Wolbachia strain diversity in a complex group of sympatric cryptic parasitoid species

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Abstract

Background

Maternally-inherited symbionts can induce pre-mating and/or post-mating reproductive isolation between sympatric host lineages, and speciation by modifying host reproductive phenotypes. The large parasitoid wasp genus *Cotesia* (Braconidae) includes a diversity of cryptic species, each specialized in parasitizing one to few related Lepidoptera host species. Here, we characterized the infection status of an assemblage of 21 *Cotesia* species by several microbial symbionts, as a step towards testing whether symbionts might provide a barrier to gene flow between the parasitoid host lineages.

Results

The symbiotic microbes *Arsenophonus*, *Cardinium*, *Microsporidium* and *Spiroplasma* were not detected in the *Cotesia* wasps. However, the endosymbiotic bacterium *Wolbachia* was present in at least eight *Cotesia* species. Some of the closely related *Cotesia* species carry similar *Wolbachia* strains, but most *Wolbachia* strains showed patterns of horizontal transfer between phylogenetically distant host lineages.

Conclusions

Consequently, we did not detect co-phylogenetic signals, suggesting *Wolbachia* is not a strictly obligatory beneficial symbiont in these insects. Instead, as a potential facultative symbiont of *Cotesia* species, *Wolbachia* may still function as a key-player in the biology of the parasitoid wasps, but its role in the evolution of this complex clade of cryptic species remains to be further investigated.

Background

At least 40% of all insect species worldwide are associated with endosymbiotic microbes, including *Arsenophonus*, *Cardinium*, Microsporidium, *Spiroplasma*, and possibly the most common one: *Wolbachia* [1]. To enhance their own fitness through transmission in their host population, these microbes can manipulate their host reproduction and other life-history traits [2–4]. For example, the bacteria *Wolbachia* and *Spiroplasma* can induce cytoplasmic incompatibility (CI), in which infected males are incompatible with females that are uninfected or infected with other incompatible symbiotic strain [5–7]. *Wolbachia* can also manipulate the behaviours of their host, such that infected and uninfected individuals have different mate or host preferences [8–11]. These symbiont-induced reproductive and behavioural alterations have thus long been proposed as key drivers of host speciation and diversity [12], via post-mating isolation [13, 14], and/or pre-mating reproductive isolation between lineages of different infection status [15, 16]. For example, Shoemaker et al. [17] showed that in the *Drosophila subquinaria* species group, *Wolbachia* induces unidirectional CI, which, coupled with mate choice preferences, establishes a reproductive barrier between *D. recens* and *D. subquinaria*. While divergence between insect species often

occurs independently of any symbiotic infection [18], the relative importance of microbial symbiont in this process is likely underestimated as the prevalence, diversity, and role of symbionts remain unknown for many insect systems.

Biogeographic studies of symbiotic diversity and prevalence, combined with phylogenetic analyses, can provide clues to the ecological and evolutionary roles of symbionts in their host species clade. For example, obligate symbionts transmitted exclusively maternally are likely to show high prevalence within their host species, and might also be conserved across the evolutionary history of their host [19–21]. In such cases, we could expect concordance between the phylogeny of the symbiont and that of their host, as it is observed between diverse beneficial *Wolbachia* strains and their bedbug or nematode hosts [22, 23]. In contrast, strong co-speciation is not expected if the symbionts are facultative or parasitic. Additionally, although endosymbionts such as *Wolbachia* are predominantly transmitted vertically from mothers to offspring, these bacteria can also be horizontally transferred between host lineages and species [24–29]. Horizontal transfer events might occur between species sharing the same niches, including between parasitoids and their prey, between preys attacked by the same parasitoid species, between predators or parasitoids sharing the same prey [30, 31], between herbivores sharing the same host plants [25, 26], or between hybridizing species [32]. These events allow the symbiotic strains to colonize divergent host species.

Parasitoids wasps in the genus *Cotesia* (Hymenoptera: Braconidae) parasitize Lepidoptera by laying a single to multiple eggs in their host caterpillars. The parasitoid larvae grow while feeding on the developing caterpillar's haemolymph, and then pupate in silken cocoons outside the body of the host [33]. The whole genus *Cotesia* accounts over 1000 named species worldwide and parasitize many Lepidoptera species [34, 35]. In some cases the *Cotesia* wasps can have dramatic effects on their host population dynamics [38]. For example, even by only infecting about 10% of the caterpillars of *Melitaea cinxia* in the Åland Islands, Finland, *Cotesia melitaearum* has been found to cause localized decline within the larger host metapopulation [39, 40]. Furthermore, multiple *Cotesia* species can co-occur, where their host species occur together in a landscape. In North-eastern Spain, seven cryptic *Cotesia* species were described to emerge from only one to two of each of the local eight related *Melitaea* and two *Euphydryas* butterfly species (Lepidoptera: Nymphalidae: Melitaeini), which sometimes share host plant species, and live in shared meadow habitats [36, 37].

