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

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Abstract

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

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Keywords: COI; *Culicoides*; mtDNA; *C. obsoletus* complex; *C. pulicaria* complex; Species-specific PCR

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1. Introduction

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

The implicated vectors of the virus in these areas are members of both the Culicoides obsoletus and Culicoides plicaris complexes, with the C. obsoletus complex member, Culicoides dewulfi (Anonymous, 2006), being suggested as a significant vector in propagating the BTV-8 in Northern Europe. The northern bluetongue outbreaks were part of four separate epizootics that occurred in Europe during 2006. Other outbreaks occurred in the mid-south, in Sardinia, involving BTV-1 and affecting sheep, and the two other outbreaks were, one in the southeast, in Bulgaria involving BTV-8 (serological evidence only), and one in the southwest in Portugal, involving BTV-4 (OIE, disease information: http://www.oie.int/eng/info/hebdo/a_dsum.htm). In total, 2047 BT outbreaks caused by BTV-8 were reported by EU member states during 2006 in Northern Europe, 456 in The Netherlands, 695 in Belgium, 885 in Germany, 6 in France, and 5 in Luxembourg. In Southern Europe, a total of 241 BT outbreaks were observed, 227 in Sardinia, 13 in Bulgaria, and 1 in Portugal (European Community animal disease notification system: http://ec.europa.eu/food/animal/diseases/adns/table_11/2006.pdf).

The contribution of Culicoides vectors to the northward expansion of bluetongue is thought to be two-fold. First, the main Old World bluetongue vector Culicoides imicola Kieffer 1913 has expanded its range to include several countries bordering the Mediterranean basin (Baylis et al., 1997; Calistrri et al., 2003; Capela et al., 2003; Miranda et al., 2003; Sarto et al., 2003, 2005; Purse et al., 2005). Second, bluetongue outbreaks where C. imicola is either rare or absent (as in parts of Northern, Central, and Eastern Europe) implicate native European Culicoides species as competent vectors. The primary candidates are member species of the C. obsoletus and C. pulicaris complexes, which are abundant and widespread in Palearctic regions particularly in farmland habitats. Circumstantial evidence of their involvement is strong. The spatial and temporal distributions of the two complexes coincide with bluetongue outbreaks (Torina et al., 2004; Purse et al., 2005); bluetongue is present in pools of wild-caught insects (Caracappa et al., 2003; De Liberato et al., 2005; Savini et al., 2005); and bluetongue virus replication to likely transmissible titres occurs in both the C. obsoletus and C. pulicaris complexes under laboratory conditions (Carpenter et al., 2006). In this respect, there are now two foci of BTV transmission in Europe, one being driven by C. imicola which is expanding northward into Southern Europe and another being driven by members of the C. obsoletus and C. pulicaris complexes which exist throughout the rest of Europe.

Further spread of bluetongue virus transmission into the western Palaearctic, and the possibility of a similar, future, incursion of African horse sickness
Improvements in risk assessment of vector-borne BTV and AHSV transmission require an expanded taxonomy of the C. obsoletus and C. pulicaris complexes that identifies species-diagnostic traits that are both easy to analyse and expressed in readily collectable life stages. DNA sequence analysis has been shown to readily identify such traits. Members of C. imicola species complex from southern Africa form distinct lineages based on RAPD markers (Sebastiani et al., 2001) or sequences of the mitochondrial COI gene (Linton et al., 2002; Nolan et al., 2004), and specimens of C. imicola from the Mediterranean basin and South Africa cluster in the same group according to phylogenetic analysis of COI (Dallas et al., 2003). Indeed, members of the C. obsoletus complex can be identified more reliably using species-specific markers in COI (Pagès et al., 2005) or in the intergenic transcribed spacer of ribosomal DNA (Gomulski et al., 2005) than using morphological traits. However, in order to allow routine entomological assessments of distribution and densities of vector species, it is necessary to develop a single diagnostic assay across both the C. obsoletus and C. pulicaris species complexes.

