

In the current work, Filée *et. al.* explore the presence and putative beneficial role of *Wolbachia* bacteria in the blood-feeding *Rhodnius* genus (Hemiptera: Triatominae). These blood-feeding insects are generally thought to be dependent on B vitamin supplementation by their *Rhodococcus* symbionts. However, there exists conflicting evidence regarding their obligate dependence on the aforementioned symbiont, as under certain blood types, no dependence is observed. The authors first used diagnostic PCR reactions in a diverse set of samples representing 17 out of 24 species currently recognised in the genus. Through the use of this diagnostic PCR, they found that, in addition to the widespread nutritional symbiotic *Rhodococcus* bacteria, a number of the samples were also found to be infected with *Wolbachia* bacteria. In addition, these *Wolbachia* symbionts seem to be closely related to the nutritional *Wolbachia* symbiont of *Cimex lectularius* (wCle) and, those within the *prolixus* group, encode for a complete biotin biosynthetic gene cluster. The hypothesis the authors put forward is that "Wolbachia may also act as a nutritional mutualist in triatomines, as observed in bedbugs, in complementation (or in rescue) to the *R. rhodnii* gut symbionts".

I find the work well done and the methods generally adequate for analysing the data. However, I have one major conceptual concern the way the current article and the conclusions are framed.

Major comments

My major concern with the article is the way the results are framed into a conclusion which I believe is not fully supported by the data. In my view, the current work fully supports that the *Wolbachia* identified in *Rhodnius* spp., could potentially provide a benefit to their hosts in the form of nutritional supplementation (namely biotin and riboflavin). I believe this is well supported by the presence of these intact pathways in the genomes of *Wolbachia* and its apparent widespread presence (albeit not necessarily fixed in any given species) across the *Rhodnius* genus (namely *pictipes* and *prolixus* groups). However, I do not believe there is enough evidence provided to claim (or favour) "a ménage à trois scenario rather than a dual symbiosis as conceived until now" nor to "speculate that *R. rhodnii* and wRho compose an ancient and dual association of co-symbionts, as seen in many other hemipteran". The reasons I believe this are the following:

- It is not unexpected, at least for me, that the relationship that *Rhodnius* spp. keep with their nutritional *Rhodococcus* symbionts is not as "intimate" as that that other blood-feeders keep with theirs (e.g. ticks and *Coxiella/Francisella*, bedbugs and *Wolbachia*, tsetse flies and *Wigglesworthia*, and even *Haementeria* leeches). This comes mainly from observations that (at least some) *Rhodnius* can feed and thrive on supplementary food sources (doi: 10.1186/s13071-016-1401-0). As suggested by the authors in the aforementioned study, this additional food source might be important in the field in relation to its richer microbiota (and so, other possible sources for B vitamins).
- In addition, and as the authors of the present study remark, *Rhodnius* spp. have also been shown to develop similarly with *R. rhodnii* strains both capable and incapable of synthesising specific B vitamins (nicotinamide, thiamin, pyridoxine, riboflavin, aminobenzoic acid [pABA], or biotin). As the authors of the present study do well in pointing out, the authors of the 1976 study did not control for other bacterial symbionts. It is therefore possible that any other bacteria capable of synthesising B vitamins could be complementing the host's diet (and not necessarily *Wolbachia*). Also, as the authors also point out, B vitamin supplementation is not necessary for *Rhodnius* when feeding on certain blood diets vs. others.
- Lastly, the presence of a B vitamin operon in *Wolbachia* is not necessarily evidence of a "mutualistic" (or better said "beneficial") relation with its host, with some examples given by the authors in the present study but also from the *Wolbachia* strains found in the spider *Oedothorax gibbosus* (doi: 10.1101/2022.05.31.494226).

Therefore, I believe there exists enough evidence to propose that while *R. rhodnii* can establish a very successful nutrition-based symbiosis with *Rhodnius* spp. (and it is very successful in infecting the new generations), its association with its host is not necessarily obligate or intimate, opening the opportunity for other symbionts to also take over the B vitamin biosynthetic role. Here is where I see that *Rhodnius*-associated *Wolbachia* strains could have been retained (due to their B vitamin biosynthetic capabilities) and co-diverged with their hosts likely given the well known capacity of *Wolbachia* spp. to be retained and both vertically and horizontally transmitted. However, I fail to see why other members of the microbiota would not similarly be providing B vitamins to their host when needed, and that *Wolbachia* has simply been more successful in spreading and being maintained, giving the impression its association is more “intimate”. Therefore, I believe even the title “*Wolbachia* genomics support a tripartite nutritional symbiosis in blood-sucking Triatomine bugs.” communicates an incorrect message and would much better read as “*Wolbachia* genomics reveals a potential for a nutrition-based symbiosis in blood-sucking Triatomine bugs”, or something in that line. This would be more cautious in not overstating the potential nature of the *Rhodnius-Rhodococcus-Wolbachia* relation without any other experimental data.

Minor comments

Line 328: The authors refer to conservation of synteny. however, when looking at the assembled files and table 2, I cannot but notice these assemblies are highly fragmented, which makes me wonder, exactly how can the authors speculate anything more than conservation of synteny at very small scale (AKA micro-synteny)? With such sort of data claiming synteny conservation across the genomes (as it is shown in Figure 3b).

