Let’s put our responses in blue below each point, or pick your own color. Also, feel free to leave notes about the papers as we read them.

Reviewer: 1

Comments to the Author

In the submitted manuscript, the authors analyse published genome sequence data from spiders and their relatives; on the basis of their own analyses of these data the authors argue that several previously published papers are in error. Several papers have argued, from multiple lines of evidence, that spiders underwent a whole genome duplication (WGD) in their ancestry. This conclusion is now widely accepted. The authors claim that that this conclusion is incorrect. When attempting to overturn accepted conclusions, I think it is important that the new analyses are more robust than previous work, and that reasons are investigated why different researchers reached different conclusions. I conclude that neither goal has been achieved.

I must first point out serious errors made in referring to previous work, which paint the robustness of previous work in an unfair light. Both in the Abstract and in the Introduction, it is stated that previous work relied primarily on duplication data for one set of genes, the Hox genes. This is not true. The same erroneous point is made again on page 10 and in the discussion. Previous papers did discuss Hox genes, since there is a community of scientists very interested in these genes, but this does not constitute the primary support for WGD presented in the cited papers. Several other methods were used in the papers cited, including within-genome synteny, yet this is overlooked. Even if we just consider ‘target gene family’ analyses, these go well beyond just Hox genes. For example, Harper et al. use three classes of genes in many species: Hox genes, Wnt genes and frizzled genes; since the latter two are dispersed, the actual number of gene families found to be duplicated is much higher. In a similar vein, Schwager et al find duplication of 48 homeobox gene families (42 excluding Hox), with 32 of these duplications shared by two species. Leite et al use the full diversity of homeobox genes in house spider, detecting duplication of around 40 different dispersed genes, not just the Hox cluster. Leite et al also report duplication of the NK gene cluster, SINE gene cluster, Emx gene pair, LIM cluster and more. These reports are important for two reasons. First, the number of duplicated gene families reported is much higher than one cluster - this evidence for WGD is greatly downplayed by the current authors. Second, it has previously been shown from comparison of amphioxus and vertebrate genomes that ‘developmental genes’ are more likely retained as multiple copies post-WGD (PMID 18562680); genes like Hox, Emx, NK cluster, SINE, LIM, other homeobox genes, plus Wnt and frizzled, are better markers of ancient WGD than most other genes.

We did indeed make an error in not correctly specifying "homeobox genes" where we instead said "Hox genes" in our original submission. The homeobox genes are a larger family of genes (that includes Hox genes), and we intended to say that there is only evidence from "homeobox" genes for the spider WGD. We apologize for this error and have updated our manuscript accordingly.

However, results on homeobox genes in Schwager et al. (2017), Leite et al. (2018), and Harper et al. (2021) are much more ambiguous in the original publications than is summarized here. To make this clearer, we have now expanded our analyses to ~140 homeobox homologs from Schwager et al. (2017) and Leite et al. (2018). Using reciprocal best BLAST hits, we were able to identify homologs for 105 of these genes in *Parasteatoda tepidariorum* (the house spider), and then used MCScanX to classify duplicates (as in Schwager et al. 2017). These results—now presented in the Results section of our paper—find 1 of these homeobox genes as a singleton, 88 as dispersed, 16 as proximal, and 28 as tandem duplicates, mirroring previous results by other authors. None were labeled as due to a whole genome duplication.

These results come with several caveats—as with any analysis of synteny—so we describe extended analyses in our responses to later comments about synteny below.

On a more minor issue, also in the Introduction, the authors paint a confusing picture with regard to diploidization after WGD. The term is used in many recent papers to mean to mean reversion to disomic inheritance of the four post-WGD alleles, not to mean gene loss or divergence to the point of being unrecognizable as paralogs. It is a point of definition perhaps, but it could cause confusion.

\*\*Lets discuss this. I elect to define diploidization as both fractionation and reversion to disomic inheritance in the paper, with us specifying each specific case later in the paper. We cover both processes at different points. What do ya’ll think?\*\*

