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SHORT COMMUNICATION

Identifying potential candidate *Culicoides* spp. for the study of interactions with *Candidatus* Cardinium hertigii

J. PILGRIM[1], S. SIOZIOS[1], M. BAYLIS[1,2], G. VENTER[3], C. GARROS[4,5] and G. D. D. HURST[1]

[1]Institute of Infection, Veterinary and Ecological Sciences, Faculty of Health and Life Sciences, University of Liverpool, Liverpool, U.K., [2]Health Protection Research Unit in Emerging and Zoonotic Infections, Liverpool, U.K., [3]Onderstepoort Veterinary Institute, Agricultural Research Council, Pretoria, South Africa, [4]ASTRE, University of Montpellier, Cirad, INRA, Montpellier, France and [5]Cirad, UMR ASTRE, Montpellier, France

Abstract. *Culicoides* biting midges (Diptera: Ceratopogonidae) are vectors responsible for the transmission of several viruses of veterinary importance. Previous screens of *Culicoides* have described the presence of the endosymbiont *Candidatus* Cardinium hertigii (Bacteroidetes). However, any impacts of this microbe on vectorial capacity, akin to those conferred by *Wolbachia* in mosquitoes, are yet to be uncovered and await a suitable system to study *Cardinium*-midge interactions. To identify potential candidate species to investigate these interactions, accurate knowledge of the distribution of the endosymbiont within *Culicoides* populations is needed. We used conventional and nested PCR assays to screen *Cardinium* infection in 337 individuals of 25 *Culicoides* species from both Palearctic and Afrotropical regions. Infections were observed in several vector species including *C. imicola* and the Pulicaris complex (*C. pulicaris*, *C. bysta*, *C. newsteadi* and *C. punctatus*) with varying prevalence. Phylogenetic analysis based on the Gyrase B gene grouped all new isolates within 'group C' of the genus, a clade that has to date been exclusively described in *Culicoides*. Through a comparison of our results with previous screens, we suggest *C. imicola* and *C. sonorensis* represent good candidates for onward study of *Cardinium*-midge interactions.

Key words. Cardinium, Culicoides, Rickettsia, symbiosis.

Worldwide, biting midges of the genus *Culicoides* (Diptera: Ceratopogonidae) are known to transmit more than 50 arboviruses (Mellor *et al.*, 2000). Notably, bluetongue virus (BTV) and Schmallenberg virus (SBV) pose a great threat to livestock welfare and have caused serious economic damage to the European livestock industry. African horse sickness virus (AHSV) causes a highly lethal disease of equids, with past epizootic outbreaks in Africa, the Middle East and southern Europe. Vaccines have been very effective in halting the transmission of *Culicoides*-borne viruses but take significant time and resources to get into at-risk populations. This creates a need to develop alternative mitigation methods and tools to counter a continued threat of emergence of BTV and other *Culicoides*-borne pathogens.

The microbiome of arthropods is known to modify host biology in a number of ways, ranging from nutritional provisioning to parasite protection. These effects are particularly pronounced in the case of endosymbionts (Su *et al.*, 2013). For example, the endosymbiont *Wolbachia* (Rickettsiales: Anaplasmataceae) leads to an arbovirus blocking effect in *Aedes aegypti* mosquitoes, which has led to their successful deployment as a dengue fever control strategy in field trials (Nazni *et al.*, 2019). The potential to harness viral blocking effects in wild populations is enabled by *Wolbachia*-induced cytoplasmic incompatibility (CI)-embryo death in mating between infected males and uninfected females. CI is exploited as a mechanism to drive the *Wolbachia* into a population to distribute this virus blocking phenotype into a naïve population; as *Wolbachia* pres-

Correspondence: Gregory D. D. Hurst, Institute of Infection, Veterinary and Ecological Sciences, Faculty of Health and Life Sciences, University of Liverpool, Liverpool, U.K. Tel.: +44 151 7954520. E-mail: g.hurst@liverpool.ac.uk

ence in a population reduces the fitness of uninfected females, the bacterium is able to reach high frequencies of infection. Importantly, this protective phenotype appears to be effective against a broad range of RNA viruses, supporting the potential use of endosymbiont-based biocontrol in major midge-borne pathogens such as BTV, SBV and AHSV.

