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Short Note

Absence of *Wolbachia* in the sub-Antarctic midge, *Eretmoptera murphyi* (Diptera: Chironomidae)

Octavia Brayley[1,2], Scott Hayward[1,3], Kirsty McCready[1], Shengwei Liu[4], Yin Chen[1], Sami Ullah[3,5], Nicholas Teets[6] and Peter Convey[1,2,7,8]

[1]School of Biosciences, University of Birmingham, Birmingham, UK; [2]British Antarctic Survey, NERC, High Cross, Cambridge, UK; [3]The Birmingham Institute of Forest Research, University of Birmingham, Edgbaston, Birmingham, UK; [4]School of Life Sciences, University of Warwick, Gibbet Hill Campus, Coventry, UK; [5]School of Geography, Earth and Environmental Sciences, University of Birmingham, Edgbaston, Birmingham, UK; [6]Department of Entomology, University of Kentucky, Lexington, KY, USA; [7]Department of Zoology, University of Johannesburg, Auckland Park, South Africa and [8]Biodiversity of Antarctic and Sub-Antarctic Ecosystems (BASE), Santiago, Chile

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Introduction

Wolbachia, a bacterial genus in the Rickettsiales, are Gram-negative endosymbiotic bacteria that are primarily found in invertebrates, particularly arthropods and nematodes (Werren *et al.* 2008). *Wolbachia* form part of the complex invertebrate microbiome, enhancing the efficiency of a range of biological systems in host organisms, including the immune system (Pimentel *et al.* 2021), nutrient acquisition (Newton & Rice 2020, Deconninck *et al.* 2024) and temperature tolerance (Hague *et al.* 2020), thereby impacting the host phenotype through parasitic and/or mutualistic relationships (Iturbe-Ormaetxe & O'Neill 2007, Manoj *et al.* 2021). No free-living members of the genus are known (Vancaester & Blaxter 2023). Perhaps one of the most well-known impacts of *Wolbachia* is its ability to manipulate the reproduction of the host (Hyder *et al.* 2024), which can, for example, initiate parthenogenesis (Werren *et al.* 2008), as well as feminization (Asgharian *et al.* 2014) and cytoplasmic incompatibility (Chen *et al.* 2020). Other known insect endosymbionts in the same order that can induce parthenogenesis include the genera *Rickettsia* and *Cardinium* (Ma & Schwander 2017). Most *Rickettsia* species that have been observed in insects are facultative symbionts, and some can cause the death of male embryos (Giorgini *et al.* 2010) in, for example, certain ladybirds (Werren *et al.* 1994, von der Schulenburg *et al.* 2001) and beetles (Lawson 2001). Of these reproductive manipulations, parthenogenicity can be advantageous in the short term as it guarantees reproduction when access to mates is limited, although there is often a loss of heterozygosity over time (Jaron *et al.* 2022). In extreme and isolated environments such as remote oceanic islands and in the polar regions, environmental constraints on individual movement have been suggested to select for parthenogenesis (Chown & Convey 2016, Leihy & Chown 2020).

