We thank the reviewers for providing thoughtful comments that have helped us to improve this manuscript. We have incorporated changes that address the reviewer’s concerns, and significantly improved the clarity of the manuscript. We hope that you find the revised manuscript suitable for publication. Below we have included each of the reviewer's comments and our responses in red.

**Reviewer 1**

A main issue is that the authors praise their current study to go deeper than a former study that looked at the impact of holocentricity at the level of orders (line 75) and indeed the authors use several genera per order. However, the results amalgamate the inferences again to an order level (same in the abstract - line 26). It would be important to assess and discuss the variation within each order also because such variation may provide hints about the underlying processes. This has been shown for Lepidoptera in De Vos et al. (2020 Philosophical Transactions of the Royal Society B) where the authors compared rates of speciation in relation to chromosomal variation.

The pervious study the reviewer mentions only assessed simple difference in chromosome number this is emphasized in lines 77-80 of the introduction.

*“This previous study was limited to an order level analysis and looked only for an absolute difference in chromosome number between monocentric and holocentric clades. A stronger test of the impact of holocentricity would be to investigate the rates of fusions, fissions, and polyploidy in clades with holocentric and monocentric chromosomes.”*

The study we present here uses a recently developed method of estimating rates of chromosome fissions and fusions [1]. Since all clades are monomorphic with regard to centromere type reviewing the data at an order level still seems appropriate (Figure 3) but this is done in addition to the insect wide analysis that incorporates all available data for all orders simultaneously (Figure 2).

With regard to variation within an order, we agree that this is an interesting idea. However, there is currently a lack of methods to evaluate rate variation within a phylogeny for the evolution of discrete traits. The only existing methods are like the one we have applied here where the investigator must a priori provide a hypothesis about what groups of species are allowed to vary in rates of evolution (e.g. in the current study we allow holocentric and monocentric lineages to have different rates of evolution). We are currently in the process of developing comparative methods that would allow for a more agnostic approach to investigating regions of increased or decreased rates of evolution in discrete traits but it is still under heavy development and testing and is not ready to be applied to any empirical analyses.

Another potential issue I see is that chromosome numbers per genus can be very variable. The authors tried to get around with this by sampling from the distribution (lines 238/239), but how robust is this and how does such variation relate to the variation in R?

The sampling approach that we use is relatively common comparative analyses (CITATIONS). However, we were unable to find any actual tests of the degree to which this may impact our inferences. For this reason, we chose to perform a new analysis of our results to test the impact of this approach on our findings. Because we performed this sampling approach once for each tree from our posterior distribution, we reasoned that bootstrapping our MCMC results based on the tree being analyzed allows us to get some sense of the degree to which this sampling approach may lead to significantly different estimates of rates. In particular we looked to determine the number of boostrapped datasets that conflicted with our major finding that the credible interval

Line 27: Here and throughout the manuscript it would be important to state in which orders polyploidy is common. It is for example highly debated if polyploidisation occurred in Lepidoptera – the authors only highlight that there were ancient whole genome duplications (line 186-188) but even that has been debated (ref 41 in the manuscript). See also Lukhtanov et al (2015, Proceedings of the Royal Society B).

The authors agree that it would be beneficial to add those orders where WGD is common when discussing polyploidization in insects, therefore we have added the following to lines XX in the manuscript: “There is support for WGDs in 18 hexapod species encompassing 12 orders (Ephemeroptera, Zoraptera, Plecoptera, Embioptera, Blattodea, Thysanoptera, Psocodea, Hymenoptera, Coleoptera, Trichoptera, Lepidoptera, Diptera). 5 of these 12 orders with WGDs are included in our study.” Additionally, the authors appreciate that there is a debate over polyploidization in Lepidoptera and have added the additional literature to the manuscript.

Line 48 onwards: It is important to note that these processes described here, e.g. Robertsonian translocations, are based on monocentric chromosomes. I would suggest to expand this section to also indicate processes involved for holocentric chromosomes (reviewed in ref 21 in the manuscript).

Read the paper. In hilicentric species, simple fusions or fissions may be more common. See why this may not apply holocentric chromosomes? (Expectaitons about centromere position) 🡪 just say fusion and fission

Line 63: However, many species are often not variable at all in terms of chromosome numbers, such as Lepidoptera where most known species show a karyotype close to the putative ancestral state (ref 36 in the manuscript and De Vos et al. 2020).

We aren’t sure what the reviewer is taking issue, we agree that segregating variation in chromosome number is rare. We are giving examples where individuals with heterozygous chromosome number have been evaluated.

