Development of a multi-locus sequence typing system helps reveal the evolution of *Cardinium hertigii*, a reproductive manipulator symbiont of insects

Corinne M. Stouthamer¹, Suzanne E. Kelly¹, Evelyne Mann², Stephan Schmitz-Esser³ and Martha S. Hunter¹*

Abstract

Background: *Cardinium* is an intracellular bacterial symbiont in the phylum Bacteroidetes that is found in many different species of arthropods and some nematodes. This symbiont is known to be able to induce three reproductive manipulation phenotypes, including cytoplasmic incompatibility. Placing individual strains of *Cardinium* within a larger evolutionary context has been challenging because only two, relatively slowly evolving genes, 16S rRNA gene and Gyrase B, have been used to generate phylogenetic trees, and consequently, the relationship of different strains has been elucidated in only its roughest form.

Results: We developed a Multi Locus Sequence Typing (MLST) system that provides researchers with three new genes in addition to Gyrase B for inferring phylogenies and delineating *Cardinium* strains. From our *Cardinium* phylogeny, we confirmed the presence of a new group D, a *Cardinium* clade that resides in the arachnid order harvestmen (Opiliones). Many *Cardinium* clades appear to display a high degree of host affinity, while some show evidence of host shifts to phylogenetically distant hosts, likely associated with ecological opportunity. Like the unrelated reproductive manipulator *Wolbachia*, the *Cardinium* phylogeny also shows no clear phylogenetic signal associated with particular reproductive manipulations.

Conclusions: The *Cardinium* phylogeny shows evidence of diversification within particular host lineages, and also of host shifts among trophic levels within parasitoid-host communities. Like *Wolbachia*, the relatedness of *Cardinium* strains does not necessarily predict their reproductive phenotypes. Lastly, the genetic tools proposed in this study may help future authors to characterize new strains and add to our understanding of *Cardinium* evolution.

Keywords: Endosymbiont, *Wolbachia*, Cytoplasmic incompatibility, Parthenogenesis induction, Feminization, MLST, Phylogenetics

Background

The life histories and evolution of many multicellular organisms are intimately entwined with the microbes they carry [1]. A large number of arthropods carry maternally inherited, intracellular bacterial symbionts that can affect their host’s reproductive outcomes in both detrimental and beneficial ways [2, 3]. These symbionts come from various bacterial phyla, but are categorized based on their associations with their hosts. Primary (or obligate) symbionts complement their hosts’ diet with essential amino acids or other limiting nutrients, are often housed in specialized structures, and are essential to their host’s reproduction (reviewed in Moran et al [2]). Secondary (or facultative) symbionts, though largely unnecessary for successful host reproduction, can provide conditional benefits to their host, have no measurable effect, or manipulate their host’s reproduction in ways that increase the spread of the symbiont [4–6].

© The Author(s). 2019 Open Access This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.
Symbiont phylogenies may offer clues to the relationship between the symbionts and their hosts. For instance, primary symbionts, such as *Buchnera* in their aphid hosts, display congruent phylogenies [7], indicating the long evolutionary history and cospeciation of these groups. Secondary symbionts generally have shorter associations with their hosts and may occur at intermediate frequencies within the host population [2]. The evolutionary phylogenies of secondary symbionts generally display many host switches and are non-congruent with their host’s phylogenies (e.g. [8]). Genera of bacteria commonly thought of as secondary symbionts may also include lineages of primary symbionts in their midst, as with *Serratia symbiotica* in aphids [9, 10]. Even the best-known secondary symbiont, *Wolbachia*, a notorious host switcher, contains a clade of symbionts that display congruent evolution and co-cladogenesis in their obligatory symbiosis with nematodes [11, 12] as well as a lineage that is required for B-vitamin production in bedbugs [13]. These patterns show that different strains within one group of secondary symbionts can differ dramatically in their relationships with their hosts.

While transitions from secondary to obligate symbiosis may be apparent in phylogenies, as shown by host and symbiont phylogenetic congruence, subtler facets of secondary symbiont life histories may also be elucidated by a well-resolved phylogeny. Horizontal transmission of secondary symbionts between hosts is key to the secondary symbiont lifestyle, yet these transmission events are rarely captured in experiments (see exceptions in Huiegens et al. [14] and Caspi-Fluger et al. [15]), and are likely to happen infrequently in nature. Phylogenies are currently the most powerful tools we have to describe these host switches. Well resolved phylogenies may also elucidate co-cladogenesis over a short evolutionary time scale, which can occur when a reproductive manipulator in essence “hijacks” a key reproductive function of their host, creating host-symbiont dependency [6, 16, 17]. In this paper, we explore evolution of the secondary symbiont of arthropods, *Cardinium hertigii* (Bacteroidetes), and address questions concerning horizontal transmission and the evolution of reproductive manipulations with a well-resolved phylogeny.

*Cardinium hertigii*, a member of the phylum Bacteroidetes, infects approximately 7–9% of arthropods [18–20] as well as at least one lineage of the plant parasitic nematode, *Heterodera glycines* [21, 22]. Although it infects many insects, particularly members of Hymenoptera and Hemiptera, much of the diversity of this symbiont genus as described so far appears to lie in arachnids, such as mites, spiders, and harvestmen as hosts [18, 23, 24]. Although the phenotype of *Cardinium* in many hosts is unknown, it has been shown to manipulate host reproduction in insects and mites, and rivals *Wolbachia* in its versatility. Strains of *Cardinium* induce at least three reproductive manipulations: parthenogenesis, feminization, and cytoplasmic incompatibility (CI).

