First detection of endosymbotic bacteria in *Culicoides pulicaris* and *Culicoides punctatus,* important Palearctic vectors of bluetongue virus

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Abstract

Heritable bacteria have been highlighted as important components of vector biology, acting as required symbionts with an anabolic role, altering competence for disease transmission, and affecting patterns of gene flow by altering cross compatibility. In this paper, we tested 8 UK species of *Culicoides* midges for the presence of 5 genera of endosymbiotic bacteria: *Cardinium*, *Wolbachia, Spiroplasma, Arsenophonus* and *Rickettsia. Cardinium* spp. was detected in both sexes of *C pulicaris* and *C. punctatus,* two known vectors of bluetongue virus. It was not detected in any other species, including the *C. obsoletus* group, the main vector of bluetongue and Schmallenberg viruses in northern Europe. The other endosymbionts were not detected in any *Culicoides* species. The *Cardinium* strain detected in the UK *Culicoides* species is very closely related to *Candidatus Cardinium hertigii* group C, previously identified in *Culicoides* in Asia. Further, we infer that the symbiont is not a sex ratio distorter and shows geographic variation in prevalence within a species. Despite its detection in several species of *Culicoides* that vector arboviruses worldwide, the absence of *Cardinium* in the *C. obsoletus* group suggests that infections of these symbionts may not be necessary for arboviral vector competence of biting midges.

Species of midges in the genus *Culicoides* (Diptera: Ceratopogonidae) are among the most abundant of haematophagous insects and are important vectors of viruses affecting humans and livestock. Over 50 viruses have been isolated from *Culicoides* to date, including bluetongue virus (BTV) and the recently emerged Schmallenberg virus (SBV). *Culicoides obsoletus* group are the main vectors of BTV and SBV in Northern Europe, and include *C. montanus, C. scoticus*, *C. obsoletus, C. dewulfi and C. chiopterus*. However, at present there is insufficient data on the vector competence of specific *Culicoides* species for SBV. BTV is observed predominantly in sheep, but can infect all ruminants. In addition to welfare implications, BTV has a devastating impact on the farming industry through loss of production and trade. SBV also infects ruminants, causing little or no clinical disease in adult animals but leading to a high frequency of abortion or developmental abnormality in newborn offspring (Mellor *et al.*, 2000; De Regge *et al.*, 2012).

Microorganisms and insects commonly form symbiotic associations, which may have implications for control of vector-borne disease. Endosymbiotic bacteria that reduce the longevity of their hosts can be used to interrupt onward viral transmission (McMeniman *et al*., 2009). In addition, endosymbionts may affect vector competence by decreasing (Hedges *et al.*, 2008), or increasing (Graham *et al.*, 2012) host susceptibility to viruses.

Endosymbionts have been detected in certain *Culicoides* species (Morag *et al.*, 2012; Nakamura *et al.*, 2009). Tests to date have focused on just two symbiont clades, *Cardinium* and *Wolbachia*. Reports to date indicate the presence of a phylogenetically distinct clade of *Cardinium* symbionts in some *Culicoides* (Nakamura *et al.*, 2009). However, there has been no study of European *Culicoides* species, and the role of *Cardinium* in midge biology remains unclear. In our study we aimed first to test UK

26 species of *Culicoides* for a variety of common endosymbionts. Further, by screening
27 male and female hosts separately, we sought to establish whether these symbionts
28 show sex biased prevalence typical of host sex ratio distorting activity.

29 *Culicoides* were collected from Leahurst Campus, University of Liverpool,
30 England and Bala, Wales, between July and October 2012. Samples were captured
31 using light traps that were active overnight, and the insects were trapped into 95%
32 ethanol for rapid preservation. *Culicoides* were identified to species as per Downes &
33 Kettle (1952) and sexed. *C. scoticus* and *C. obsoletus* females cannot be separated
34 morphologically and so were grouped together. A total of 173 *Culicoides* midges of 8
35 species were collected, including both vectors and non-vectors of BTV.

