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1 **Rapid diagnostic PCR assays for members of the *Culicoides obsoletus* and *Culicoides***
2 ***pulicaris* species complexes, implicated vectors of bluetongue virus in Europe**

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16 Short title: PCR identification of members of the *C. obsoletus* and *C. pulicaris* complexes

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21 **Abstract**

22

23 Biting midges of the *Culicoides obsoletus* Meigen and *Culicoides pulicaris* L. species
24 complexes (Diptera: Ceratopogonidae) are increasingly implicated as vectors of bluetongue
25 virus in Palearctic regions. However, predicting epidemiological risk and the spread of
26 disease is hampered because whilst vector competence of *Culicoides* is expressed only in
27 adult females, morphological identification of constituent species is only readily applicable to
28 adult males and some species distinguishing traits have overlapping character states.
29 Furthermore, adult males are typically rare in field collections, making characterisation of
30 *Culicoides* communities impossible. Here we highlight the utility of mitochondrial
31 cytochrome oxidase subunit I (COI) DNA sequences for taxonomic resolution and species
32 identification of all species within *C. obsoletus* and *C. pulicaris* complexes. *Culicoides* were
33 collected from 18 sites in the UK and Continental Europe, and identified to species level, or
34 species complex level, based on morphological characters. The sample comprised four
35 species from the *C. obsoletus* complex ($n = 88$) and five species from the *C. pulicaris*
36 complex ($n = 39$). The DNA sequence of the 5' end of the COI gene was obtained from all
37 individuals. Each member species formed a well-supported reciprocally monophyletic clade
38 in a maximum likelihood phylogeny. Levels of DNA sequence divergence were sufficiently
39 high between species to allow the design of species-specific PCR primers that can be used in
40 PCR for identification of members of the *C. pulicaris* complex or in a multiplex PCR to
41 identify members of the *C. obsoletus* complex. This approach provides a valuable diagnostic
42 tool for monitoring species composition in mixed field collections of *Culicoides*.

43

44 **Keywords:** COI; *Culicoides*, mtDNA; *C. obsoletus* complex; *C. pulicaris* complex; species-
45 specific PCR

46 **1. Introduction**

47

48 Midge of the genus *Culicoides* Latreille 1809 are major vectors of bluetongue virus
49 (BTV) and African horse sickness virus (AHSV), the etiological agents of the infectious, non-
50 contagious diseases, bluetongue and African horse sickness, respectively. BTV infects all
51 ruminants but causes severe clinical disease primarily in certain fine-wool breeds of sheep and
52 deer species (Taylor, 1986), while AHSV is among the most lethal infections of equids
53 (Mellor et al., 2000). Bluetongue is endemic in tropical latitudes worldwide and African
54 horse sickness is endemic in sub-Saharan Africa. Although traditionally regarded as diseases
55 with stable endemic ranges, since the mid-1980's there has been a gradual northerly spread of
56 confirmed cases of both AHSV and BTV. The AHSV outbreaks in Iberia during 1987-1991
57 were of unprecedented scale and duration (Mellor, 1994; Mellor and Boorman, 1995), and the
58 largest bluetongue outbreaks on record have occurred since 1998 in Mediterranean countries
59 and central Europe (Baylis and Mellor, 2001; Georgiev et al., 2001; Baylis, 2002; Mellor and
60 Wittmann, 2002). During 1998-2005 outbreaks of bluetongue affected 12 countries in the
61 Mediterranean region extending some 800 km further northwards in some areas of mainland
62 Europe than previously recorded, with seven separate introductions involving serotypes BTV-
63 1, 2, 4, 9 and 16 (Purse et al., 2005). In 2006, additional bluetongue outbreaks were observed
64 further north with confirmed cases occurring for the first time in the Netherlands, Belgium,
65 Germany, France and Luxembourg. These outbreaks involved a new serotype in Europe,
66 BTV-8, producing clinical symptoms and mortalities in cattle as well as sheep. Severe clinical
67 symptoms and mortalities are unusual in cattle but may occur when highly susceptible bovine
68 populations are exposed to the first introduction of a new serotype in a particular region.

69 The implicated vectors of the virus in these areas are members of both the *C. obsoletus*
70 and *C. pulicaris* complexes, with the *C. obsoletus* complex member, *C. dewulfi* (Anonymous,

71 2006), being suggested as a significant vector in propagating the BTV-8 in northern Europe.
72 The northern bluetongue outbreaks were part of four separate epizootics that occurred in
73 Europe during 2006. Other outbreaks occurred in the mid-south, in Sardinia, involving BTV-1
74 and affecting sheep, and the two other outbreaks were, one in the southeast, in Bulgaria
75 involving BTV-8 (serological evidence only), and one in the southwest in Portugal, involving
76 BTV-4 (OIE, disease information: http://www.oie.int/eng/info/hebdo/a_dsum.htm). In total,
77 2047 BT outbreaks caused by BTV-8 were reported by EU member states during 2006 in
78 northern Europe, 456 in the Netherlands, 695 in Belgium, 885 in Germany, 6 in France, and 5
79 in Luxembourg. In southern Europe, a total of 241 BT outbreaks were observed, 227 in
80 Sardinia, 13 in Bulgaria, and 1 in Portugal (European Community animal disease notification
81 system: http://ec.europa.eu/food/animal/diseases/adns/table_11/2006.pdf).

