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PROCEEDINGS B

Evolution of the nitric oxide synthase family in vertebrates and novel insights in gill development

Giovanni Annona, Iori Sato, Juan Pascual-Anaya, David Osca, Ingo Braasch, Randal Voss, Jan Stundl, Vladimir Soukup, Allyse Ferrara, Quenton Fontenot, Shigeru Kuratani, John H. Postlethwait and Salvatore D'Aniello

Article citation details
Proc. R. Soc. B **289**: 20220667.
http://dx.doi.org/10.1098/rspb.2022.0667

Review timeline

Original submission:	2 August 2021
1st revised submission:	2 June 2022
2nd revised submission:	30 June 2022
Final acceptance:	19 July 2022

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

Review History

RSPB-2021-1549.R0 (Original submission)

Review form: Reviewer 1

Recommendation
Reject – article is not of sufficient interest (we will consider a transfer to another journal)

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

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

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

Is the length of the paper justified?
No

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?
No

Is it clear?
No

Is it adequate?
No

Do you have any ethical concerns with this paper?
No

Comments to the Author
This manuscript reports a phylogenetic analyses of Nitric Oxyde synthase genes in vertebrates, particularly taking advantage of recently released genomes from early diverging ray-finned fishes such as bichir, as well as chondrichthyans. They suggest that all three NOS subfamilies have originated through the vertebrate WGDs, but have been subsequently lost or alternatively heavily duplicated in some lineages. Then, the author hypothesise an ancestral role in gills in gnathosztomes based on in situ hybridisation in shark and early diverging ray-finned fishes (sturgeon and bichir).
The data presented and the methodology seems satisfying, however, the manuscript does not provide a definitive mechanistic explanation of the functional shifts of NOS in the different organs and tissues, like fish gills. What is their function there? What is the ancestral functional in vertebrates?

I also think it would be really important to have some ideas about the genes that regulate the expression of NOS in the different tissues. There are available regulatory profiling data in zebrafish for instance, that could be used to determine which transcription factors are responsible. Moreover, I also find that the authors do not use any quantitative gene expression data, like RNA-seq, in situs are beautiful and inspiring but they also sometimes only reveal one side of the story. Finally, there is little discussion of gene expression of the duplicates within fishes, which is another interesting aspect of the paper: what are all the fish duplicates doing. I am sure there are RNA-seq datasets of different organs that could illuminate this question. The author published previously several papers on the topic, including expression in amphioxus and some phylogenetic analysis. In sum, I think this is decent quality work, but that it does not really provide an in-depth approach to understand the evolution of paralogues role and regulation after duplication, which strongly limits its scope.
Other remarks:
- I think it would be great to have a ML tree as well. Are the domains of the protein conserved across paralogues? Any clue of differential evolution at the protein level (selection or reduced constraints). Like a PAML site or branch site analysis, etc.. Also, please, make the alignments available.
- L426-438: this is very speculative, the hagfish could very well have a similar situation than lamprey.
-

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?
Acceptable

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?
Yes

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
See Appendix A.

Review form: Reviewer 3

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

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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
In this MS entitled 'Evolution of the nitric oxide synthase family in vertebrates and novel insights in gill development', Giovanni Annona and colleagues perform and exhaustive phylogenetic analysis of the nitric oxide synthase family in vertebrates, paying special attention to gnathostomes and cyclostomes, revealing a complex evolutionary history of gene duplications and losses. The MS, moreover, provides valuable expression analyses in a variety of species that help to clarify the function of each member of the Nos family,. The phylogenetic methods and the synteny analyses is solid, providing little doubt about the robustness of the results. The expression analyses is also solid and clear. The results are interpreted correctly, and discussed in the proper context of other publish works, as far as I know. The final model proposed for the evolutionary history of this complex family is relevant for the scientific community interested in gene family evolution, as well as the role of nos in embryo development and adult organ homeostasis, paving therefore the way for future functional analyses by gene modification. I have no major revisions, and I have only find some minor points that can be addressed by the authors at their own judgement without further revision.

