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Poorly differentiated XX/XY sex chromosomes are widely shared across skink radiation

Alexander Kostmann, Lukáš Kratochvíl and Michail Rovatsos

Article citation details
Proc. R. Soc. B **288**: 20202139.
http://dx.doi.org/10.1098/rspb.2020.2139

Review timeline

Original submission:	29 August 2020
1st revised submission:	27 November 2020
2nd revised submission:	23 December 2020
Final acceptance:	23 December 2020

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

Review History

RSPB-2020-2139.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?
Excellent

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?
N/A

Is it clear?
N/A

Is it adequate?
N/A

Do you have any ethical concerns with this paper?
No

Comments to the Author
RSPB-2020-2139

This study establishes that 13 species forming a monophyletic group among Scincidae, and covering a large spectrum of lineages, share the same XY sex-determining system, with a small sex-determining genomic region homologous to a portion of Podarcis muralis chromosome 10 (and chicken chromosome 1). Their sex chromosomes are thus clearly old (between 85 and 150 My), but have nevertheless remained homomorphic. These results show that GSD is more widespread among squamates than previously thought, they provide support for the idea that sex chromosomes in amniotes are often conserved over long evolutionary times, and also that, despite this, they do not necessarily degenerate.

The work is scientifically sound and competent, the results are nice and mostly well discussed. I only have a few comments

Main comments

Abstract : The first sentence introduces the idea that sex-chromosome differentiation increases the rate of species diversification. This idea is mentioned again end of this abstract, and also developed in the intro (top of page 5) and the discussion (top of p.16). Apparently, the authors take it as an important selling point. However, I don't think it has any statistical support. Among vertebrates, for instance, there are much more species of fishes (>30'000) than of mammals (~5000) and birds (~9600), despite the fact that fish mostly lack differentiated sex chromosomes. With ~7600 species, amphibians, which also lack differentiated sex chromosomes, are also more numerous than mammals. Regarding the rationale, furthermore, I don't see how Y chromosome degeneration might increase diversification and adaptability. I would actually expect genomic degeneration to lower adaptive potential. Fast-X and fast-Z effects also stem essentially from enhanced genetic drift, which clearly does not favor adaptive radiation.

p.5, last paragraph: the contrast between endotherms and ectotherms mentioned in this paper [15] actually refers to the state of sex-chromosome differentiation (homo- vs heteromorphic), and not to their evolutionary stability. The point of this paper is in fact to suggest that, through occasional sex reversal (and ensuing XY recombination), sex chromosomes might remain homomorphic despite long-term stability. The same paper is also cited p. 15, (with a different ref number [81]), also with a misunderstanding: the point of this paper is not that poorly differentiated sex chromosome are young; quite to the contrary, it suggests a mechanisms by

which old sex chromosomes might remain undifferentiated over long evolutionary times. By the way, this same mechanism (sex reversal and XY recombination) might possibly also account for the situation documented here in skinks. Maybe worth mentioning in your paper.

Last sentence of the discussion: this reference to sex differences in recombination comes a little bit from nowhere. You should either develop the idea, or drop any mention to it.

Detail comments
Bottom of p.6. "and undermined long-term stability of GSD". Not sure to follow the logic. As presently written, your sentence means that the fact that earlier reports of ESD were found to be unreliable undermines the long-term stability of GSD, which does not make much sense. Do you actually mean that these earlier reports undermined the idea of a long-term stability of GSD, but that this idea is now restored, given that these reports have been shown to be unreliable?

p.14, 2nd paragraph: sex reversal is a consequence of the environmental effect on sex determination, not an explanation of it.

p. 16, bottom: grammatical typo: "…have thus potentially had…" drop the "have"

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?
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

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 work uses cytogenetic and genetic techniques to identify a homologous sex chromosome system for several species of skinks encompassing their phylogenetic breadth, allowing the authors to conclude that all skinks share a common sex chromosome system. This result was unexpected given the overall diversity of sex chromosome systems in squamate lizards, but the authors argue that squamates actually tend to have conserved sex chromosomes system within families. Their data are convincing and provide a molecular sex test for skink. I list suggestions for improvement below.