82

83 The contribution of *Culicoides* vectors to the northward expansion of bluetongue is
84 thought to be two-fold. First, the main Old World bluetongue vector *Culicoides imicola*
85 Kieffer 1913 has expanded its range to include several countries bordering the Mediterranean
86 basin (Baylis et al., 1997; Calistri et al., 2003; Capela et al., 2003; Miranda et al., 2003; Sarto
87 I Monteys and Saiz-Ardanaz, 2003; Purse et al., 2005; Sarto I Monteys et al., 2005). Second,
88 bluetongue outbreaks where *C. imicola* is either rare or absent (as in parts of northern, central,
89 and eastern Europe) implicate native European *Culicoides* species as competent vectors. The
90 primary candidates are member species of the *C. obsoletus* and *C. pulicaris* complexes, which
91 are abundant and widespread in Palearctic regions particularly in farmland habitats.
92 Circumstantial evidence of their involvement is strong. The spatial and temporal distributions
93 of the two complexes coincide with bluetongue outbreaks (Torina et al., 2004; Purse et al.,
94 2005); bluetongue is present in pools of wild-caught insects (Caracappa et al., 2003; De
95 Liberato et al., 2005; Savini et al., 2005); and bluetongue virus replication to likely

96 transmissible titres occurs in both the *C. obsoletus* and *C. pulicaris* complexes under
97 laboratory conditions (Carpenter et al., 2006). In this respect, there are now two foci of BTV
98 transmission in Europe, one being driven by *C. imicola* which is expanding northward into
99 southern Europe and another being driven by members of the *C. obsoletus* and *C. pulicaris*
100 complexes which exist throughout the rest of Europe.

101

102 Further spread of bluetongue virus transmission into the western Palaearctic, and the
103 possibility of a similar, future, incursion of African horse sickness virus (which relies on
104 similar epidemiological conditions and vectors), poses obvious risks for animal health (Mellor
105 and Hamblin, 2004; Ducheyne et al., 2005; Purse et al., 2005). An adequate risk assessment
106 requires both accurate identification of which members of the *C. obsoletus* and *C. pulicaris*
107 complexes are competent vectors and knowledge of their geographic distributions. The present
108 taxonomy for the *C. obsoletus* and *C. pulicaris* complexes is based on morphological traits that
109 require a highly specialised and specific knowledge of insect morphology, and has two main
110 weaknesses for species identification to inform risk assessment. First, the members of the *C.*
111 *obsoletus* complex are only easily distinguishable using species-diagnostic morphological
112 traits in adult males, which are rarely caught in large numbers and not involved in viral
113 transmission. Second, the traits on which the taxonomy is based are only semi-diagnostic in
114 some cases, e.g., *Culicoides pulicaris* L. and *Culicoides punctatus* Meigen in the *C. pulicaris*
115 complex overlap for wing morphology (Lane, 1981).

116

117 Improvements in risk assessment of vector-borne BTV and AHSV transmission
118 require an expanded taxonomy of the *C. obsoletus* and *C. pulicaris* complexes that identifies
119 species-diagnostic traits that are both easy to analyse and expressed in readily collectable life
120 stages. DNA sequence analysis has been shown to readily identify such traits. Members of *C.*

121 *imicola* species complex from southern Africa form distinct lineages based on RAPD markers
122 (Sebastiani et al, 2001) or sequences of the mitochondrial COI gene (Linton et al., 2002,
123 Nolan et al., 2004), and specimens of *C. imicola* from the Mediterranean basin and South
124 Africa cluster in the same group according to phylogenetic analysis of COI (Dallas et al.,
125 2003). Indeed, members of the *C. obsoletus* complex can be identified more reliably using
126 species-specific markers in COI (Pagès and Sarto I Monteys, 2005) or in the intergenic
127 transcribed spacer of ribosomal DNA (Gomulski et al., 2005) than using morphological traits.
128 However, in order to allow routine entomological assessments of distribution and densities of
129 vector species, it is necessary to develop a single diagnostic assay across both the *C. obsoletus*
130 and *C. pulicaris* species complexes.

131

132 The aims of the present study were 1) to generate a phylogeny for all species within
133 the *C. obsoletus* and *C. pulicaris* species complexes based on DNA sequence variation at the
134 mitochondrial COI gene 2) develop a PCR assay for rapid species identification of unknown
135 individuals.

136 2. Materials and methods

137

138 2.1 Insects

139

140 *Culicoides* specimens were collected at 18 sites: 11 in the UK, 2 in Bulgaria, 3 in Italy,
141 and 1 each in Morocco and Greece (Table 1 and Fig. 1). Insects were collected using
142 downdraught suction UV (8 Watt, 350nm) light traps into 200–300 ml of either water
143 containing 2-3 drops of detergent or 2.5 M NaCl, 0.25 M EDTA, pH 8. The catch at each site
144 contained 99.3-100% females. *Culicoides* specimens were identified to species according to
145 wing morphology of adults of both sexes and genital morphology of adult males (Campbell
146 and Pelham-Clinton, 1960), and then stored in 95% ethanol at -20°C.

147

148 2.2. DNA extraction

149

150 Total DNA was extracted from single midges using the DNeasy Tissue Kit (Qiagen,
151 Crawley, UK), according to manufacturer's instructions, with DNA elution into 55 µL of
152 sterile water.