Minor points:
L3: remove comma before "and"
L71: "which" should be "whose"(?)
L114: the number of examined available genomes should be stated
L130: "is" should be "was"?
L144: the authors say "with no clear orthology relationship to any specific gnathostome Nos1, Nos2, Nos3". I disagree, however, since the tree suggest that NosA/B clade originated in the cyclostome genome, and this clade is pro-ortholog (according to PMID: 10597641) to the clade Nos1/2/3. I would ask the authors to evaluate the strength of this clade's relationship, and if they consider it solid enough, I encourage the authors to consider whether cyclostome Nos A and B

should be renamed as Nos1/2/3a and Nos1/2/3b. In fact, the same should be used for cephalochordate and tunicate sequences, if no clear ortholog of nos1, nos2 or nos3 is present in any of those group of organisms.
L168: remained “in”?
Figure 1a: If the names of species and tree branches were colored according to their groups, it would facilitate their visualization. The code of colors could be maintained in Fig 1b and c.
Micro-synteny figures: please, specify in the legends what is the minimal distance to be considered “long” as indicated by // (>100 kb). Are the indicated genes consecutive? I also wondered if other genes can be in-between the colored genes (please, clarify in the legend) if so.
L228: Clarify, please, why is unexpected.
L374: In the context of the discussion of the Nos genes and the VGD, reference [8] should be cited.
Discussion: I wonder if there is any evidence on Nos expression in the gills or pharynx of tunicates or cephalochordates that could enrich the evolutionary origin of this expression domain and function. Please, comment.
L431: as correctly discussed in this line, I would suggest to remove the word “likely” from L47 in the abstract, and use a less strong statement “may have arisen”

Decision letter (RSPB-2021-1549.R0)

04-Oct-2021

Dear Dr D'Aniello:

I am writing to inform you that your manuscript RSPB-2021-1549 entitled "Evolution of the nitric oxide synthase family in vertebrates and novel insights in gill development" has, in its current form, been rejected for publication in Proceedings B.

This action has been taken on the advice of the Associate Editor and the 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.
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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 Loeske Kruuk
mailto: proceedingsb@royalsociety.org

Associate Editor
Board Member: 1
Comments to Author:
Three experts in the field have provided feedback on the manuscripts, and they are overall positive. However, reviewer #1 had some concerns about the lack of mechanistic explanation on what drives the expression of NOS in different tissues in zebrafish and on the phylogenetic analysis. I agree with the reviewer that discussing what is known about the different T.F.s driving the expression on NOS and the ML trees would strengthen the M.S.
In conclusion, considering the reviewers' comments, I cannot recommend the M.S. for publication on Proc. B. in its current form.

Reviewer(s)' Comments to Author:
Referee: 1
Comments to the Author(s)
This manuscript reports a phylogenetic analyses of Nitric Oxyde synthase genes in vertebrates, particularly taking advantage of recently released genomes from early diverging ray-finned fishes such as bichir, as well as chondrichthyans. They suggest that all three NOS subfamilies have originated through the vertebrate WGDs, but have been subsequently lost or alternatively heavily duplicated in some lineages. Then, the author hypothesise an ancestral role in gills in gnathosztomes based on in situ hybridisation in shark and early diverging ray-finned fishes (sturgeon and bichir).
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- L426-438: this is very speculative, the hagfish could very well have a similar situation than lamprey.
-

I think the alignments should be present in a downloadable form with proper annotation. The phylogeny constitutes one of the resources of the paper.

Referee: 2
Comments to the Author(s)
See attached document (Appendix A).

Referee: 3
Comments to the Author(s)
In this MS entitled 'Evolution of the nitric oxide synthase family in vertebrates and novel insights in gill development', Giovanni Annona and colleagues perform and exhaustive phylogenetic analysis of the nitric oxide synthase family in vertebrates, paying special attention to gnathostomes and cyclostomes, revealing a complex evolutionary history of gene duplications and losses. The MS, moreover, provides valuable expression analyses in a variety of species that help to clarify the function of each member of the Nos family,. The phylogenetic methods and the synteny analyses is solid, providing little doubt about the robustness of the results. The expression analyses is also solid and clear. The results are interpreted correctly, and discussed in the proper context of other publish works, as far as I know. The final model proposed for the evolutionary history of this complex family is relevant for the scientific community interested in gene family evolution, as well as the role of nos in embryo development and adult organ homeostasis, paving therefore the way for future functional analyses by gene modification. I have no major revisions, and I have only find some minor points that can be addressed by the authors at their own judgement without further revision.