The endosymbiont *Candidatus* Cardinium hertigii (Bacteroidetes) has been found to be widely associated with *Culicoides* (Morag *et al.*, 2012; Mee *et al.*, 2015; Pagès *et al.*, 2017). Although the biological role of *Cardinium* in biting midges is undetermined, data from different host species demonstrate various *Cardinium*-dependent reproductive alterations including parthenogenesis-induction and CI (Zchori-Fein *et al.*, 2001; Hunter *et al.*, 2003), with the latter providing a potential drive mechanism for future introductions into *Culicoides* populations. Importantly, potential effects relating to pathogen-blocking and fitness (e.g., fecundity) remain underexplored.

A key requirement for studying *Cardinium* impacts in midges stems from the need for a model system. The main barrier to such a model is the difficult laboratory mass-rearing of midges, with only *C. nubeculosus* and *C. sonorensis* colonies currently in existence. Despite this, vector competence studies of field-caught *Culicoides* are still possible, such that identifying populations containing both *Cardinium* infected and uninfected individuals (for negative controls) is also of interest. In light of this, we investigated the distribution and prevalence of *Cardinium* infections in *Culicoides* populations from Palearctic and Afrotropical regions, using a conventional and nested PCR screen approach. These data are then added and compared to previous screen data to assess what we know about the distribution of *Cardinium* in field and laboratory populations and the suitability of specific midge species for further investigation pertaining to the endosymbiont.

Culicoides specimens were collected between 2007 and 2016 from sites spanning France, South Africa and the United Kingdom (Table 1). Details of *Culicoides* collection methods, storage and species identification can be found in Pilgrim *et al.* (2017). DNA extracts from a previous study by Lewis *et al.* (2014), describing positive *Cardinium* infection in *C. pulicaris* and *C. punctatus* from the United Kingdom, were used to validate screening assays.

Amplification of the host *COI* gene was initially assessed as a means of quality control by conventional PCR assay. *COI* amplicons from the Pulicaris complex were Sanger sequenced to determine cryptic species, which are difficult to distinguish morphologically. To this end, individuals were designated as belonging to a particular species if their *COI* haplotype had >99% sequence identity to previously designated DNA barcodes. DNA extracts, which passed quality control, were then screened for *Cardinium* using conventional and nested primers amplifying partial sequences of the Gyrase B (*GyrB*) gene (Primer sequences and PCR cycling conditions in File S1). Prevalence estimates were defined by the nested PCR data and categorised into no infection (0%), polymorphic infection (>0.1% and < 100%) and fixation (100%). Collection sites of *Culicoides* populations were then plotted against *Cardinium* infection prevalence based on the nested PCR assay and mapped geographically. For populations containing both males and females, Fisher's exact test (two-tailed) was used to investigate possible associations between endosymbiont and sex with a significance cut-off of $P < 0.01$.

The relatedness of *Cardinium* strains from different host species was analysed using nucleotide sequences of amplicons derived from conventional PCR. The Gyrase B gene (*GyrB*) was chosen for phylogenetic analysis because it has a higher level of divergence when compared to the conserved 16S rRNA gene, another gene used in *Cardinium* phylogeny reconstruction. Amplicons were purified enzymatically (ExoSAP) before being sequenced using a BigDye® Terminator v3.1 kit (Thermo Scientific, Waltham, MA, U.S.A.), and capillary sequenced through both strands on a 3500 xL Genetic Analyser (Applied Biosystems, Austin, TX, U.S.A.). Sequences were aligned using the LINSI algorithm in MAFFT v7 (Katoh & Standley, 2013). A maximum likelihood (ML) phylogeny was then inferred with RAxML v8 (Stamatakis, 2014) using 1000 rapid bootstrap replicates and using the GTR + I + G model, which was selected with jModelTest 2 (Darriba *et al.*, 2012) using the Akaike information criterion, with the topology search taking the best of Subtree Pruning and Nearest Neighbour Interchange rearranging.