153

154 2.3. Mitochondrial DNA COI gene

155

156 A 472 segment of the mitochondrial COI gene was PCR amplified from individual
157 midges using the primers C1-J-1718 and C1-N-2191 (Dallas et al., 2003). The reaction
158 volume was 25 µL, consisting of 1 x NH₄ reaction buffer (Bioline, London, UK), 2.5 mM
159 MgCl₂, 200 µM dATP, dCTP, dGTP and dTTP, 1 µM of each primer, and 0.5 units of Taq
160 DNA polymerase (Bioline). The thermal profile consisted of 95°C for 5 min to activate the
161 Immolase Taq, an initial denaturation step at 92°C for 2 min 15 s, followed by 30 cycles of

162 92°C for 15 s, 50°C for 15 s, 72°C for 30 s, and ending with a final elongation step at 72°C
163 for 1 min. PCR products were purified using QIAquick spin columns (Qiagen, Crawley, UK)
164 and were sequenced in both directions using the same PCR primers by MWG-Biotech
165 (Ebersberg, Germany). The COI sequences are deposited in Genbank under accession
166 numbers AM236652–AM236671 (*C. obsoletus* s.s.), AM236625–AM236651 (*C. scoticus*),
167 AM236672–AM236707 (*C. dewulfi*), AM236747–236751 (*C. chiopterus*),
168 AM236708–236716 (*C. pulicaris*), AM236717–AM236725 (*C. impunctatus*),
169 AM236726–AM236732 (*C. grisescens*), AM236733–AM236737 (*C. punctatus*), and
170 AM236738–AM236746 (*C. newsteadi*).

171

172 2.4. Data analysis

173

174 A COI sequence was obtained for each midge by alignment of the forward and reverse
175 sequences using Se-Al (available at evolve.zoo.ox.ac.uk/software/Se-Al/main.html).
176 Sequences from different midges were then aligned using Clustal X (Thompson et al., 1997).
177 No insertions or deletions were present in both the forward and reverse sequences. The first
178 base of the *Culicoides* sequences corresponded to the last base in codon 82 of the *Anopheles*
179 *gambiae* homologue, and no inferred stop codons were detected. Transition/transversion
180 ratios and pairwise genetic distance values were calculated using MEGA v 3.1 (Kumar et al.,
181 2004), and values for base composition, haplotype diversity and nucleotide diversity were
182 calculated using DnaSP v 3.99 (Rozas et al., 2003).

183 The phylogenetic relationships among individual sequences were determined using a
184 maximum likelihood (ML) phylogeny generated within PAUP v 4b10 (Swofford, 1998). The
185 most appropriate model for DNA substitution among haplotypes was identified using
186 MODELTEST v 3.06 (Posada and Crandall, 1998). The Tamura-Nei (TrN) model chosen
187 incorporated base frequencies of A, C, G, T of 0.3437, 0.1923, 0.0867, 0.3774 respectively,

188 with proportion of invariable sites (I) = 0.5244 and gamma distribution shape parameter (γ) =
189 0.7650. The stability of resultant clades was assessed using bootstrap analysis (10,000
190 iterations) within a neighbour joining tree constructed with the same model of sequence
191 evolution as the ML analysis.

192

193 2.5. Species-specific PCR

194

195 Sequence alignments of the four members of the *C. obsoletus* and five members of *C.*
196 *pulicaris* complexes found in the UK were used to design species-specific primers using the
197 Primer 3 software (Rozen and Skaletsky, 2003). The identified primers given in Table 4 were
198 tested in 25 μ L PCR reactions, consisting of 1 x NH_4 reaction buffer (Bioline, London, UK),
199 2.5 mM MgCl_2 , 200 μ M dATP, dCTP, dGTP and dTTP, 1 μ M of each primer and 0.5 units of
200 Immolase DNA polymerase (BioLine). The PCR reactions were carried out on a PTC-100
201 Programmable Thermal Controller, (MJ Research Inc., Watertown, MA, USA) under the
202 following conditions: an initial denaturation step at 92°C for 2 min 15 s, followed by 30
203 cycles of 92°C for 15 s, X°C (where X is the annealing temperature indicated for each
204 species-specific primer, Table 4) for 15 s, 72°C for 30 s, and ending with a final elongation
205 step at 72°C for 1 min. PCR products were examined using electrophoresis on a 2.0%
206 agarose gel with ethidium bromide staining.

207 Each species-specific primer in combination with the C1-N-2191 primer were tested
208 against members of the same species complex and other commonly collected *Culicoides*
209 species to confirm specificity of each species-specific primer. The multiplex PCR for the *C.*
210 *obsoletus* complex was identical to above, except, 1 μ M of UOAobsF, UOAcoF, UOAchiF,
211 UOAdeW and C1-N-2191 primers were used and the annealing temperature was 61°C.
212 Products were examined using electrophoresis on a 3.0% AquaPor HR GTAC agarose gel

213 (National Diagnostics, Atlanta, GA, USA) with ethidium bromide staining.

