Dear recommender and reviewers,

We are grateful for the positive feedback and the thorough reviews that help substantially improving our manuscript. We considered all your comments, suggestions, as well as typos, syntax and grammar corrections. We paid particular attention to clarifying all aspects that were questioned or reported unclear. In addition, we carefully restructured the introduction, clarified the hypotheses, and reformulated the discussion related to the antiquity of open vegetation in northern-Madagascar.

We hope that our revision of the manuscript will satisfy the recommender and the reviewers and that the new version will reach PCI-Evol-Biol standards for recommendation.

You will find below our detailed point-by-point answer to your questions and comments. Note that additional minor typos, syntax and grammar errors were corrected according to the recommender and reviewers suggestion but are not reported here to keep the document focus on major comments and to maintain its length to a reasonable size.

Sincerely,

Jordi Salmona and Guillaume Besnard, on behalf of all co-authors.

Recommender: Miguel Navascués, 2021-01-07 21:56

I have now received the reviews for your manuscript. While they are in general positive, both point out few concerns regarding the methods, conclusions and writing that require further work on the manuscript. Please consider all their comments carefully before submitting a revised version for evaluation. I believe most of these points can be addressed by working on the text and no further analysis will be necessary. However, pay special attention to properly justify the absence of new analysis regarding the concerns on the methodology.

Both reviewers highlight the lack of explanation for the reduction of samples from sampling to genotyping. Please revise your text so it is completely clear for the reader why there are such differences. In addition, clarify how the survey of Noronhia was conducted and if N. spinifolia was identified in the field. Were all Noronhia spp. sampled? In the main text it is unclear if the 220 samples are from Noronhia spp. or from N. spinifolia only (in the supplementary text it is clearly stated, but I think the reader should not need to go there for this basic information).

> We thank the recommender for the positive feedback as well for his suggested strategy concerning additional analyses. We modified the manuscript to better explain the sample selection, and to clarify, in the main text, the crucial aspects of sampling. Briefly, the reduction of samples from sampling to genotyping was motivated by DNA extraction yields and quality, as well as by the genotyping depth/cost trade-of. These important aspects were clarified in the main text (§M&M: Laboratory procedures).

Another question that seems to require a more clear explanation is the discussion of the age of forest fragmentation in the area. I believe that some of the confusion stems from the better fit of the genetic distances with data on recent vegetation cover (2000's) but a conclusion that forest fragmentation structuring the genetic diversity of N. spinifolia must be ancient. It might not be clear for the reader why data on older vegetation cover does not explain better the genetic structure of N. spinifolia.

> On the basis of the reviewers and the recommender comments, we have extensively revised and clarified the antiquity of forest fragmentation discussion. Specifically, we restructured this paragraph to make sure that each idea is clearly stated, and included the wise suggestions of the reviewers. We further toned down the implications of our results, we maintain that our results supports the antiquity of open habitats in northern Madagascar, but discarded the mention over-

stating that “our results corrode the narrative that human habitat alone changed the island’s landscape”. §Discussion: The antiquity of forest fragmentation in northern Madagascar

I also have some points I would like you to address:

1) Regarding the discussion on the role forest cover for seed dispersal (lines 497-509), it is not obvious to me why patterns of mtDNA and cpDNA haplotype networks would be incompatible with the signal found by IBR analysis on organelle data. I would appreciate if you could develop this point.

We thank the recommender for raising this point. The phrasing and the structure of the paragraph was indeed pointing towards an unnecessary dichotomy among the results of IBR and haplotype networks. We thoroughly developed, rephrased and restructured this discussion section to highlight, instead, the complementarity of the information brought by both types of analyses. §Discussion: On seed-mediated gene flow: the organellar DNA testimony.

2) Supplementary data availability: In addition to making available the missing Table S1, please consider making supplementary tables S6, S7 and S8 available through reputed repositories (such as institutional repositories, Zenodo, DataDryad, supplementary material in bioRxiv...) that can assure long term accessibility to those files (instead of Google Drive/Docs service). Also, I could not find reference PRJNA632767 in the Sequence Read Archive (SRA) NCBI database, make sure the reference is correct and it is accessible.

All the supplementary tables previously included as Google Drive links (some of which appeared to be non-functional) were moved to BioRxiv supplementary material. In addition, the project PRJNA632767 in the Sequence Read Archive (SRA) NCBI database was made public.