Abstract: "cytogenetically hardly distinguishable" might be better as 'cytogenetically indistinguishable".

Page 4: "GSD is very common in animals and has evolved in them multiple times, it is estimated that this has occurred independently up to 40-times just within amniotes." Should be 2 sentences.
Page 4: "Surprisingly, in spite of over a century of research [2], the adaptive significance and consequences of sex chromosomes and their differentiation, the progressive cessation of recombination and divergence of sequences between chromosomes in a sex chromosome pair, are still rather controversial". "Poorly understood" might be better than "controversial".

Page 4: "however, the sex-specific specialization and particularly, the role in the resolution of the conflict between sexes over trait expression have been considered crucial for differentiation and evolutionary stability of sex chromosomes". Should be "however, the sex-specific specialization, and particularly the role in the resolution of the conflict between sexes over trait expression, have been considered crucial for differentiation and evolutionary stability of sex chromosomes"

Page 4: "Higher mortality and a reduced lifespan in individuals of the heterogametic sex leading to a biased adult sex ratio attributed to degeneration of the Y and W were reported across animal lineages". Oddly phrased.

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Page 10: Figure 3 is discussed before Figures 1 or 2. Change figure order to reflect their place in the manuscript.

Page 11: "Notably, 37 of these genes are linked to Pu. Muralis chromosome 10, covering a chromosomal region of approximately 7 million base pairs" How big is the chromosome? Is this a large region or small?

Page 12: "In pathological conditions, the ectopic expression of sox10 in embryonic gonad leads to upregulation of transcriptional targets of the sox9 gene, triggering the male differentiation pathway, and resulting in sex-reverted XX males in humans and mice." Should be sex-reversed, not reverted.

Page 12: “Notably, sbf1 is nested in the 7 million base pair chromosomal region” Are ep300 and sox10 not also nested in this region?

Page 12: “However, other lineages such as the caenophidian and non-caenophidian snakes, the xantusiid lizard Xantusia henshawi, the pygopodid geckos and skinks [63–66] have evolved sex chromosomes from syntenic blocks not forming sex chromosomes in other amniotes”. Caenophidian snakes, pythons, and Aristelliger geckos sex linkage groups are all homologous to Anolis chromosome 6 / chicken chromosome 2. See Gamble et al. 2017, The Discovery of XY Sex Chromosomes in a Boa and Python, and Keating et al. 2020, Conserved ZZ/ZW sex chromosomes in Caribbean croaking geckos (Aristelliger: Sphaerodactylidae).

Page 13: The sex chromosomes are described as “poorly differentiated” but a large number of genes were identified as X-specific due to halved read depth and lack of SNPs in males. This suggests the Y-linked genes are differentiated from the X-linked genes or lost altogether, suggesting genetic differentiation even if morphologically/cytogenetically the chromosomes do not appear different. The term homomorphic might be a better fit instead of differentiated to describe the lack of morphological differences between the sex chromosomes. A discussion of whether the 500 X-specific genes found here is a large or small number relative to similar studies would fit in well here and add context to the degree of differentiation.

Page 13 / Fig. 2: “The orthologs of X-specific genes of the common sandfish showed a pseudoautosomal or autosomal pattern in the outgroups from the three families (Cordylidae, Gerrhosauridae and Xantusiidae).” Were all PMU10 genes, whether or not they were pseudoautosomal or X-specific in skinks, grouped into the orange bar for the outgroups in Fig 2? The figure make it seem as though the authors did not test the sex-linked X-specific genes for the outgroups, and only tested putatively pseudoautosomal genes. If both the PMU10 genes with pseudoautosomal position and PMU10 genes with X-specific position were tested for the outgroups, they should be divided into two separate bars to show there is no gene dose difference between males and females for either category for the outgroups.

Fig 2: Did the PMU10 pseudoautosomal genes not work for all the skink species? Why are some species missing the orange bar? Did all 10 X-specific genes have lower gene dose ratio in males, causing them to be lumped into the red bar? If so, consider adding numbers next to the bars so readers know how many genes fall into each category for each species. See comment above as well.

Fig 3: There does seem to be some difference in the strength of the rDNA signal between the chromosome 12 pair for T. baconi. Is this a significant difference?