Corresponding author: Octavia Brayley Email: oxb233@student.bham.ac.uk

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Although *Wolbachia* has a global distribution (Werren & Windsor 2000), infecting ~40% of arthropods (Nugapola *et al.* 2017) and up to 60% of terrestrial insect species (Sazama *et al.* 2017), to date it has not been detected in Continental Antarctic, Maritime Antarctic or sub-Antarctic invertebrates (e.g. Charlesworth *et al.* 2019, McQueen *et al.* 2023, Serga *et al.* 2024), including in the only insect from the Antarctic continent studied to date: the non-biting flightless midge *Belgica antarctica* (Diptera: Chironomidae; Maistrenko *et al.* 2023). *Eretmoptera murphyi* (Fig. 1) is another non-biting flightless midge that molecular analysis shows to be very closely related to *B. antarctica* (Allegrucci *et al.* 2012). It is native and endemic to the sub-Antarctic island of South Georgia (Chown & Convey 2016), but it is now also well established and considered an invasive species on Signy Island in the Maritime Antarctic (South Orkney Islands; Hughes & Worland 2010, Bartlett *et al.* 2020). Unlike *B. antarctica*, which is a sexual species, *E. murphyi* reproduces parthenogenetically, and no male specimens have ever been reported (Cranston 1985, Bartlett *et al.* 2018, Kozeretska *et al.* 2021). The hypothesis that *Wolbachia* could be responsible for the different reproductive strategies of the two species has not been investigated to date. However, recently, Serga *et al.* (2024) provided some new data and highlighted the absence of *Wolbachia* amongst all Antarctic terrestrial invertebrates whose genomes and/or microbiomes have been sequenced and are publicly available. Serga *et al.* (2024) specifically emphasized that, to date, no study has investigated the presence of *Wolbachia* in *E. murphyi*. Therefore, the aim of this short publication is to directly address this knowledge gap so as to contribute further insights into *Wolbachia* in Antarctica and to consider the possibility of other candidate bacteria that may be involved in *E. murphyi*'s parthenogenetic life history.

Methods

As part of a wider insect microbiome study, *E. murphyi* larvae were collected from the 'Backslope' (unofficial name) adjacent to the British Antarctic Survey's Signy Island research station in

Figure 1. Adult (left) and larval (right) *Eretmoptera murphyi*. Photographs by British Antarctic Survey.

February 2022 during the summer. They were stored under field conditions on Signy (~2–4°C; Convey *et al.* 2025) before being placed in 96% ethanol and kept at -80°C on the return ship to the UK. They were maintained at this temperature at the University of Birmingham until analysis. At the University of Warwick, DNA was extracted from five individuals using a DNeasy PowerSoil Pro Kit (QIAGEN) following the manufacturer's protocol. Universal primers were used to target and amplify the bacterial V4 region of the 16S rRNA; 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). Polymerase chain reaction (PCR) involved cycles being carried out to amplify the extracted DNA (95°C for 30 s, 53°C for 30 s, 72°C for 30 s). The target region of ~300 bp was obtained by gel electrophoresis, followed by extraction and purification using a QIAquick Gel Extraction Kit (QIAGEN) following the manufacturer's protocol. Library preparation and sequencing were carried out on an Illumina PE250 platform by Novogene, UK. A total of 333 809 bacterial sequences were obtained. The *DADA2* pipeline (V1.32.0; Callahan *et al.* 2016) was used for quality trimming, error rate estimation, merging, chimera removal and amplicon sequence variant (ASV) feature table construction. Primer sequences were removed from the 5′ region of forward and reverse reads (19 and 20 bp, respectively), and reads were truncated at the first instance of a quality score of ≤ 10. Following dereplication, merging and chimera removal, 69.7–73.1% of bacterial reads were retained for further analysis. ASVs classified as chloroplast, mitochondria or archaea were removed from the samples. To remove sequences potentially arising from the host species (which could result in contamination and possibly reduce the microbial output, especially if the volume of DNA is low; Heravi *et al.* 2020), a local BLAST search was performed against the closely related Antarctic chironomid *Belgica antarctica* (Kelley *et al.* 2014). ASVs with > 90% sequence similarity across 90% of the query length were removed to ensure host sequences were removed (Lagunas *et al.* 2023). Bacterial samples were rarefied to depths of 42 723 and 31 182, respectively, using the *rrarefy* function from the *R* package *vegan* v 2.6-6.1, with default settings (Oksanen *et al.* 2022). Taxonomy was assigned using SILVA 138.1 prokaryotic small subunit (SSU) taxonomic training data formatted for *DADA2*, *Zenodo* (McLaren & Callahan 2021). We searched for *Wolbachia* sequences in a database of 16S amplicon sequences amplified from whole larvae of *E. murphyi*.

