

Dear Recommender and Reviewers,

Once again, we thank the recommender and the reviewers for their careful and constructive comments, which helped us to improve the manuscript. We address each of the comments (reviewer 2 and recommender) in detail below.

Yours sincerely,

Dominique Colinet

### **Reviewer 2:**

- Comment 1:

I suggest that the new BLAST and Exonerate search analyses, conducted in response to my earlier feedback, should be detailed either in the main text or Supplementary Text. This inclusion is important to transparently convey the absence of sequences similar to the new exon, even if the results are negative.

Authors' response:

L212-214 and L411-414: Some details of the analysis performed to identify the possible origin of the signal peptide of *Leptopilina* venom RhoGAPs are given. No significant sequence similarity was found between the exon(s) preceding the RhoGAP domain coding sequence of *Leptopilina* venom RhoGAPs and other sequences in the *Leptopilina* genomes that could provide an indication of the origin of the signal peptide. The sequences used for the analysis and the results of the similarity searches (using Exonerate, which is more suitable for this type of analysis than BLAST) are given in Supplementary Dataset 2.

- Comment 2:

Additionally, the authors have not presented a gene family-wide, unified phylogeny in the revised Figure 1. Such a phylogeny is essential to clearly demonstrate the independent origins of the two venom protein lineages within the same protein family. While I believe its inclusion would greatly enhance readers' understanding, the decision to omit it ultimately lies with the authors.

Authors' response:

A unified phylogenetic analysis was performed using either protein- or codon-based alignment of the RhoGAP domain, and the resulting phylogenies are shown in Supplementary Figure S4.

L457-467: The results of the phylogenetic analyses are presented in relation to the independent duplication hypothesis. The analysis confirmed a relationship between either *Leptopilina* venom RhoGAPs or *V. canescens* calyx RhoGAPs with RacGAP1 since

they all form a robust monophyletic group. In the codon-based phylogeny, *V. canescens* calyx RhoGAPs formed a robust monophyletic group with VcRacGAP1, suggesting that they originated from an independent duplication event. However, in the protein-based phylogeny, the *V. canescens* calyx RhoGAPs did not form a robust monophyletic group with VcRacGAP1. Similarly, the *Leptopilina* venom RhoGAPs did not form a robust monophyletic group with *Leptopilina* RacGAP1 in either phylogeny. This was not unexpected due to the high divergence of *Leptopilina* venom RhoGAPs and *V. canescens* calyx RhoGAPs compared to RacGAP1. Nevertheless, like reviewer 1 in the round 1 revision, we are convinced of the hypothesis of independent duplication in both *Leptopilina* and *Venturia*, since these are very distant and the presence of RhoGAP family proteins in the maternal fluids of parasitoid wasps has not been described outside of *Leptopilina* and *Venturia* to date (see comparison of hypotheses made in the discussion and described below).

L625-630: A comparison was made between the independent duplication hypothesis and the alternative hypothesis of a single ancestral duplication. The latter would imply thousands or even hundreds of thousands of loss events, since (i) the last common ancestor of *Leptopilina* and *Venturia* dates back to the early radiation of parasitoid wasps (over 200 million years ago), and (ii) *Leptopilina* and *Venturia* are the only parasitoid wasps for which the presence of RhoGAP family proteins in the maternal fluids has been observed. The hypothesis of independent duplication events of RacGAP1 in both *Leptopilina* and *Venturia* seems much more parsimonious than the alternative hypothesis of a single ancestral duplication of RacGAP1.

#### **Recommender:**

- Comment 1:

I would nevertheless agree with reviewer#2 regarding the need for a higher-level phylogenetic integration of the results. Such an analysis should include in the same inference the proposed orthologs for all species studied. Otherwise the evolutionary relationships are inferred by comparison between parallel analyses, as it is the case in the present version of the text.

[...]