Line 38-39: The authors talk about complete and almost complete genomes. I do not see how was this assessed. Where assembly graphs inspected for completeness of sequences belonging to an isolated *Wolbachia* graph? Otherwise, I would stay away from categorising these genomes as “complete” or “nearly complete”

In the methods, it is unclear to me if the authors performed mapping and reassembly following the extraction of bins of *Wolbachia* contigs/scaffolds. Did the authors do that? I was surprised by the number of contigs from each assembly (sometimes well over a thousand), especially so when I myself have performed assemblies with this sort of sequencing data and rarely results in these large number of contigs. This might also help get more contiguous assemblies to better assess synteny.

I would suggest to explicitly group Table 1 species by group, as it makes it more comparable and easier to read by a non-Triatominae expert.

Line 175-178: [...] BLAST hit and sequence identity >90% for *Wolbachia* and >99% for *R. rhodnii*. How were the percentage thresholds calculated

Line 191-194: If authors note the difference in coverage between host and *Wolbachia* contigs, why was this not also used when binning? It might have helped them retrieve more *Wolbachia* contigs. At least I myself often use this criteria to complement BLAST-based (and graph-based) binning, as it can help also retrieve extrachromosomal sequences not easily identified by BLAST-based binning.

Line 217-219: I understand the logic behind searching for flanking regions of *Wolbachia* insertions. But I see that many of the claimed *Wolbachia* HGTs have very small distances to the end of the “host” contigs. In my opinion, this cannot assure these are *bona-fide* HGTs, as these regions can well originate from chimeric sequences artefact from the sequencing technology. Moreover, if they were *bonna-fide* Horizontally-transferred regions, why would they very often (30% of the times) land in contig ends (as repeats do)?

Line 217-219: The authors referred to “masked” genomes. Masked for what? repeats? low complexity regions?

Is table 2 missing a caption? I did not see the explanation of what * stood for.

Line 301: I would suggest displaying the coverages in a box plot format with semi-transparent colouring of dots on top of it. This would make it much more readable and easier to interpret.

Line 336-338: Do not see how having only a very small draft genome (likely missing most of the genome) would make wRobQ cluster with the *pictipes* group. Only thing I can think of is a lot of missing data in that genome making it cluster “erroneously” with the *pictipes* group. Is this correct? Did the authors encode a lot of missing data for this genome in the alignment? Otherwise, I would probably think that its clustering is correct.

Line 387-389: Couldn't the erratic distribution of B vitamin genes might simply come from the highly fragmented (and likely incomplete) *Wolbachia* assemblies making it hard to detect these genes?

Line 496: Didn't Mesquita *et al.* (2015) reported 25 HGTs, not 21?

Line 502: I do not understand the statement that “most all the *Rhodnius* samples have been infected by wRho at one time”. Do the authors mean the common ancestor of *Rhodnius* spp.? It now reads as if all the samples, rather than the species, have been infected at one time, which is not necessarily not true.

Line 522-523: I would stay away from doing such divergence estimations with these sort of data, especially when comparing infections across distantly related hosts.

Line 540-543: I would stay away, with current evidence, from making any sort of suggestion regarding “ a direct *Wolbachia* transfer between an ancestor of bedbugs and an ancestor of the *Rhodnius* triatomine”, as as the authors rightly point, there is simply not enough genetic nor genomic data from the *Wolbachia* F supergroup.

Line 544-549: Has cleptohaematophagy been observed between *Rhodnius* and bedbugs? Otherwise, it would seem unlikely, right? I guess the fact that they feed on similar hosts is much better evidence for a possible transmission route of their microbiota. Are there any studies revealing *Wolbachia* can be found in sterile blood after a *Rhodnius* or bedbug has fed on it?

Line 556-558: I would not say that just because two *Wolbachia* strains belong to the same supergroup they are both necessarily sharing the phenotype of being beneficial nutritional symbionts, especially so in distantly-related hosts. This is just not good evidence for a specific type of symbiotic relationship

Edits

Particularly in the abstract, it reads strange when using the past tense when referring to what is shown in the article. For example “In this study, we showed that *Wolbachia* symbionts were also widely distributed in the *Rhodnius* genus”. As it stands, it reads that the symbiont were (and thus, not any more) widely distributed. I would suggest to change to the present tense. For example “In this study, we show that *Wolbachia* symbionts are also widely distributed in the *Rhodnius* genus”

When referring to nutritional mutualism, better to refer to it as a phenomenon rather than a “process”. I can the the process of “genome reduction”, but not the process of “nutritional mutualism”

It would be good to offer a general genome/assembly characteristics table early in the manuscript. I believe it would make the paper easier to read.

As a general comment on the figures, please provide better quality ones. Using PDF figures is much better than pre-rendered ones. It was often difficult to read small text or images due to pixelation.

I suggest to change the black vs. grey triangle differentiation to a filled/unfilled one. It makes it easier to read especially with such small triangles.

I would suggest the authors to go through the manuscript one last time to correct some typos and strange phrases across the manuscript such as (not an extensive list):

- In most case(s), *Wolbachia* [...]
- A subsample of 36 specimens including *Wolbachia*-free and contaminated insects were used f. replace for “infected”
- biotine (remove trailing e)
- We cannot rule(**d**) out a whole *Wolbachia* lateral transfer/replacement

I believe that after addressing these comments and making necessary clarifications, corrections, and changes, the article would be a very interesting addition to both the *Wolbachia*- and the blood-feeding symbiosis- literature.

Sincerely,

Alejandro Manzano Marín