36 DNA from individual specimens was extracted using the Wizard® SV 96
37 Genomic DNA Purification System (Promega) into two 96 well plates, each with 2
38 positive and 2 negative controls. The DNA quality of each sample was tested using a
39 PCR amplification of part of the COI gene in the mtDNA of its host (Folmer *et al.*,
40 1994), and this assay was used to optimize DNA dilution where necessary. All
41 *Culicoides* that passed this initial assessment were tested for the presence of
42 endosymbiotic bacteria in the genera *Wolbachia, Cardinium, Spiroplasma, Rickettsia*
43 and *Arsenophonus*. To test for the presence of *Wolbachia* a PCR based assay was
44 undertaken using primers 81F/691R designed to amplify part of the wsp gene.
45 *Spiroplasma* presence was tested using PCR assay with primers GPO-1/MGSO that
46 amplify part of the 16S rRNA gene from Mollicutes only, and *Cardinium* assays
47 utilized Car-sp-F/Car-sp-R which amplify part of the 16S rRNA gene. For *Rickettsia*,
48 PCR assay utilized primers R1/R2 based on the 17kDa omp and for *Arsenophonus*
49 primer pair ArsF/ArsR2 that amplifies part of the 16S rRNA gene. Details of primer
50 sequences and amplification conditions can be found in Duron *et al.* (2008)

(*Wolbachia, Rickettsia, Arsenophonus*), van Kuppeveld *et al*. (1992) (*Spiroplasma*) and Nakamura *et al.* (2009) (*Cardinium*). PCR assays included positive controls from insect material known to be infected with the relevant symbiont (taken from Duron *et al.* (2008)), and negative (water) controls. It should be noted that our screening, because it relies on single PCR assays for each microbe, may create a low rate of false negative results (Simoes *et al.*, 2011). However, it does permit direct comparison with the results of other screens.

When amplicons were obtained in PCR assays, the sequence of the amplicons was obtained to confirm that the result represented a true positive. To this end, PCR products deriving from one male and female midge of each species that was positive for a symbiont were purified using an ExoSAP digest to remove unincorporated primers and nucleotides, and cycle sequencing was performed according to the Sanger method using each of the initial primers separately. The products were visualised on an ABI automated sequencing machine at the University of Liverpool, and aligned using MEGA5 (Tamura *et al*., 2011).

PCR screening revealed 2 out of 8 species of *Culicoides* were positive for *Cardinium* infection (Table 1). The prevalence in the two infected species was significantly different (Fisher exact test: $p \leq 0.05$, d.f.=1). In *C. punctatus*, *Cardinium* prevalence was nearly fixed at 0.960 (Binomial Confidence Interval (CI): $0.796 \leq p \leq 0.999$), whilst in *C. pulicaris* the endosymbiont was at a lower prevalence of 0.256 (Binomial CI: $0.130 \leq p \leq 0.471$). For three of the *Cardinium* negative species, reduced availability of material allows us to conclude that there is no high prevalence/fixed infection, but do not give sufficient power to establish absence of low prevalence infection (<30%). The 16S rRNA sequences for the two infected species were identical, and were 99% similar to *Candidatus Cardinium hertigii* group

C, previously discovered in Japanese *Culicoides* (Nakamura *et al.*, 2009) (Accession codes: HG380245, HG531389). No other endosymbionts were detected in any of the samples.

This is the first detection of *Cardinium* in UK *Culicoides.* The 16S rRNA gene is slow evolving, and thus we additionally obtained the sequence of the GyraseB gene of our detected strains to produce a more fine grained phylogenetic analysis in comparison to other *Cardinium* strains in the clade (Nakamura *et al.*, 2009). 1200 bp of this gene were amplified using primer pair gyrB23F (5' GGA GGA TTA CAT GGY GTG GG) and gyrB1435R (5' GGA GGA TTA CAT GGY GTG GG). PCR amplifications were performed under the following conditions: initial denaturation at 95°C for 2 minutes, 35 cycles of denaturation (94°C, 15 seconds), annealing (57°C, 60 seconds), extension (72°C, 90 seconds) and a final extension at 72°C for 5 minutes. The product was then purified and sequenced through both strands using the original and two internal primers. The *Cardinium gyrB* sequence was identical in *C. punctatus* and *C. pulicaris*, and forms a monophyletic clade with *Cardinium* reported from other species of *Culicoides* (Accession codes: HG380244, HG531390) (Figure 1).

In our study, *Cardinium* infection occurs in a higher proportion of species than is generally observed in insects. To date, 6 of 33 (18%) *Culicoides* species tested have been observed to carry *Cardinium* across two surveys. This compares to past unbiased surveys which report *Cardinium* global incidence of 4.4% of arthropod species (n=136), and 0% of sampled insect species (n=100) (Duron *et al*., 2008)(Fisher exact test: *Culicoides* vs all insects, $p<0.001$). It is notable that the strains identified in our study confirmed the presence of a particular clade of *Cardinium* (elsewhere termed clade C) that is present in this group, and not observed to date in other arthropod

100 species. This conclusion is based on information from two markers (16S rRNA and
101 *gyrB*) and awaits confirmation from further markers.