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214 **3. Results**

215

216 *3.1. Member species of C. obsoletus and C. pulicaris complexes*

217

218 *Culicoides* specimens ($n = 127$) belonging to the *C. obsoletus* and *C. pulicaris* species
219 complexes were obtained in insect collections. From morphological analysis, the insect
220 collections represented four member species of the *C. obsoletus* complex (*Culicoides*
221 *obsoletus* sensu stricto Meigen 1818, *Culicoides scoticus* Downes & Kettle 1952, *Culicoides*
222 *dewulfi* Goetghebuer 1936 and *Culicoides chiopterus* Meigen 1830) and five members of the
223 *C. pulicaris* complex (*Culicoides pulicaris* sensu stricto L. 1758, *Culicoides impunctatus*
224 Goetghebuer 1920, *Culicoides punctatus* Meigen 1804, *Culicoides grisescens* Edwards 1939
225 and *Culicoides newsteadi* Austin 1921).

226

227 *3.2. COI sequences of C. obsoletus and C. pulicaris complexes*

228

229 A total of 45 COI haplotypes (472 bp) were obtained from the 127 *Culicoides*
230 specimens (108 females and 19 males, Table 2). Haplotype diversity within each member
231 species ranged from 0.222 ± 0.166 (*C. newsteadi*) to 0.900 ± 0.161 (*C. chiopterus*) (Table 2).
232 The *C. obsoletus* s.s. sequences contained three nonsynonymous changes; a C↔T transition at
233 position 189, and A↔T transversions at positions 47 and 260. The *C. scoticus* sequences
234 contained one nonsynonymous change at position 397, an A↔T transversion, and the *C.*
235 *dewulfi* sequences contained three nonsynonymous changes, an A↔G transition at position
236 107, and C↔T transitions at positions 366 and 384. All other intraspecific substitutions were
237 synonymous. The base composition of the *Culicoides* COI sequences was A+T-biased.
238 Mean A+T content within species was 60.8-65.4%, and A+T content was highest at the third
239 codon position (51.6-58.6% at position 1, 55.4-57.3% at position 2 and 71.5-84.8% at position
240 3).

241

242 3.3. Sequence divergence

243

244 Observed transition/transversion ratios for comparisons between pairs of COI
245 sequences from the nine species (mean = 1.02, range 0.52 – 1.54) were comparable to values
246 previously observed among members of the *C. imicola* species complex (Linton et al., 2002).
247 The most common substitutions were A↔T transversions (31.1% of the total) and C↔T
248 transitions (37.5% of the total). Most substitutions between pairs of COI sequences occurred
249 at inferred third codon position (average percentages of substitutions were 18% at position 1,
250 2% at position 2 and 80% at position 3). From pairwise TrN+I+ γ distances among COI
251 haplotypes, the most similar species within the *C. obsoletus* complex were *C. obsoletus* s.s.
252 and *C. scoticus*, and *C. dewulfi* and *C. chiopterus* were the most different. The most similar
253 species within the *C. pulicaris* species complex were *C. punctatus* and *C. newsteadi*, and the
254 most different were *C. pulicaris* and *C. grisescens* (Table 3). The distance values were no
255 higher for pairwise comparisons between the species complexes than for comparisons within
256 them. A low level of polymorphism was observed within the same species from distant
257 geographical regions, which may indicate moderately continuous populations and large scale
258 gene flow.

259

260 3.4. Phylogenetic analysis

261

262 The ML analysis showed strong bootstrap support (95-100%) for nine clades, each of
263 which corresponded to a single, different species (Fig. 2). The analysis also yielded moderate
264 bootstrap support (71%) for the clustering of the trio *Culicoides obsoletus* s.s., *Culicoides*
265 *scoticus* and *Culicoides chiopterus*, but with no support for clustering at any higher level
266 among the nine species. A topology characterised by reciprocal monophyly for each species
267 was also observed using distance and parsimony based optimality criterion (topologies not

268 shown). The resolved topologies were significantly more likely than any topology
269 constrained to be paraphyletic or polyphyletic for any species pair in a Shimodaira-Hasegawa
270 test ($P < 0.001$, Shimodaira and Hasegawa, 1999).

271

272 3.5. Species-specific PCR

273

274 PCR reactions using the species-specific primers designed for the four members of the

275 *C. obsoletus* complex commonly found in the UK (*C. obsoletus* s.s., *C. scoticus*, *C. dewulfi*

276 and *C. chiopterus*), in combination with C1-N-2191 serving as a common reverse primer,

277 were performed using adult specimens belonging to the *C. obsoletus* complex. The agarose

278 gel analysis showed amplification of the target template species for each species-specific

279 primer and no amplified products in non-target species (Fig. 3A-D). The length of PCR

280 products amplified ranged from approx. 200-500bp as expected (Table 4). No amplification

281 was observed in the case of the *C. imicola* sample and the no-template negative controls (Fig.

282 3A-D, lanes 9 & 10). No PCR products were amplified in 13 other *Culicoides* species

283 outwith the *C. obsoletus* complex: *C. acrayi* Kettle & Lawson, 1955, *C. brevitarsis* Kieffer

284 1917, *C. bolitinos* Meiswinkel 1989, *C. circumscriptus* Kieffer, 1918, *C. festipennis* Kieffer,

285 *C. grisescens* Edwards 1939, *C. impunctatus* Goetghebuer 1920, *C. newsteadi* Austen 1921,

286 *C. pulicaris* Linné, 1758, *C. punctatus* Meigen, 1804, *C. puncticollis* Becker, 1903, *C.*

287 *salinaris* Kieffer 1914, *C. stigma* Meigen 1818 (data not shown).