Collection and screening of 337 specimens of 35 midge populations consisting of 25 species from both Palearctic and Afrotropical regions indicated varying prevalence of *Cardinium* between species (Table 1). All specimens were female apart from some males collected for *C. imicola*, *C. punctatus*, *C. impunctatus* and *C. bolitinos* (File S1). Most populations (23/35) did not show any signs of *Cardinium* infection, although low collection size in some cases tempers against a hard conclusion that infection is absent from the population. Of the remaining 12 populations, seven were at fixation for *Cardinium* (although a few of these had low sample sizes) with the other five showing signs of polymorphism, with a mix of infected and uninfected individuals. This prevalence range is similar to the study of Australian *Culicoides* by Mee *et al.* (2015), in which 3/26 positive species had a fixed infection, with the remainder being of intermediate prevalence. Notably, Mee's study (Mee *et al.*, 2015) detected at least one *Cardinium* positive individual in each population screened, which is at odds to our findings and another study by Pagès *et al.* (2017), investigating *Cardinium* distribution in *Culicoides* from Spain.

It is possible that the apparent 'hotspot' in Australia is as a result of a more sensitive assay, as the qPCR method used by Mee *et al.* (2015) is sometimes preferred to nested PCR in screening. Alternatively, variation in thermal environment could explain the prevalence discrepancies observed between these bioclimatic zones. Morag *et al.* (2012) found a positive correlation between land surface temperature (LST) and *Cardinium* prevalence in *Culicoides imicola* in Israel, with higher prevalence at higher mean LST. Furthermore, there is a known relationship between *Cardinium* presence and latitude more globally, with *Cardinium* infection being more common for hosts near the equator (Charlesworth *et al.*, 2019).

In order to investigate *Cardinium–Culicoides* interactions, candidate model midge species must be identified, which combine the ability to vector etiological agents with either lab culturability or a natural polymorphism in endosymbiont presence that permits comparison between infected and uninfected

Table 1. *GyrB* conventional and nested PCR results of *Cardinium* identification in *Culicoides* spp. collected in different Palearctic and Afrotropical regions.