In symbiont-induced parthenogenesis, genetic males turn into genetic females during embryogenesis. Parthenogenesis has been shown or associated with *Cardinium* infection in several parasitoid wasps in the genus *Encarsia* [20, 25] and with the oleander scale, *Aspidiotus nerii* [26]. In feminization, as has been shown in *Brevipalpus* mites, *Cardinium* causes infected genetic males to be converted into functional females [27]. Finally, *Cardinium* is able to induce cytoplasmic incompatibility in several wasps, mites, planthoppers and a thrips [28–35], where infected females produce both male and female offspring, but uninfected females mated with infected males produce few or no offspring (in diploid systems) or few or no daughters (in haplodiploid systems). Of all reproductive manipulators, so far only *Cardinium*, *Wolbachia*, and a recently discovered Alphaproteobacterium [36] have been found to induce CI, although genomic evidence of the *Cardinium* strain cEper1, found in the parasitic wasp *Encarsia Suzanneae*, suggest that at least *Wolbachia* and *Cardinium* independently evolved this trait [37]. In addition to the reproductive manipulations, *Cardinium* has been shown to affect other host fitness traits as well. In the planthopper *Sogatella furcifera*, *Cardinium* infection is associated with faster nymphal developmental times [34] and in the parasitoid wasp *Encarsia inarum*, *Cardinium* infection is associated with increased longevity of female wasps [38].

Despite the diverse impacts *Cardinium* can have on key aspects of its host’s survival and reproduction, few resources have been devoted towards developing better genetic tools for assessing the evolutionary history of this genus, leaving open some intriguing questions about the symbiont’s evolution and ecological interactions with its hosts. Some of the enduring mysteries involving secondary symbionts, and *Cardinium* in particular, are how these reproductive manipulations evolved. For example, are the genes coding for these manipulations largely horizontally transmitted between strains or do they evolve independently, perhaps repeatedly, within lineages? Additionally, *Cardinium* horizontal transmission rate at a genus-wide level is poorly understood. With weakly resolved phylogenies, it is not clear whether *Cardinium* displays the same low level of host affinity as most other secondary symbionts, or whether the shorter list of host taxa with which it is associated than, for example, the cosmopolitan *Wolbachia*, is indicative of fewer host switches among host lineages. While a total of six *Cardinium* genomes have now been sequenced [39–44] genetic resources that enable broad comparisons among many taxa are still needed. We present four
sets of primers from single locus housekeeping genes that each amplify 450-700 bp of DNA in order to more fully resolve the evolutionary relationships of the divergent Cardinium strains. By providing primers for the community of Cardinium researchers to use to diagnose Cardinium and discriminate among as yet uncharacterized strains, the study provides a framework for future studies of this versatile symbiont.

Results
MLST primers
Most of the arthropod Cardinium in our set of host taxa (Table 1) could be amplified by the MLST primers (Table 2), including members from groups A (the largest arthropod group), C (biting midges in the Culicoides group), and D (Opiliones group). All primers amplified products for Cardinium residing in Opiliones and Culicoides spp. The EF-G primers worked on all samples, the SufB and GyrB primers worked on most samples in group A and all in group C and E. For the GroEL primers, two sets of forwards were used (Table 2), depending on which amplified better, but only sequences from the inner forward primer (groel_346F) were used for the phylogenies.

Phylogenetic trees
The phylogeny of concatenated MLST loci supports the monophyly of Cardinium as a genus (Figs. 1 and 2). While the individual gene trees are not completely topologically congruent (Figs. 3, 4, 5, and 6), all phylogenies suggest that groups A and C are each supported as monophyletic groups, as proposed by Nakamura et al. [24]. In addition, the suggestion that group E, with hosts in the Opiliones, is a separate clade [23] is also supported by both individual gene trees as well as the concatenated tree. Evidence of host affinity of related Cardinium strains is also shown across phylogenies. This is shown particularly in group A in the Cebaeus spider clade, and in a smaller clade showing the sister relationship between strains in the two mites, E. suginamensis and T. pueraricola. Further, group C is now populated entirely by Culicoides hosts, and group E contains entirely Opiliones hosts.

Discussion
This study aimed to better understand the evolution of the diverse arthropod symbiont Cardinium, and provide genetic tools to better identify individual strains within this group. Phylogenies based on sequences derived from four loci across a representative set of Cardinium strains show a greater resolution of Cardinium clades in this diverse genus than single gene trees using more slowly evolving DNA such as the 16S rRNA gene. Direct sequencing and analysis of the genes selected for the MLST and phylogenetic analyses suggested that they were almost always single copy genes, although there appeared to be two copies of SufB in one host species, the planthopper Sogatella furcifera. The single gene trees were not entirely congruent with each other (Figs. 3, 4, 5, and 6), as is most common in bacterial multilocus sequence analyses [45], and underscores the value of combining data from multiple genes. There are several potential reasons for non-congruence of gene trees and lineage trees [46]. They include lateral gene transfer, which is common in bacterial endosymbionts. However, none of the MLST genes are among the 68 Cardinium genes that showed evidence of horizontal transfer in the Cardinium genome eEperI [37]. Lineage sorting, where polymorphisms in a gene precede the lineage split is another possible cause of non-congruence, although lineage sorting is more likely when population sizes are large [46], and endosymbionts typically have small effective population sizes [2]. Lastly, gene duplication and extinction is another process that can lead to non-congruence of gene trees with lineage trees, since different loci may be represented in different terminal taxa, even in single copy genes [46].