288 The *C. obsoletus* species-specific primers were used in combination in a single-tube

289 multiplex PCR. Multiplex PCR reactions using one individual adult specimen of each of the

290 four main members of the *C. obsoletus* species complex yielded PCR products of expected

291 length (Fig. 4A). Analysis of a mixed sample containing one specimen of each of the four

292 main members of the *C. obsoletus* complex amplified PCR products of the correct estimated

293 length for each species (Fig. 4B).

294 PCR reactions using the species-specific primers designed for the five members of the
295 *C. pulicaris* complex found in the UK (*C. pulicaris* s.s., *C. punctatus*, *C. impunctatus*, *C.*
296 *grisescens* and *C. newsteadi*) were performed using adult specimens belonging to the *C.*
297 *obsoletus* species complex. The agarose gel analysis showed amplification of the target
298 template species for each species-specific PCR and no amplified products for each of the non-
299 target species (Fig. 5A-D), except in the case of the specific primer for *C. newsteadi* which
300 also detected a COI amplicon in *C. impunctatus* (Fig. 5E). Identification of *C. newsteadi* is
301 therefore achieved by performing PCR with each of the *C. newsteadi* and *C. impunctatus*
302 specific primers on the specimen of interest: amplification of a specific product in only the
303 PCR using the *C. newsteadi* specific primer confirms this species. The length of the PCR
304 products amplified ranged from approx. 150-400bp as expected (Table 4). No amplification
305 was observed in the case of the *C. imicola* sample and the no-template negative controls (Fig.
306 5A-E, lanes 6 & 7).

307 **4. Discussion**

308

309 The four species of the *C. obsoletus* species complex and five species of the *C.*
310 *pulicaris* species complex from the UK and continental Europe formed reciprocally
311 monophyletic clades in COI phylogenies. In all cases specimens morphologically identified
312 and from different geographical sources grouped together within these phylogenetic clades.
313 There was no evidence of intraspecific differences existing within the members of the *C.*
314 *obsoletus* complex, *C. scoticus* and *C. dewulfi*, indicating separate species types as observed
315 using the internal transcribed spacer region 2 (ITS2) in a previous study (Gomulski et al.,
316 2005). We observed a clustering of the trio *Culicoides obsoletus* s.s., *Culicoides scoticus* and
317 *Culicoides chiopterus* within the *C. obsoletus* complex with *C. dewulfi* lying outside of this
318 cluster. Although the bootstrap value is low, when combined with the branch length of the *C.*
319 *dewulfi* cluster this may indicate further support for this species to be considered a separate
320 monophyletic lineage within *Avaritia* (as suggested in; Gomulski et al., 2005). In addition,
321 members of the *C. pulicaris* complex showed no intraspecific variation in COI in contrast to
322 ITS2 for *C. newsteadi* (Gomulski et al., 2006). Specimens of *C. scoticus* and *C. dewulfi* were
323 analysed from different geographical localities in the UK, and Greece, and *C. newsteadi* from
324 three sites in Italy, and no intraspecific variation was observed based on COI. Overall, this
325 study illustrates the congruence between the morphological identification and COI molecular
326 characterisation for member species of both the *C. obsoletus* and *C. pulicaris* species
327 complexes, it illustrates the utility of DNA barcoding based on COI (Hebert et al., 2003) in
328 future studies requiring species identification of *Culicoides* species complexes and other
329 genera of arbovirus vector.

330 Our results show that COI is a source of species-diagnostic characters for studies of
331 vector competence in morphologically cryptic members of *Culicoides* species complexes. We
332 have developed species-specific PCR assays for members of the *C. obsoletus* and *C. pulicaris*

333 complexes and a one-tube multiplex PCR assay for members of the *C. obsoletus* complex.
334 This one-tube multiplex PCR can be applied to the analysis of the presence or absence of
335 these species within routine survey collections. Additionally, the species-specific primers
336 may be useful for the identification of *Culicoides* larvae, which can often only be performed
337 readily to genus-level as morphological based differences between species are not well
338 defined. Species identification of the larval stage would allow year round monitoring of
339 *Culicoides* and could be used to study changes in larval biodiversity at farm sites aiding our
340 understanding of bluetongue vector ecology and refining risk assessment at a small
341 geographic scale.

342 It seems clear that the implication of members of the *C. obsoletus* and *C. pulicaris*
343 complexes as vectors of bluetongue is stimulating the systematics of these groups. In the
344 most recent bluetongue outbreaks in northern Europe, *C. dewulfi* was implicated as the major
345 vector (Anonymous, 2006). The process of integrating the morphological taxonomy with
346 molecular characterisation is establishing a useful framework for further studies on vector
347 competence and larval identification. Questions of whether particular species or indeed
348 populations of the same species determine vector competence require further investigation
349 presently to aid risk assessment for potential future incursions of bluetongue in the UK.

350 The species-specific diagnostic assays developed in this study will provide a more
351 rapid, cost effective, and reliable monitoring tool, allowing the geographical distribution of
352 members of the *C. obsoletus* and *C. pulicaris* species complexes across Europe to be further
353 investigated. Additionally, these assays will aid vector competence studies allowing direct
354 studies linking vector susceptibility to infection with bluetongue virus and species
355 identification.