Subgenus	*Culicoides* species	Locality (site name)	Year of collection	Proportion of *Cardinium* positive conventional PCR (n) [95% confidence interval]	Proportion of *Cardinium* positive nested PCR (n) [95% confidence interval]
Avaritia	*C. bolitinos*	South Africa (Onderstepoort)	2016	0 (19) [0–0.21]	0 (19) [0–0.21]
	C. huambensis	South Africa (Koeburg)	2007	0 (1) [0–0.95]	0 (1) [0–0.95]
	C. imicola	Corsica (Site 1)	2015	0 (46) [0–0.1]	0 (46) [0–0.1]
		Corsica (Site 2)	2015	0 (27) [0–0.16]	0 (27) [0–0.16]
		South Africa (Onderstepoort)	**2016**	**0.3 (33) [0.16–0.49]**	**0.3 (33) [0.16–0.49]**
	C. tuttifrutti	South Africa (Chintsa)	2016	0 (10) [0–0.34]	0 (10) [0–0.34]
	C. obsoletus	U.K. (Neston)	2012–2015	0 (33) [0–0.13]	0 (33) [0–0.13]
Beltranmyia	*C. salinarius*	Sweden (Unknown site)	2009	0 (1) [0–0.95]	0 (1) [0–0.95]
	C. sphagnumensis	**Sweden (Axvalla)**	**2008**	**1 (1) [0.05–1]**	**1 (1) [0.05–1]**
Culicoides	***C. bysta***[a]	**Corsica (Site 2)**	**2015**	**0.5 (10) [0.24–0.76]**	**1 (10) [0.66–1]**
	C. impunctatus	Sweden (Torsås)	2008	0 (20) [0–0.2]	0 (20) [0–0.2]
		U.K. (Kielder)	2016	0 (13) [0–0.28]	0 (13) [0–0.28]
	C.magnus	South Africa (Koeburg)	2007	0 (1) [0–0.95]	0 (1) [0–0.95]
	***C. newsteadi* N1**[b]	**Corsica (Site 1)**	**2015**	**0 (4) [0–0.6]**	**0.75 (4) [0.22–0.99]**
		Corsica (Site 2)	**2015**	**0 (2) [0–0.8]**	**1 (2) [0.2–1]**
	***C. newsteadi* N2**[b]	**Corsica (Site 2)**	**2015**	**1 (2) [0.2–1]**	**1 (2) [0.2–1]**
	C. newsteadi N3[b]	Sweden (Unknown site)	2008–2010	0 (4) [0–0.6]	0 (4) [0–0.6]
	***C. newsteadi* N6**[c]	**Corsica (Site 1)**	**2015**	**0 (12) [0–0.3]**	**0.42 (12) [0.16–0.71]**
		Corsica (Site 2)	2015	0 (3) [0–0.69]	0 (3) [0–0.69]
	C. pulicaris	U.K. (Canterbury)	2014	0 (3) [0–0.69]	0 (3) [0–0.69]
		U.K. (Hereford)	2014	0 (1) [0–0.95]	0 (1) [0–0.95]
		U.K. (Luton)	2014	0 (1) [0–0.95]	0 (1) [0–0.95]
		U.K. (Wolverhampton)	**2013**	**0 (15) [0–0.25]**	**0.07 (15) [0–0.34]**
		U.K. (Worcester)	2014	0 (7) [0–0.44]	0 (7) [0–0.44]
	C. punctatus	**Sweden (Torsås)**	**2008**	**0.44 (25) [0.25–0.65]**	**0.8 (25) [0.59–0.92]**
		U.K. (Luton)	**2014**	**0 (2) [0–0.8]**	**1 (2) [0.2–1]**
		U.K. (Wolverhampton)	**2014**	**0.4 (5) [0.3–0.99]**	**1 (5) [0.46–1]**
Meijerehelea	*C. leucostictus*	South Africa (Kuleni)	2014	0 (1) [0–0.95]	0 (1) [0–0.95]
Monoculicoides	*C. stigma*	Sweden (Unknown site)	2008	0 (3) [0–0.69]	0 (3) [0–0.69]
Oecacta	*C. clastrieri*	Sweden (Unknown site)	2009	0 (2) [0–0.8]	0 (2) [0–0.8]
	C. duddingstoni	Sweden (Bara)	2008	0 (4) [0–0.6]	0 (4) [0–0.6]
Silvaticulicoides	***C. achrayi***	**Sweden (Axvalla)**	**2009**	**0 (1) [0–0.95]**	**1 (1) [0.05–1]**
	C. subfascipennis	Sweden (Romakloster)	2008	0 (12) [0–0.3]	0 (12) [0–0.3]
Silvicola	*C. grisescens*	Sweden (Torsås)	2008	0 (6) [0–0.48]	0 (6) [0–0.48]
Synhelea	*C. similis*	South Africa (Alexandria)	2016	0 (6) [0–0.48]	0 (6) [0–0.48]
Wirthomyia	*C. reconditus*	Sweden (unknown site)	2008	0 (1) [0–0.95]	0 (1) [0–0.95]

[a] *C. bysta* was originally designated by Sarvašová *et al.* (2017).

[b] *C. newsteadi* haplotypes N1, N2 and N3 originally designated by Pagès *et al.* (2009).

[c] *C. newsteadi* N6 previously undesignated.

Pulicaris complex species were distinguished using *COI* DNA barcoding. COI accessions are KY765346, KY765347, KY765348, KY765350, KY765356, KY765357, KY765358. Bold entries are of species identified as being infected with *Cardinium*.

individuals. Conventional PCR detected *Cardinium* in four putative vector species of bluetongue virus (BTV): *C. imicola*, *C. newsteadi*, *C. bysta* and *C. punctatus* (Table 1). Additionally, evidence of a low-level *Cardinium* infection was detected in one individual of the vector species *C. pulicaris* when screened with the nested assay. The infection patterns within the Pulicaris complex species (*C. bysta*, *C. newsteadi*, *C. pulicaris* and *C. punctatus*) suggest field populations could be suitable for future *Cardinium* work. However, the lack of polymorphic infection observed in some populations from this study, alongside the difficulties in species differentiation, could prove problematic. Furthermore, the utility of *C. pulicaris* as a candidate should be met with caution due to the conflicting reports of *Cardinium* prevalence within this species in past study (Lewis *et al.*, 2014; Pagès *et al.*, 2017). Notably, our more sensitive nested PCR assay detected a very low *Cardinium* frequency of 1/27 for *C. pulicaris*, compared to Lewis *et al.* (2014) (the higher frequency, 10/29, in Lewis *et al.* represents a laboratory artefact; see doi: 10.6084/m9.figshare.13187069).