After sequencing and rarefaction revealed 13 sequences belonging to the order Rickettsiales, some of which have known impacts on insect reproduction (Hagimori *et al.* 2006, Giorgini *et al.* 2010, Owashi *et al.* 2024), these were selected for phylogenetic comparison with reference sequences. These reference sequences were obtained through BLAST analysis, which was performed for each ASV using the 16S ribosomal RNA database. The top results for each ASV (with the highest percentage similarity) were used as reference sequences in generating the phylogenetic tree. To place the ASVs in a broader context, three *Wolbachia* strains (all insect endosymbionts) were also selected from the SILVA 138.2 SSU database. These were chosen based on sequence quality, species-level annotation and host diversity to reflect phylogenetic variation across strains. To explore potential links to reproductive manipulation, two additional sequences from taxa that are known to facilitate parthenogenesis were sourced from GenBank and included in the phylogenetic analysis. These were *Rickettsia* sp. (accession no. L28944.1), which induces parthenogenesis in the larval endoparasitoid *Neochrysocharis formosa* (Hagimori *et al.* 2006), and *Rickettsia bellii* strain RML369-C (accession no. NR_074484.2), which facilitates cytoplasmic incompatibility (a similar reproductive method to parthenogenesis that is shared with *Wolbachia*) in the green mirid *Nesidiocoris tenuis* (Owashi *et al.* 2024) and parthenogenesis in the parasitoid wasp *Pnigalio soemius* (Giorgini *et al.* 2010). Our ASV subset and reference sequences were aligned using the *AlignSeqs*() function in the *DECIPHER* package. A multiple sequence alignment was converted into a phylogenetic data object using *phyDat*() from the *phangorn* package, and a pairwise genetic distance matrix was computed with *dist.ml*() based on a maximum likelihood model of nucleotide substitution. These values were used to infer genetic similarity between our ASVs and the reference sequences, with lower values indicating greater genetic similarity. The phylogenetic tree was constructed using *NJ*().

Results

A total of 2013 bacterial ASVs were generated across all samples, and 1971 were classified to at least the phylum level. The average number of unique ASVs in each sample was 677. A search of the database found no *Wolbachia* sequences present in the samples of *E. murphyi*. Thirteen ASVs were assigned to the order Rickettsiales (Table I), to which *Wolbachia* belongs (Serga *et al.* 2024). Of these, five were assigned to the genus *Rickettsia* and eight were unassigned beyond the order level. None were assigned to species

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Table I. Taxonomic assignment of the Rickettsiales amplicon sequence variant (ASV) subset using the SILVA 138.1 prokaryotic small subunit (SSU) taxonomic training data formatted for *DADA2*, *Zenodo*. No ASVs were assigned to species level. Closest BLAST search results to each ASV and the associated percentage identities are shown. Closest reference sequences for the Rickettsiales ASV subset with the associated genetic distances (measured as estimated substitutions per nucleotide site, calculated using maximum likelihood models) were used to construct the phylogenetic tree.