Decision letter (RSPB-2020-2139.R0)

06-Nov-2020

Dear Dr Rovatsos:

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 reviewers and the Editors have raised some concerns with your manuscript and we would like to invite you to revise your manuscript to address them.

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.

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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.

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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.

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It is a condition of publication that you make available the data and research materials supporting the results in the article. Please see our Data Sharing Policies (https://royalsociety.org/journals/authors/author-guidelines/#data). 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.

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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. 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,
Dr Sasha Dall
mailto: proceedingsb@royalsociety.org

Associate Editor
Board Member: 1
Comments to Author:
The manuscript has now been reviewed by two expert reviewers and both viewed the work quite favorably. I have also read the manuscript and agree that this is an interesting piece of work that, with some careful editing, could be publishable in Proc B.

Both reviewers provide helpful comments that should increase the clarity of the manuscript. In particular, one reviewer noted concerns with how some of the material in the discussion links with the rest of the manuscript. They also counter the authors assertion that differentiation of sex chromosomes should drive species diversification, and so a more careful treatment of this idea is warranted. The other reviewer found the presentation of figure 2 to be somewhat lacking. A better link between the data and the conclusions drawn from it is needed.

Reviewer(s)' Comments to Author:

Referee: 1
Comments to the Author(s)
RSPB-2020-2139

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Fig 3: There does seem to be some difference in the strength of the rDNA signal between the chromosome 12 pair for T. baconi. Is this a significant difference?

Author's Response to Decision Letter for (RSPB-2020-2139.R0)

See Appendix A.

Decision letter (RSPB-2020-2139.R1)

11-Dec-2020

Dear Dr Rovatsos

I am pleased to inform you that your manuscript RSPB-2020-2139.R1 entitled "Poorly differentiated XX/XY sex chromosomes are widely shared across skink radiation" has been accepted for publication in Proceedings B.

The referees and AE have recommended publication, but also suggest some minor revisions to your manuscript. Therefore, I invite you to respond to the AE's 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

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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:
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• Phylogenetic data: TreeBASE accession number S9123
• Final DNA sequence assembly uploaded as online supplemental material
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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,
Dr Sasha Dall
Editor, Proceedings B
mailto:proceedingsb@royalsociety.org

Associate Editor:
Board Member
Comments to Author:
The authors have done a nice job of addressing concerns raised by the reviewers. I have no additional substantive changes to suggest, but do note two minor syntax issues.

1. line 90-91 "even subterraneous" should simply read subterranean
2. line 299 "to be sexed" should simply read to sex

Decision letter (RSPB-2020-2139.R2)

23-Dec-2020

Dear Dr Rovatsos

I am pleased to inform you that your manuscript entitled "Poorly differentiated XX/XY sex chromosomes are widely shared across skink radiation" 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.

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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

Editor: The manuscript has now been reviewed by two expert reviewers and both viewed the work quite favorably. I have also read the manuscript and agree that this is an interesting piece of work that, with some careful editing, could be publishable in Proc B. Both reviewers provide helpful comments that should increase the clarity of the manuscript. In particular, one reviewer noted concerns with how some of the material in the discussion links with the rest of the manuscript. They also counter the authors assertion that differentiation of sex chromosomes should drive species diversification, and so a more careful treatment of this idea is warranted. The other reviewer found the presentation of figure 2 to be somewhat lacking. A better link between the data and the conclusions drawn from it is needed.

Authors: Thank you for your positive evaluation of our manuscript. We read carefully the comments of both reviewers, which were very helpful, and we revised the manuscript accordingly. We agree that the idea that sex chromosome differentiation can drive species diversification is too speculative, and since it is not crucial for the interpretation of the results in this paper we decided to remove it entirely from the manuscript. The figure 2 was updated according to the Reviewer´s suggestions. We are grateful for your time and effort to improve our manuscript.