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357

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364

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509 **Figure legends**

510

511 Fig. 1. Locations of the sampling sites in the UK and Europe. Abbreviations of locations are
512 explained in Table 1.

513

514 Fig. 2. Phylogenetic relationships among COI haplotypes of 127 midges of the *Culicoides*
515 *obsoletus* and *Culicoides pulicaris* species complexes. Maximum likelihood tree constructed
516 using a TrN substitution model with a proportion of invariable rates and a γ -distributed rate.
517 Numbers on the nodes represent bootstrap values (10,000 replicates) obtained under distance
518 criterion of a maximum likelihood setting with fast stepwise addition, 4-5 morphologically
519 identified male specimens were used in the case of the *C. obsoletus* complex, in the case of
520 the *C. pulicaris* complex all specimens used were morphologically identified females.

521

522 Fig. 3. Validation of diagnostic PCR primers for identifying members of the *C. obsoletus*
523 species complex. M is hyperladder IV (Bioline), Lanes 1 & 2, *C. chiopterus*, 3 & 4, *C.*
524 *scoticus*, 5 & 6, *C. dewulfi*, 7 & 8, *C. obsoletus* s.s., 9, *C. imicola*, and 10, no-template
525 negative control. The species-specific F primers were: (A) UOAchiF, (B) UOAscoF, (C)
526 UOAdewF and (D) UOAobsF, and the common R primer was C1-N-2191.

527

528 Fig. 4. Validation of a multiplex PCR assay for identifying members of the *C. obsoletus*
529 species complex. Lane 1, *C. dewulfi*, 2, *C. chiopterus*, 3, *C. obsoletus* s.s., 4, *C. scoticus*. (A)
530 Individual adult specimens and (B) mixture of DNA of all four species. M is hyperladder IV
531 (Bioline). The multiplex PCR contained UOAchiF, UOAscoF, UOAdewF, UOAobsF and
532 C1-N-2191 primers.

533

534 Fig. 5. Validation of diagnostic PCR primers for identifying members of the *C. pulicaris*
535 complex. M is hyperladder IV (Bioline), Lane 1, *C. pulicaris*, 2, *C. punctatus*, 3, *C.*
536 *impunctatus*, 4, *C. grisescens*, 5, *C. newsteadi*, 6, *C. imicola*, and 7, no-template negative
537 control. The species-specific F primers were: (A) UOApulF, (B) UOApunF (C) UOAimpF
538 (D) UOAgriF and (E) UOAnewF, and the common R primer was C1-N-2191.

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539 **Tables**

540

541 Table 1

542 Locations and years of sampling of *Culicoides*

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Country	Site	Latitude	Longitude	Year	Site code	N	Species present
UK	Chobham	51° 20' 49'' N	00° 40' 16'' W	2004	CHO	30	OBS (14), SCO (16)
	Ewyas	51° 57' 33'' N	02° 53' 15'' W	2005	EWY	1	CHI
	Keele	53° 00' 01'' N	02° 16' 45'' W	2004	KEE	31	SCO (7), DEW (24)
	Maud	57° 29' 09'' N	02° 11' 04'' W	2004	MAU	8	DEW (4), GRI (1), IMP (2), PUL (1)
	Low Moorhead	53° 59' 57'' N	02° 42' 06'' W	2005	LMH	2	CHI
	Milltimber	57° 11' 02'' N	02° 11' 03'' W	2004	MIL	4	PUL
	Normandy	51° 20' 49'' N	00° 37' 56'' W	2004	NOR	1	SCO
	Ormsary	55° 53' 13'' N	05° 37' 00'' W	2004	ORM	9	SCO (1), PUL (4), GRI (3), PUN (1)
	Rothiemay	57° 33' 02'' N	02° 43' 09'' W	2004	ROT	11	DEW (3), GRI (1), IMP (7)
	Rowett	57° 11' 06'' N	02° 11' 26'' W	2004	ROW	10	DEW (4) , GRI (2), PUN (4)
Wye	51° 17' 07'' N	00° 56' 31'' W	2005	WYE	2	CHI	
Morocco	El Jadida	33° 03' 29'' N	08° 32' 50'' W	2004	JAD	2	SCO
Italy	San Giuliano	43° 44' 20'' N	10° 19' 57'' E	2005	SGI	5	NEW
	Grosetto	42° 43' 45'' N	11° 00' 29'' E	2005	GRO	1	NEW
	Colle Salvetti	43° 35' 03'' N	10° 21' 24'' E	2005	CSA	3	NEW
Bulgaria	Blagoevgrad	41° 35' 18'' N	24° 02' 21'' E	2004	BLA	2	OBS
	Montana	43° 17' 50'' N	22° 57' 55'' E	2004	MON	2	OBS
Greece	Drama	41° 23' 34'' N	24° 16' 23'' E	2004	DRA	3	SCO (2), DEW (1)

544 N, number of insects yielding COI haplotypes. The species present at each site are indicated
 545 using the codes in Table 2.