Notwithstanding, as reviewer #2 indicates, there is no analysis that allows for introducing polarity in the tree, and confidently establishing the independence and the order of the initial duplication events (e.g. an analysis of the phylogenetic relationships of NVIT RACGAP1, *Leptopilina* and *Venturia* RacGaps, and some representatives of the *Leptopilina* and the *Venturia* venom GAPs, chosen based on the relationships depicted in figs S2 and S4). Given the quality of the amino-acid based alignment and the increased information available at the nucleotide level (as inferred from the genetic distance matrices) I would suggest to run the phylogenetic inference at the nucleotide level (or at the codon level including codon-based nucleotide partitions), so that the fine relationships (mostly in the VcGAP analyses) can be resolved. It is also my impression that an analysis aiming at identifying and removing rogue taxa in the dataset (probably the case of VcGAP5) will help clarify the relationships.

Authors' response:

As described above in response to reviewer 2's comment 2, a unified phylogenetic analysis was performed using either protein- or codon-based alignment of the RhoGAP domain, and the resulting phylogenies are shown in Supplementary Figure S4. The results of the phylogenetic analyses are presented in relation to the independent duplication hypothesis (L457-467) and a comparison was made between the independent duplication hypothesis and the alternative hypothesis of a single ancestral duplication (L625-630) (see above).

The phylogenetic analysis included all *Nasonia* RhoGAPs and not only RacGAP1 to confirm the relationship of either *Leptopilina* venom RhoGAPs or *V. canescens* calyx RhoGAPs with RacGAP1. The phylogenetic analysis included all *Leptopilina* venom RhoGAPs or *V. canescens* calyx RhoGAPs since we could not find a suitable criterion to select among them. Furthermore, we believe that the analysis was more informative when all sequences were included.

- Comment 2:

Overall, this analysis should allow establish:

1) whether RacGAP gene-based relationships for *Leptopilina*, *Venturia* and *Nasonia* recapitulate the species relationships (it would be good to have for this the LvRacGAP, but this is a minor detail).

Authors' response:

The relationships for RACGAP1 in *Leptopilina*, *Venturia* and *Nasonia* do not perfectly recapitulate the species relationships. According to Peters et al. (2017), *Nasonia* and *Leptopilina* shared a common ancestor (more than 200 million years ago) before sharing one with *Venturia*, whereas *Venturia* and *Nasonia* RACGAP1 were grouped together in the phylogenies (although this was not robust in the protein-based phylogeny). However, we do not feel it is relevant enough to discuss explanations for this discrepancy in the manuscript.

There was an error regarding LvRacGAP1, as we could not identify its sequence from the transcriptomic data we obtained. The manuscript has been corrected.

2) whether VcGAPs and *Leptopilina* venom RhoGAPs are respectively monophyletic (this seems to be the case for *Leptopilina*, but less clear for *Venturia*), and what their respective relationships are with VcRacGAP1 and with the *Leptopilina* RacGAP1 (i.e. whether they have actually appeared after independent duplication events after the speciation between the *Venturia* and the *Leptopilina* clades).

Authors' response:

This point is discussed in the response to reviewer 2's comment 2 (see above).

3) whether Lb/mGAPs 1,3,4 and 5 are absent in Lh/c/v; should this be the case, all the subsequent duplication events in this lineage would be exclusive to the Lby/m (most likely after loss of the duplicate in Lh/c/v).

Authors' response:

See combined answer below.

4) whether Lb/mGAP and Lb/mGAPs2 and 6 are sister taxa to Lc/h/vGAPs; should this be the case, further duplication events may have occurred in the Lc, Lh and Lv lineage, e.g. (all this needs to be interpreted in the light of ITS2-based relationships in fig1).

Authors' response (points 3 and 4):