To date, *Wolbachia* is, to our knowledge, the only endosymbiont that has been previously screened for, and detected from *Cotesia* species. The bacterium has been found in *C. glomerata* (Linnaeus) and *C. vestalis* (Haliday) (synonym of *C. plutellae* (Kurdjumov)) [41], and in *C. sesamiae* from Cameron and from Kenya [42, 43]. Branca et al. [43] demonstrated that *Wolbachia* induces unidirectional CI in *C. sesamiae* from Sub-Saharan Africa, which influenced the host specialization, genetic structure, and biogeography. In the *C. melitaearum* clade, molecular characterizations based on small number of genes have shown that specialization and competitive interactions in local *Cotesia* are associated with the emergence of several cryptic sympatric *Cotesia* species [36, 44]. The role of *Wolbachia* in this clade however remains to be investigated. In our study, we screened for the presence of different endosymbiotic microbes in 15

Cotesia species and cryptic species parasitizing Melitaeini butterfly species across different geographic locations. After identifying *Wolbachia* as the only detectable symbiont in the *Cotesia*, we characterized the *Wolbachia* strains diversity and phylogeny, including strains identified from an additional six *Cotesia* species from which genomic data was publicly available on NCBI. Our study provides an overview of the prevalence and diversity of common endosymbiotic microbes of insects in *Cotesia* wasps, which is a step towards evaluating the roles such symbionts might play in the evolution of parasitoid wasps.

Material and Methods

Material

We analysed 323 *Cotesia* specimens (Table S1) originally collected and used for earlier studies of the phylogeny and butterfly-host specialization of *Cotesia* species associated with checkerspot/fritillary butterflies (*Melitaea* and *Euphydryas*) [36, 44, 45]. Ten specimens were included in duplicates to control for false positive or negative results.

To put our findings in a larger phylogenetic context we expanded the host species range and *Wolbachia* strain diversities of our study by screening for *Wolbachia* genomic material in the genomic data from *Cotesia* sequencing projects publicly available from NCBI Sequence Read Archive (SRA) database (https://www.ncbi.nlm.nih.gov/sra), and from the NCBI nucleotide database (https://www.ncbi.nlm.nih.gov/nucleotide). We searched the SRA database using the keyword "*Cotesia*", selecting "DNA" as source and "Genome" as strategy. With this approach, we identified 28 genome sequencing projects including both short-read and long-read data and representing six different *Cotesia* species (*C. congregata*, *C. flavipes*, *C. glomerata*, *C. rubecula*, *C. sesamiae*, and *C. vestalis* as synonym of *C. plutellae*) (Table S2).

Similarly, to increase the list of *Wolbachia* strains included in our phylogeny, we screened the NCBI nucleotide database for any of the five *Wolbachia* Multilocus Sequence Typing (MLST) markers [46] and *wsp* (*Wolbachia surface protein*) gene [47] from any *Cotesia*, and some of their known butterfly host species. This provided an additional 40 MLST and *wsp* sequences from four *Cotesia* species (*C. flavipes*, *C. glomerata*, *C. sesamiae* and *C. vestalis*), and four Lepidoptera host species (*Melitaea didyma*, *Chilo partellus*, *Pieris rapae*, *Plutella xylostella*). The sample size and geographic sampling locations are provided in Table S1 and Figure S1.

Molecular work on lab-stored DNA extracts

The DNA from all field collected wasps was extracted using NucleoSpin Tissue Kit (Macherey-Nagel) for the purpose of phylogenetic studies of the *Cotesia* wasp species in the early 2000s by Kankare and colleagues [36, 44, 45, 48]. The DNA extracts have since been preserved in the freezer (-20°C) at the University of Helsinki, Finland. The quality of each DNA extract was tested by PCR amplification of the mitochondrial cytochrome C oxidase subunit I gene (*COI* - primer pair LCO/HCO) [49]. The DNA extracts that did not amplify with the primers LCO/HCO after two PCRs were removed from further analyses.

We first screened 56 *Cotesia* specimens for infection with five microbial symbionts (Table S1). We screened for the bacteria *Spiroplasma* and *Cardinium* using the 16S ribosomal RNA (16S rRNA) gene [50–52], for the bacterium *Arsenophonus* by targeting the 23S rRNA gene [53], for *Wolbachia* using *Wolbachia*-specific primers amplifying the *wsp* gene and up to five conserved MLST genes: *coxA*, *fbpA*, *ftsZ*, *gatB* and *hcpA* [46], and for the fungal symbiont *Microsporidium* by amplifying the 18S rRNA gene [54]. We later screened the remaining *Cotesia* specimens for infection by *Wolbachia* only. One negative control (water sample) and one positive control from a *Wolbachia*-infected *Ischnura elegans* specimen [55] were included in each PCR. All primer sequences are given in Table S3. We Sanger sequenced the amplified genes on an ABI-3730 DNA Sequencer (Applied Biosystems) at the University of Helsinki, Finland, using only the forward primers for each gene. All *Wolbachia* MLST loci and *wsp* gene sequences were identified by comparing the resulting assemblies against the PubMLST database (https://pubmlst.org) with BLAST [56].