Using 16S rDNA and gyrase B, Nakamura et al. [24] grouped Cardinium into three groups: A, which contains Cardinium strains infecting insects, mites, and other arthropods, B, which contains the Cardinium strain infecting the plant parasitic nematode, Heterodera glycines; and C, which contains Cardinium infecting biting midges in the genus Culicoides. These groups are supported in the current study using the concatenated sequence of four loci. Chang et al. [23] suggested that the Cardinium found in the harvestmen clade (Leibunnum spp., Opiliones) might be an independent group, based on a phylogeny constructed using a partial 16S rRNA sequence. However, because the 16S rRNA gene displays a relatively slow rate of evolution, the phylogeny provided limited support for this idea. The current phylogeny using the concatenated loci provides more robust support for a separate clade of Cardinium found in the Leibunnum Opiliones. Following the convention of Nakamura et al. [24] this clade is designated group E, with clade D reserved for Cardinium in Daphnia, water fleas [47].

The monophyly of Cardinium in the oleander scale, Aspidiotus nerii (Diaspididae), Encarsia parasitic wasps, and whiteflies is supported. Species of Encarsia that harbor these Cardinium parasitize either whiteflies (E. hispida, E. suzannae, E. tabacivora, E. inaron (IT and US)) or armored scale insects in the Diaspididae (E. perni- ...
| Host organism             | Cardinium strain | Collection information           | Reproductive phenotype                                      |
|--------------------------|------------------|----------------------------------|-------------------------------------------------------------|
| Aleurothrixus floccosus  | cAflo1           | Israel                           | Unknown                                                     |
| Encarsia suzannae        | cEper1           | Texas, USA                       | CI (Hunter et al., 2003)                                    |
| Encarsia hispida         | cHis1            | San Diego, USA                   | PI (Zchori-Fein et al., 2004)                               |
| Encarsia tabacivora      | cEper2           | Brazil                            | PI association (Zchori-Fein et al., 2001)                   |
| Encarsia inaron (IT), high density strain (HIT) | cEina2           | Italy                            | One of two strains co-infecting a host with a CI phenotype (Gebiola et al., 2016). This strain does not cause CI (Stouthamer, et al. unpubl.) |
| Encarsia inaron, (IT), low density strain (LIT) | cEina3           | Italy                            | One of two strains co-infecting a host with a CI phenotype (Gebiola et al., 2016). This strain causes CI (Stouthamer et al. unpubl.) |
| Encarsia inaron (USA)    | cEina1           | USA                              | No CI, no PI (White et al., 2009)                           |
| Aspidiotus nerii         | cAnet            | University of California, Riverside culture | Associated with parthenogenetic host (Provencher et al., 2005) |
| Bemisia tabaci, Q1 species | cBtQ1           | Valencia, Spain                  | No CI, no PI (Fang et al., 2014)                            |
| Ixodes scapularis cell line | cIsca1           | Nantucket Island (Massachusetts), USA | Unknown                                                     |
| Indozucriell dantur      | cIdan1           | Japan                            | Unknown                                                     |
| Sagatella furcifera      | cShf1            | China                            | Unknown                                                     |
| Sagatella furcifera      | cShf2            | Japan                            | CI (Nakamura et al., 2009)                                  |
| Eotetranychus suginamensis | cEsu1           | Taiwan                           | CI (Gotoh et al., 2007)                                    |
| Oligonychus coffeae      | cOcof1           | Japan                            | Unknown                                                     |
| Oligonychus gotohi       | cOgot1           | Japan                            | Unknown                                                     |
| Panonychus mori          | cPmor1           | Japan                            | CI (Gotoh et al., 2003)                                    |
| Tetranynchus pueraricola | cTpeue1          | Japan                            | No CI, no PI (Gotoh et al., 2003)                           |
| Culicoides arakawae      | cCar1            | Kagoshima Pref. or Okinawa Pref., Japan | Unknown                                                     |
| Culicoides ohmorni       | cCohm1           | Kagoshima Pref., Japan           | Unknown                                                     |
| Culicoides peregrinus    | cCper1           | Yonaguni Isl., Okinawa Pref., Japan | Unknown                                                     |
| Culicoides punctatus     | cCpun1           | Leahurst Campus, University of Liverpool, UK | Unknown                                                     |
| Cybaeus eutypus          | cCeat            | Vancouver Island, Canada         | Unknown                                                     |
| Cybaeus signifer         | cCsig1           | Vancouver Island, Canada         | Unknown                                                     |
| Cybaeus chauliodus       | cCchao1          | Northern California, USA         | Unknown                                                     |
| Cybaeus somesbar         | cCosom1          | Northern California, USA         | Unknown                                                     |
| Cybaeus sanbruno         | cCsan1           | North central California, USA    | Unknown                                                     |
| Cybaeus morosus          | cCmor1           | British Columbia, Canada         | Unknown                                                     |
| Cybaeus hesper           | cChes1           | North central California, USA    | Unknown                                                     |
| Cybaeus multnoma         | cCmul1           | Oregon, USA                      | Unknown                                                     |
| Cybaeus penedentatus     | cCpen1           | North central California, USA    | Unknown                                                     |
| Culicoides imicola       | cCimi1           | Unknown                          | Unknown                                                     |
| Metaseiulus occidentalis | cMooc1           | Washington and Oregon, USA       | CI (Roush and Hoy, 1981)                                   |
| Leiosunum sp 1           | cLsp2            | Georgetown Island, Maine, USA    | Unknown                                                     |
Table 1 Collection localities of Cardinium strains and their associated reproductive phenotypes (Continued)

| Host organism | Cardinium strain | Collection information | Reproductive phenotype |
|---------------|------------------|------------------------|------------------------|
| Leiobunum sp 2| cLsp3            | N. Monmouth, Maine, USA| Unknown                |
| Leiobunum     | cLsp1            | Ellison Park, Monroe County, New York, USA | Unknown                |
| Brevipalpus californicus | cBcal1 | Minas Gerais, Brazil | Feminization (Groot and Breeuwer, 2006) |
| Brevipalpus phoenicas | cBpho1 | Minas Gerais, Brazil | Feminization (Groot and Breeuwer, 2006) |
| Macrosteles quadrilineatus | cMque1 | Unknown | Unknown |
| Encarsia peminosi | cEper3 | Tijuana River Valley Park, San Diego, USA | Associated with parthenogenetic host (Stouthamer and Luck, 1991) |
| Pezothrips keliganus | cPKel1 | Australia | CI (Nguyen et al., 2017) |