102 The 'hotspot' presence of *Cardinium* in sampled *Culicoides* contrasts with
103 *Wolbachia. Wolbachia* predominates in both arthropods and insects (22.5% of
104 arthropod species tested, 18% of insects: Duron *et al*., 2008). In contrast, *Culicoides*
105 appears to be a 'cold spot' for *Wolbachia* infection; the symbiont has only been
106 detected in 1 of the 33 screened *Culicoides* species (Nakamura *et al*., 2009) (Fisher
107 exact test *Culicoides* incidence vs all insects: $p<0.05$).

108 Our results also suggest the presence of geographical variability in the
109 *Culicoides*-*Cardinium* interaction. In our study, the prevalence of infection in *C.*
110 *punctatus* is 96%, which contrasts with absence of infection in this same species in
111 Japan (0/7) (Test of null hypothesis of same prevalence in each population: Fisher
112 exact test: $p<0.0001$). This was also seen with *C. oxystoma*, which was infected with
113 *Cardinium* in Israel but not in Japan (Morag *et al.*, 2012; Nakamura *et al.*, 2009).
114 Geographic differentiation within a species is common for heritable symbionts
115 (Duron *et al.*, 2008), but the drivers of geographical variation are commonly not
116 known.

117 It is interesting to observe that the results of this study produced two different
118 'types' of *Cardinium* infection, one nearly fixed, and one with low prevalence. This
119 echoes the results of the study by Nakamura *et al*. (2009), where three out of four
120 species that tested positive had a fixed infection, and one carried *Cardinium* in a
121 minority of individuals sampled. The fixed infections are reminiscent of those causing
122 a cytoplasmic incompatibility (CI) phenotype, as this reproductive alteration drives
123 the bacterium to high prevalence within the population, with infection found in both
124 sexes (Brelsfoard & Dobson, 2009). The factors maintaining the low prevalence

125 infection are more enigmatic. The presence of infected male hosts make sex ratio
126 distortion an unlikely explanation, and the precise phenotype of *Cardinium* in *C.*
127 *pulicaris* requires further research.

128 Phylogenetic analysis has grouped both *C. pulicaris* and *C. punctatus* within
129 the subgenus *Culicoides*. *C. impunctatus*, the Scottish biting midge, is also within this
130 subgenus (Meiswinkel *et al.*, 2004). Both *C. pulicaris* and *C. punctatus* are vectors of
131 BTV and this study showed they are both infected with *Cardinium*. *C. impunctatus*
132 however is not a vector, and this study showed it is not infected. This is an interesting
133 result as it may suggest the bacterium is associated with vector competence. However,
134 there are contrasting results in our study within the subgenus *Avaritia*. In Israel it was
135 demonstrated that *C. imicola*, a major vector of BTV, harbors *Cardinium* (Morag *et*
136 *al.*, 2012). However, all tested species belonging to the subgenus were uninfected
137 with *Cardinium* despite all of these species acting as vectors of BTV. Overall, it is
138 interesting to note that although *Cardinium* is not present in all vectors (and thus may
139 not be necessary for competence), the 4 species in the Western Palearctic in which it
140 is detected are all vectors (*C. punctatus, C. pulicaris, C. imicola, C. oxystoma*).
141 Further research is required to determine if *Cardinium* does influence the ability of
142 *Culicoides* biting midges to transmit viruses.

143 This study confirms *Culicoides* carry a clade of *Cardinium* that on the basis of
144 the sequence of two markers forms a monophyletic assemblage found only within
145 biting midges, and there is evidence for geographic variation in infection in a species.
146 It is not known what drives *Cardinium* infection into *Culicoides* populations; however
147 presence in an equal fraction of male and female hosts are not consistent with
148 *Cardinium* acting as a sex ratio distorter, a phenomenon commonly seen with other
149 endosymbionts. Although *Cardinium* was detected in two species that are vectors of

BTV, failure to detect *Cardinium* in the major vector in Europe, the *C. obsoletus* group, suggests this endosymbiont may not be necessary for BTV vector competence of *Culicoides*.

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