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

2. Materials and methods

2.1. Insects

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

2.2. DNA extraction

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

2.3. Mitochondrial DNA COI gene

A 472 bp segment of the mitochondrial COI gene was PCR amplified from individual midges using the primers C1-J-1718 and C1-N-2191 (Dallas et al., 2003). The reaction volume was 25 μL, consisting of 1× NH4 reaction buffer (Bioline, London, UK), 2.5 mM MgCl2, 200 μM dATP, dCTP, dGTP and dTTP, 1 μM of each primer, and 0.5 units of Taq DNA polymerase (Bioline). The thermal profile consisted of 95 °C for 5 min to activate the Immolase Taq, an initial denaturation step at 92 °C for 2 min 15 s, followed by 30 cycles of 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 for 1 min. PCR products were purified using QIAquick spin columns (Qiagen, Crawley, UK) and were sequenced in both directions using the same PCR primers by MWG-Biotech (Ebersberg, Germany). The COI sequences are deposited in Genbank under accession numbers AM236652–
Table 1
Locations and years of sampling of Culicoides

<table>
<thead>
<tr>
<th>Country</th>
<th>Site</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Year</th>
<th>Site code</th>
<th>N</th>
<th>Species present</th>
</tr>
</thead>
<tbody>
<tr>
<td>UK</td>
<td>Chobham</td>
<td>51°20'49&quot;N</td>
<td>0°40'16&quot;W</td>
<td>2004</td>
<td>CHO</td>
<td>30</td>
<td>OBS (16), SCO (14)</td>
</tr>
<tr>
<td></td>
<td>Ewyas</td>
<td>51°57'33&quot;N</td>
<td>02°53'15&quot;W</td>
<td>2005</td>
<td>EWY</td>
<td>1</td>
<td>CHI</td>
</tr>
<tr>
<td></td>
<td>Keele</td>
<td>53°00'01&quot;N</td>
<td>02°16'45&quot;W</td>
<td>2004</td>
<td>KEE</td>
<td>31</td>
<td>SCO (7), DEW (24)</td>
</tr>
<tr>
<td></td>
<td>Maud</td>
<td>57°29'09&quot;N</td>
<td>02°11'04&quot;W</td>
<td>2004</td>
<td>MAU</td>
<td>8</td>
<td>DEW (4), GRI (1), IMP (2), PUL (1)</td>
</tr>
<tr>
<td></td>
<td>Low Moorhead</td>
<td>53°59'57&quot;N</td>
<td>02°42'06&quot;W</td>
<td>2005</td>
<td>LMH</td>
<td>2</td>
<td>CHI</td>
</tr>
<tr>
<td></td>
<td>Milltimber</td>
<td>57°11'02&quot;N</td>
<td>02°11'03&quot;W</td>
<td>2004</td>
<td>MIL</td>
<td>4</td>
<td>PUL</td>
</tr>
<tr>
<td></td>
<td>Normandy</td>
<td>51°20'49&quot;N</td>
<td>00°37'56&quot;W</td>
<td>2004</td>
<td>NOR</td>
<td>1</td>
<td>SCO</td>
</tr>
<tr>
<td></td>
<td>Ormsary</td>
<td>55°53'13&quot;N</td>
<td>05°37'00&quot;W</td>
<td>2004</td>
<td>ORM</td>
<td>9</td>
<td>SCO (1), PUL (4), GRI (3), PUN (1)</td>
</tr>
<tr>
<td></td>
<td>Rothiemay</td>
<td>57°33'02&quot;N</td>
<td>02°43'09&quot;W</td>
<td>2004</td>
<td>ROT</td>
<td>11</td>
<td>DEW (3), GRI (1), IMP (7)</td>
</tr>
<tr>
<td></td>
<td>Rowett</td>
<td>57°11'06&quot;N</td>
<td>02°11'26&quot;W</td>
<td>2004</td>
<td>ROW</td>
<td>10</td>
<td>DEW (4), GRI (2), PUN (4)</td>
</tr>
<tr>
<td></td>
<td>Wye</td>
<td>51°17'07&quot;N</td>
<td>00°56'31&quot;W</td>
<td>2004</td>
<td>WYE</td>
<td>2</td>
<td>CHI</td>
</tr>
<tr>
<td>Morocco</td>
<td>El Jadida</td>
<td>33°03'29&quot;N</td>
<td>08°32'50&quot;W</td>
<td>2004</td>
<td>JAD</td>
<td>2</td>
<td>SCO</td>
</tr>
<tr>
<td>Italy</td>
<td>San Giuliano</td>
<td>43°44'20&quot;N</td>
<td>10°19'57&quot;E</td>
<td>2005</td>
<td>SGI</td>
<td>5</td>
<td>NEW</td>
</tr>
<tr>
<td></td>
<td>Grosseto</td>
<td>42°43'45&quot;N</td>
<td>11°00'29&quot;E</td>
<td>2005</td>
<td>GRO</td>
<td>1</td>
<td>NEW</td>
</tr>
<tr>
<td></td>
<td>Colle Salvetti</td>
<td>43°35'03&quot;N</td>
<td>10°21'24&quot;E</td>
<td>2005</td>
<td>CSA</td>
<td>3</td>
<td>NEW</td>
</tr>
<tr>
<td>Bulgaria</td>
<td>Blagoevgrad</td>
<td>41°35'18&quot;N</td>
<td>24°02'21&quot;E</td>
<td>2004</td>
<td>BLA</td>
<td>2</td>
<td>OBS</td>
</tr>
<tr>
<td></td>
<td>Montana</td>
<td>43°17'50&quot;N</td>
<td>22°57'55&quot;E</td>
<td>2004</td>
<td>MON</td>
<td>2</td>
<td>OBS</td>
</tr>
<tr>
<td>Greece</td>
<td>Drama</td>
<td>41°23'34&quot;N</td>
<td>24°16'23&quot;E</td>
<td>2004</td>
<td>DRA</td>
<td>3</td>
<td>SCO (2), DEW (1)</td>
</tr>
</tbody>
</table>