5) lines 324-5: It is unclear what the values “m=4, M=5, N=8” are. Please clarify: a brief description/definition (such as in figure S5) should be included in the main text when and in the supplementary materials (Method S6) when they are first mentioned.

A brief description of the parameters has been included at their first mention in the manuscript and supplementary material. §Results: Organellar DNA genotyping and nuclear catalog construction

Additional minor typos, syntax and grammar errors were corrected according to the recommender suggestion but are not reported here to keep the document focus on major comments and to maintain its length to a reasonable size.

Review by Katharina Budde, 2021-01-06 16:31

The Manuscript „How ancient forest fragmentation and riparian connectivity generate high levels of genetic diversity in a micro-endemic Malagasy tree“ written by Salmona et al. deals with an interesting topic in a very interesting and understudied ecosystem. The authors used chloroplast SSRs and RADseq loci from the chloroplast, mitochondrial and nuclear genomes to study the genetic diversity and genetic structure of a narrow endemic tree species, Noronhia spinifolia in Madagascar. The study presents interesting data and results and is well written, nevertheless I have some concerns that I will lay out in detail below and that I hope will be helpful for the authors to improve the manuscript.

We thank Katharina Budde for her positive feedback and for her detailed review that helped significantly improve the manuscript.

Study species: In this section I am missing some more information e.g. about the flowering and fruit phenology of the study species. Is this a monoecious or dioecious species? Are the flowers hermaphroditic or are female and male flower parts separated on the same individual? Please also

add information about the possible pollen dispersal and seed dispersal mechanisms. If no literature is available for this particular species the flower and fruit morphology can already give an idea.

We thank the reviewer for spotting this missing piece of information. It has been included in the material and methods (study species): "The plant has cream-white, urceolate, small (> 7 mm long), and hermaphroditic flowers, as well as small (> 10 mm long) and drupaceous fruits that have a thin mesocarp and a rather crustaceous endocarp (Hong-Wa, 2016). Flowering and fruiting typically occur from October to May, during the rainy season. Flower and fruit characteristics, along with observational accounts, suggest insect pollination (e.g. bees) and animal dispersal (e.g. birds, lemurs, rodents)." §Material and methods: Study species. We additionally linked this information to the general introduction of the species and the description of the hypotheses. §Introduction

Sample numbers: 220 leaf samples collected, DNA extracted from 137 samples and after quality control only 72 samples were used for genotyping and sequencing. Why could only so few samples be used at the end?

This aspect was also pointed out by the recommender. We modified the manuscript to better explain the sample selection, and to clarify, in the main text, the crucial aspects of sampling. Briefly, the reduction of samples from sampling to genotyping was motivated by DNA extraction yields and quality, as well as by the genotyping depth/cost trade-of. These important aspects were clarified in the main text (§M&M: Laboratory procedures).

The methods appear mostly sound but sometimes I am missing details:

A minimum coverage of 4x for the RADseq seems very low to me and I am wondering why the authors chose such a low threshold. Anyway, it is good that the authors used both called genotypes and genotype likelihoods! Could a higher threshold for the minimum coverage have improved the genotype calling in STACKs?

The minimum depth of 4× questioned by the reviewer was in fact the minimum read depth used by stacks to build a stack, not the depth considered to call or consider SNPs. The comment of the reviewer pointed out a lack of details and clarity in some parts of our method section. We therefore improve the details and the structure of the Method S7 section "SNP calling & Genotype Likelihood". The minimum depth used to consider a SNP (10×) and other unclear details were clarified in Method S7.

What about linkage disequilibrium at nuclear loci? I could only find information for the organellar loci. And which markers were used for NgsAdmix and ADMIXTURE? Did the authors take care to remove loci showing high LD?

The reviewer is right, we did not formally test linkage disequilibrium between the >22 000 nuclear variant RAD-loci. We did however test the effect of removing loci with high LD on ADMIXTURE analyses. Specifically, we used PLINK to keep one SNP per locus. The analyses with or without SNP under LD did not show substantial differences. This is expected, considering the large number of loci, which present a good representation of the genome, and the high number of SNP per loci, that balances possible LD effect across most loci. The reviewer's comment also highlighted that our LD analyses needed being better detailed. Therefore, we paid attention to improving their presentation in the new version of the manuscript. §Method S7 section "SNP calling & Genotype Likelihood"

I am missing Table S1 (if there is a link in blue, it did not work for me).