Referee#1: This study establishes that 13 species forming a monophyletic group among Scincidae, and covering a large spectrum of lineages, share the same XY sex-determining system, with a small sex-determining genomic region homologous to a portion of Podarcis muralis chromosome 10 (and chicken chromosome 1). Their sex chromosomes are thus clearly old (between 85 and 150 My), but have nevertheless remained homomorphic. These results show that GSD is more widespread among squamates than previously thought, they provide support for the idea that sex chromosomes in amniotes are often conserved over long evolutionary times, and also that, despite this, they do not necessarily degenerate. The work is scientifically sound and competent, the results are nice and mostly well discussed. I only have a few comments.

Authors: Thank you for your positive evaluation and the effort to further improve our manuscript. We followed all recommendations and we updated our manuscript accordingly.

Referee#1: Abstract: The first sentence introduces the idea that sex-chromosome differentiation increases the rate of species diversification. This idea is mentioned again end of this abstract, and also developed in the intro (top of page 5) and the discussion (top of p.16). Apparently, the authors take it as an important selling point. However, I don't think it has any statistical support. Among vertebrates, for instance, there are much more species of fishes (>30'000) than of mammals (~5000) and birds (~9600), despite the fact that fish mostly lack differentiated sex chromosomes. With ~7600 species, amphibians, which also lack differentiated sex chromosomes, are also more numerous than mammals. Regarding the rationale, furthermore, I don't see how Y chromosome degeneration might increase diversification and adaptability. I would actually expect genomic degeneration to lower adaptive potential. Fast-X and fast-Z effects also stem essentially from enhanced genetic drift, which clearly does not favor adaptive radiation.

Authors: The basic idea was that lineages with highly differentiated sex chromosomes might have higher rates of speciation because of the accumulation of sexual antagonist alleles, due to fast X/Z (even if it is by drift) and due to faster mutation rates in pseudoautosomal regions. This idea was mainly driven by data from birds and snakes, where related lineages differing in the degree of differentiation of sex chromosomes sharply differ in the number of species (we discussed it briefly in our paper on snakes; Rovatsos et al. *Proc. R Soc. Lond B* 2015). Nevertheless, we conclude in the current manuscript that skinks do not support this hypothesis. We agree that the hypothesis is mainly a speculation lacking rigorous statistical testing at this stage, and as this point is not crucial for the current work on skinks, we removed it from the manuscript.

Referee#1: p.5, last paragraph: the contrast between endotherms and ectotherms mentioned in this paper [15] actually refers to the state of sex-chromosome differentiation (homo- vs heteromorphic), and not to their evolutionary stability. The point of this paper is in fact to suggest that, through occasional sex reversal (and ensuing XY recombination), sex chromosomes might remain homomorphic despite long-term stability. The same paper is also cited p. 15, (with a different ref number [81]), also with a misunderstanding: the point of this paper is not that poorly differentiated sex chromosome are young; quite to the contrary, it suggests a mechanisms by which old sex chromosomes might remain undifferentiated over long evolutionary times. By

the way, this same mechanism (sex reversal and XY recombination) might possibly also account for the situation documented here in skinks. Maybe worth mentioning in your paper.
Authors: We apologize for the mistake with the double reference, thank you for pointing to it. We added a note that the mechanism suggested by Nicolas Perrin in his *Evolution* paper (2009) can explain a long-time maintence of poorly differentiated sex chromosomes in ectotherms. Thank you very much for this note! Previously, we had in mind that poorly differentiated sex chromosomes might be prone to turnover, we agree that we were not very precise and explicit at this point and that we should better cite another paper suggesting the contrast between endotherms and ectotherms in the stability of sex chromosomes. Into the current version, we put to this specific part the reference to the paper by Grossen, Neuenschwander and Perrin (*Evolution* 2011) explicitly stating it in the Introductory part ("Birds and mammals display a strictly genotypic sex determination (GSD), with highly differentiated sex chromosomes (Graves2008). […] Patterns are strikingly different in other vertebrates. First, sex determination is often extremely labile..."