Minor points:
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L168: remained "in"?
Figure 1a: If the names of species and tree branches were colored according to their groups, it would facilitate their visualization. The code of colors could be maintained in Fig 1b and c.
Micro-synteny figures: please, specify in the legends what is the minimal distance to be considered "long" as indicated by // (>100 kb). Are the indicated genes consecutive? I also wondered if other genes can be in-between the colored genes (please, clarify in the legend) if so.
L228: Clarify, please, why is unexpected.
L374: In the context of the discussion of the Nos genes and the VGD, reference [8] should be cited.
Discussion: I wonder if there is any evidence on Nos expression in the gills or pharynx of tunicates or cephalochordates that could enrich the evolutionary origin of this expression domain and function. Please, comment.
L431: as correctly discussed in this line, I would suggest to remove the word "likely" from L47 in the abstract, and use a less strong statement "may have arisen"

Author's Response to Decision Letter for (RSPB-2021-1549.R0)

See Appendix B.

RSPB-2022-0667.R0

Review form: Reviewer 3

Recommendation
Accept as is

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?
Yes

Is it clear?
Yes

Is it adequate?
Yes

Do you have any ethical concerns with this paper?
No

Comments to the Author
After reading the new version of the MS and the responses to the reviewers, and I am fully satisfied with the responses to my previous questions, and I also agree with the authors' responses to the other reviewers. I therefore, in agreement with my previous review, I recommend this MS for publication.

(PS: In line 123 I found a typo: "unprecented" should be "unprecedented")

Decision letter (RSPB-2022-0667.R0)

23-Jun-2022

Dear Dr D'Aniello,

I am pleased to inform you that your Review manuscript RSPB-2022-0667 entitled "Evolution of the nitric oxide synthase family in vertebrates and novel insights in gill development" has been accepted for publication in Proceedings B.

The referee and Associate Editor have recommended publication with no further changes required, from what we can see of the revised manuscript. However, the one issue that needs to be addressed is that we were not able to view the figures in the new manuscript (it looks like they were uploaded in a zip file?). Therefore, please proof-read your manuscript carefully and upload your final files for publication, making sure that the figures are accessible. 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.

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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/

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It is a condition of publication that data supporting your paper are made available either in the electronic supplementary material. Authors must complete the 'data accessibility' section in the submission system. 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

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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 include the Dryad DOI in the Data Accessibility section and reference in the paper's bibliography.

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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,
Professor Loeske Kruuk
mailto:proceedingsb@royalsociety.org

Associate Editor
Comments to Author:
the authors have addressed all the concerns with the manuscript. Thereby following the reviewer's suggestions I am delighted to recommend the manuscript for publication in its current form.

Reviewer(s)' Comments to Author:
Referee: 3
Comments to the Author(s).
After reading the new version of the MS and the responses to the reviewers, and I am fully satisfied with the responses to my previous questions, and I also agree with the authors' responses to the other reviewers. I therefore, in agreement with my previous review, I recommend this MS for publication.

(PS: In line 123 I found a typo: "unprecented" should be "unprecedented")

Decision letter (RSPB-2022-0667.R1)

19-Jul-2022

Dear Dr D'Aniello,

I am pleased to inform you that your manuscript entitled "Evolution of the nitric oxide synthase family in vertebrates and novel insights in gill development" 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

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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.

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,
Professor Loeske Kruuk
Editor, Proceedings B
mailto: proceedingsb@royalsociety.org

Associate Editor:
Board Member
Comments to Author:
(There are no comments.)

Appendix A

The paper describes gains and losses of Nos paralogs within Actinopterygii. The findings are clear and the conclusions are largely supported by the analyses. I only have a few suggestions to improve readability.