Cleaning, and processing sequence material from NCBI repository

We used different bioinformatic tools to access and use the additional *Wolbachia* sequence material retrieved from NCBI repository. We first processed the short-read (Illumina) sequencing samples with Prinseq-lite (version 0.20.4) [57] to remove all sequences with at least one ambiguous nucleotide. The resulting reads were adapter trimmed and quality filtered using Trimmomatic (version 0.39) [58]. Quality assessment reports were obtained with FastQC [59] and summarized by MultiQC [60]. In contrast, the long-read sequences (Oxford Nanopore, ONT) were quality filtered using NanoFilt (version 2.7.1) [61], which excluded sequences with a mean base quality lower than ten and lengths lower than 1 kb. The quality of the processed specimens was evaluated with NanoStat (version 1.5.0) [61].

We then screened the Illumina samples for *Wolbachia* infection using Kraken2 (version 2.0.8) provided with a custom database of 142 *Wolbachia* publicly available reference genomes [62] (140 reference genomes from GenBank and two (*w*Di and *w*Ls) from http://nematodes.org/) (See Table S4). Samples with at least 1000 reads classified as *Wolbachia* according to Kraken2 [63] were then mapped against our *Wolbachia* reference genomes database using Bowtie2 (version 2.4.4) [64]. In contrast, the ONT sequencing data were directly aligned to *Wolbachia* reference genomes by Minimap2 (version 2.21) [65]. We used SAMtools (version 1.13) [66] to extract, merge, and sort reads properly mapped as pairs (mapping quality of 20) from the SAM file generated in the alignment step. For each alignment, the per-base read depth across two *Wolbachia* reference genomes (*w*MelPop strain GenBank CP046921.1 and *w*PipPel strain GenBank AM999887.1) was calculated using the SAMtools depth function and plotted in R with ggplot2 [67] (Fig. S2-3). Mapped reads belonging to samples from the same BioSample were also processed as merged reads.

Finally, we built *Wolbachia* genome assemblies by individually assembling mapped reads from short- and long-read sequencing using the Unicycler pipeline (version 0.4.9) [68]. The quality and the completeness of the resulting genome assemblies were estimated by QUAST (version 5.0.2) [69] and BUSCO (version

5.4.3, Rickettsiales odb10 database) [70]. The assemblies, along with the two *Wolbachia* reference genomes mentioned above, were analysed using FastANI (version 1.3) [71]. FastANI estimates the Average Nucleotide Identity (ANI) metric, enabling the clustering of genomes from different individuals/organisms. This method facilitates the inference of the supergroup placement of *Wolbachia* strains by utilizing their entire genomes, and is a more comprehensive approach compared to using a limited set of markers. Annotation of *Wolbachia* assemblies and reference genomes was performed with Prokka (version 1.4.6) [72] using default settings. Subsequently, the protein sequences predicted by Prokka were uploaded into the OrthoVenn3 web server (https://orthovenn3.bioinfotoolkits.net); accessed date: 15 July 2023) for identification and comparison of orthologous clusters (Fig. S4). All final assemblies are available from Zenodo at https://doi.org/10.5281/zenodo.8422079.

Identifying the CI-associated genes

To explore whether the *Wolbachia* strains analysed here may be causing CI in their hosts, we searched for the CI-associated genes, *cifA* and *cifB*, in the newly assembled *Wolbachia* genomes, using BLAST. For this purpose, we downloaded the amino acid sequences of CifA and CifB from various *Wolbachia* strains in the NCBI database (Organism: *Wolbachia*, Source: RefSeq only) using the keywords “cytoplasmic incompatibility CifA” or “cytoplasmic incompatibility CifB”. Subsequently, these amino acid sequences served as queries in two distinct searches: TBLASTN against *Wolbachia* assemblies and BLASTP against proteomes derived from the same assemblies. Homologs covering at least 70% of the length of the query, with an identity of at least 50%, and having an E-value cut-off of 10^{-10} were aligned to the intact Cif homologs identified by Martinez et al. [73] using MUSCLE [74].