Line 70 onwards: This argument makes only sense if holocentricity would result in fission events, yet fusion is also possible.

First, even if fusions and fissions are both equally higher in holocentric lineages maximum chromosome number will on average increase. For instance, imagine a Brownian motion process where increases and decrease are equal and thought the expected value remains constant the maximum value observed across many simulations will remain higher as Brownian motion parameter increases. Second, much of the previous literature surrounding holocentricity has focused on the ability to segregate fragmented chromosomes (cite) Therefore, we focus in this paragraph on explanations for high chromosome number including one of our previous analyses. Finally, we agree that we might expect both rates to increase and that these rates are really much closer to what it is we are interested in. Indeed being able to answer this question was one of several motivating desires in the development of this software that we are now applying to this question.

Line 101: Perhaps remind the reader what rate you are looking at.

Corrected

Line 106: Clarify that you refer to the tree of Misof et al.

Corrected

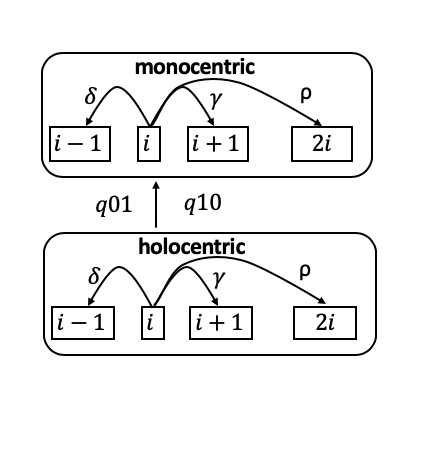
**Reviewer 2**

First, the title conclusion is too broad given the scope of the paper. The title should be changed to:  
  
Chromosome number evolves at equal rates in holocentric and monocentric insects.

We agree that the title was a bit broad originally. We have changed it to “Chromosome number evolves at equal rates in holocentric and monocentric clades”. We do not believe that it is necessary to specify insects in the title. The information that has informed our understanding of the differences in monocentric and holocentric chromosomes comes from both plants, insects, and non insect animals and the segregation behavior in all of these groups is largely similar.

Second, the machinery for rate estimation is too opaque. In particular, the authors should provide a simplified version of the core likelihood equation that is being used.

Because this method has already been published, rather than including a likelihood equation, we decided to include a graphical depiction of the Markov model being used (Supplemental figure 2).



Some clarification should be provided on how the polyploidization rate is estimated. In particular, would a polyploidization even be inferred when there is a doubling of chromosome number inferred in an ancestor? Presumably there is no other karyotypic signal identified. What signal in the phylogeny provides the support for these estimates? Some brief discussion would be helpful.

As depicted in the figure above polyploidy provides a route for an immediate doubling of chromosome number. Because of this, anytime the data suggests a very rapid transition from i to 2i chromosomes it will support a higher rate of polyploidy. This approach to inferring polyploidy has been quite well studied [1-5]. We believe that the inclusion of the Markov model figure from above will make this easier for readers to understand.

Finally, since the machinery for estimating fusion, fission and polyploidization rates is novel, it would be worth running some simulations on the tree that is being used, with fixed rates on subclades, followed by rate estimation on the simulated tip values for chromosome number. The degree to which the machinery used is good at estimating rates from simulated data is important to know.

The model for estimating these rates is not novel and is already published [1]. This paper contains the simulations that are being suggested here.

**Reviewer 3**

My main concern about the paper is that not enough attention is devoted to the underlying  
HMM model, and to the differences in results between models with and without polyploidy.  
Although the model is explained in an earlier publication (Blackmon et al. 2019), the current  
manuscript would perhaps be easier to understand for the reader if at least a minimal  
conceptual schematic was provided, showing how the eight parameters of the full model are  
related to each other.

The authors appreciate that more attention needs to be given to the underlying HMM model. Therefore, Supplemental Figure 2 has been added to the manuscript to better describe the model.

In the order-level analysis, the exclusion of the polyploidy parameter  
leads to an opposite conclusion. The basic reason for it seems obvious and there is some  
discussion in lines 185-193. However, what is the relation between size of a clade, rate of  
polyploidy and number of chromosomes?

The authors have added a sentence into the discussion to reinforce the take home that while inclusion of this parameter is important and we see the difference when comparing with or without this parameter, it does not alter that the HPD is overlapping and there is not significant difference between the two traits.