Fig. 1 Bayesian phylogeny with of all Cardinium strains from this study using concatenated loci: gyrB, subB, EF-G, and groEL. Node support of > 0.99 posterior probability is indicated by an asterisk. Cardinium strains are labeled by the host taxon species name and colored by the host taxon order or sub-class. Acari are pink, Diptera are mustard yellow, Opiliones are green, Thysanoptera are grey, Hemiptera are light blue, Hymenoptera are orange, and Araneae are deep blue. Symbols refer to reproductive phenotype when it has been investigated: filled squares indicate cytoplasmic incompatibility (CI) has been shown, empty squares indicate CI has been looked for and not found, filled triangles indicate feminization, filled circles indicate parthenogenesis-induction has been shown, and hatched circles indicate an association with a parthenogenetic host.
transmission events between host and parasitoids, and perhaps among parasitoids, have occurred, although the
directionality of these events can only be discerned with
a deeper sampling of both hosts and parasitoids. Addi-
tionally, none of the closely related strains of Cardinium
residing in whiteflies, and two related species of Encarsia
(C. oshikiri, C. arakawa, C. pierginus, C. acridum sp.2,
C. acridum sp.1, C. acridum sp.3, P. mori) are known to
cause CI or any other reproductive manipulations [48, 49].
This suggests that the ancestral strain of Cardinium in
this group either spread with a phenotype other than some type of
reproductive manipulation, or lost its ability to manipu-
late in each new host after it spread. The Cardinium
group in this clade is the clearest example of closely related
Cardinium strains residing in distantly related hosts, in contrast to the previously observed pattern of
closely related Cardinium strains residing in closely related hosts [24, 50–52], a pattern which is generally sup-
ported in the phylogenies of the current study as well.

Interestingly, some patterns appear at least superfi-
cially similar between Cardinium and Wolbachia. The
reproductive manipulations that Cardinium is able to in-
duce overlap with Wolbachia. Strains that cause the
same reproductive manipulations do not clearly form
one monophyletic clade, except perhaps in the case of
the mite strains causing feminization, but this might
change when further examples of feminizing Cardinium
are discovered. Additionally, closely related Cardinium
strains do not necessarily cause the same reproductive
manipulations, as exemplified by the sister strains
cEper1, which causes CI, and the parthenogenesis-
inducing (PI) strain, cEper2 [25, 30]. Similarly, cEsug1,
which causes CI, and cTpue1, which does not cause CI
or PI, are sister taxa [29]. This pattern also occurs in
Wolbachia; closely related Wolbachia strains in Acraea
butterflies have shown multiple transitions between sex
ratio distorting and CI-inducing Wolbachia strains [53].
Additionally, in *Drosophila*, wMel, causing CI, and wAu, having no phenotype, are also very closely related [54]. These similar patterns between *Wolbachia* and *Cardinium* trees are not necessarily expected; recently, it has been suggested that the horizontal transfer of the CI phenotype may be linked to the *Wolbachia*’s WO phage, which can cross-infect *Wolbachia* strains [55, 56]. So far, sequenced genomes of *Cardinium* do not show the presence of phage DNA. Unlike *Wolbachia*, however, many *Cardinium* strains do harbor plasmids [37, 40], which may serve a similar function in horizontal transmission of reproductive manipulation genes [57, 58].

We fully expect the phylogeny of *Cardinium* to become better resolved when more *Cardinium* genomes are published, as have *Wolbachia* genomes [59]. There are currently six *Cardinium* genomes published [37, 40–44], three of them published in the last couple of years, so it is reasonable to expect more in the near future. Indeed, the high genetic diversity within this genus made the design of a single MLST, a scheme designed for strains within a bacterial species [60], challenging. However, while full genomes are always going to be better for inferring phylogenies and group placements [61], the cost of sequencing, the sequencing depth necessary for symbionts that may exist at relatively low titer in their hosts, and the expertise in assembling symbiont genomes from metagenome data can still be a limiting factor for many laboratories. For ecological studies and surveys in particular, the ability to relatively quickly type *Cardinium* strains meets the objective of giving the strain an identity and fitting it into the *Cardinium* phylogeny. This MLST offers a relatively low-cost way to differentiate between strains of *Cardinium* and is a starting point for researchers considering the study of *Cardinium*. In particular, the utility of the *Cardinium* MLST will be valuable in answering questions concerning relatively recent biogeographic or host switching events.

Characterizing a strain of a symbiont with an MLST allelic profile may be difficult when more than one strain
co-infects individual host individuals. If both alleles are amplified, direct sequencing may not be possible, and cloning may be required prior to sequencing. Even more challenging is determining which sequenced allele at a particular locus belongs to which strain. There are a couple of potential solutions to this problem. When multiple strains are present in different combinations among individuals, one can logically examine the sets of alleles in multiply infected and singly infected individuals to allow assignment of allelic profiles to strains, a system known as Allelic Intersection Analysis [62]. This may be particularly relevant in complex situations like that found in the apple maggot, Rhagoletis pomonella, where up to four Wolbachia strains have been found in multiple combinations [63]. Another tool that could be useful when co-infecting symbiont strains are found at different titers is quantitative PCR. It may be possible to design specific qPCR primers for each allele and quantify the relative titer of each. If the titer is consistently higher in one set of alleles than the other, one can presume the alleles in that set belong to the same strain. In the host Encarsia inaron (from Italy) co-infecting strains cEina2 and cEina3 are found at high and low titers, respectively (Table 1).