ASV number	Kingdom	Phylum	Class	Order	Family	Genus	Closest BLAST hit	Percentage identity	Closest phylogenetic reference	Genetic distance
ASV_1	Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	Rickettsiaceae	*Rickettsia*	*Rickettsia hoogstraalii*; Alphaproteobacteria; Rickettsiales; strain Croatica	98.88	*Rickettsia* sp. 16S ribosomal DNA sequence (L28944.1)	0.0150
ASV_2	Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	Rickettsiaceae	*Rickettsia*	*Rickettsia hoogstraalii*; Alphaproteobacteria; Rickettsiales; strain Croatica	98.88	*Rickettsia* sp. 16S ribosomal DNA sequence (L28944.1)	0.0112
ASV_584	Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	Rickettsiaceae	-	*Rickettsia conorii*; Alphaproteobacteria; Rickettsiales; strain Malish 7	98.50	*Rickettsia* sp. 16S ribosomal DNA sequence (L28944.1)	0.0188
ASV_1143	Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	Rickettsiaceae	*Rickettsia*	*Rickettsia hoogstraalii*; Alphaproteobacteria; Rickettsiales; strain Croatica	98.50	*Rickettsia* sp. 16S ribosomal DNA sequence (L28944.1)	0.0188
ASV_1321	Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	Rickettsiaceae	*Rickettsia*	*Rickettsia hoogstraalii*; Alphaproteobacteria; Rickettsiales; strain Croatica	98.51	*Rickettsia* sp. 16S ribosomal DNA sequence (L28944.1)	0.0150
ASV_1327	Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	Rickettsiaceae	-	*Rickettsia rhipicephali*; Alphaproteobacteria; Rickettsiales; strain 3–7-female6-CWPP	95.13	*Rickettsia* sp. 16S ribosomal DNA sequence (L28944.1)	0.0579
ASV_1461	Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	SM2D12	-	*Litorimonas haliclonae*; Alphaproteobacteria; Rhodobacterales; strain MAA42	86.52	*Rickettsia* sp. 16S ribosomal DNA sequence (L28944.1)	0.1989
ASV_1714	Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	SM2D12	-	*Haematospirillum jordaniae*; Gammaproteobacteria; Burkholderiales; strain H5569	89.59	*Rickettsia* sp. 16S ribosomal DNA sequence (L28944.1)	0.1751
ASV_1715	Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	Rickettsiaceae	-	*Rickettsia peacockii*; Alphaproteobacteria; Rickettsiales; strain Skalkaho	93.26	*Rickettsia* sp. 16S ribosomal DNA sequence (L28944.1)	0.0783
ASV_1734	Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	Rickettsiaceae	-	*Rickettsia honei*; Alphaproteobacteria; Rickettsiales; strain RB	91.85	*Rickettsia* sp. 16S ribosomal DNA sequence (L28944.1)	0.0992
ASV_1876	Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	SM2D12	-	*Desulforegula conservatrix*; Deltaproteobacteria; Desulfuromonadales; strain Mb1Pa	79.70	*Rickettsia* sp. 16S ribosomal DNA sequence (L28944.1)	0.3353
ASV_1922	Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	SM2D12	-	*Desulfuromonas palmitatis*; Deltaproteobacteria; Desulfuromonadales; strain SDBY1	87.64	*Rickettsia* sp. 16S ribosomal DNA sequence (L28944.1)	0.2087
ASV_1927	Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	Rickettsiaceae	*Rickettsia*	*Conexibacter woesei*; Actinobacteria; Solirubrobacterales; strain DSM 14684	86.19	*Rickettsia hoogstraalii* strain Croatica 16S ribosomal RNA, partial sequence (NR_104877.1)	0.2316

