to present high-resolution plots of diversity over selected smaller (20 Mb) regions (Fig. S2) as opposed to visualizing entire chromosomes, some of which each are ~200 Mb. Moreover, by inspecting Fig. S2, one can imagine how striking genomic signatures at this scale might seem insignificant if viewed at 1/10th the scale. To complement these close-up plots and as further illustrative tools, we provide several descriptive plots that summarize and compare genome-wide data (Figs. 2, 3, S1, and S3) and several tables that provide granular information about $N_{interact}$ outliers across the whole genome (Tables S3, S4, S5, S6) that will facilitate comparison to other studies.

Overall, we have intensively (and appreciatively) considered this suggestion. We feel that we have communicated this information in most readily interpreted and precise way we can.

Secondly, I was wondering how you reached the decision of analysing the data in 100 kb windows. 100 kb sounds like quite a large window size as genes in mammals (to my knowledge) very rarely exceed 30 kb. Since gene density seem to be an important covariate in these analyses, I am wondering whether using a smaller window size may lead to more evenly distributed number of genes (among the windows which will still contain genes), and also ensure that the genome statistics are actually driven by the $N_{interact}$ genes rather than neighboring ones. Showing consistent results with the current ones, while using another window size might also be a good argument to back up the claims in this paper.

Response: We agree. In this revision we performed and report a complementary analysis with 30kb windows for Fst, pi, Tajima's D and Fay and Wu's H. The results were essentially identical (including there still being a strong correlation between gene density and Fst, pi, Tajima's D and Fay and Wu's H in most species). Results using 30kb windows are now highlighted in the main text and discussed in detail in the Supplement. We agree that the consistency of these results with the 100kb analyses provides further support for our conclusions.

Thirdly, did you consider constructing gene trees from the $N_{interact}$ genes and use e.g. the PAML package to test directly for positive selection? To me, tests for positive selection on a gene level would be more convincing than the current approach of quite large genome windows. Calculating total branch length within gene trees may also give an estimate of gene divergence, which could be compared to a subset of non-$N_{interact}$ genes.

Response: Thank you for this constructive suggestion, which was also independently suggested by another reviewer. As discussed in detail below, we have performed a genome-wide analysis of dN and dN/dS. Excitingly, and in agreement with our other findings, the conclusions strongly support atypically strong natural selection on Ninteract genes as compared to other genes.

Below you will find some line-specific comments:

Lines 38-39: I would rephrase the way the term “genome sequence” is being used throughout this manuscript. The phrasing “Using 29 new genome sequences from eight species” sounds odd to me. I suggest changing to something like: “Using genomic data from 29 individuals from eight species”.

Response: We agree and have modified the main text and supplement to follow this suggestion.

Line 154-159: Can you provide a statistical test to see if this is a significant difference?

Response: This is a very useful suggestion that we should have implemented for the first submission. In this revision, we report p-values from binomial tests for upper and lower outliers for all analyses using the non-Ninteract proportions as the expectation. The main text and methods in the supplement have been revised to reflect this (the results are significant for all tests for all statistics).

Line 177: Please provide test statistics for this statement in the main text.

Response: As above, we performed binomial tests for this statement and have inserted p-values and a statement of statistical significance.

Line 226-233: The X chromosome (or Z chromosome) has been shown to have higher intraspecies divergence than autosomes across a wide range of species, which do not have the social system of these monkeys. Given this knowledge, I would be careful in phrasing this as a test of “expectations associated with the social system of these monkeys”. Is this not more likely to be an effect of for example genetic drift, which is stronger on sex chromosomes than autosomes?

Response: We completely agree and thank you for pointing this out – this point was also independently raised by another reviewer. We have removed this expectation from the manuscript and moved the Fst figure to the supplement. In this revision, we report the observation as a prelude to the introgression analyses.

Line 259-263: Should this not be mentioned only in the discussion?

Response: We agree. We have removed this statement from the Results.

Figure 2: Consider changing the format of this plot. As it is now, it is very hard to get an idea of the number of data points in each group, and the differential distribution of Fst values among $N_{interact}$ and non-$N_{interact}$ windows. Perhaps a grouped boxplot?

Response: We have added a grouped box plot to the Supplement to complement this figure (Fig. S2). A problem with using only a grouped box plot is that this type of graph does not capture the relationship between *Fst* and gene density that is depicted in Fig. 2. To further facilitate interpretation of Fig. 2, we have added clarifying text (in bold) to the manuscript: “This suggests that the higher F_{ST} of all $N_{interact}$ windows is largely attributable to gene density rather than to the presence of $N_{interact}$ genes **(e.g., pink and gray dots in Fig. 2 overlap extensively)**, an observation that is not consistent with our expectations.” In addition, to clarify sample sizes of each group, we have added this statement to the legend of Fig. 2: “Because F_{ST} was not calculated for some windows due to genotype quality filtering, the sample size of $N_{interact}$ windows (pink, red, and blue dots) is 204 and of non-$N_{interact}$ windows (gray dots) is 9,121.”.