Phylogenetic analyses

We inferred the phylogenetic relationships between the different *Wolbachia* strains of *Cotesia* wasps using the characterized *Wolbachia* MLST (*coxA*, *fbpA*, *ftsZ*, *gatB* and *hcpA*) and *wsp* genes sequences. Individual MLST and *wsp* genes, and their concatenated alignments were produced using MAFFT [75], and manually curated in AliView [76]. We performed the phylogenetic analyses using RAxML [77] in raxmlGUI 2.0 [78] applying a general time reversible model with gamma-distributed rate variation across sites and a proportion of invariable sites (GAMMAGTR + I) on individual genes and concatenated alignments (Fig. S5 and S6). In all cases, node support was calculated by the rapid bootstrap feature of RAxML (100 replicates). The *Wolbachia* phylogeny also includes 12 reference *Wolbachia* strains belonging to A-, B-, D- or F- supergroups and originating from different host species (https://pubmlst.org, *w*Au, *w*Bm, *w*Bol1, *w*Clec-F, *w*Ha, *w*Irr, *w*MelPop, *w*No, *w*Pel, *w*Ri, *w*Stri, *w*Vit), one strain from the butterfly *Danaus chrysippus (Nymphalidae)*, and three from the parasitoid wasp *Hyposoter horticola (Hymenoptera: Ichneumonidae)* (Table S1). The *Wolbachia* strains *w*Bm [79] and the *w*Cle [80], which belong to the D- and F-supergroup, respectively, were used as outgroups to root the *Wolbachia* trees.

To infer whether *Wolbachia* strain diversification is concordant with the *Cotesia* wasp species diversification, we inferred the phylogenetic relationships between *Cotesia* species using the *COI* sequences of *Cotesia* species, employing a maximum likelihood approach. We sampled all *COI*

sequences deposited in GenBank for 39 different *Cotesia* species, including sequences from our *Cotesia* specimens previously deposited by [44, 45] (Table S5). As outgroups, we selected three species belonging to the *Microgaster* genus (Hymenoptera, Braconidae), namely *Microgaster nobilis*, *M. deductor* and *M. subcompletus* (see Table S5). We generated a *COI* sequence alignment of 606 bp with MAFFT, that we manually curated for misaligned regions using AliView. We constructed a maximum likelihood phylogeny from this alignment using IQTREE [81] under the best-fit model automatically selected by ModelFinder [82] (Fig. 1 and Fig. S7). Node support was estimated using ultrafast bootstrapping with 1,000 replicates [83].

The CifA and CifB proteins have previously been classified into at least five distinct phylogenetic clades (types I–V) with different degrees of compatibility [73, 84–86]. To determine the group to which the annotated Cif homologs from *Wolbachia* found in *Cotesia* hosts belong, we performed a phylogenetic analysis. The best-fit substitution model for the protein multiple sequence alignment was estimated using Modeltest-NG [87] in raxmlGUI 2.0 and based on the Akaike information criterion (AIC), it was determined to be a JTT + G4 + F model. A maximum likelihood phylogenetic tree was built using RAxML in raxmlGUI 2.0 software with 100 rapid bootstraps (Fig. S8). Tree visualization and figures were obtained with ITOL [88] using the bipartitions output trees produced by RAxML and the bootstrap consensus tree from IQTREE analysis.

Results

Endosymbionts in Cotesia

1_ From DNA extracts

Out of the 323 DNA extracts selected for *Wolbachia* screening, 282 were of good quality based on *COI* amplification, suggesting most of the specimens had been sufficiently preserved since extraction [44, 45].

The PCR amplifications for *Arsenophonus*, *Spiroplasma* or *Microsporidium* from 56 *Cotesia* specimens from four countries were negative (Table S1). There was one amplification using the *Cardinium 16S*rRNA primers in one unique specimen of *C. melitaearum* cryptic sp. H from Spain. However, our attempts at sequencing this amplificon were not successful, thus we could also not confirm the presence of *Cardinium* in our *Cotesia* samples. In contrast, out of the 282 *Cotesia* samples of good quality, 50 (17.7%) carried the symbiotic bacterium *Wolbachia* (Table 1, S1), representing at least eight *Cotesia* species parasitizing Melitaeini butterfly species (Fig. 1). As of the 10th January 2023, there was no record of *Wolbachia* strain from *Cotesia* species in the PubMLST database.

2_ From genome projects available in NCBI

By screening the 28 *Cotesia* genome projects (i.e. SRA projects) available on NCBI, we also identified 14 specimens (50%) containing at least 1000 reads classified as *Wolbachia* (Table S6). Ten specimens (six specimens from *C. glomerata*, one from *C. sesamiae* and three from *C. vestalis*) included *Wolbachia*

reads distributed throughout the *Wolbachia* reference genomes (Fig. S2-3), while the last four specimens only included reads with patchy coverage across the *Wolbachia* reference genomes. These last four projects were considered as potential false positive results for *Wolbachia* infection, with the *Wolbachia* reads representing potential contamination, or insertions of *Wolbachia* sequences in the *Cotesia* host genomes.

