Dear Pr. Ponts, Pr. Nègre and Pr. Almudi,

We have uploaded on *bioRxiv* a revision of our manuscript entitled '**Chromosome-level genome assembly and annotation of two lineages of the ant *Cataglyphis hispanica*: steppingstones towards genomic studies of hybridogenesis and thermal adaptation in desert ants**'. We were pleased to learn that you found our work interesting and acceptable for recommendation with minor revisions. We appreciated the constructive comments made to improve our manuscript. A detailed point-by-point response to all the issues raised is given below.

We followed *PCI*'s rules and have made all data available to readers (on NCBI and Figshare). A text file containing annotated command-lines for all analyses presented in the manuscript has also been added to the Figshare repository. Finally, we disclosed at the end of the manuscript that "J.F. Flot is one of the PCI Genomics recommenders".

We hope that you will find this revised version acceptable for recommendation by PCI Genomics.

Yours sincerely,

Hugo Darras, Natalia de Souza Araujo, Lyam Baudry, Nadège Guiglielmoni, Pedro Lorite, Martial Marbouty, Fernando Rodriguez, Irina Arkhipova, Romain Koszul, Jean-François Flot, Serge Aron

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NP-1: "Notably, as noted by both reviewers, discussion putting the findings back in perspective with current biological knowledge and questions is lacking."

We have followed reviewers' suggestions. Results and discussion putting findings in perspective have been added to the manuscript (see our answer to comments ***NN-1*** and ***IA-1***).

NP-2: "As an additional specific comment, considering the level of bioinformatics performed, a schematic drawing of the pipeline describing the sequential analyses performed would be very much welcome as supplemental data for example."

We agree with this suggestion. We have added a flowchart detailing our genome assembly and annotation pipeline in the supplementary material (Figure S1)

NN-1: "My main criticism of the manuscript is that it does not use this resource to provide some answers to the questions described in the introduction section. Indeed, the only scientific result described is about the change in chromosome numbers. But, unfortunately, while the number of chromosomes has been cytologically confirmed for one lineage, it has not been done for the other one. Thus an additional cytological validation of the translocation cannot be provided. If I had only one request to enhance the paper, would be to provide the karyotype for the Chis1 population as well. From the same biological material, comparative DNA resequencing (to identify CNVs) and RNA-Seq, could have been attempted to detect differences between the chis1 and chis2 lineages that could explain their divergence.

Thus, while the manuscript is robust, it is my opinion it could have had much more impact with additional analyses focused on answering a biological question."

We agree with this comment and have decided to bring more perspective to our results.

To obtain karyotype data for the Chis1 lineage, we analyzed new samples and added this result in the revised manuscript. Pure-lineage offspring are only produced during a short period of the year, in small quantities and only in some of the nests. Despite a large sampling, we were not able to obtain pure-lineage Chis1 pupae from the field. Furthermore, they cannot be obtained in laboratory conditions. As a result, we resorted to use worker samples instead to obtain Chis1 karyotypes, which is a strategy also suggested in ***IA-2***. Workers are first generation hybrids and therefore carry a set of chromosomes from each lineage. We found good metaphases in three worker samples out of 83 individuals analyzed (one from Merida and two from Bonares). The worker from Merida carried odd numbers of chromosomes (2n=26+27) as expected. Surprisingly, the two additional workers analyzed carried even number of chromosomes (2n=54) indicating that both lineages carry 27 chromosomes in the population studied (a different population that the one used for genome sequencing). This new result contradicts our previous hypothesis that the two lineages are fixed for different chromosome polymorphism (n=26 and n=27), which could have played a role in the evolution of social hybridogenesis in this species. We have updated the results and discussion of the manuscript to consider this new data (see lines 133-169).

We provide additional comparative analyses for the two lineages, i.e., the distribution of inter-lineage sequence divergence across the genomes, the distribution of sequences that are specific to each assembly and the number of genes annotated in Chis2 that were not found in the Chis1 assembly. The distribution of small polymorphisms and large indels among lineages appeared uniformly distributed across the chromosomes. Although 6.4-6.6 % of the chromosome sequences were unique to each lineage assembly, the vast majority of genes annotated on the Chis2 chromosomes (99.7 %) could be mapped on the Chis1 assembly. We put these findings back in perspective with our current understanding of social hybridogenesis. No region with elevated differentiation between the two assemblies was revealed in our analyses. This pattern of distribution of polymorphism across the chromosomes of the two lineages allow us to reject the previously formulated hypothesis that hybridogenetic lineage pairs might be determined by ancient non-recombining regions (Linksvayer et al. 2013. *Bioessays* 35: 10.1002/bies.201300038). This finding could help steering future research into more fruitful venues and we added discussion regarding this point (see lines 220-240).