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

AM236671 (C. obsoletus s.s.), AM236625–AM236651 (Culicoides scoticus), AM236672–AM236707 (C. dewulfi), AM236747–AM236751 (Culicoides chiopterus), AM236708–AM236716 (C. pulicaris), AM236717–AM236725 (C. impunctatus), AM236726–AM236732 (Culicoides grisescens), AM236733–AM236737 (C. punctatus), and AM236738–AM236746 (Culicoides newsteadi).

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2.4. Data analysis

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

The phylogenetic relationships among individual sequences were determined using a maximum likelihood (ML) phylogeny generated within PAUP v 4b10 (Swofford, 1998). The most appropriate model for DNA substitution among haplotypes was identified using MODELTEST v 3.06 (Posada and Crandall, 1998). The Tamura-Nei (TrN) model chosen incorporated base frequencies of A, C, G, T of 0.3437, 0.1923, 0.0867, 0.3774, respectively, with proportion of invariable sites (I) = 0.5244 and gamma distribution shape parameter (\( \gamma \)) = 0.7650. The stability of resultant clades was assessed using bootstrap analysis (10,000 iterations) within a neighbour joining tree constructed with the same model of sequence evolution as the ML analysis.

2.5. Species-specific PCR

Sequence alignments of the four members of the C. obsoletus and five members of C. pulicaris complexes found in the UK were used to design species-specific primers using the Primer 3 software (Rozen and Skaletsky, 2003). The identified primers given in Table 4 were tested in 25 \( \mu \)L PCR reactions, consisting of 1\( \times \) NH\(_4\) reaction buffer (Bioline, London, UK), 2.5 mM MgCl\(_2\), 200 \( \mu \)M dATP, dCTP, dGTP and dTTP, 1 \( \mu \)M of each primer and 0.5 units of Immolase DNA polymerase (BioLine). The PCR reactions were carried out on a PTC-100 Programmable Thermal Controller (MJ Research Inc., Water-}

town, MA, USA) under the following conditions: an initial denaturation step at 92°C for 2 min 15 s, followed by 30 cycles of 92°C for 15 s, X°C (where X is the annealing temperature indicated for each species-specific primer, Table 4) for 15 s, 72°C for 30 s, and ending with a final elongation step at 72°C for 1 min. PCR products were examined using electrophoresis on a 2.0% agarose gel with ethidium bromide staining.

Each species-specific primer in combination with the C1-N-2191 primer were tested against members of the same species complex and other commonly collected Culicoides species to confirm specificity of each species-specific primer. The multiplex PCR for the C. obsoletus complex was identical to above, except, 1 \( \mu \)M of UOAbFsF, UOAsoF, UOAchIF, UOAdeWF and C1-N-2191 primers were used and the annealing temperature was 61°C. Products were examined using electrophoresis on a 3.0% AquaPor HR GTAC agarose gel (National Diagnostics, Atlanta, GA, USA) with ethidium bromide staining.