Wolbachia strain diversity

Using the ten *Cotesia* genome projects found infected with *Wolbachia*, we partially assembled nine *Wolbachia* genomes. Three assemblies isolated from *C. glomerata*, exhibited BUSCO completeness of 86.8% (SRR13990441), 87.7% (SRR13990442), and 41.8% (SAMEA7283786) with corresponding total sizes of 1.10 Mbp, 1.08 Mbp, and 0.52 Mbp, respectively (See Table S7-8), while all other *Wolbachia* assemblies had a low number of BUSCO genes and were < 0.1 Mbp in size (Table S7-8). We were only able to extract between three and six MLST and *wsp* markers from the three largest *Wolbachia* assemblies.

Combining results obtained by direct amplification of the *Wolbachia* markers by PCRs and by screening the *Wolbachia* genomic assemblies built from *Cotesia* genomic sequences available on NCBI for those same markers, we obtained sequences from one to six markers for 38 (out of 61) *Wolbachia*-infected specimens (Table S1). We identified a total of 14 alleles for the *ftsZ* gene, nine for the *hcpA* gene, five for the *coxA* gene, six for *fbpA*, and six for *gatB* (See Table S1 for further details). This resulted in a concatenated alignment of 2559 bp, which allowed us to discriminate ten *Wolbachia* strains from ten *Cotesia* species (Table 1). We did not detect multiple infections in any of the individual *Cotesia* specimens, but two species carried several *Wolbachia* strains. Specimens of *C. koebelei* reared from *E. editha* carried either a supergroup A or a B *Wolbachia* strain, and Spanish specimens of *C. bignellii* carried a A-supergroup strain, while French specimens of the same species carried one of two B-supergroup strains (Fig. 1).

Table 1

Metadata for the Cotesia species and cryptic species found to be infected with *Wolbachia*: their butterfly host species, country of origin, and *Wolbachia* prevalence. Rows in grey highlight the specimens that were screened for all five symbionts (Table S1), while rows in white include the specimens screened for *Wolbachia* only.

Species	Host species reared from	Country	Infection rate (infected/total tested)	Strains detected
C. acuminata cryptic sp. B	*Melitaea phoebe*	Spain	24.4% (5/17)	Uncharacterized
C. bignellii	*Euphydryas aurinia*	France	100.0% (2/2)	*w*Cbig
C. bignellii cryptic sp. C	*Euphydryas aurinia*	Spain	50.0% (3/6)	*w*CbigC
C. koebelei	*Euphydryas editha*	USA	100.0% (2/2)	*w*CkoeA, *w*CkoeB
C. melitaearum cryptic sp. D	*Euphydryas aurinia*	Spain	10.8% (4/37)	*w*CmelD
C. melitaearum cryptic sp. F	*Melitaea didyma*	Spain	100% (12/12)	*w*CmelF
C. melitaearum cryptic sp. G	*Melitaea trivia*	Spain	92.9% (13/14)	*w*CmelG
C. melitaearum cryptic sp. H	*Melitaea cinxia*	Finland	11.1% (6/54)	*w*CmelH1
C. melitaearum cryptic sp. H	*Melitaea cinxia*	Russia	27.3% (3/11)	Uncharacterized
C. glomerata	*Pieris sp.*		75% (6/8)	*w*Cglo
C. sesamiae	*Stem boring moths*		100% (1/1)	Uncharacterized
C. vestalis	*Plutella sp.*		75% (3/4)	Uncharacterized

Analyses of the Wolbachia genomic assemblies

By comparing the predicted proteomes of our two largest *Wolbachia* assemblies with an > 50% BUSCO completeness against those of the two *Wolbachia* reference genomes (*w*MelPop and *w*PipPel) using Prokka, we identified 954 protein-coding genes, 30 tRNAs, and one rRNA in the SRR13990441 assembly, and 996 protein-coding genes, 32 tRNAs, and three rRNAs in the SRR13990442 assembly (Table S9). In contrast, the two reference genomes, *w*MelPop and *w*PipPel, contained 1304 and 1410 protein-coding genes, 34 tRNAs, and three rRNAs, respectively (Table S9). The comparison using the Orthovenn 3 web server showed a total of 1057 conserved orthologs in all four strains, with 590 of these being single copy. All four strains shared 639 ortholog clusters (Fig. S4). The SRR13990442 assembly contains 876 orthologs, while SRR13990441 has 875, and they both share 71 unique orthologs with the reference B-

supergroup *Wolbachia w*PipPel, but only 21 with the A-supergroup *Wolbachia w*MelPop reference (Fig. S4). Similarly, the ANI analysis, which calculates the average nucleotide identity among orthologous gene pairs shared between two genomes, revealed a high similarity between *w*PipPel, SRR13990441, and SRR13990442, with ANI values around 98% in pairwise comparisons (Table S10). In contrast, *w*MelPop displayed a lower ANI (~ 85%) in pairwise comparisons with *w*PipPel, SRR13990441, and SRR13990442 (Table S10). Altogether, these results suggest the two *Wolbachia* assemblies from *Cotesia* belongs to the B-supergroup *Wolbachia*.