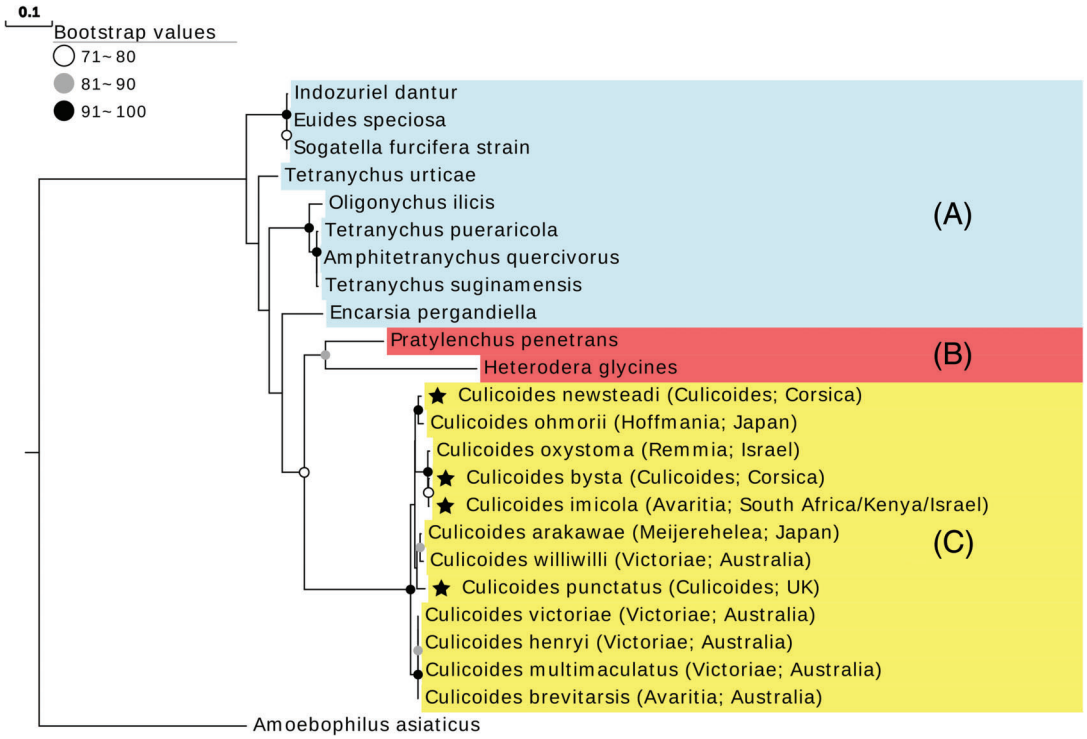

Fig. 1. Maximum likelihood phylogeny of *Cardinium* strains. Phylogeny is based on a 1300 bp region of the *GyrB* gene generated in RAxML using 1000 rapid bootstrap replicates and using the GTR + I + G substitution model. Groups A (blue) B (red) and C (yellow) are designated based on *Cardinium* taxonomic convention. Stars represent sequences generated in this study. Brackets indicate subgenus and country of origin. [Colour figure can be viewed at wileyonlinelibrary.com].

Phylogenetic analysis based on a 1300 bp region of the *GyrB* gene confirmed a monophyletic clade of *Cardinium*, grouping all *Culicoides Cardinium* isolates from this study in group C of the genus (Nakamura *et al.*, 2009), in which all previously described sequences of *Culicoides* clustered (Fig. 1) (Genbank accession numbers [https://www.ncbi.nlm.nih.gov/genbank/]: LR877462-LR877465). The *C. imicola GyrB* sequence obtained from South Africa in this study (LR877465) was identical to both of those obtained from Kenya (KR026927) and Israel (JN166963), indicating a global infection. Likewise, the *Cardinium* sequences obtained from *C. punctatus* from Wolverhampton, U.K. (LR877464) had 100% identity to the sequence reported in the same species by Lewis *et al.* (2014; HG380244). Mee *et al.* (2015) have previously suggested that *Cardinium* strains group by geography; by contrast, we find a sporadic distribution of strains with respect to location. For example, the *C. bysta* and *C. newsteadi* strains (LR877462 and LR877463) identified from Corsica, group with strains from Israel and Japan, respectively (JN166964 and AB506792). Furthermore, no clear pattern of *Cardinium* distribution was observed between the three geographical regions of this study, though the small sample size for some species forbids any formal test of the presence or absence of heterogeneity (File S2).