Conclusion
Cardinium evolution appears to be driven by both ecological opportunity and host specialization. Cardinium has frequently switched between parasitoids and their hosts, even though they are physiologically quite different, causing these strains to form a clade. In contrast, the Cardinium in Cybaeus spiders, Culicoides spp., and Leobinium spp. appear to be quite specialized to particular host lineages, without distantly related hosts breaking up these clades. Similar to Wolbachia, the relatedness of Cardinium strains does not necessarily predict their reproductive phenotypes. Overall, the new genetic tools proposed in this study allow for clearer strain delimitation and a more detailed picture of the evolution of Cardinium, one that will keep unfolding the more the MLST primers are used to characterize strains and add taxa to the Cardinium phylogeny.

Methods
Gene selection
Four genes with the highest amino acid identity between the sister group to Cardinium, Amoebophilus asiaticus, and the sequenced Cardinium strain, cEper1, were chosen to develop a Multi Locus Sequence Typing
(MLST) system with other strains [37]. We did not attempt to choose genes that are evenly spaced around the Cardinium chromosome. While, in more conserved lineages, linkage among loci is often avoided by choosing MLST genes that are evenly spaced [60], in Cardinium there is little shared synteny, even between the two related sequenced genomes, cBtQ1 and cEper1 [40]. In addition to making even spacing of chosen genes unworkable across the genus, the low level of synteny suggests frequent gene rearrangements in this lineage, and a low probability of linkage among loci. The genes selected for this study were: Elongation Factor G, a protein responsible for coordinating the movement of tRNA and mRNA during translation [64]; gyrase B, a topoisomerase that unwinds DNA during DNA replication [65]; Iron Sulfur Cluster Assembly Protein (SufB), a protein involved in generating Fe-S complexes mainly involved in electron transfer [66] and the Heat shock protein GroEL, a chaperone protein essential in stress-related responses [67].

**DNA extractions**

Arthropods with confirmed Cardinium infections and DNA samples were received from cooperators around the world (Table 1). From Japan (H. Noda), we received planthopper, mite, and biting midge DNA, extracted as described in Nakamura et al. [24]. Cardinium from the Ixodes cell line ISE6 (T. Kurtti) was processed by shearing the cells and filtering them through a 1.5 μm syringe, then extracting the lysate with 3 μl of 20 mg/ml proteinase K and 50 μl of water with 10% w/v chelex beads [49]. Cybeus spiders (S. Perlman) were extracted using Qiagen DNeasy extraction kits. All other samples of alcohol-preserved specimen were also extracted using the chelex extraction protocol.

**Primer design, PCR, and sequencing**

Primers were iteratively designed as sequenced products from strains were added to sequence alignments. Initially, general primers were designed based on the only.
two sequenced (and closely related) *Cardinium* strains (cEper1, cBtQ1) and the sister taxon to *Cardinium*, *Amoebophilus asiaticus* 5a2. These initial primers were designed using cEper1 as the reference strand in Primer3 [68, 69] with ambiguities based on the other strains added manually. Amplification of some gene products was not successful from all strains using these initial primers, particularly from strains divergent with respect to cEper1 and cBtQ1, such as those in the biting midges, *Culicoides* spp. In these instances, strain-specific primers were designed once a small segment of the gene was sequenced. These strain-specific primers were then used in conjunction with the initial degenerate primers to obtain more sequence. When more than three bacterial strains were used for primer design, areas of conservation were manually detected and these potential primer

**Table 2** MLST primers and their suggested melting temperatures for PCR

| Primer name | Primer sequence (5′ – 3′) | Tm (°C) | Gene length (bp) | Amplified nucleotide range of gene (bp) | MLST fragment size (bp) |
|-------------|--------------------------|---------|------------------|----------------------------------------|------------------------|
| gyrb_859F   | ATGCAYGTMACBDGGDITATARAA  | 50      | 1950             | 859–1637                               | 736                    |
| gyrb_1637R  | TARAGTGGGRGARGARGCAAT     |         |                  |                                        |                        |
| groel_346F  | VTHAARCGBGGBATWGACAA      | 52      | 1638             | 346–842                                | 476                    |
| groel_287F* | CNCARCKCTWTWRYVRCATGG     |         |                  |                                        |                        |
| groel_842R  | TGGGBAYAGAAGRAARCCGTATG   |         |                  |                                        |                        |
| sufB_806F   | CTACNGTDCAATTTTGATATCC    | 50      | 1443             | 806–1289                               | 451                    |
| sufB_1289R  | ADYTGRCYKRCRRATTT        |         |                  |                                        |                        |
| EF_1689R    | AAABCCYTYYGAATIGCTGG      | 52      | 2142             | 1689–1162                              | 482                    |
| EF_1162F    | GCGNTRIGTIGGTTAAARARRATTA |         |                  |                                        |                        |

*Alternative forward primer for groEl*
regions were checked for hairpins and tendency to form primer dimers in Primer3 [69] against every strain. All primers were selected by minimizing the number of ambiguities and maximizing the number of conserved base pairs in the 3’ primer region, and M13 tags were added to the primers for ease of sequencing [70].

Although the melting temperature varied depending on the primer pair (Table 1), PCR conditions were generally as follows: 15 μl reaction volume with New England Biolabs buffer and Taq at 1X concentration, 5 mM dNTPs, 0.76 mM MgCl₂, 1.1 μM primers with 2 μl of DNA. From mite extractions, 4 μl of DNA was added (similar to Groot and Breeuwer (2006)). The initial melting temperature was 94 °C for 2 min; this was followed by 40 cycles of 94 °C for 45 s, the annealing temperature (Table 1) for 45 s, and extension at 68 °C for 45 s. The final extension was at 68 °C for 7 min.