Finally, we partially extracted the CI-associated genes from our *Wolbachia* assemblies. With this, we identified one copy of a Type I CifA in the SRR13990441 assembly (Table S11, Fig. S8), and a truncated/partial copy of *cifB* in both the SRR13990441 assembly (contig 109, position 1492–3201) and the SRR13990442 assembly (contig 221, position 1-1624). The sequences of the *cif*B gene from our *Wolbachia* assemblies were highly similar to that previously characterized from the fig wasp *Kradibia gibbosae* (Hymenoptera: Chalcidoidea) (WP_275944372.1), without any report of the role played by the symbiont in this host species [89].

Phylogenetic analyses

Our phylogenetic tree of the *COI* mitochondrial gene of 39 *Cotesia* species shows that the *Cotesia* wasps parasitizing Melitaeini butterflies belong to three distinct clades (See Fig. 1, S5-6:

- Clade 1 includes *C. melitaearum* cryptic species (D, E, F, G, H, I, M, N),
- Clade 2 includes *C. koebelei*,
- Clade 3 includes *C. bignellii* cryptic species C, and *C. acuminata* cryptic species (A, B, and K).

This phylogenetic pattern is consistent with previous studies on the same clade [45]. Our phylogeny also preserves the previous grouping of *C. xylina* with *C. yakutabensis* [90]. Although the three *Cotesia* clades are specialists to the Melitaeini butterflies, the phylogeny suggests this host associated clustering is not conserved across the genus *Cotesia*. Indeed, closely related *Cotesia* species to each of the three clades have been described as parasitoids of divergent non-Melitaeini butterflies. For instance, *Pieris sp*. butterflies are host to *C. glomerata*, *Lampides boeticus* is host to *C. specularis*, and diverse moths host other *Cotesia* species (ie. *Chilo sp*. for *C. flavipes*, or *Plutella sp*. for *C. vestalis*). Furthermore, each of these butterfly species feeds on a wide diversity of host plants.

The *Wolbachia* phylogeny confirms that all *Wolbachia* strains characterized from *Cotesia* belonged to the A- and B-supergroups, with the majority (49/53, 92.4%) belonging to the B-supergroup (Fig. 1). Despite fewer representative taxa per phylogeny and lower resolution, phylogenies based only on individual gene alignments maintained similar sample groupings, with conserved strain assignment to supergroups A and B (Fig. S5), thus suggesting no recombination has occurred between the strains of the two supergroups in these *Cotesia* species. A visual comparison supports the lack of congruence and co-phylogeny between the maximum likelihood trees of *Cotesia* and their symbiotic strains. Phylogenetically

close *Wolbachia* strains were found in phylogenetically distant *Cotesia* host species (Fig. 1). This was true for both the concatenated alignment as well as for the individual marker alignments inferring the symbiont phylogenies (Fig. 1, S5-6). The *wsp* gene tree suggests that the *Wolbachia* strain found in the butterfly host *M. didyma* is phylogenetically close to the *Wolbachia* strain from their *Cotesia* parasitoid, which could have occurred through horizontal transfer between the host and parasitoid. No similar pattern was observed for the *Wolbachia* strains we were able to extract from the other Lepidoptera hosts including *Chilo partellus*, *Pieris rapae*, or *Plutella xylostella*.

Discussion

To bring some light on the possible role(s) of endosymbionts on the evolutionary history of *Cotesia* parasitoid wasps, we screened > 200 *Cotesia* DNA-extracts from field collected samples, as well as 28 *Cotesia* genomic projects publicly available on NCBI, for diverse symbiotic infections. Although we did not detect the symbionts *Arsenophonus, Cardinium, Microsporidium* and *Spiroplasma*, *Wolbachia* was detected in 61 (17.9%) of all samples, covering 11 Lepidoptera host species (52.4%) out of 21 included in the study. Concordant with previous studies on *Cotesia* wasps species [41–43], and in insects in general [91, 92], such *Wolbachia* prevalence is still likely an underestimate of the true infection prevalence in *Cotesia* wasps. This is because our study covers only a small number of *Cotesia* species and individuals representing only part of their geographic distributions [35]. Additionally, the commonly used MLST markers have been criticised for being too conserved to allow reliable strain differentiation or infer precise phylogenetic relationships of closely related *Wolbachia* strains [93]. We provide here the first partial assemblies of *Wolbachia* strains from *Cotesia* hosts. Access to whole genome sequences will, in the future, allow more holistic estimates of both host's and symbiont's patterns of diversity.