Unfortunately, the unified phylogenetic analysis performed using either protein or codon-based alignment of the RhoGAP domain does not allow the complete evolutionary history of *Leptopilina* venom RhoGAPs to be accurately described. *Leptopilina* venom RhoGAPs form a monophyletic group in the protein-based phylogeny (although not robust), but not in the codon-based phylogeny, and *L. boulardi* venom RhoGAPs do not form a monophyletic group in any phylogeny. This could be explained by the high divergence of *Leptopilina* venom RhoGAPs even at the codon level compared to RacGAP1. Furthermore, we are not sure that we found all venom RhoGAPs in *Leptopilina* species other than *L. boulardi* due to the quality of the transcriptomes and/or genomes we analyzed. For example, we did not find the classical RacGAP1 in the transcriptome of *L. victoriae*, so there may be venom RhoGAPs other than LvGAP1 and LvGAP2 in this species. However, our goal in including venom RhoGAPs from *Leptopilina* species other than *L. boulardi* was not to describe the complete evolutionary history of *Leptopilina* venom RhoGAPs. Our goal was to determine whether the occurrence of venom RhoGAPs was not exclusive to *L. boulardi* and likely originated from an ancestral duplication in the ancestor of the *Leptopilina* genus. The relationship of all *Leptopilina* venom RhoGAPs to classical RacGAP1 and the common domain organization with a signal peptide followed by a RhoGAP domain (whereas RacGAP1 has a different domain organization) are consistent with the hypothesis of an ancestral duplication.

Although an accurate and complete description of the evolutionary history of *Leptopilina* venom RhoGAPs was not our goal and would be challenging based on the phylogenies obtained, some specific points are already discussed in the manuscript. In addition, we have added a sentence regarding the observation that some of the *L. boulardi* venom RhoGAPs form robust monophyletic groups, suggesting that some of the duplication events following the initial duplication of the RacGAP1 gene in the ancestor of the *Leptopilina* genus appear to be specific to *L. boulardi* and would explain the large number of venom RhoGAPs found in this species (L415-417).

- Comment 3:

Regarding the mass spectrometry analyses, it is unclear from the results whether the database used for searching included only the *Leptopilina* venom RhoGAP sequences or all *Leptopilina* known CDSs. This may be important to understand the multiple matches of a same band to several protein sequences, and of a same protein to several bands, especially when considering very closely related proteins, as in LgmGAP1/2/3.x.

Authors' response:

As explained in the Methods section (L318-320) and Results section (L435-436), the coding sequences of *Leptopilina* venom RhoGAPs were used to perform Mascot searches on the mass spectrometry data. However, this has been clarified in the legend of Figure 2.

The occurrence of two or more venom RhoGAPs to the same protein band, and of two or more protein bands to the same venom RhoGAP, was a common observation in our analysis of venom proteins from various parasitoid wasps using 1D gel electrophoresis. This can be attributed to the abundance of some proteins in the venom and the high similarity between some of the *L. boulardi* venom RhoGAPs. However, our objective was to investigate whether we could hypothesize that venom RhoGAPs from *L. boulardi* and *L. heterotoma* are associated with venosomes following their secretion in the venom (and transported by these vesicles to target host cells, as previously shown for LbmGAP and LbmGAP2). We do not believe that a discussion on the occurrence of multiple venom RhoGAPs associating with the same protein band or vice versa would be relevant within the manuscript.

- Comment 4:

It would finally be interesting to project onto the phylogeny and to discuss the differential location vesicular/supernatant, which are puzzling for orthologs (LbmGAP4 and LbyGAP4; LbyGAP6 and LbyGAP), and also the vesicular-only detection of the LhGAP1/2/3.

Authors' response:

In the manuscript, we showed that all *L. boulardi* and *L. heterotoma* venom RhoGAPs were detected in the vesicular fraction, where most of them were enriched compared to the supernatant fraction. This led us to hypothesize that *L. boulardi* and *L. heterotoma* venom RhoGAPs are associated with venosomes following their secretion in the venom. The variations among some of the *L. boulardi* ISm and ISy orthologs can be attributed to differences in the abundance of venom proteins, as described in our previous works. For example, we previously evidenced that LbmGAP is abundant in the venom of *L. boulardi* ISm, while LbyGAP is present in much lower quantities in the venom of *L. boulardi* ISy, due to differences in transcription levels. The detection of *L. heterotoma* venom RhoGAPs only in the vesicular fraction can be attributed to their low abundance (described previously). However, we believe that a detailed discussion regarding such variations

among some *L. boulardi* orthologs and the vesicular-only detection of some of the venom RhoGAPs is beyond the scope of this manuscript.