Phylogenetic analysis

DNA sequences were quality-controlled and aligned using CLC Main Workbench 6 (Qiagen) and MUSCLE [71]. jModelTest was used to select the optimum model of evolution based on the Akaike information criterion [72]. Bayesian trees were constructed using MrBayes with one million Markov Chain Monte Carlo (MCMC) generations and sampled every 1000 generations [73]. Maximum likelihood trees were constructed using RaxML with 1000 rapid bootstraps. Both Bayesian and ML methods used the GTR + I + G model of nucleotide evolution with a total of 2145 bp from Gyrase B (gyrB), translation elongation factor G (EF-G), Iron Sulfur cluster assembly protein (sufB), and heat shock protein (groEL) for each taxon, partitioned by gene and codon position. Phylogenetic tree figures were generated in Mesquite [74].

Abbreviations

CI: Cytoplasmic incompatibility; MCMC: Markov chain Monte Carlo; MLST: Multi-locus sequence typing; PCR: Polymerase chain reaction; PI: Parthenogenesis-induction; qPCR: Quantitative polymerase chain reaction

Acknowledgements

We are very grateful to a number of people who sent us many Cardinium-infected specimens: Tim Kurtt, Marjorie Hoy, Johannes Breeuwer, Hiroaki Noda, Steve Perlman, Jack Werren, Yuval Gottlieb, Nîr Nitanel, and Markus Riegler. We are also grateful to Wendy Moore, who provided comments on an earlier version of the M5 and consulted on the phylogenetic analysis, as did James Robertson. Garrett Hughes also consulted on tree building.

Authors’ contributions

CMS analyzed sequences, designed primers, performed the experiments and phylogenetic analyses and wrote the first draft of the manuscript. SEK assisted with PCR, helped develop and test methods, and revised drafts of the manuscript. EM consulted on experimental methods, made figures and revised drafts of the manuscript. SSE consulted on experimental methods, served as a mentor in the design and performance of the research and revision of the project and worked with CMS on the writing and revision of all drafts of the manuscript. All authors read and approved the final manuscript.

Funding

Funding for this study was provided by an NSF grant (IOS-1256905) to MSH and SSE, and by the center for Insect Science at the University of Arizona to CMS. Neither funding body had any role in the design of the study or collection, analysis, and interpretation of data or in writing the manuscript.

Availability of data and materials

The sequence datasets generated and/or analyzed during the current study have been deposited in the NCBI repository, under accession numbers MK264778-MK264911 [https://www.ncbi.nlm.nih.gov/nuccore/].

Ethics approval and consent to participate

NA

Consent for publication

NA

Competing interests

The authors declare that they have no competing interests.

Author details

1Department of Entomology, University of Arizona, 410 Forbes Building, Tucson, AZ 85721, USA. 2Milk Technology and Food Science, Institute for Milk Hygiene, University of Veterinary Medicine, Vienna, Austria. 3Department of Animal Science, Iowa State University, Ames, Iowa, USA.

Received: 17 January 2019 Accepted: 12 November 2019
Published online: 27 November 2019

References

1. McFall-Ngai M, Hadfield MG, Bosch TCG, Carey HV, Domazet-Loso T, Douglas AE, et al. Animals in a bacterial world, a new imperative for the life sciences. Proc Natl Acad Sci U S A. 2013;110(32):12926–31.
2. Moran NA, McCutcheon JP, Nakabachi A. Genomics and evolution of heritable bacterial symbionts. Annu Rev Genet. 2008;42:165–90.
3. O’Neill SL, Hoffmann AA, Werren JH, editors. Influential passengers. New York: Oxford University Press; 1997.
4. Stouthamer R, Breeuwer JAU, Hurst GG. Wolbachia papai: microbial manipulator of arthropod reproduction. Annu Rev Microbiol. 1999;53:71–102.
5. Werren JH. Biology of Wolbachia. Annu Rev Entomol. 1997;42:587–609.
6. Zug R, Hammerstein P. Bad guys turned nice? A critical assessment of Wolbachia mutualisms in arthropod hosts. Biol Rev. 2015;90(1):89–111.
7. Clark MA, Moran NA, Baumann P, Wernegreen JJ. Coevolution between bacterial endosymbionts (Buchnera) and a recent radiation of aphids (Uroleucon) and pitfalls of testing for phylogenetic congruence. Evolution. 2000;54(2):517–25.
8. Novakova E, Hyspa V, Moran NA. Arsenophonus, an emerging clade of intracellular symbionts with a broad host distribution. BMC Microbiol. 2009;9:143. https://doi.org/10.1186/1471-2180-9-143.
9. Burke GR, Moran NA. Massive genomic decay in Serratia symbiotica, a recently evolved symbiont of aphids. Genom Biol Evol. 2011;3:195–208.
10. Manzano-Marín A, Lamelas A, Moya A, Latorre A. Comparative genomics of Serratia spp.: Two paths towards endosymbiotic life. PLoS One. 2012;7(10).
11. Fenn K, Blaxter M. Are filarial nematode Wolbachia obligate mutualist symbionts? TREE. 2004;19(14):163–6.
12. Fenn E, Bain O, Barbuto M, Martin C, Lo N, Uni S, et al. New insights into the evolution of Wolbachia infections in filarial nematodes inferred from a large range of screened species. PLoS One. 2011;6(5):e18043.
13. Nikoh N, Hosokawa T, Moriyama M, Oshima K, Fukatsu T, et al. Evolutionary origin of insect-Wolbachia nutritional mutualism. Proc Natl Acad Sci U S A. 2014;111(28):10257–62.
14. Hugens ME, de Almeida RP, Booms PAH, Luck RF, Stouthamer R. Natural interspecific and intraspecific horizontal transfer of pathogen-inducing Wolbachia in Tricho gramm a wasps. Proc R Soc Lond B. 2004; 271(1538):509–15.
15. Caspi-Fluger A, Inbar M, Mozess-Daube N, Kastir N, Portnoy V, Belausov E, et al. Horizontal transmission of the insect symbiont Rickettsia is plant mediated. Proc R Soc Lond B. 2012;279(1734):1791–6.
16. Dedeine F, Bouletreau M, Vare T, Wolbachia requirement for coevolution: occurrence within the genus Asobara (Hymenoptera, Braconidae) and evidence for intraspecific variation in A. stideli. Heredity. 2005;95(5):394–400.
17. Dedeine F, Vavr F, Soeckmaer DD, Bolleau M. Intra-individual coexistence of a Wolbachia strain required for host coenosis with two strains inducing cytoplasmic incompatibility in the wasp Asobara tabida. Evolution. 2004;58(10):2167–74.