Referee#1: Last sentence of the discussion: this reference to sex differences in recombination comes a little bit from nowhere. You should either develop the idea, or drop any mention to it.
Authors: This idea is developed in the Introduction and further in the Discussion, but we agree that it is not easy to understand just from the Conclusion, we delete the sentence and change the Conclusions accordingly.
Referee#1: Bottom of p.6. "and undermined long-term stability of GSD". Not sure to follow the logic. As presently written, your sentence means that the fact that earlier reports of ESD were found to be unreliable undermines the long-term stability of GSD, which does not make much sense. Do you actually mean that these earlier reports undermined the idea of a long-term stability of GSD, but that this idea is now restored, given that these reports have been shown to be unreliable?
Authors: Exactly. Previous reports on erroneously identified "ESD" species inside GSD lineages gave the misleading impression that GSD was not stable in the long-term with respect to ESD. For easier reading, we rephrased the sentence in question to "Nevertheless, many earlier reports of ESD were found to be unreliable based on recent cytogenetic or molecular evidence [36–39]. These erroneous reports of ESD in actually GSD species caused an overestimation of

the number of GSD to ESD transitions among amniotes and undermined the long-term stability of GSD."

Referee#1: p.14, 2nd paragraph: sex reversal is a consequence of the environmental effect on sex determination, not an explanation of it.
Authors: We rephrased this sentence accordingly.

Referee#1: p. 16, bottom: grammatical typo: "…have thus potentially had…" drop the "have"
Authors: Thank you, the typo is now corrected.

Referee#2: This work uses cytogenetic and genetic techniques to identify a homologous sex chromosome system for several species of skinks encompassing their phylogenetic breadth, allowing the authors to conclude that all skinks share a common sex chromosome system. This result was unexpected given the overall diversity of sex chromosome systems in squamate lizards, but the authors argue that squamates actually tend to have conserved sex chromosomes system within families. Their data are convincing and provide a molecular sex test for skink. I list suggestions for improvement below.
Authors: Thank you for the positive evaluation of our manuscript and your effort to improve it. We took all comments into consideration and we revised the manuscript accordingly.

Referee#2: Abstract: "cytogenetically hardly distinguishable" might be better as 'cytogenetically indistinguishable".
Authors: Thank you, we followed this recommendation.

Referee#2: Page 4: "GSD is very common in animals and has evolved in them multiple times, it is estimated that this has occurred independently up to 40-times just within amniotes." Should be 2 sentences.
Authors: Thank you, we split the long sentence.

Referee#2: Page 4: “Surprisingly, in spite of over a century of research [2], the adaptive significance and consequences of sex chromosomes and their differentiation, the progressive cessation of recombination and divergence of sequences between chromosomes in a sex chromosome pair, are still rather controversial”. “Poorly understood” might be better than “controversial”.
Authors: Thank you, we followed this recommendation.

Referee#2: Page 4: “however, the sex-specific specialization and particularly, the role in the resolution of the conflict between sexes over trait expression have been considered crucial for differentiation and evolutionary stability of sex chromosomes”. Should be “however, the sex-specific specialization, and particularly the role in the resolution of the conflict between sexes over trait expression, have been considered crucial for differentiation and evolutionary stability of sex chromosomes”
Authors: Thank you, we followed this recommendation.

Referee#2: Page 4: “Higher mortality and a reduced lifespan in individuals of the heterogametic sex leading to a biased adult sex ratio attributed to degeneration of the Y and W were reported across animal lineages”. Oddly phrased.
Authors: We rephrased the sentence.

Referee#2: Paged 5: “The expectation of the faster differentiation (or degeneration) of Y was based on assumptions of a stronger selection in males” Sexual selection?
Authors: Yes, the term “sexual selection” is now added to the sentence.

Referee#2: Page 10: “For qPCR, we designed specific primers for sandfish X-specific genes using Primer3 software [51] for the amplification of a 120– 200 bp fragment of three single-copy autosomal genes (abarb2, eef1a1, mecom) and 10 X-specific gene”. How were these 10 X-specific genes chosen? Are these genes lacking male SNPs?
Authors: We designed primers for genes with the male to female genome coverage ratio around 0.5 lacking SNPs in the male of *Scincus scincus,* which is the expected ratio for X-specific

genes. Primers were designed for 10 such genes and all were proven by qPCR to be X-specific. This part is now clarified in the manuscript.

Referee#2: Page 10: Figure 3 is discussed before Figures 1 or 2. Change figure order to reflect their place in the manuscript.
Authors: Thank you, it is now corrected.