In early studies, Kankare et al. [36, 44] suggested that direct competition for the Melitieni butterfly host species between *Cotesia* wasps might have driven the divergence between the parasitoid species and cryptic species. Our study does not identify any co-phylogenetic patterns between the *Cotesia* hosts and their *Wolbachia* strains, suggesting *Wolbachia* is not at the origin of the divergence between all *Cotesia* lineages. However the symbiont could still be involved in the restricted gene flow between some of the sympatric *Cotesia* lineages [94]. For example, CI could occur between *Cotesia* lineages that carry divergent *Wolbachia* strains, such as *C. melitaearum* sp. F and G, or between *Cotesia* lineages of different infection status, such as *C. melitaearum* sp. D and E. Although we isolated a complete homolog of the *cifA* gene and a partial homolog of *cifB* gene, which code for the *Wolbachia*-induced CI phenotype in other species [84, 95–98], only experimental rearing [99, 100][101, 102] and crossing between lineages would confirm the expression of CI between different host lineages. Alternatively, because CI causes visible morphological abnormalities of sperm in the testes of infected males [8, 103–105], or cytological embryonic defects, microscopic approaches may be used to confirm post-mating isolation between *Cotesia* lineages, as shown previously in the flies *Culex pipiens, Drosophila simulans* and the parasitoid wasp, *Nasonia* [106–112].

Nonetheless, the comparison of the phylogenetic trees from *Cotesia* hosts and their *Wolbachia* symbionts clearly showed that distantly related *Cotesia* species share similar *Wolbachia* strains. Such lack of concordance between the host and the symbiont phylogenies has been previously described in diverse systems [24, 31]. This pattern suggests that *Wolbachia* strains have transferred horizontally between *Cotesia* lineages. Thus, the bacteria are unlikely to be obligate mutualistic symbionts driving speciation of the *Cotesia* wasps, as strict transmission vertically across generations would show as associated host and symbiont phylogenies [24, 31, 32, 113]. Because many *Cotesia* species occur in sympatry, sharing either their geographical ranges, their local habitats, their hosts, which is some cases also share the same host plants [36, 114, 115], the Cotesia species complex offers many opportunities for *Wolbachia* to transfer horizontally:

1. First, divergent *Cotesia* wasps might have acquired their *Wolbachia* infections while parasitising infected caterpillars, as was suggested by [116] between whiteflies and their parasitoids. Although *Wolbachia* was previously detected in *M. didyma* [117, 118], *M. athalia*, *M. britomartis* [119], *M. phoebe*, *M. ornata* [120] and *M. cinxia* [24], genetic sequences for those strains were not available. However, we did find that sequence from the *Wolbachia* infecting *C. melitaearum* parasitizing *M. didyma* caterpillars was very similar to the *wsp* sequence from a strain infecting *M. didyma*. This suggests that *Wolbachia* might transfer between *Cotesia* wasps and their Lepidoptera hosts. But this is not always the case as other strains characterized from other Lepidoptera species (i.e. *w*Cpar from *Chilo partellus*, *w*Prap from *Pieris rapae*, and *w*Pxyl from *Plutella xylostella*) were phylogenetically divergent from the strains found in the *Cotesia* wasps infecting those Lepidoptera (*C. flavipes*, *C. glomerata*, *C. vestalis*, respectively).
2. Second, *Wolbachia* could be exchanged between parasitoids simultaneously parasitising the same host caterpillar, as shown by [31] in other parasitoid wasps. In Åland, *M. cinxia* is commonly parasitized by several parasitoid wasps [121]. Out of these, *Hyposoter horticola* is known to carry *Wolbachia* [122], and this *Wolbachia* strain (*w*Hho) is phylogenetically closely related to the *Wolbachia* characterized from *C. melitaearum*. These results suggest that at least some *Wolbachia* might transfer horizontally even between divergent parasitoid species sharing the same Lepidoptera hosts.

Abbreviations

CI
Cytoplasmic Incompatibility
MLST
Multi Locus Sequence Typing

Declarations

Ethics approval and consent to participate

The data does not include any personal data. The collection of the samples dated prior to 2004, and the author list includes a member of a Spanish institution, which in line with the current requirement of the Nagoya protocol on Access and Benefit-sharing.

Consent for publication

All authors consent in the publication of this manuscript.

Availability of data and materials

All sequence alignments and final assemblies are available from Zenodo at https://doi.org/10.5281/zenodo.8422079.

All sequences were deposited on NCBI with the accession numbers OR597552-OR597565 (wsp), OR608488 - OR608500 (hcpA), OR640995-OR641006 (coxA), OR641007-OR641026 (ftsZ), OR641031-OR641044 (gatB), and OR641048-OR641057 (fbpA) (Also see Table S12 for more details).

Competing interests

Authors declare no conflict of interest.

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Authors' contributions

A.D. designed the study. A.D., C.M., C.S., and M.K. collected the data. F.V. analyzed the data and prepared all figures. A.D. and F.V. wrote the main manuscript. All authors reviewed the manuscript.