The table S1, S6, S7, S8, have been added to the bioRxiv supplementary material repository associated with the manuscript.

The authors mention that they focus on the mature trees. Why did they make this choice? For the future, I think a comparison between mature and young trees might be interesting to evaluate the effect of more recent forest fragmentation.

We thank the reviewer for the suggestion of comparing the genetic diversity of mature and young trees. We selected mature trees for the two following reasons (also clarified in the main text): **First**, because much of the density-dependent mortality takes place before maturity in trees, their effective population size contributing to the genetic diversity is closer to the actual adult census size than to the entire population including young trees and seedling (Dodd & Silvertown 2000; Petit and Hampe 2006). Therefore, the regional patterns of diversity are expected to be better represented by adult samples. **Second**, since we also ought to infer the demographic history of *N. spinifolia*, we also made sure to genotype fully-grown reproductively mature trees that would represent the current effective population. Now clarified in §Material and Methods: Plant Sampling

We, however, agree with the reviewer that an additional analysis of seedling and young trees surrounding mature ones would bring fine scale and temporal resolution on seed and pollen dispersal processes. Such sampling was originally considered, but not retained, in the context of this study, it would have required sampling and genotyping a large number of young trees per adults (cost limitation), and be little compatible with sampling the whole diversity of *Noronhia* (all sp) trees in the region (time limitation). This is now briefly mentioned in §Discussion: Further prospects and conservation implications

l. 289 I suggest to substitute "diversity" with "structure" as the authors do not test the effect of environmental factors on genetic diversity estimates but rather the effect on the genetic distance or relationship matrices. Is this correct? It might be helpful to actually mention which measures were tested for correlation. In the following paragraph the authors only mention "genetic matrices".

The reviewer is right; to test the effect environmental factors (EF) on the structure of the genetic diversity we compared it (EF) to matrices of genetic distances and relationship matrices. We modified the sentence according to the reviewer's suggestion. The measures tested for correlation were initially mentioned in the "genetic distances" and "Landscape variables" paragraph above. We now added a reference to these paragraphs to improve clarity. § M&M: Statistical procedures.

l. 391 What is the null IBD layer? Do you mean a null model only taking into account IBD? Please clarify.

The reviewer is right; the null IBD layer is indeed a null model only taking IBD into account. We modified the sentence to improve its clarity. § Results: Landscape genetics

Figure 4C Is "Percent of tree cover geographic distance" referring just to a pair-wise distance or is it a conductance measure? Please clarify! I find it sometimes difficult to follow the authors to which kind of estimate (pair-wise distance, resistance etc.) they are referring. Please revise the manuscript accordingly.

The legend has been modified to "Percent tree cover conductance". We also homogenized and revised terminology use through the manuscript, with regards to the reviewers comment. § Fig4C

l. 447-450 Is it meaningful to directly compare the genetic diversity levels between species with such different life history traits as a tree species and mammal species (here mouse lemurs and plains zebras)? Also, why did the authors pick these species and not others? Tree species are characterized by harboring huge amounts of standing genetic variation (e.g. Petit & Hampe 2006, https://doi.org/10.1146/annurev.ecolsys.37.091305.110215). The authors have already illustrated well the big difference in diversity levels in the sentences before by comparing with other tree species. Therefore, I would suggest to remove these sentences.

We agree with the reviewer that it can seem meaningless to directly compare the genetic diversity levels between species with such different life history traits. However it is meaningful, on a technical level, to compare estimates obtained with the exact same data (RADseq) and analytical procedure (GL-estimates). It is to that purpose that we present these comparisons that were kept and better justified in the revised version. The high standing genetic variation of trees and the useful reference suggested by the reviewer were also included to the discussion. §Discussion: *Noronhia spinifolia*, a highly diverse Malagasy micro-endemic.

The authors explain their results by a long-standing forest fragmentation in the LM region (l. 521) which is in line with other studies suggesting that forests were already fragmented before intensified human use in some regions of Madagascar. Do they have any idea if the areas surrounding the current forest patches might have been historically covered by a different vegetation type where N. spinifolia was not present? Are there other environmental factors that are correlated with forest cover, e.g. soil type that might be less suitable for N. spinifolia?