3. Results

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

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

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

A total of 45 COI haplotypes (472 bp) were obtained from the 127 Culicoides specimens (108 females and 19 males, Table 2). Haplotype diversity within each member species ranged from

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Table 2

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

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

N, number of insects yielding COI haplotypes; N_m, number of insects identified to species using morphology; in the case of the *C. obsoletus* species complex these were morphologically identified male specimens, in the case of *C. pulicaris* species complex morphologically identified female specimens, N_hap, number of COI haplotypes; H, haplotype diversity values; π, nucleotide diversity values.

0.222 ± 0.166 (C. newsteadi) to 0.900 ± 0.161 (C. chiopterus) (Table 2). The *C. obsoletus* s.s. sequences contained three nonsynonymous changes; a C → T transition at position 189, and A → T transversions at positions 47 and 260. The *C. scoticus* sequences contained one nonsynonymous change at position 397, an A → T transversion, and the *C. dewulfi* sequences contained three nonsynonymous changes, an A → G transition at position 107, and C → T transitions at positions 366 and 384. All other intraspecific substitutions were synonymous. The base composition of the *Culicoides* COI sequences was A + T-biased. Mean A + T content within species was 60.8–65.4%, and A + T content was highest at the third codon position (51.6–58.6% at position 1, 55.4–57.3% at position 2 and 71.5–84.8% at position 3).

3.3. Sequence divergence

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

3.4. Phylogenetic analysis

The ML analysis showed strong bootstrap support (95–100%) for nine clades, each of which corresponded to a single, different species (Fig. 2). The analysis also yielded moderate bootstrap support (71%) for the clustering of the trio *C. obsoletus* s.s., *Culicoides scoticus* and *C. chiopterus*, but with no support for clustering at any higher level among the nine species. A topology characterised by reciprocal monophyly for each species was also observed using distance and parsimony based optimality criterion (topologies not shown). The resolved topologies were significantly more likely than any topology constrained to be paraphyletic or polyphyletic for any species pair in a Shimodaira–Hasegawa test (*P* < 0.001, Shimodaira and Hasegawa, 1999).
3.5. Species-specific PCR

PCR reactions using the species-specific primers designed for the four members of the *C. obsoletus* complex commonly found in the UK (*C. obsoletus* s.s., *C. scoticus*, *C. dewulfi* and *C. chiopterus*), in combination with C1-N-2191 serving as a common reverse primer, were performed using adult specimens belonging to the *C. obsoletus* complex. The agarose gel analysis showed amplification of the target template species for each species-specific primer and no amplified products in non-target species (Fig. 3A–D). The length of PCR products amplified ranged from approximately 200 to 500 bp as expected (Table 4). No amplification was observed in the case of the *C. imicola* sample and the no-template negative controls (Fig. 3A–D, lanes 9 and 10). No PCR products were amplified in 13 other *Culicoides* species outwith the *C. obsoletus* complex: *C. acrayi* Kettle and Lawson, 1955, *C. brevitarsis* Kieffer 1917, *C. bolitinos* Meiswinkel 1989, *C. circumscriptus* Kieffer, 1918, *C. festipennis* Kieffer, 1913, *C. grisescens* Edwards 1939, *C. impunctatus* Goetghebuer 1920, *C. newsteadi* Austen 1921, *C. pulicaris* Linné, 1758, *C. punctatus* Meigen, 1804, *C. puncticollis* Becker, 1903, *C. salinaris* Kieffer 1914, *C. stigma* Meigen 1818 (data not shown).

The *C. obsoletus* species-specific primers were used in combination in a single-tube multiplex PCR. Multiplex PCR reactions using one individual adult specimen of each of the four main members of the *C. obsoletus* species complex yielded PCR products of expected length (Fig. 4A). Analysis of a mixed sample containing one specimen of each of the four main members of the *C. obsoletus* complex amplified PCR products of the correct estimated length for each species (Fig. 4B).