18. Russell JA, Funaro CF, Giraldo GF, Goldman-Huetars B, Suh D, Kumsner DC, et al. A veleti menagerie of heritable bacteria in ants, butterflies, and beyond: Broad molecular surveys and a systematic review. PLoS One. 2012;7(12):e51027.

19. Weeks AR, Veltin R, Stouthamer R. Prevalence of a new sex ratio distorting endosymbiotic bacterium among arthropods. Proc R Soc Lond Ser B. 2003;270:1857–65.

20. Zchori-Fein E, Perlman SJ, Kelly SE, Katzin N, Hunter MS. Characterization of a ‘Bacterioides’ symbiont in Encarsia wasps (Hymenoptera: Aphelinidae): proposal of ‘Candidatus Cardinium hertigii’. Int J Syst Evol Microbiol. 2004;54:961–8.

21. Noel GR, Atila-Benta N. ‘Candidatus Paenicardinium endonii’, an endosymbiont of the plant-parasitic nematode Heterodera glycines (Nematoda: Tylenchida), affiliated to the phylum Bacteroidetes. Int J Syst Evol Microbiol. 2006;56:1697–702.

22. Showmaker KC, Walden KKO, Fields CJ, Lambert KN, Hudson ME. Genome sequence of the soybean cyst nematode (Heterodera glycines) endosymbiont “Candidatus Cardinium hertigii” strain chgTN10. Genome Announc. 2018;6:e00624–18.

23. Chang J, Masters A, Avery A, Werren JH. A divergent Cardinium found in daddy long-legs (Arachnida: Opiliones). J Invertebr Pathol. 2010;105(3):220–7.

24. Nakamura Y, Kawai S, Yuyuhiro F, Isu S, Gotoh T, Kismoto R, et al. Prevalence of Cardinium bacteria in planthoppers and spider mites and taxonomic revision of ‘Candidatus Cardinium hertigii’ based on detection of a new Cardinium group from biting mites. Appl Environ Microbiol. 2009;75(21):6757–63.

25. Zchori-Fein E, Gottlieb Y, Kelly SE, Brown JK, Wilson JM, Carr TL, et al. A newly discovered bacterium associated with parthenogenesis and change in host selection behavior in parasitoid wasps. Proc Natl Acad Sci U S A. 2001;98(22):12555–60.

26. Provencher LM, Morse GE, Weeks AR, Normark BB. Panthenogenotes in the Aspidotus neri complex (Hemiptera: Diaspididae): a single origin of a worldwide, polyphagous lineage associated with Cardinium bacteria. Ann Entomol Soc Am. 2005;98(5):629–35.

27. Groot TVM, Breeuwer JAJ. Cardinium symbionts induce haploid thelytoky in most clones of three closely related Brevipalpus species. Exp Appl Acarol. 2005;34(4):257–71.

28. Geobiota M, White JA, Cass BN, Kozuch A, Harris LR, Kelly SE, et al. Cryptic diversity, reproductive isolation and cytoplasmic incompatibility in a classic biological control success story. Biol J Linn Soc. 2016;117(2):217–30.

29. Gotot T, Noda H, Ito S. Cardinium symbionts cause cytoplasmic incompatibility in spider mites. Heredity. 2007;98(1):13–9.

30. Hunter MS, Perlman SJ, Kelly SE. A bacterial symbiont in the Bacterioides clade of alphaproteobacterial endosymbions induces complete cytoplasmic incompatibility in the coconut beetle. Proc Natl Acad Sci U S A. 2017;114(23):6105–10.

31. Perez T, Schmitz-Esser S, Kelly SE, Cass BN, Muller A, Wayne T, et al. Comparative genomics suggests an independent origin of cytoplasmic incompatibility in Cardinium hertigii. PLoS Genet. 2012;8(10):e1003012.

32. White JA, Kelly SE, Cockburn SN, Perlman SJ, Hunter MS. Endosymbiont costs and benefits in a parasitoid infected with both Wolbachia and Cardinium. Heredity. 2011;106(4):585–91.

33. Perez T, Horn M, Schmitz-Esser S. The genome of the amoeba symbiont ‘Candidatus Amoebophisculus australis’ encodes an alf-like prophage possibly used for protein secretion. Virulence. 2010;1(6):541–5.

34. Santos-Garcia D, Rollatt-Farnier PA, Beita F, Zchori-Fein E, Vavr F, Mouton L, et al. The genome of Cardinium cBTQ1 provides insights into genome reduction, symbiont motility, and its settlement in Bemisia tabaci. Genome Biol Evol. 2014;6(4):1013–30.