It is a very interesting point raised by the reviewer. Indeed, the effects of human colonization in Madagascar, and the expansion of agropastoralism through Madagascar ~1 ky ago, may have had drastic effects on fire ignition frequency and regime. Fire-prone open-habitat, grasslands and savannas may therefore have changed more rapidly than closed-canopy forests. Furthermore, areas easy to access (along ancient trails), cultivated (rivers banks, fertile land) and with constant water resources may also have been occupied and modified earlier than remote close canopy mountainous forests. This is now briefly covered in the §Discussion: The antiquity of forest fragmentation in northern Madagascar

Concerning *N. spinifolia* soil's preferences, we unfortunately did not collect data nor assessed it. Further study including high quality soils characteristics layers, and / or may soil characterization in the field may shed light on its influence on *N. spinifolia*'s settlements and preferences. This is now mentioned in the §Discussion: Further prospects and conservation implications. We also added a mention of *N. spinifolia* soil preferences in the §M&M: Study species.

Another or additional possible explanation for the high genetic diversity and structuring, as also mentioned by the authors, might be hybridization with closely related species and I very much agree with this. Could it also be a case of cryptic radiation as it has been reported for several groups on Madagascar (but see also Pillon et al. 2014; doi: 10.1111/nph.12677)? Noronhia habors many micro-endemic species in Madagascar and I agree that a more exhaustive sampling of all possible closely related species and taking into account morphological and ecological features in future analyses might shed further light on hybridization, ecotype differentiation and speciation.

The reviewer's hybridization comment is in line with our understanding of the genus evolutionary history. Our understanding, however, is that the strong linear relationship between geographic and genetic distance precludes cryptic radiation (Pillon *et al.*, 2014) and microgeographic adaptation (Scotti *et al.*, 2016) to be the major drivers of the observed genetic diversity. In addition, our cpSSR data do not show any shared haplotypes with close relatives, suggesting that hybridization, if any, was relatively ancient (see discussion). This is mentioned in the §Discussion: *Noronhia spinifolia*, a highly diverse Malagasy micro-endemic.

Additional minor typos, syntax and grammar errors were corrected according to the reviewer suggestion but are not reported here to keep the document focus on major comments and to maintain its length to a reasonable size.

Reviewed by Yurena Arjona, 2021-01-04 22:23

The manuscript entitled "How ancient forest fragmentation and riparian connectivity generate high levels of genetic diversity in a micro-endemic Malagasy tree" by Salmona et al. evaluates the influence of landscapes variables in the genetic differentiation of the olive species Noronhia spinifolia, micro-endemic to northern Madagascar. The main result shows the forest cover as the most important variable modulating genetic differentiation of this species.

The manuscript is well written, and the analyses are appropriate and thoroughly performed. I particularly appreciate the effort made in analyzing separately the organelle and nuclear genetic signals. However, I have two main concerns. The first one is about the emphasis of an ancient forest fragmentation that, from my point of view, has little support on the results (see the last comment below). The second main concern is about the landscape variables used and how the resistance layers were created from them (see below for more details), but I am sure that with some clarification and modifying a bit the text, it will be easily solved.

In addition, I have some suggestions about the structure of the manuscript, particularly regarding the introduction. The introduction is a bit disconnected from the other sections, particularly when referring to the landscape effects on the genetic differentiation of N. spinifolia. During the introduction, some landscape variables are referred as important for other species, such as the forest matrix, or the Manankolana River. In Methods, it is said that little information on the landscape variables that may affect N. spinifolia connectivity is available and hence all available landscape variables were assessed (L. 266-269). However, in the discussion I could learn that from fruit and flower morphology, and from knowledge of close related species some hypotheses may be formulated, and consequently, some expectations about the landscape variables as well. Including these hypotheses and expectations in the introduction may help the flow of the text between the different sections. Without these previous hypotheses and expectations a wide range of possibilities is open. For example, the authors include rivers and streams as landscape variables, but they did not consider the directionality of the water flow which could be connecting upstream forest patches with downstream ones. The same happens with wind direction and slope (upslope vs downslope movements).