Referee#2: Page 11: "Notably, 37 of these genes are linked to Pu. Muralis chromosome 10, covering a chromosomal region of approximately 7 million base pairs" How big is the chromosome? Is this a large region or small?
Authors: The length of PMU10 chromosome is approx. 76 million base pairs. Only a small part of this chromosome seems to be orthologous to the X-specific region in the skink. With the applied methods, we cannot distinguish if the rest of PMU10 chromosome is homologous to pseudoautosomal or autosomal regions in the skink. Taking into account that the size of the smallest chicken microchromosome (GGA38) is around 2.9 million bp (Corrêa Mendonça et al., 2016, Caryologia 69: 201-206), we can conclude that the X-specific region of the skink is indeed small.

Referee#2: Page 12: "In pathological conditions, the ectopic expression of sox10 in embryonic gonad leads to upregulation of transcriptional targets of the sox9 gene, triggering the male differentiation pathway, and resulting in sex-reverted XX males in humans and mice." Should be sex-reversed, not reverted.
Authors: Thank you, the word is corrected now.

Referee#2: Page 12: "Notably, sbf1 is nested in the 7 million base pair chromosomal region" Are ep300 and sox10 not also nested in this region?
Authors: As we do not know the exact position of genes in the X chromosome of *S. scincus*, our suggestions about candidate sex-determining genes in skinks are based on the topology of orthologs in *Podarcis muralis* (PMU). The majority of the *S. scincus* X-specific genes have homologs linked to the region of approx. 7 Mbp in PMU10 (in total 37 genes). *Sbf1* is nested inside this region. Nevertheless, several other genes are scattered across PMU10. The genes

ep300 and *sox10* are located near each other in a different region of PMU10 close to orthologs of four other X-specific genes of *S. scincus*. This paragraph of Discussion is meant as a suggestion of candidate sex-determining genes in skinks to be explored in future studies.

Referee#2: Page 12: "However, other lineages such as the caenophidian and non-caenophidian snakes, the xantusiid lizard Xantusia henshawi, the pygopodid geckos and skinks [63–66] have evolved sex chromosomes from syntenic blocks not forming sex chromosomes in other amniotes". Caenophidian snakes, pythons, and Aristelliger geckos sex linkage groups are all homologous to Anolis chromosome 6 / chicken chromosome 2. See Gamble et al. 2017, The Discovery of XY Sex Chromosomes in a Boa and Python, and Keating et al. 2020, Conserved ZZ/ZW sex chromosomes in Caribbean croaking geckos (Aristelliger: Sphaerodactylidae).
Authors: Thank you very much, during the writing we did not include the recent study on *Aristelliger* geckos. In the python and caenophidian snakes, we assumed that their sex chromosomes might be homologous although they these snakes made a turnover between male and female heterogamety. Nevertheless, we meanwhile discovered and published as a preprint that the skink sex-linked region is also sex-linked in the gecko genus *Coleonyx* (available at https://www.preprints.org/manuscript/202011.0213/v1). Therefore, this part of Discussion describing that the skink sex-linked region have not been found in sex chromosomes of other amniotes was deleted from the new version.

Referee#2: Page 13: The sex chromosomes are described as "poorly differentiated" but a large number of genes were identified as X-specific due to halved read depth and lack of SNPs in males. This suggests the Y-linked genes are differentiated from the X-linked genes or lost altogether, suggesting genetic differentiation even if morphologically/cytogenetically the chromosomes do not appear different. The term homomorphic might be a better fit instead of differentiated to describe the lack of morphological differences between the sex chromosomes. A discussion of whether the 500 X-specific genes found here is a large or small number relative to similar studies would fit in well here and add context to the degree of differentiation.
Authors: The terms homomorphic/heteromorphic and differentiated/undifferentiated are often used in a confusing way. We consider that "homomorphic/heteromorphic" describes the chromosome morphology (this term originated from the "classical" cytogenetic era describing