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Figures

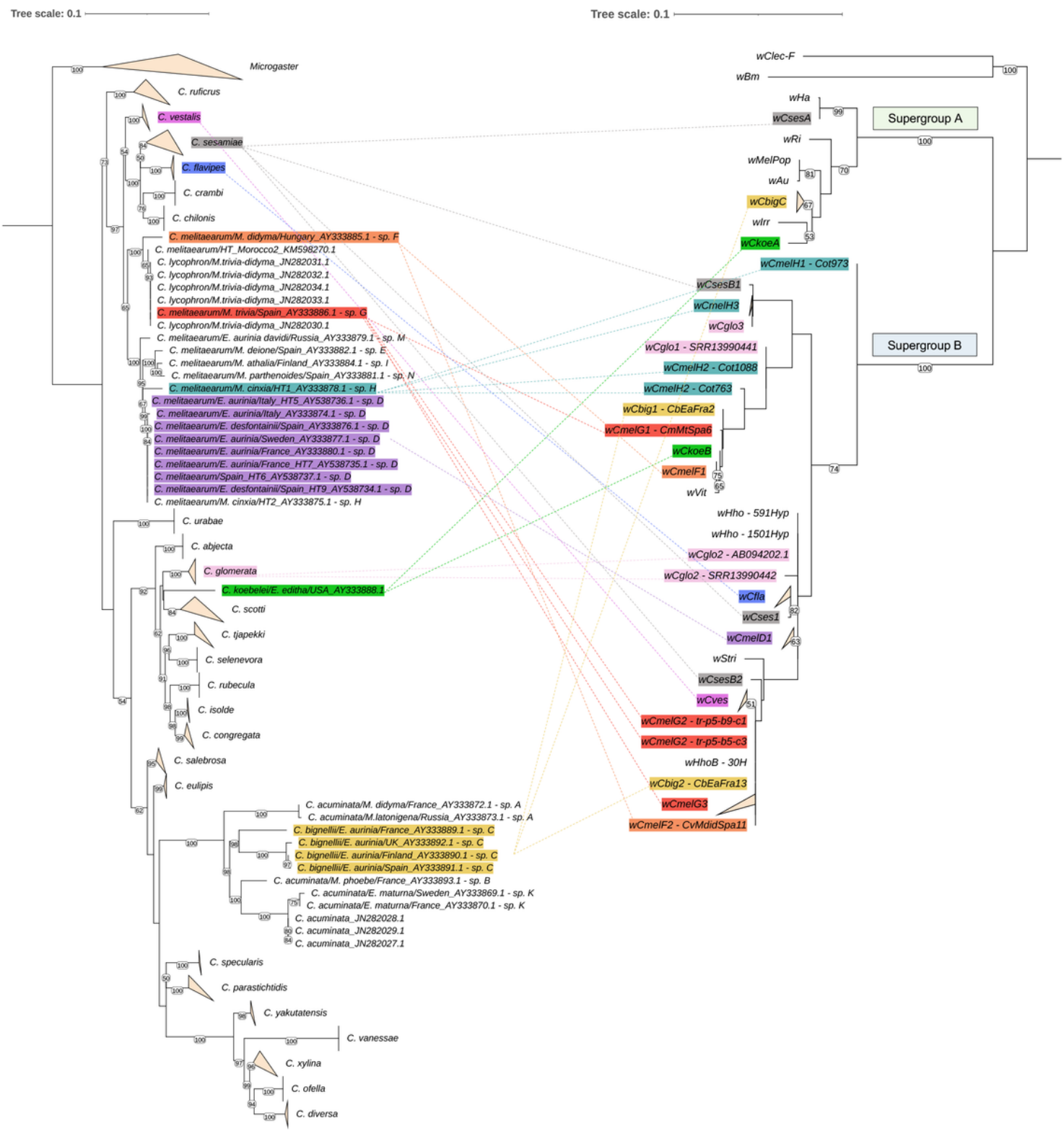

Figure 1

Comparison between *Cotesia*parasitoid lineages and *Wolbachia* strains from *Cotesia* species. The *Cotesia* maximum likelihood phylogenetic tree was inferred from the nucleotide sequence alignment (606 bp) of the mitochondrial *COI* gene. The *Wolbachia* maximum likelihood tree was based on concatenated alignment (2,559 bp) of the MLST and *wsp* markers and rooted using reference genomes from *Wolbachia* strains *w*Bm and *w*Clec belonging to the supergroups D and F, respectively. *Cotesia*species labelled A

through N correspond to cryptic species described in [45]. The coloured lines link *Cotesia* host species to their respective *Wolbachia* strain infections; with a unique colour for each host species. Branches corresponding to different sequences obtained from different specimens within the same species, and sequences from different species but within the same genus (only in the case of the outgroup *Microgaster*), were collapsed and represented as orange triangles for visual clarity. Bootstrap support values >50 are displayed at each node.

Supplementary Files

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