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Table 2
COI haplotype characteristics and levels of diversity in nine *Culicoides* species

Taxon	Taxon code	N	N _m	N _{hap}	H	π
<i>C. obsoletus</i> complex						
<i>C. obsoletus</i> s.s.	OBS	20	4	9	0.779±0.085	0.00929±0.00261
<i>C. scoticus</i>	SCO	27	5	7	0.655±0.086	0.00299±0.00078
<i>C. dewulfi</i>	DEW	36	5	7	0.510±0.096	0.00174±0.00040
<i>C. chiopterus</i>	CHI	5	5	4	0.900±0.161	0.00551±0.00162
<i>C. pulicaris</i> complex						
<i>C. pulicaris</i> s.s.	PUL	9	9	5	0.861±0.087	0.00365±0.00075
<i>C. impunctatus</i>	IMP	9	9	5	0.833±0.098	0.00259±0.00056
<i>C. punctatus</i>	PUN	5	5	2	0.400±0.237	0.00085±0.00050
<i>C. grisescens</i>	GRI	7	7	4	0.857±0.102	0.00504±0.00138
<i>C. newsteadi</i>	NEW	9	9	2	0.222±0.166	0.00047±0.00035

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569 N, number of insects yielding COI haplotypes; N_m, number of insects identified to species
570 using morphology; in the case of the *C. obsoletus* species complex these were morphologically
571 identified male specimens, in the case of *C. pulicaris* species complex morphologically
572 identified female specimens, N_{hap}, number of COI haplotypes; H, haplotype diversity values;
573 π , nucleotide diversity values.

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Table 3

Pairwise Tamura-Nei+I+ γ genetic distances for COI haplotypes within and between nine *Culicoides* species

	<i>C. obsoletus</i> complex				<i>C. pulicaris</i> complex				
	OBS	SCO	DEW	CHI	PUL	IMP	PUN	GRI	NEW
<i>C. obsoletus</i> s.l.									
OBS	0.010±0.002								
SCO	0.140±0.017	0.003±0.001							
DEW	0.269±0.024	0.247±0.023	0.002±0.001						
CHI	0.172±0.019	0.167±0.019	0.354±0.027	0.006±0.002					
<i>C. pulicaris</i> s.l.									
PUL	0.255±0.022	0.276±0.023	0.272±0.023	0.241±0.022	0.004±0.002				
IMP	0.276±0.024	0.275±0.024	0.285±0.024	0.296±0.025	0.235±0.022	0.003±0.001			
PUN	0.225±0.020	0.227±0.021	0.320±0.026	0.253±0.022	0.249±0.022	0.256±0.024	0.001±0.001		
GRI	0.239±0.022	0.244±0.022	0.292±0.026	0.249±0.022	0.319±0.026	0.249±0.024	0.224±0.022	0.005±0.002	
NEW	0.222±0.022	0.223±0.021	0.288±0.025	0.245±0.023	0.289±0.024	0.227±0.022	0.220±0.020	0.257±0.023	0.000±0.000

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Taxa are indicated according to the codes in Table 2. Within-species distances are shown in boldface on the diagonal.

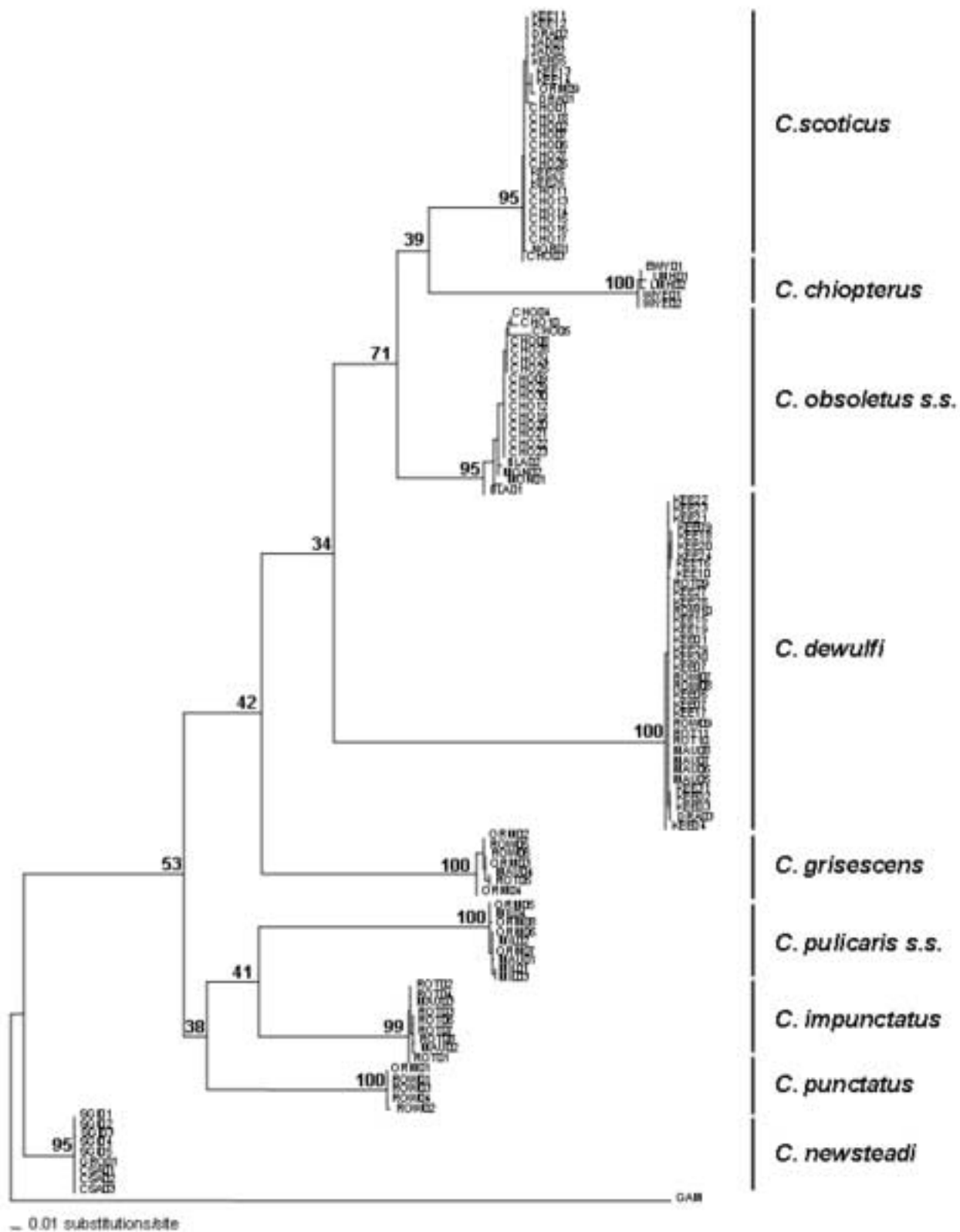
582 Table 4
 583 Species-specific primers for members of the *C. obsoletus* and *C. pulicaris* complexes
 584