The first analysis presented in the Results is based on tree reconciliation. In essence, tree reconciliation methods ask whether the majority of gene paralog divergence nodes (as judged by a tree) map to particular phylogenetic nodes. There is an intuitive appeal to tree reconciliation, but it is likely to be a flawed method when dealing with ancient (auto)polyploidy events. The first reason is that even if a WGD event occurred, this does not imply that most retained gene duplication events happened as a result of the WGD. Tandem duplication is always the commonest method of gene duplication, whether or not a WGD event occurred in the ancestry of a clade. Second, this effect is exaggerated by the extreme rates of loss following WGD - shown to be around 80% loss in post-WGD teleosts (PubMed PMID: 26578810 & 24732281) or 88% in post-WGD yeast (PMID: 9192896). If something similar happened after a putative spider WGD, the signal from tandem duplication events would easily be greater than that from retained WGD duplicates. Third, tree reconciliation is based on estimating the point of initial sequence divergence between duplicated paralogs. But finding dates of sequence divergence is not the same as dating the time of duplication under autopolyploidy. Sequence divergence commences after WGD (sometimes tens of millions of years after) and furthermore it is not expected to commence at the same time across chromosomes; hence dating sequence divergence is absolutely not the same as dating gene duplication under WGD (see PMID: 28615063 & 37208359). Hence, the tree reconciliation analysis does not disprove WGD, and it is certainly not “definitive” to use the authors’ term.

This is a complex set of statements and claims, so we take each of the three in turn (numbered as in the reviewer's comment), plus one additional comment of our own at the end.

1) This comment may be due to a misunderstanding: the reconciliation method used here (GRAMPA) does not require the majority (or any plurality) of duplicates to map to a particular node/branch in order to identify a WGD. It is perfectly possible for the vast majority of duplicates to be due to tandem duplication and for our method to still identify a WGD. We now state this explicitly in the text (line XX).

2) Our method is able to deal with extreme rates of loss following WGD. For example, GRAMPA can find the WGD in the history of Saccharomyces fungi even though only ~500 genes (out of ~6,000) retain ohnologs in any particular species due to this event (Thomas et al. 2017).

3) It is absolutely correct that most reconciliation methods find the point of initial sequence divergence between paralogs, and therefore could confuse auto- and allopolyploids. This is why GRAMPA takes this distinction into account—see Figure 1 in Thomas et al. (2017). The methods employed here can therefore detect and distinguish either type of WGD, which we now also state in the text (line XX).

Finally, we would be remiss if we did not point out that reconciliation has been used in multiple of the papers cited by the reviewer in support of the spider WGD (though it is not always called "reconciliation"). Schwager et al. (2017) use reconciliation of gene trees to the species tree to both date duplication events (their Figure 4) and to test hypotheses about WGDs (their Figure 7). Harper et al. (2021) carry out a very similar analysis (their Figure 2). So it is certainly not the case that all researchers feel that reconciliation is "a flawed method when dealing with ancient (auto)polyploidy events."

The next section of the results is based on intra-genomic synteny. In my view, this is potentially the most powerful way to detect ancient WGD, if complications such as gene loss and rearrangement are accounted for. The submitted manuscript has not detected a strong signal of syntenic blocks. This can be a preliminary suggestion of no WGD, but it falls short of strong evidence for several reasons. It is not sufficient to overturn the evidence from previously published analyses. What perhaps should have been considered is (1) an investigation of different parameters to see how they affect this result, including limitation of the analysis only to duplicate genes to account for possible extensive gene loss, (2) comparison to previous papers such as Schwager et al that did detect some intragenomic synteny in spider, (3) it would have been informative to zoom in to the known duplicated gene clusters (Hox, SINE, NK etc) to see where they lie in the analysed genomic regions, and then examine and compare the chromosomal regions around them, (4) and inter-genomic analyses to see if there is any signal of ‘double conserved synteny’, where one genomic region in an outgroup matches two genomic regions in the ingroup - this last method can potentially overcome the issue of extensive post-WGD gene loss, which as noted above could be as high as 80 or 90%. The last method proved powerful in revealing ancient WGD in teleost fish and ancient 2xWGD in vertebrates. These tests have not been conducted in the submitted work.

Thank you for these suggestions to improve the rigor of our synteny analyses. To further explore synteny in the house spider genome, we did the following (again using the numbering of the reviewer's comments):

1) We ran MCScanX on further relaxed parameters to account for increased fractionation. We did so by both increasing the gap size to 50 genes (20 default) and decreasing the minimum syntenic block size to 3 genes (5 default). We also used a second synteny finding tool, synmap.pl from COGE, an alternative implementation of DAGchainer. Neither of these analyses found significantly more syntenic blocks consistent with WGD compared to our prior analyses. These results are now reported in our manuscript (lines XX).

2) Prior analyses used a few different methods for detecting syntenic blocks in Chelicerate genomes. Schwager et al. (2017) used MCScanX to annotate the mode of duplication for homeobox clusters. Similar to our results using MCScanX reported above, their supplementary Additional Figure 1 reports 50 genes (<1% of coding genes) as segmental/WGD. Our results using MCScanX therefore recovered a similar number of collinear genes to their analysis, neither of which showed strong evidence for WGD.