We thank Yurena Arjona for her positive feedback and for her detailed review that helped us to significantly improve the manuscript. We considered her comments with care and modified the manuscript to clarify both the ancient forest fragmentation and methodological concerns. In addition we took advantage of her structural suggestions to improve the introduction as well as the flow among sections.

Following the manuscript flow, see below my detailed comments, most of them minor comments:

The minor typos, syntax and grammar errors were corrected according to the reviewer suggestion but are not reported here to keep the document focus on major comments and to maintain its length to a reasonable size.

L. 72-79. This paragraph changes the focus from the importance of studying species genetic diversity and connectivity across landscapes and landscape features affecting them, to the importance of the antiquity of open-canopy environment. Although I have no doubts on the importance of confirming the antiquity of this kind of habitat, I recommend to remove this paragraph as it is not the main objective of the study and is disconnected from the other paragraphs.

We understand the reviewer's concern, and we agree that this paragraph was imperfectly integrated to the §Introduction flow. To overcome this issue we have substantially modified the structure of this paragraph, to strengthen our point, i.e. "the genetic diversity of an organism is the combined result of its distribution structure and history, it is "therefore" crucial to assess the antiquity of landscapes which can be questioned from genetic data". We also improved its flow with the

previous and following paragraph. We thank the reviewer for pointing out this "flow disconnection" that helped us to improve the introduction.

L. 93-94. Tree species usually have long generation times, so changes in the landscape will need long time to be reflected by these species and hence the effect of recent changes will not be noticeable. I would rather expect scrubs or herbs associated with the forest and with shorter generation times to be better models for studies in fragmented habitats.

We agree with the reviewer's comment. Since it is not our aim to compare the quality of potential models in the introduction, we toned our sentence down by introducing the idea that their long generation time is a limit to being the best models. In complement we included the reviewer's suggestion in the discussion section.

L. 189-192: I find confusing that 220 leaves were collected (from 220 different trees, based on Methods S1), 137 samples were used to extract DNA, and 72 were selected for genotyping. Why was it not possible to use all collected samples?

This aspect was also pointed out by the recommender and the other reviewer. We modified the manuscript to better explain the sample selection, and to clarify, in the main text, the crucial aspects of sampling. Briefly, the reduction of samples from sampling to genotyping was motivated by DNA extraction yields and quality, as well as by the genotyping depth/cost trade-of. These important aspects were clarified in the main text (§M&M: Laboratory procedures).

L. 202-208: Some information is missing here to fully understand this paragraph. Particularly, what the authors called "loci identified in silico". In Methods S5 is clearly explained but the methods section in the main text should be comprehensible by itself.

We thank the reviewer for pointing out that this paragraph needed clarifications. We clarified in the § Method S5 that the *in silico* analyses were mentioned at the beginning of the paragraph.

L. 268: I recommend to specify the ten landscape variables used. A table may be a good way to show all of them and a summary of the results, or at least specifying which ones were discarded for further analyses (L. 391).

We thank the reviewer for this wise suggestion. The suggested table has been added as Table 1.

L. 276: It is not clear how the authors created the resistance layers. It says that 14 conductance-resistance values were tested, and in Methods S10 this values are 1:20, 1:15, 1:10, 1:8, 1:5, 1:4, 1:2, 2, 4, 5, 8, 10, 15, 20. From the table S6 I assume that each of these 14 values is assigned to the landscape feature (cost variable) and to the rest of the surface (cost non-variable), and a resistance surface is created from each combination of two values. The x-axis in figures S21-S24 is the logarithm of the ratio between cost variable and cost non-variable. If I am right, discrete features only have one cost level? i.e. discrete forest cover was considered as forest vs non-forest, independently of the density of the forest cover. How the procedure is for continuous variables? About the categorization of variables in discrete and continuous, slope and wind speed were considered as discrete variables, why? This part of the methods should be clarified in the manuscript.

We thank the reviewer for pointing out these particular aspects that needed clarifications. The table included thanks to the previous comment partly clarified this matter (Table 1). The variable categorization description in Method S10 contained an error that led to the reviewer's question, it has been corrected (5 discrete and 7 continuous were tested). We further clarified the S21 and S24 legends, and the methodology (in §Method S10) by re-using the explanation suggested by the reviewer.