PCR reactions using the species-specific primers designed for the five members of the *C. pulicaris* complex found in the UK (*C. pulicaris* s.s., *C. punctatus*, *C. impunctatus*, *C. grisescens* and *C. newsteadi*) were performed using adult specimens belonging to the *C. obsoletus* species complex. The agarose gel analysis showed amplification of the target template species for each species-specific PCR and no amplified products for each of the non-target species (Fig. 5A–D), except in the case of the specific primer for *C. newsteadi* which also detected a COI amplicon.
Fig. 2. Phylogenetic relationships among COI haplotypes of 127 midges of the *Culicoides obsoletus* and *Culicoides pulicaris* species complexes. Maximum likelihood tree constructed using a TrN substitution model with a proportion of invariable rates and a $\gamma$-distributed rate. Numbers on the nodes represent bootstrap values (10,000 replicates) obtained under distance criterion of a maximum likelihood setting with fast stepwise addition. 4–5 morphologically identified male specimens were used in the case of the *C. obsoletus* complex, in the case of the *C. pulicaris* complex all specimens used were morphologically identified females.

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

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

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequences (5′–3′)</th>
<th>$T_m$ (°C)</th>
<th>Annealing temperature(^a) (°C)</th>
<th>Product length(^b) (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. obsoletus</em> s.s.</td>
<td>UOAobsF TGCAGGAGCTTCTGTAGATTTG</td>
<td>59</td>
<td>61</td>
<td>335</td>
</tr>
<tr>
<td><em>C. scoticus</em></td>
<td>UOAscoF ACCGGCATAACTTTTGATCG</td>
<td>60</td>
<td>61</td>
<td>229</td>
</tr>
<tr>
<td><em>C. chiopterus</em></td>
<td>UOAchiF TACCGCCCTCTATACCCCTA</td>
<td>59</td>
<td>61</td>
<td>435</td>
</tr>
<tr>
<td><em>C. dewulfi</em></td>
<td>UOAdewF ATACTAGGAGCGCCGGACAT</td>
<td>61</td>
<td>61</td>
<td>493</td>
</tr>
<tr>
<td><em>C. pulicaris</em> s.s.</td>
<td>UOApulF CATCCGTAGACTTGGCCATT</td>
<td>60</td>
<td>62</td>
<td>327</td>
</tr>
<tr>
<td><em>C. punctatus</em></td>
<td>UOApunF CTCTTTCGGCCAATGTATCC</td>
<td>60</td>
<td>62</td>
<td>357</td>
</tr>
<tr>
<td><em>C. impunctatus</em></td>
<td>UOAimpF GGAGCATCAGTCGATCTAGCA</td>
<td>61</td>
<td>64</td>
<td>331</td>
</tr>
<tr>
<td><em>C. grisescens</em></td>
<td>UOAgrif CCCCAGTCTTAGCAGGAGCCATT</td>
<td>61</td>
<td>62</td>
<td>150</td>
</tr>
<tr>
<td><em>C. newsteadi</em></td>
<td>UOAnewF CCCCCTTTTCAGCAAATATC</td>
<td>60</td>
<td>64</td>
<td>361</td>
</tr>
<tr>
<td>C1-N-2191(^c)</td>
<td>CAGGTAAAATATTTATAATAATTTCTTGG</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) In combination with the reverse primer, C1-N-2191.

\(^b\) In combination with the reverse primer, C1-N-2191.

\(^c\) Reverse primer C1-N-2191 (Dallas et al., 2003).
in *C. impunctatus* (Fig. 5E). Identification of *C. newsteadi* is therefore achieved by performing PCR with each of the *C. newsteadi* and *C. impunctatus* specific primers on the specimen of interest: amplification of a specific product in only the PCR using the *C. newsteadi* specific primer confirms this species. The length of the PCR products amplified ranged between approximately 150 to 400 bp as expected (Table 4). No amplification was observed in the case of the *C. imicola* sample and the no-template negative controls (Fig. 5A–E, lanes 6 and 7).

4. Discussion

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

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Our results show that COI is a source of species-diagnostic characters for studies of vector competence in morphologically cryptic members of Culicoides species complexes. We have developed species-specific PCR assays for members of the C. obsoletus and C. pulicaris complexes and a one-tube multiplex PCR assay for members of the C. obsoletus complex. This one-tube multiplex PCR can be applied to the analysis of the presence or absence of these species within routine survey collections. Additionally, the species-specific primers may be useful for the identification of Culicoides larvae, which can often only be performed readily to genus-level as morphological based differences between species are not well defined. Species

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

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identification of the larval stage would allow year round monitoring of Culicoides and could be used to study changes in larval biodiversity at farm sites aiding our understanding of bluetongue vector ecology and refining risk assessment at a small geographic scale.

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

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

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References