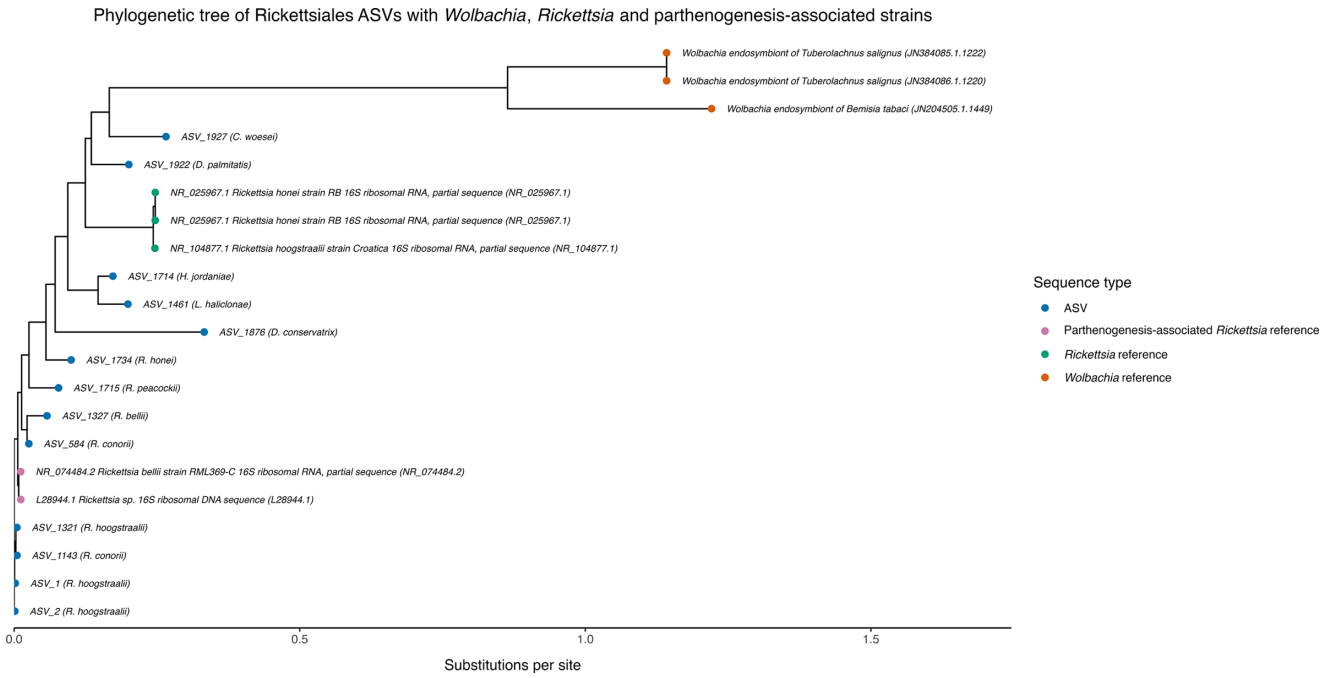

Figure 2. Neighbour-joining phylogenetic tree showing genetic distance values (measured as estimated substitutions per nucleotide site, calculated using maximum likelihood models) between the 13 amplicon sequence variants (ASVs) assigned to the order Rickettsiales in our study and the reference sequences. Tip labels next to ASVs correspond to the closest reference sequence. Shorter branch lengths or lower substitution values represent fewer genetic differences, indicating greater similarity between sequences.

level. BLAST results indicated that 9/13 of our Rickettsiales ASVs were most closely matched to known *Rickettsia* species with high sequence identities (Table I). In particular, ASV_1, ASV_2, ASV_1143 and ASV_1321 showed the highest similarity (98.50–98.88%) to *Rickettsia hoogstraalii* strain Croatica, whereas ASV_584 and ASV_1143 were closely matched to *Rickettsia conorii* strain Malish 7 (98.50%). At lower similarity, ASV_1327 aligned most closely with *Rickettsia rhipicephali* (95.13%), ASV_1715 with *Rickettsia peacockii* (93.26%) and ASV_1734 with *Rickettsia honei* (91.85%). Phylogenetic reconstruction confirmed that the majority of ASVs clustered with a parthenogenesis-associated *Rickettsia* sp. (accession L28944.1), with pairwise genetic distances ranging from 0.0112 to 0.2087 (Fig. 2 & Table I), corresponding to ~79–99% sequence similarity (Yarza *et al.* 2014). No ASVs showed close similarity to *Wolbachia* reference strains.