The authors do a good job of describing taxonomic groups and what belongs to them in the methods and results, but improvements could be made here. For example, Clupeocephala is weakly defined and Morymyridae is undefined. Otomorpha and Protacanthopterygii are kind of defined in Fig 1 and the legend of Fig 6. Neoteleostei is not defined anywhere in the paper, which is not helpful since there is a major focus on the loss of Nos2 in Neoteleostei. A simple remedy may be to extend the supplemental table 1 to include taxonomic classifications. I would also encourage the authors to make supplemental table 1 an editable spreadsheet (excel or other). Working with pdf tables for replication purposes is quite painful.

I'm not very good with fish taxonomy (zebrafish -> *Danio rerio* is about as good as I can do) and using scientific names in fig 1, but common names in fig 2 (and Fig S1) isn't very helpful when I want to link duplication events in fig 1 to synteny in fig 2. I would like to see a naming standardization, it's probably easier to replace common names with scientific names in fig 2 and fig S1.

It may be worth citing https://doi.org/10.1038/s41467-021-24573-z regarding cyclostome duplications and the vertebrate 2R. Interpreting cyclostome duplications could benefit.

I appreciate the authors attempt to incorporate the Italian flag into figures 1 and 6a. However, these figures (especially Fig 6a) may be difficult to interpret for those with red/green color blindness. I would encourage the authors to use a different palette to provide better accessibility/interpretability.

Reference to human Hox genes is surprising in Fig 6b. Are Nos paralogs close to the Hox clusters in fishes without additional genome duplications (i.e. spotted gar)? Are genome drafts in fishes intact enough to demonstrate this? If not, I would understand the use of human, but there's currently no rationale for using human.

Minor fixes

I think it should be clearer in the methods that all paralogs were tested for expression among species.

In fig 1 Nos is presented as NOS in human and anole, but Nos for the others and it's not clear why. For aesthetic purposes, Nos should be consistent?

Line 47: replace 'suggest' with 'suggests' and the comma after suggest is probably unnecessary.

Line 63: remove 'and in' before neurodegenerative.

Line 121: Replace 'last' with 'lastly'.

Line 121-125: The statement: "we named nos2.1 and nos2.2 paralogs…" could be better rewritten as: "and lastly, the two nos2 paralogs…..were named nos2.1 and nos2.2".

Line 128: remove 'latter'.

Line 168: add ‘in’ between ‘remained’ and ‘the’

Line 211: reference supplemental figure 2 in the legend since nos2a isn’t shown in fig 2.

Line 256: “Surprisingly, nos1 and nos2 were expressed in gills of sturgeon, bichir, and shark.” I think you should remove the sentence. It’s confusing as it suggests both nos1 and nos2 are expressed in all 3 species when the next sentence clarifies what’s actually happening.

Line 359: Clupeocephala is misspelled.

Appendix B

04-Oct-2021

Dear Dr D'Aniello:

I am writing to inform you that your manuscript RSPB-2021-1549 entitled "Evolution of the nitric oxide synthase family in vertebrates and novel insights in gill development" has, in its current form, been rejected for publication in Proceedings B.

This action has been taken on the advice of the Associate Editor and the 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.
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Sincerely,

Professor Loeske Kruuk

mailto: proceedingsb@royalsociety.org

Response to referees document.

Associate Editor
Board Member: 1
Comments to Author:
Three experts in the field have provided feedback on the manuscripts, and they are overall positive. However, reviewer #1 had some concerns about the lack of mechanistic explanation on what drives the expression of NOS in different tissues in zebrafish and on the phylogenetic analysis. I agree with the reviewer that discussing what is known about the different T.F.s driving the expression on NOS and the ML trees would strengthen the M.S.
In conclusion, considering the reviewers' comments, I cannot recommend the M.S. for publication on Proc. B. in its current form.

Reviewer(s)' Comments to Author:

Referee: 1

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The data presented and the methodology seems satisfying, however, the manuscript does not provide a definitive mechanistic explanation of the functional shifts of NOS in the different organs and tissues, like fish gills. What is their function there? What is the ancestral functional in vertebrates?

I also think it would be really important to have some ideas about the genes that regulate the expression of NOS in the different tissues. There are available regulatory profiling data in zebrafish for instance, that could be used to determine which transcription factors are responsible.
As far as we know there are no available data regarding which transcription factor regulates the expression of Nos genes in different tissues. Nevertheless such a predictive study would be too speculative without the support of *in vivo* functional experiments. Therefore, although we agree it would be interesting, we think that it is out of the scope of the present study and consider this suggestion useful for our future work.