Primer	Sequences (5'-3')	T _m (°C)	⁺ Annealing temperature (°C)	[‡] Product length (bp)
<i>C. obsoletus</i> s.s.				
UOAobsF	TGCAGGAGCTTCTGTAGATTTG	59	61	335
<i>C. scoticus</i>				
UOAscoF	ACCGGCATAACTTTTGATCG	60	61	229
<i>C. chiopterus</i>				
UOAchiF	TACCGCCCTCTATCACCCCTA	59	61	435
<i>C. dewulfi</i>				
UOAdeWf	ATACTAGGAGCGCCCGACAT	61	61	493
<i>C. pulicaris</i> s.s.				
UOApulF	CATCCGTAGACTTGGCCATT	60	62	327
<i>C. punctatus</i>				
UOApunF	CTCTTTCGGCCAATGTATCC	60	62	357
<i>C. impunctatus</i>				
UOAimpF	GGAGCATCAGTCGATCTAGCA	61	64	331
<i>C. griseus</i>				
UOAgriF	CCCAGTCTTAGCAGGAGCCATT	61	62	150
<i>C. newsteadi</i>				
UOAnewF	CCCCCTCTTTCAGCAAATATC	60	64	361
* C1-N-2191	CAGGTAAAATTAATAAATAAACTTCTGG			

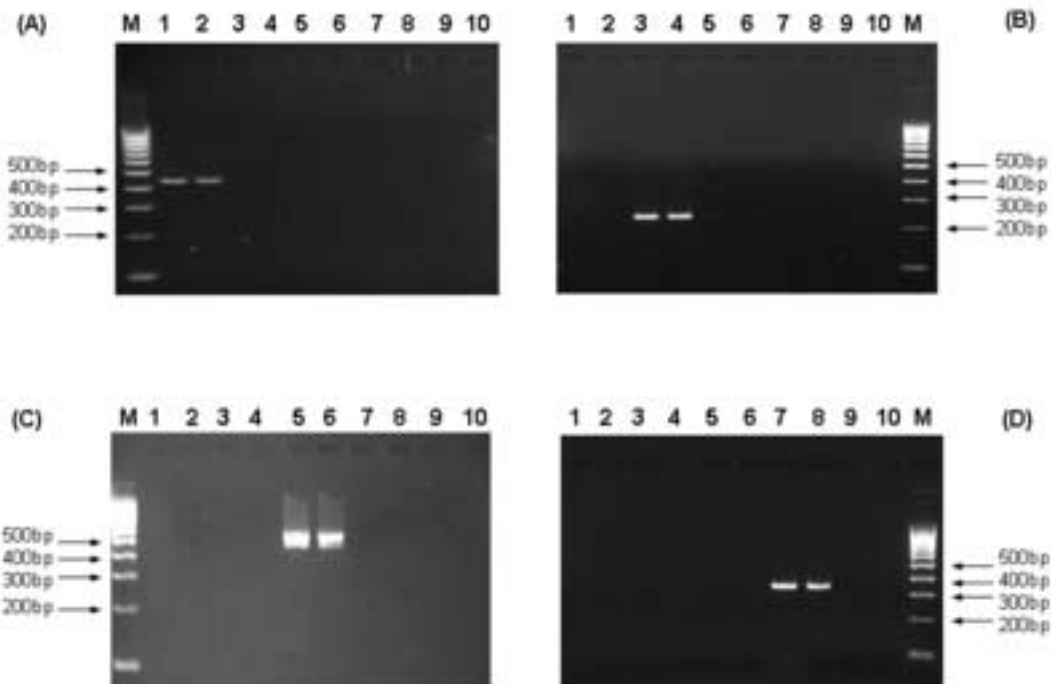
585 * Reverse primer C1-N-2191 (Dallas et al., 2003); [‡] in combination with the reverse primer, C1-N-2191.

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