Discussion

The absence of *Wolbachia* in the microbiome sequences obtained from *E. murphyi* builds upon the findings of Serga *et al.* (2024), which suggest that *Wolbachia* may be entirely absent from Antarctic terrestrial invertebrates. We can find only one putative record of *Wolbachia* being reported in Antarctic soil: an environmental DNA study of soils at Mars Oasis on Alexander Island (72°S) in the southern Maritime Antarctic (Pearce *et al.* 2012). It is plausible that the earlier sequencing methods used at this time misassigned *Wolbachia* at this site, especially considering invertebrate taxa that could potentially host *Wolbachia*, such as mites, springtails and nematodes, are generally present at low densities at Mars Oasis (Convey & Smith 1997, Maslen & Convey 2006). As noted by Serga *et al.* (2024), there are no known records of any of these groups anywhere in Antarctica, so its presence at the extreme southern limit of the Maritime Antarctic would seem surprising.

The absence of *Wolbachia* in *E. murphyi* does not support the hypothesis that it plays a role in parthenogenesis in this species, although this finding does not rule out the possibility that a different, perhaps undescribed bacteria could influence the insect's reproductive system. In particular, we identified representatives of the genus *Rickettsia* (a close relative of *Wolbachia*) in our database, some members of which are known to induce thelytokous parthenogenesis in parasitoid wasps (Hagimori *et al.* 2006, Giorgini *et al.* 2010) and cytoplasmic incompatibility in true bugs (Owashi *et al.* 2024). Furthermore, our phylogenetic analysis and associated low genetic distances revealed that most of our ASVs assigned to Rickettsiales are most closely related to *Rickettsia* sp. (accession L28944.1), which has been shown to induce parthenogenesis in the larval endoparasitoid *N. formosa* (Hagimori *et al.* 2006), although this is a parasitoid within the Hymenoptera rather than the Diptera. Even though the ASVs are closely related to this *Rickettsia* sp., the similarity values suggest that they probably represent distinct *Rickettsia* taxa; in particular, ASV_1876 was highly divergent and may be a novel *Rickettsia* lineage (Yarza *et al.* 2014). As this is the first study that has assessed some of the bacterial communities associated with *E. murphyi*, this result is not unexpected. Nonetheless, the phylogenetic analyses confirm that *Wolbachia* is not involved in the parthenogenetic life history of *E. murphyi*, but they do not discount the notion that other species within the Rickettsiales may facilitate this reproductive mechanism. To test this hypothesis, future studies could utilize antibiotic treatments to target *Rickettsia* taxa in order to determine whether this group is involved in altering reproduction (Giorgini *et al.* 2010), isolate and culture the specific bacteria to carry out more detailed phylogenetic analysis (Owashi *et al.* 2024) and conduct microscopy to track the bacteria from adult to egg in order to infer possible function (Hagimori *et al.* 2006). There are other possible causes for parthenogenesis, including spontaneous differential gene expression (Sperling *et al.* 2023), developmental

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changes and alterations to cell cycle mechanisms (Sperling & Glover 2023), as well as strong selective forces leading to the evolution of obligate asexual reproduction (Chown & Convey 2016, Leihy & Chown 2020).

Various potential explanations for the absence of *Wolbachia* in Antarctic invertebrates and insects, including whether this observation is simply the result of a lack of targeted research, were raised by Serga *et al.* (2024). For example, as *Wolbachia* is known to be sensitive to temperature (Charlesworth *et al.* 2019), it is possible that the polar regions are simply too cold for *Wolbachia* to persist within its hosts, which is consistent with observations that some widely distributed species host *Wolbachia* at lower but not higher latitudes (Chrostek *et al.* 2021). However, research has yet to directly investigate whether low temperature impacts *Wolbachia* survival (Serga *et al.* 2024). Polar terrestrial environments in Antarctica are also generally nutrient-limited (Lambrechts *et al.* 2019), which may mean that it is too costly to the host to harbour *Wolbachia* in these regions, as suggested by Serga *et al.* (2024). Further research on the bacterial diversity (particularly representatives of *Rickettsia*) associated with *E. murphyi*, including expansion of the taxonomic databases representing Antarctic microbes, is required to understand how parthenogenesis developed and persists in this midge.

Supplementary material. To view supplementary material for this article, please visit http://doi.org/10.1017/S0954102025000197.

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