Moreover, I also find that the authors do not use any quantitative gene expression data, like RNA-seq, in situs are beautiful and inspiring but they also sometimes only reveal one side of the story.
Unfortunately comparable RNA-seq data (development, gill) are not available for model animals treated in the present study. Anyway, our purpose was to know which Nos paralog was expressed in gills during development, more than a speculation on their level of expression.

Finally, there is little discussion of gene expression of the duplicates within fishes, which is another interesting aspect of the paper: what are all the fish duplicates doing. I am sure there are RNA-seq datasets of different organs that could illuminate this question.
There are not enough developmental expression data available for most of fishes, unless for zebrafish for which we used a single-cell RNA-seq database from 1 to 5 dpf (cited in the manuscript). The two nos2 paralogs (nos2a and nos2b) show differences in the expression pattern. The nos2a is expressed only in 5 cells, mainly in intestine and thymus. The nos2b is expressed in 156 cells of different tissues as periderm, fins, pharyngeal epidermis, nervous system and intestine. Therefore, this suggests the possible occurrence of a subfunctionalization event, although more data are necessary to clarify it.

The author published previously several papers on the topic, including expression in amphioxus and some phylogenetic analysis. In sum, I think this is decent quality work, but that it does not really provide an in-depth approach to understand the evolution of paralogues role and regulation after duplication, which strongly limits its scope.

Other remarks:

- I think it would be great to have a ML tree as well. Are the domains of the protein conserved across paralogues? Any clue of differential evolution at the protein level (selection or reduced constraints). Like a PAML site or branch site analysis, etc.. Also, please, make the alignments available.
We performed an ML tree analysis using the same alignment of the MrBayes tree. The ML tree method confirmed the topology shown in the Bayesian tree (Figure 1) and is available in the new Electronic supplementary material, figure S7. We now prepared a new Figure 1 in which for each node we reported the posterior probability values for MrBayes (left) and the bootstrap support for ML (right).
- Regarding Nos protein organization, we confirm that all domains are conserved across all paralogs, and we specified this in the new version of the ms.
- The alignment used is now available, we are sorry that it was not in the previous version of the manuscript.
- We now included a dedicated section of the Nos family evolution at the protein level using Branch Model (BM) analysis, as requested. We demonstrated that all three Nos genes are under purifying (negative) selection although at a different rate, based on significant ω (non-synonymous to synonymous substitution rate ratio) values. We explained this in Result and Discussion sections, and reported the methodology used.

- L426-438: this is very speculative, the hagfish could very well have a similar situation than lamprey.
We agree, so we shortened the sentence and generalize it as a possible loss of all cyclostomes, instead of distinguish the situation in lamprey and in hagfish. Nevertheless, we still need information to conclude that hagfish do not have Nos expression in the gills.

- I think the alignments should be present in a downloadable form with proper annotation. The phylogeny constitutes one of the resources of the paper.
Right, we are sorry for that. We couldn't upload the alignment file at the moment of the submission, but it was not our intention not make it available.

Referee: 2

Comments to the Author(s). See attached document.

The paper describes gains and losses of Nos paralogs within Actinopterygii. The findings are clear and the conclusions are largely supported by the analyses. I only have a few suggestions to improve readability.

The authors do a good job of describing taxonomic groups and what belongs to them in the methods and results, but improvements could be made here. For example, Clupeocephala is weakly defined and Morymyridae is undefined. Otomorpha and Protacanthopterygii are kind of defined in Fig 1 and the legend of Fig 6. Neoteleostei is not defined anywhere in the paper, which is not helpful since there is a major focus on the loss of Nos2 in Neoteleostei. A simple remedy may be to extend the supplemental table 1 to include taxonomic classifications. I would also encourage the authors to make supplemental table 1 an editable spreadsheet (excel or other). Working with pdf tables for replication purposes is quite painful.
We thank the reviewer for the good suggestion; we now improved the taxonomic definitions in the Electronic supplementary material, Table S1 and prepared a new Electronic supplementary material, Figure S1 in which we clarified the taxonomic classification of all the species used in this study in order to better understand the species relationship.
Moreover, we saved the Electronic supplementary material, Table S1 and Table S2 as excel editable spread sheets.