To overcome possible methodological differences, we manually compared the location of the homeobox genes reported in Schwager et al (2017) to their location in the *P. tepidariorum* assembly. Using reciprocal best blast hits, we were able to locate the focal hox cluster in Figure 4 from Schwager et al. (2017) in the *P. tepidariorum* annotation; however, these were dispersed across five scaffolds. This may be a result of assembly differences between the two studies, or low divergence among the homeobox gene clusters making homology detection difficult. Finally, MCScanX labeled these clusters as tandem duplicates, whereas Schwager et al. (2017) concluded they were not tandemly duplicated. The choice of Schwager et al. (2017) to omit the syntenic results from MCScanX in favor of SatusumaSynteny may be a reflection of this result, as MCScanX masks tandem duplicates when running DAGChainer.

Schwager et al. (2017) also used SatusumaSynteny to assess syntenic blocks within the *P. tepidariorum* genome. For context, we think it is important to consider what the prior results that led to the conclusion of a WGD looked like. Here is the figure on this from Schwager et al. (their Figure 5):

A grid with purple dots

Description automatically generated

SatsumaSynteny may be able to recover more synteny than MCScanX, as it is reported to handle sparser gene densities than MCScanX (Liu et al. 2018). To date, the performance of these methods have not been compared for intraspecific comparisons. However, SatsumaSynteny is typically recommended for interspecific comparisons of low divergence, such as comparing multiple individuals in a population (Liu et al. 2018), and may be prone to error at high divergence.

3) The homeobox genes likely are not syntenic (i.e. due to WGD) as MCScanX labeled many of them as tandem duplicates. Tandem duplicate genes are masked when DAGChainer is used to detect collinear blocks. This may be representative of these genes being the results of small scale duplications, or highly fractionated genomes, as the reviewer suggests. However, they clearly do not represent strong evidence for a WGD.

4) As suggested by the reviewer, we used MCScanX to overcome these limitations by comparing *P. tepidariorum* to *Tetranychus urticae.* Although we did recover some collinear genes in this comparison (<1% of the CDS), none of them were homeobox genes. It is possible that the significant difference between Schwager et al. (2017) and our analyses are a result of significant fractionation and the sensitivity of each method. However, it is important to report that the typical genome-wide signatures of WGD are absent from the spiders and scorpions.

Finally the manuscript searches for peaks of Ks. While appealing, this again confuses the start of sequence divergence with the time of gene duplication; in an autopolyploidy these are not the same thing (PMID: 28615063 & 37208359). Nor does this analysis take into account the signal from tandem duplication.

We would like to thank the reviewer for highlighting the nuance of synonymous divergence in paralogs formed by auto- and allopolyploidy. Both modes of duplication will form peaks in Ks plots representing when duplicated genes begin to diverge (again, see Figure 1 in Thomas et al. 2017). In the case of allopolyploids, peaks represent the divergence of the parental species, whereas divergence in autopolyploids begins when loci return to diploid inheritance patterns.

The teleost duplication cited by the reviewer is a special case of such a pattern, where diploidization has been delayed in different regions of the genome over millions of years. This delayed diploidization could mask signatures of WGD in K*S* plots by producing broader peaks that are more difficult to discern from the background exponential distribution of small-scale duplicates, including tandem duplications. The machine learning method used in our paper, SLEDGe (cite), was trained to discern peaks formed by WGD from the background distribution of small-scale duplications across a wide range of duplication and loss rates. It is possible that fractionation is so significant in spiders that the signal is too weak to discern in K*S* data and with methods like SLEDGe. However, similar to our syntenic results, we think it is important to report in our paper that widely used methods for detecting genome wide signatures of WGD (e.g. the Ks-plot in Figure 6 of Schwager et al. 2017) do not recover evidence of WGD shared by the spiders.

The Discussion section greatly overstates the conclusions that can be safely drawn from this study. Again the authors claim that previous studies relied heavily on the Hox cluster, which is false. The authors also mis-quote the studies on insect Hox clusters - these cited papers do not report cases of Hox gene cluster duplication, they discuss tandem duplication within Hox gene clusters which is not the same thing. The insect data is not relevant and does not add support to the authors’ argument. The present manuscript does not critically evaluate the differences between the current work and previous work, and fails to provide strong evidence to overturn previous conclusions. Several lines of investigation presented do not constitute strong tests of the hypothesis under investigation.