differences in morphology of chromosomes in a chromosome pair) and "differentiated/undifferentiated" refers to the degree of difference in sequences, e.g. in gene content, and in other structural changes (e.g. degree of heterochromatinization) between X/Z and Y/W chromosomes reflecting the arrest of recombination. Notably, the sex chromosomes can be homomorphic (i.e. indistinguishable in morphology) but at the same time highly differentiated (e.g. with significant differences in gene content), as can be found for instance in many lacertids. In the case of skinks, sex chromosomes are homomorphic and considering the relatively small X-specific region within otherwise quite large sex chromosomes, they can be assigned as poorly differentiated (although this term is rather subjective and the objective criteria for the degree of differentiation are highly discussed - see e.g. the recent manuscript by D. Charlesworth minutely discussing just this issue - available at https://matthiasstoeckdotorg.files.wordpress.com/2020/07/dcharlesworth_accversion.pdf). This part is now clarified in the manuscript.

Referee#2: Page 13 / Fig. 2: "The orthologs of X-specific genes of the common sandfish showed a pseudoautosomal or autosomal pattern in the outgroups from the three families (Cordylidae, Gerrhosauridae and Xantusiidae)." Were all PMU10 genes, whether or not they were pseudoautosomal or X-specific in skinks, grouped into the orange bar for the outgroups in Fig 2? The figure make it seem as though the authors did not test the sex-linked X-specific genes for the outgroups, and only tested putatively pseudoautosomal genes. If both the PMU10 genes with pseudoautosomal position and PMU10 genes with X-specific position were tested for the outgroups, they should be divided into two separate bars to show there is no gene dose difference between males and females for either category for the outgroups.
Fig 2: Did the PMU10 pseudoautosomal genes not work for all the skink species? Why are some species missing the orange bar? Did all 10 X-specific genes have lower gene dose ratio in males, causing them to be lumped into the red bar? If so, consider adding numbers next to the bars so readers know how many genes fall into each category for each species. See comment above as well.
Authors: All three autosomal genes and 10 X-specific genes of *S. scincus* (with homologs linkeds to PMU10) were tested across all 13 skink and four outgroup species. Autosomal genes serving as a control were identified as such from the coverage analysis - their homologs are

linked to other syntenic blocks than the X-specific genes and have male to female ratios around 1.0 in the coverage analysis. These autosomal genes appeared indeed autosomal (or pseudoautosomal) in all skinks and outgroups and are depicted by blue bars.
The genes from the X-specific region of *S. scincus* determined by the coverage analysis and tested by qPCR showed clearly bimodal pattern in the male to female ratios: they have either ratios around 0.5 consistent with the X-specificity (these are depicted in red), or around 1.0 consistent with autosomal or pseudoautosomal position (orange bars). All the genes X-specific in the *S. scincus* are in the latter category in outgroups (orange bars), which points to non-homologous sex chromosomes between skinks and the outgroups. Inside the family Scincidae, few genes that are X-specific in *S. scincus* occasionally appear autosomal or pseudoautosomal in other skinks. This can be explained by (i) translocations of genes from the X-specific to autosomal or pseudoautosomal regions by chromosomal rearrangements and/or (ii) rare events of recombination between X and Y, occurred during the species diversification after the emergence of differentiated sex chromosomes. Such chromosomal rearrangements are expected to be found when comparing species across an old radiation (see similar cases in lacertid lizards in Rovatsos et al. *Mol. Ecol.* 2016 and *Sci. Rep.* 2019). Nevertheless, only a few such cases are detected and different genes are involved in them in skinks (see Table S3 for the full list of qPCR values). Some skink species are missing the orange bar, as we did not detect in them autosomal or pseudoautosomal position of the genes from *S. scincus* X-specific region. Also, some genes were not amplified successfully as determined largely by melting curve analyses in qPCR. Following the Reviewer's recommendation, we report the number of genes in each bar in the figure now, and we updated the figure legend to be easier for the readers to follow. Thank you very much for this very good suggestion.

Referee#2: Fig 3: There does seem to be some difference in the strength of the rDNA signal between the chromosome 12 pair for *T. baconi*. Is this a significant difference?
Authors: There is a small difference in the accumulation of rDNA loci between the two chromosomes of the pair in this male of *T. baconi*. We have analyzed more individuals of *T. baconi* and several species of skinks, and we have not identified a sex-specific pattern. Such polymorphism in the intensity of signal of rDNA loci is commonly reported in amniotes. It is now commented in the text.