I'm not very good with fish taxonomy (zebrafish -> Danio rerio is about as good as I can do) and using scientific names in fig 1, but common names in fig 2 (and Fig S1) isn't very helpful when I want to link duplication events in fig 1 to synteny in fig 2. I would like to see a naming standardization, it's probably easier to replace common names with scientific names in fig 2 and fig S1.
We agree that the fish taxonomy is quite tough and to avoid confusion we adopted name standardization among all figures. In the specific, we preferred to add the scientific names to the common names in Figure 2, rather than to substitute scientific names with common names in the tree of Figure 1. In any case now readers have the proper information to link data presented.

It may be worth citing https://doi.org/10.1038/s41467-021-24573-z regarding cyclostome duplications and the vertebrate 2R. Interpreting cyclostome duplications could benefit.

We added this very recent publication on early vertebrate evolution.

I appreciate the authors attempt to incorporate the Italian flag into figures 1 and 6a. However, these figures (especially Fig 6a) may be difficult to interpret for those with red/green color blindness. I would encourage the authors to use a different palette to provide better accessibility/interpretability.
We improved the figure taking into account the visual difficulties using Color Oracle program in order to find friendly colors for red/green blindness. We used the blue for Nos1 (color code 76ccfc), yellow for Nos2 (color code fdf152), and red for Nos3 (color cose fc8d62).

Reference to human Hox genes is surprising in Fig 6b. Are Nos paralogs close to the Hox clusters in fishes without additional genome duplications (i.e. spotted gar)? Are genome drafts in fishes intact enough to demonstrate this? If not, I would understand the use of human, but there's currently no rationale for using human.
We checked for the linkage between *Hox* clusters and *Nos* genes in *Lepisosteus oculatus* and detected it exclusively for *Nos3* and *HoxA* cluster that lie on chromosome LG11. It is possible that in the lineage of Actinopterygii chromosomal rearrangements occurred so that we cannot observe linkage between the other two *Nos* genes and Hox clusters. For this reason the human genome arrangement is the best support we found for a 2R origin of *Nos* genes.

Minor fixes
I think it should be clearer in the methods that all paralogs were tested for expression among species.
Done.

In fig 1 Nos is presented as NOS in human and anole, but Nos for the others and it's not clear why. For aesthetic purposes, Nos should be consistent?
In the present work we adopted the gene/protein nomenclature used in zebrafish. As observed by the reviewer we used NOS according to the "community-based genetic nomenclature for anole lizards" (see Kusumi K et al., *BMC Genomics* 2011. doi:10.1186/1471-2164-12-554).

Line 47: replace 'suggest' with 'suggests' and the comma after suggest is probably unnecessary.
Done.

Line 63: remove 'and in' before neurodegenerative.
We prefer leave as it is because we want to distinguish the physiological processes from disease and cancer.

Line 121: Replace 'last' with 'lastly'.
Done.

Line 121-125: The statement: "we named nos2.1 and nos2.2 paralogs…" could be better rewritten as: "and lastly, the two nos2 paralogs…..were named nos2.1 and nos2.2".
Done.

Line 128: remove 'latter'.
Done.

Line 168: add 'in' between 'remained' and 'the'
Done.

Line 211: reference supplemental figure 2 in the legend since nos2a isn't shown in fig 2.
Done.

Line 256: "Surprisingly, nos1 and nos2 were expressed in gills of sturgeon, bichir, and shark." I think you should remove the sentence. It's confusing as it suggests both nos1 and nos2 are expressed in all 3 species when the next sentence clarifies what's actually happening.
We agree that it was misleading, therefore we slightly modify the sentence.

Line 359: Clupeocephala is misspelled.
Corrected.