As mentioned above, we do apologize for saying "Hox" genes when we meant "homeobox"—this has been fixed throughout. We have also clarified what it is exactly that previous studies of duplication in Hox gene clusters have found. We hope the additional analyses described in the updated manuscript make a comparison of the current work and previous work clearer.

Reviewer: 2

Comments to the Author

This manuscript by Thomas and colleagues represents a examination of the evidence for an ancient whole genome duplication in the spider-scorpion ancestor. The study importantly considers multiple lines of evidence to support its claim which contradicts quite a few others now, including Ks distributions, and gene tree and synteny analyses. The work will be important to those interested in genome duplication and its inference, as well as to those studying arachnid biology and evolution. I commend the authors on re-examining this issue now that improved genome data are available.

I have some suggestions and comments that the authors may wish to consider:

1. The title is perhaps a little strong for me. Although this work supersedes past evidence supporting the WGD, strictly speaking 'A comprehensive examination of chelicerate genomes' is not yet possible since we do not have genomes for even all of the major lineages yet (including some possible closest outgroups to the proposed WGD as well as some other archnopulmonate lineages proposed to share the WGD event), perhaps this section of the title might be dropped? This is not a failure of the current study, rather simply a recognition of the state of play for chelicerate genomics.

We appreciate this point, and have now changed the title to "XX".

2. Somewhat related to the above point, Ballesteros et al 2022 is referenced for the placement of horseshoe crabs as sister to spiders and scorpions. To the best of my knowledge that result is not recovered in that study and has never been strongly recovered anywhere. While in the context of the sampled genomes in this study that may be true it is not representative of that study or of phylogenomic evidence to date when more lineages are considered. Acknowledging the improved number and quality used in this study, but still incomplete sampling of chelicerate genomes is warranted in this context. I would like to note however a recent study that does propose a sister group relationship between horseshoe crabs and arachnopulmonates (i.e. made up primarily of, but not only of, spiders and scorpions), and suggests (though without much in the way of evidence) a single WGD may be shared between these lineages and horseshoe crabs (Noah et al. 2020, evolutionary bioinformatics). The authors may wish to discuss/test this further. Similarly, the traditional hypothesis of arachnid monophyly has also been recovered in some recent and very detailed phylogenomic analyses (e.g. Lozano-Fernandez et al 2019), it might be best to acknowledge this as I suspect this difficult phylogenetic problem is far from solved.

We acknowledge that Ballesteros et al. (2022) do not conclude that horseshow crabs are sisters to spiders and scorpions, but neither did it propose a strong alternative phylogenetic hypothesis. Regardless, to clarify this situation we now cite Noah et al. (2020) and discuss its implications for a shared WGD (lines XX). We also now cite Lozano-Fernandez et al. (2019) in the context of XX...

3. Substantial alignment and sequence filtering is performed, as well as relatively high bootstrap values are applied for the Notung step. I do not mean to suggest these are inappropriate but given that the study aims to provide evidence for a negative it might be wise to examine how unfiltered data or data filtered using different parameters or software appear in downstream analyses. I feel that this is especially pertinent given that various factors can affect our ability to infer or discount WGD, e.g. extensive gene loss, rediploidization patterns, species sampling, age of events, genome quality etc.

We have carried out the analyses suggested by the reviewer. In particular, we have re-run our reconciliation analyses after using 1) a bootstrap cutoff of 80 (the original was 90), and 2) no bootstrap cutoff. For the bootstrap cutoff of 80, the species tree is again the best tree found by GRAMPA, while the tree with the horseshoe crab WGD is second-best. For the no-bootstrap runs, the species tree is the sixth-best tree, but none of the top 5 trees include a WGD confined to spiders and scorpions. Instead, they all imply biologically unrealistic hybridizations leading to allopolyploids (none of the trees imply the horseshoe crab WGD). As possibly predicted by the reviewer, filtering was likely the best course. Nevertheless, we now report all of these results in the main text (lines XX).

4. For MCScanX (line 157-162) it is not clear what input data were used, was an all-against-all blast applied for the self-self syntenic analysis or were intraspecies duplicates carried forward from the filtered phylogenetic analyses?

Thank you for pointing out this omission in our methods section. We have amended the text in the methods section to specify we performed all-against-all blast searches for the interspecific syntenic comparisons (lines XX).

5. Line 120-121: it is not clear what is meant by the phrase 'correcting for inconsistencies resulting from the data originating from various sources'. What inconsistencies and sources were corrected for and how should be described.

(I have no idea --MWH)

Line 299: should metazoans be vertebrates/teleosts?

Thank you for catching this. The text has now been updated to read "vertebrates".