I find very interesting considering the wind as landscape variable, as it is not quite usual. However, I wonder why the authors considered only wind speed and not direction, as direction may be even

more important in modulating the connectivity by wind. If the authors find it relevant, more information can be extracted from analyzing wind connectivity between patches and I recommend using rWind (Fernández-López & Schliep, 2018) for this purpose [Fernández López, J., & Schliep, K. (2018). -rWind: download, edit and include wind data in ecological and evolutionary analysis. Ecography, 42(4)].

We agree with the reviewer that wind-direction may be pivotal in modulating the connectivity by wind. We actually thought of using wind-direction, initially. Since the wind-direction was complex to integrate within our framework already testing granularity, cost and movement models, iteratively, it was unfortunately not included in our study. We however added a mention of this limitation of our analyses, in the §Discussion: Further prospects and conservation implications; to point toward these analyses in future work.

L. 391-392: "showing uniform response at varying costs" is this variable response showed in Fig. S21-S24? If it is the case, slope exhibit a uniform response and based on Table S6 it was not discarded, why?

We modified the text to clarify the point raised by the reviewer. "To build multivariate models, we retained in priority landscape variables showing a better fit (R^2) than the null model considering IBD alone, and exhibiting sensitivity to cost values (e.g. % forest cover)." This new version is closer to what we ended-up doing, while the previous one was closer to what we originally intended to do. §Results: Landscape genetics.

L. 521-525: this difference between years is not relevant since all of them have a strong relationship with the genetic differentiation of the species, the 2000's layer has better resolution that could bias the result (L. 528), and since the hypothesis of ancient forest fragmentation is been discussed.

We understand the point of the reviewer. However the difference between these layers is rather strong, and some layers (Veg_1958) do not even show higher fit than IBD. The point we make in the first sentence is indeed relevant at the scale of Madagascar where recent forest loss (in the past 70 years) reach >40%, with some regions showing much higher levels. In regions with deforestation rates >40%, we do not expect to recover a better fit of the most recent forest cover even considering the potential resolution bias. We clarified this aspect by modifying the text of that section. §Discussion: The antiquity of forest fragmentation in northern Madagascar.

L. 539-540: I find this conclusion rather strong. How many generations would be necessary to detect the genetic consequences of a recent fragmentation? I find difficult to detect a recent forest fragmentation event considering the long generation time of N. spinifolia. For example, if it has occurred at the beginning of the XX century only between two and five generations have passed, few to detect its effects.

As mentioned above, on the basis of the reviewer (and the recommender) comments, we have extensively revised and clarified the antiquity of forest fragmentation discussion. Specifically, we restructured this paragraph to make sure that each idea is clearly stated and explained. We further toned down the implications of our results, we maintain that our results support the antiquity of open habitats in Madagascar, but discarded the mention over-stating that "our results corrode the narrative that human habitat alone changed the island's landscape". §Discussion: The antiquity of forest fragmentation in northern Madagascar.

To answer specifically the point raised by the reviewer, the particular number of generations does not matter very much here. If the effect of forest fragmentation was not detectable, we would expect to recover a better fit of the isolation by distance alone (compared to vegetation covers). As the reviewer points, if the region was covered by forest 2-5 generations ago, the re-shuffling of allele frequencies, in each forest patch, over this period would not allow detecting an effect of the current forest cover, but rather would favor IBD alone. It is, therefore, the strong effect of the current forest cover, which is sound evidence that the landscape is of similar forest cover shape for

a long enough number of generation that allowed imprinting the allele frequencies of *N. spinifolia*'s populations. We thank the reviewer for questioning a weakness in the discussion, which question allowed us to strengthen our conclusions. We modified the $ Discussion: The antiquity of forest fragmentation in northern Madagascar, as follows: "The time lag for a particular landscape feature to imprint its effects in the genetic diversity of a species, has been little studied (Landguth et al., 2010; Mona et al., 2014). However, in *N. spinifolia*, based on the strength of the signal, the high level of diversity and of gene-flow, the re-shuffling of allele frequencies after fragmentation can be roughly expected to last at least 40 generations, before harboring the signature of the new geographical pattern. This suggests that the landscape changes leading to the current forest cover are at least ~800 years (40 generations x 20 years), i.e. long pre-dating the most ancient available layer (1953)."