Referee: 3

Comments to the Author(s)
In this MS entitled 'Evolution of the nitric oxide synthase family in vertebrates and novel insights in gill development', Giovanni Annona and colleagues perform and exhaustive phylogenetic analysis of the nitric oxide synthase family in vertebrates, paying special attention to gnathostomes and cyclostomes, revealing a complex evolutionary history of gene duplications and losses. The MS, moreover, provides valuable expression analyses in a variety of species that help to clarify the function of each member of the Nos family,. The phylogenetic methods and the synteny analyses is solid, providing little doubt about the robustness of the results. The expression analyses is also solid and clear. The results are interpreted correctly, and discussed in the proper context of other publish works, as far as I know. The final model proposed for the evolutionary history of this complex family is relevant for the scientific community interested in gene family evolution, as well as the role of nos in embryo development and adult organ homeostasis, paving therefore the way for future functional analyses by gene modification. I have no major revisions, and I have only find some minor points that can be addressed by the authors at their own judgement without further revision.

Minor points:
L3: remove comma before "and"
Done.

L71: "which" should be "whose"(?)
Done.

L114: the number of examined available genomes should be stated
Since the number of available genomes for this clade is limited, and possibly not representative of the whole clade, we prefer to state that *nos2* was not found in "any available genomic or transcriptomic data".

L130: "is" should be "was"?
Right.

L144: the authors say "with no clear orthology relationship to any specific gnathostome Nos1, Nos2, Nos3". I disagree, however, since the tree suggest that NosA/B clade originated in the cyclostome genome, and this clade is pro-ortholog (according to PMID: 10597641) to the clade Nos1/2/3. I would ask the authors to evaluate the strength of this clade's relationship, and if they consider it solid enough, I encourage the authors to consider whether cyclostome Nos A and B should be renamed as Nos1/2/3a and Nos1/2/3b. In fact, the same should be used for cephalochordate and tunicate sequences, if no clear ortholog of nos1, nos2 or nos3 is present in any of those group of organisms.
Although the reviewer is technically correct we would prefer to keep the names NosA and B instead of Nos1/2/3a and Nos1/2/3b for the following reason: Nos1, 2 and 3 are the only componentes of the Nos family in jawed vertebrates, and therefore there is no room for confusion for a putative orthology with other members. In other gene families, like for instance the Pax family, this is not the case, so we favour the inclusion of the original ganthosome members for which cyclostomes have pro-orthologs: for instance, there are genes for Pax2/5/8, Pax6, etc to distinguish their pro-orthology relationships with different gnathostome groups of counterparts. As we say, that possible confusion doesn't exist in the case of Nos genes, thus in order to keep the gene names as short as possible we prefer to keep our original nomenclature.

L168: remained "in"?
Done.

Figure 1a: If the names of species and tree branches were colored according to their groups, it would facilitate their visualization. The code of colors could be maintained in Fig 1b and c.
We would prefer to maintain our original figure organization using the coloured background to gene groups. Regarding to colours, we have used a colour-blind friendly visualization according to suggestions of reviewer 2.

Micro-synteny figures: please, specify in the legends what is the minimal distance to be considered "long" as indicated by // (>100 kb). Are the indicated genes consecutive? I also wondered if other genes can be in-between the colored genes (please, clarify in the legend) if so.
We now indicated the minimal distance on chromosomes as // means >600 kb. Additionally, sintenic genes indicated in the figures are consecutive (no other gene in-between), therefore we now reported it in the figure legend.

L228: Clarify, please, why is unexpected.
We meant that *Nos* expression has never been detected so early in development. Nevertheless, considering that amphioxus NosC is expressed early in pharynx area, we eliminated “unexpectedly”.

L374: In the context of the discussion of the Nos genes and the VGD, reference [8] should be cited.
Done.

Discussion: I wonder if there is any evidence on Nos expression in the gills or pharynx of tunicates or cephalochordates that could enrich the evolutionary origin of this expression domain and function. Please, comment.
In the discussion we reported that *nos* expression has been detected in cephalochordate’s developing pharynx, among other tissues, while the single *nos* gene in tunicates has been investigated in *Ciona robusta* (previously named *Ciona intestinalis*) in developmental and larval stages and it is not reported any expression in the pharyngeal area.

L431: as correctly discussed in this line, I would suggest to remove the word "likely" from L47 in the abstract, and use a less strong statement "may have arisen"
We agree on this point and modified as suggested.