As for questions regarding thermal adaptation, we refrained from making links between inferred gene content changes in *Cataglyphis* and their biology since this approach is known to be error prone; the danger of false-positive correlations is especially high when comparing genomes assembled and annotated using different methods as is the case for current ant data (Weismann et al. *biorxiv*, https://doi.org/10.1101/2022.01.13.476251). We believe that meaningful insights could later be obtained by leveraging the resources described in this manuscript to study *Cataglyphis'* transcriptomic and proteomic responses to heat stress.

NN-2: "Fig1A: In the legend the queen is marked with a red arrow, but I can't see the red arrow (even though the queen individual is obvious)."

Corrected.

NN-3: "Fig.1B: the legend is confusing because the introduction talks about 2 lineages but we immediately see three dots. WGS is defined but not 3C-seq or RNA-seq. And at the end I was confused about which sample was used for which purpose and which one was chis1 and/or chis2. Can it be made clearer on this figure so that the reader understands more easily which material was used ?"

Figure 1B and its legend have been modified to clarify our sampling strategy.

NN-4: "l. 247: typo on homozygous"

Corrected.

IA-1: "I am missing a bit a phylogenetic perspective, given that the authors performed some comparative genomics analyses. It would be nice if they mention the phylogenetic relationships of the ant species they use for their analyses. They only mentioned that L. niger is closer to Cataglyphis in phylogenetic terms, but there is nothing about the other species used. They could include a phylogenetic tree with these species highlighted and order them accordingly in the different figures. Perhaps, this would help them to draw some hypotheses regarding the presence of repetitive elements in the distinct genomes."

A phylogenetic tree derived from our orthology analyses (see methods) has been added to Figure 4 and Figure 5. The species were ordered accordingly to their phylogenetic positions. No phylogenetic pattern could be observed. This is now explained in the manuscript (line 220-222).

IA-2: "Having the karyotype of the Chis1 lineage would reinforce the results of the manuscript. I understood that it was not possible to collect Chis1 males, would it be possible to obtain a karyotype from hybrid workers, just to confirm that they have 26+27 chromosomes?"

Karyotypes from workers are now presented in the manuscript (see comment NN-1)

IA-3: "Could authors clarify what do they mean in lane 160: "no protein similarity and no functional information"? Are they using only ant genomes? Did they search outside ants? Functional information as protein domains?"

To avoid confusion, we have rewritten the sentence as follows: "*Among these, 11,101 gene models showed significant similarity to proteins predicted in other ant species (blastp against 18 ant proteomes from the RefSeq collection) and 10,543 had functional information inferred through sequence orthology with the eggnog v5.0 database which covers more than five thousands organisms (Huerta-Cepas et al. 2017, 2019). We filtered out all gene models non validated by at least one of these databases to obtain a final dataset of 11,290 high quality gene models, 11,033 (98%) of which are placed within the 27 chromosome-scale scaffolds."*

IA-4: "Fig 1. Red arrow pointing towards the queen is missing"

Corrected.

IA-5: "Fig 2. There is some discrepancy between the main text and figure 2. In the figure it is stated that Chr 1 from Chis1 is split in Chr 5 and Chr 9, whereas in the text (lane 111), it says that Chr. 1 is split in Chr5 and Chr 10."

This was a typo. The figure showed the correct chromosome names for Chis1 (#5 and #9). We have corrected the names in the main text.

IA-6: "Lane 139. Authors claim that chromosomes have small sizes, it would be good if they included some measurements and a scale bar in Figure 3."

We have added a scale bar to Figure 3.

IA-7: "Lane 178. There is a mention to Table S1, but I think the authors meant Table S2"

We did intend to mention Table S1, which lists the accessions of the 19 published ant genomes used for the comparison of repeat contents. To avoid confusion, we have modified the mention as follows: "*(see genome accessions in Table S1)."*

IA-8: "Figure S4. There is an overlap of the graph legend with the graph"

Corrected.

IA-9: "Lane 196. "smaler " instead of "smaller""

Corrected.