35. Showmaker KC, Walden KKO, Fields CJ, Lambert KN, Hudson ME. Genome sequence of the soybean cyst nematode (Heterodera glycines) endosymbiont “Candidatus Cardinium hertigii” strain chgTN10. Microbiol Resour Announcements. 2018;6(26).

36. Siosios S, Pilgrim J, Darby AC, Baylis M, Hurst GDD. The draft genome of strain cCpun from biting mites confirms insect Cardinium are not a monophyletic group and reveals a novel gene family expansion in a symbiote. PeerJ. 2019.

37. Brown AMW, Wasala SK, Howe DK, Peetab AT, Zasada IA, Denver DR. Comparative genomics of Wolbachia-Cardinium dual endosymbioses in a plant-parasitic nematode. Front Microbiol. 2018.

38. Zeng Z, Fu YT, Guo DY, Wu YX, Aiay OE, Wu QF. Bacterial endosymbiont Cardinium cBTQ1 genome sequence provides insights for understanding the symbiotic relationship in Sogatella furcifera host. BMC Genomics. 2018;19.

39. Glaeser SP, Kampfer P. Multilocus sequence analysis (MLSA) in prokaryotic taxonomy. Syst Appl Microbiol. 2015;38(4):257–45.

40. Maddison WP. Gene trees in species trees. Syst Biol. 1997;46(3):523–36.

41. Edlund A, Ek K, Breitholtz M, Gordkhova E. Antibiotic-induced change of bacterial communities associated with the copped Nipomor spines. PLoS One. 2012;7(3).

42. Fang YW, Liu LY, Zhang HL, Jiang DF, Chu D. Comparative analysis of Wolbachia-Candidatus symbiosis in a single-parasitic nematode. Front Microbiol. 2018.

43. White JA, Kelly SE, Perlman SJ. Distribution of the bacterium Cardinium in arthropods. Mol Ecol. 2004;13(18):2009–16.

44. Lewis SE, Rice A, Hurst GDD, Baylis M. First detection of endosymbiotic bacteria in biting mites. Culex pipiens and Culex punctatus, important Palearctic vectors of bluetongue virus. Med Vet Entomol. 2014;28(4):463–6.

45. Perlman SJ, Magnus SA, Copley CR. Pervasive associations between Cyttobius spiders and the bacterial symbiont Cardinium. J Invertebr Pathol. 2010;103(3):150–5.

46. Zchori-Fein E, Perlman SJ. Distribution of the bacterial symbiont Cardinium in arthropods. Mol Ecol. 2004;13(18):2009–16.

47. Jiggins FM, Bemley JK, Majerus MN, Hurst GDD. Recent changes in phenotype and patterns of host specialization in Wolbachia bacteria. Mol Ecol. 2002;11(8):1275–83.

48. Hoffmann AA, Clancy D, Duncan J. Naturally-occurring Wolbachia infection in Drosophila simulans that does not cause cytoplasmic incompatibility. Heredity. 1996;76:1–8.

49. Bordenstein SR, Bordenstein SR. Eukaryotic association module in phage WO genomes from Wolbachia. Nat Comm. 2016.

50. Le Page DP, Metcalf JA, Bordenstein SR, On JM, Perlmuter JI, Shropshire JD, et al. Phage WO genomes recapitulate and enhance Wolbachia-induced cytoplasmic incompatibility. Nature. 2017;543(7644):243 +.

51. Partridge SR. Analysis of antibiotic resistance regions in gram-negative bacteria. FEMS Microbiol Rev. 2011;35(5):820–55.

52. Stokes HW, Gillings MR. Gene flow, mobile genetic elements and the recruitment of antibiotic resistance genes into gram-negative pathogens. FEMS Microbiol Rev. 2011;35(5):790–819.

53. Gerth M, Gansauge MT, Weigert A, Bleidorn C, Moder K, Miller WJ, et al. Allelic Intersection Analysis: A Novel Tool for Multi Locus Sequence Assignment in Multiply Infected Hosts. PLoS One. 2011;6(7).
63. Schuler H, Arthofer W, Bertheau C, Krumbock S, Koppler K, et al. Multiple Wolbachia infections in Rhagoletis pomonella. Entomol Exp Appl. 2011;139(2):138–44.
64. Shoji S, Walker SE, Fredrick K. Ribosomal translocation: one step closer to the molecular mechanism. ACS Chem Biol. 2009;4(2):93–107.
65. Reece RJ, Maxwell A. DNA Gyrase – structure and function. Crit Rev Biochem Mol Biol. 1991;26(3–4):335–75.
66. Ayala-Castro C, Saini A, Outten FW. Fe-s cluster assembly pathways in bacteria. Microbiol Mol Biol Rev. 2008;72(1):110–4.
67. Fayet O, Ziegelhoffer T, Georgopoulos C. The GroES and GroEL heat shock gene products of Escherichia coli are essential for bacterial growth at all temperatures. J Bacteriol. 1989;171(3):1379–85.
68. Koressaar T, Remm M. Enhancements and modifications of primer design program Primer3. Bioinformatics. 2007;23(10):1289–91.
69. Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, et al. Primer3: new capabilities and interfaces. Nucleic Acids Res. 2012;40(15).
70. Messing J. New M13 vectors for cloning. Methods Enzymol. 1983;101:20–78.
71. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 2004;32(5):1792–7.
72. Posada D. jModelTest: Phylogenetic model averaging. Mol Biol Evol. 2008;25(7):1253–6.
73. Ronquist F, Huelsenbeck JP. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics. 2003;19(12):1572–4.
74. Maddison W, Maddison D. Mesquite: A modular system for evolutionary analysis. Version 3.51 2018 [Available from: http://www.mesquiteproject.org].

**Publisher’s Note**
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.