Lines 188: “Even a small number of polyploidy events  
[…] could lead to much higher variance […]” – how small and how much higher?

This is a general statement. How small and how much will depend on the specific phylogeny that is being analyzed for any given situation.

Furthermore, as multiple alternative explanations are provided in the Discussion, how difficult  
would it be to incorporate them into the current model? Since the authors offer “a way  
forward” (line 223 how close are we to evaluating other factors discussed? Are the data there?

The authors believe that while binary traits can currently be modelled, when discussing truly continuous traits such as population size, divergence time estimates, meiotic drive, etc. these may be a little more difficult. However, it is possible to discretize these continuous variables and with present data use this in the way we offer up as a way forward.

4,393 are many species, but not that many among insects. How well are different clades  
represented? Is the distribution of sampled species relatively even across the  
phylogeny? Just something to clarify.

In our study we used 4,393 species which is relatively small in comparison to the predicted ~900,000 insect species. However, this includes 10 of 24 known insect orders where we have over 20 genera sampled. The authors have added the following in the manuscript (line 189) to cover this point: “The number of orders with sufficient data is small (10) in comparison to the size of this group (24). However, this is largely due to insufficient data present for the remaining orders.”

line 101: what is the extent of difference between the two phylogenies? “Some clades”  
is vague.

We understand that this wording does vaguely describe the differences that could be seen among these two sets of trees. Therefore, the authors have updated the manuscript to include the clarification on how the trees differ in lines XXX. “The difference between these two trees is in the age estimates in older nodes. The Misof tree favors more recent branching events while the Rainford tree ages the nodes much later.”

Line 132-144: when discussing “intermdiate”, “lower” and so on rates, why not report  
the means and/or confidence intervals of those? (183-195)

XXX

Line 133: why is 20 the cutoff?

Only using those orders with sampling over 20 allowed for accurate prediction of rates rather than bias due to undersampling?

line 143 takeN

Corrected

line 236: a 100 trees is not many for a posterior. How much variability Is there among  
them?

100 trees is a fairly typical number of trees to fit complex models from a posterior [6-10]. Additionally, when looking at the variation among trees through several methods (bootstrapping, HPD) we find that our estimates are generally consistent across trees. There is no concerning variation among trees.

Lines 260-265: if high rates are not “biologically realistic” (BTW – is there a reference for  
this assumption?), why not limit the uniform prior to low rate values?

We have added in a citation for why it would not be biologically realistic to see really high rates of evolution. Additionally, we did limit the uniform prior to favor low rate values as stated in lines 132-134.

Line 291: perhaps should be: “…statistic, where …”

Corrected

Line 293 – monocentric clades evolve slower?

Corrected

The authors should be commended for reproducibility of the analysis. However, everything  
hinges on the R package chromePlus. Yet its repository  
([https://urldefense.com/v3/\_\_https://github.com/coleoguy/chromePlus\_\_;!!KwNVnqRv!SJMwQ5KcSvFhwbhRc3AQzSw3KnfRZl7MAxncX6NYuNJuNVYfl3Gq6g1fb38SNsp-$](https://urldefense.com/v3/__https:/github.com/coleoguy/chromePlus__;!!KwNVnqRv!SJMwQ5KcSvFhwbhRc3AQzSw3KnfRZl7MAxncX6NYuNJuNVYfl3Gq6g1fb38SNsp-$) ) states “This package is in the early stages of  
development and should not be used for any analysis at this point.” Not encouraging!

The readme for the GitHub repository had not been updated previously despite changes in the files and ultimately publication of the package. The package readme file has now been updated to “if you have questions or problems please let me know [coleoguy@gmail.com](mailto:coleoguy@gmail.com).

chromePlus should be cited as: Blackmon, H., Justison, J., Mayrose, I. and Goldberg, E.E., 2019. Meiotic drive shapes rates of karyotype evolution in mammals. Evolution, 73(3), pp.511-523.

.”

In terms of methods and data, the paper relies heavily on previous work by the same authors  
(references 5, 6). This is fine, but especially when it comes to the Markov model, a little more  
information on the guiding principles behind the model would be helpful to the reader.  
Incidentally, the website for the R package containing the statistical model states “This  
package is in the early stages of development and should not be used for any analysis  
at this point.”, which is unhelpful for anyone interested in further developments suggested at  
the end of the Discussion.

The authors have added clarification to the Markov model that is being used with the addition of Supplemental Figure 2. In addition, as stated above the readme file has been updated to reflect the publication of the package and confidence in future use.

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