By contrast with other endosymbiont–insect associations, the biological significance of *Cardinium* in *Culicoides* remains unknown and requires further research. Despite a likely vertical transmission route, our observed lack of sex-bias in infection observed in this study (*C. punctatus*; Fisher's exact, $P = 0.31$ and *C. imicola*; Fisher's exact, $P = 0.68$) corroborates previous studies (Morag *et al.*, 2012; Mee *et al.*, 2015; Pagès *et al.*, 2017) and suggests the induction of parthenogenesis, feminisation or male-killing is unlikely to be associated with *Cardinium*. Other *Cardinium* strains have been implicated in cytoplasmic incompatibility (CI), including in the wasp *Encarsia pergandiella* (Hunter *et al.*, 2003). A recent publication of the *C. punctatus Cardinium* genome by Siozios *et al.* (2019) has suggested possible unique genes related to CI. Overall, these observations suggest potential *Cardinium*-induced CI should be a priority of investigation in the future due to its possible role in driving the endosymbiont (and any desired effects) into midge populations.

The major Palearctic vector of BTV, *C. obsoletus*, has previously been shown to contain *Cardinium* at 0.4% prevalence (Pagès *et al.*, 2017). We did not observe the endosymbiont in the one *C. obsoletus* population screened in this study, although the sample size was too small to conclude the absence of infection, and lack of DNA barcodes in this case prevents matched comparison. Nevertheless, a low prevalence indicates *Cardinium* is likely not relevant to vector biology at a population level and thus this species complex does not appear to be a suitable candidate for investigation.

By contrast, two other important vector species, *C. imicola* and *C. sonorensis*, have been reported to carry *Cardinium* infection (Morag *et al.*, 2012; Möhlmann, 2019). Our results demonstrated the presence of *Cardinium* in one population of

C. imicola from South Africa (Onderstepoort), whereas specimens of two populations from Corsica were negative for the presence of *Cardinium*, a pattern likely associated with properties of founder individuals (Pilgrim *et al.*, 2021). Thus, these species appear to be the most promising candidate species for investigating *Cardinium* effects on vectorial capacity. As certain *C. imicola* and *C. sonorensis* populations are polymorphically infected (Morag *et al.*, 2012; Möhlmann, 2019), the two species both present naturally occurring negative controls without the need for the confounding effects of curing through antimicrobials. In favour of *C. imicola* as a model is the ease of obtaining large field catches, but barriers in laboratory cultivation are still to be overcome. However, *C. sonorensis* laboratory colonies already exist, suggesting this species is the most promising candidate for investigating symbiont–virus interactions and symbiont-mediated reproductive effects. It will be worthwhile, additionally, to screen other *Culicoides* species, which may become colonised in the near future, such as *C. stellifer* (Erram & Burkett-Cadena, 2020).

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

File S1. Primer attributes, PCR conditions and statistical test results of sex-based infection patterns.

File S2. Distribution of *Cardinium* positive *Culicoides* populations.

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Author contributions

JP, GDDH, SS and MB assisted in the conception and design of the study. Field work was undertaken by JP, CG and GV. Lab work was undertaken by JP. Analyses and interpretation of the data were undertaken by JP, GDDH, MB, SS, CG and GV, as well as drafting of the manuscript.

Data availability statement

The data that support the findings of this study are openly available in figshare at: http://doi.org/10.6084/m9.figshare.13187069, http://doi.org/10.6084/m9.figshare.13142684, and http://doi.org/10.6084/m9.figshare.13130306. Accession numbers generated in this study: LR877462-LR877465.

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