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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. pulicarus* 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. pulicaris* 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. pulicaris* 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*.

Keywords: COI; *Culicoides*; mtDNA; *C. obsoletus* complex; *C. pulicaris* 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 ASHV and BTV. The ASHV 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 pulicaris* 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; Calistri 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

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

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× NH_4 reaction buffer (Bioline, London, UK), 2.5 mM $MgCl_2$, 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–

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Table 1
Locations and years of sampling of *Culicoides*

Country	Site	Latitude	Longitude	Year	Site code	*N*	Species present
UK	Chobham	51°20′49″N	00°40′16″W	2004	CHO	30	OBS (16), SCO (14)
	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)

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*).

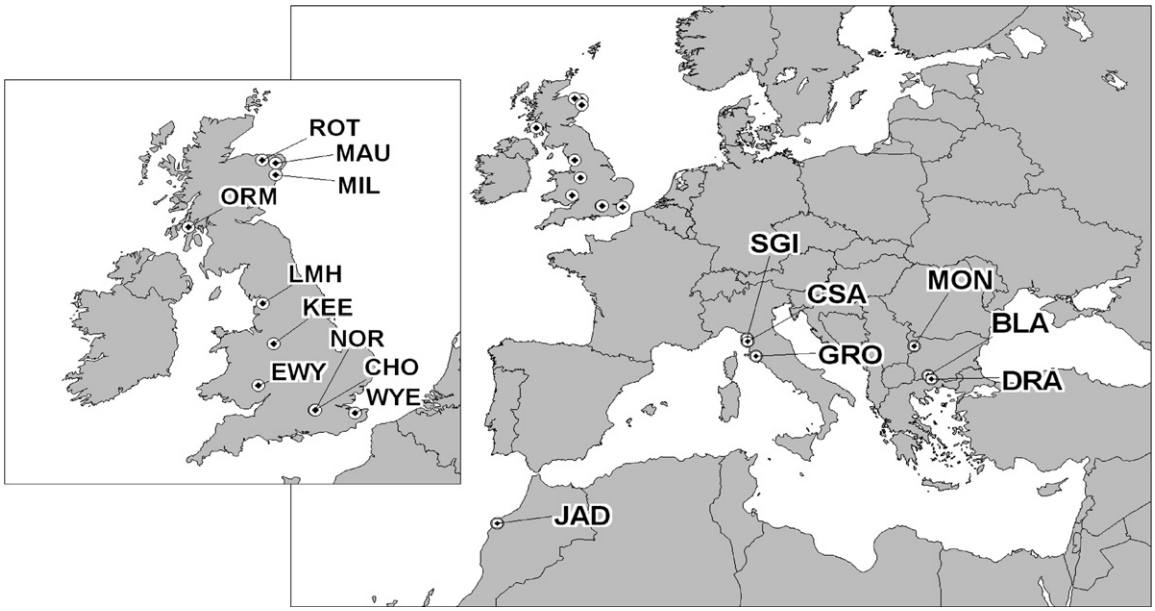

Fig. 1. Locations of the sampling sites in the UK and Europe. Abbreviations of locations are explained in Table 1.

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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 (γ) = 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 μL PCR reactions, consisting of 1× NH_4 reaction buffer (Bioline, London, UK), 2.5 mM $MgCl_2$, 200 μM dATP, dCTP, dGTP and dTTP, 1 μM 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., Watertown, 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 μM of UOAobsF, UOAscoF, 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

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

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

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 (toplogies not shown). The resolved toplologies 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).

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Table 3
Pairwise Tamura-Nei + I + γ genetic distances for COI haplotypes within and between nine *Culicoides* species

	C. obsoletus complex				*C. pulicaris* complex				
	OBS	SCO	DEW	CHI	PUL	IMP	PUN	GRI	NEW
C. obsoletus 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**					
C. pulicaris 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**

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

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, *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

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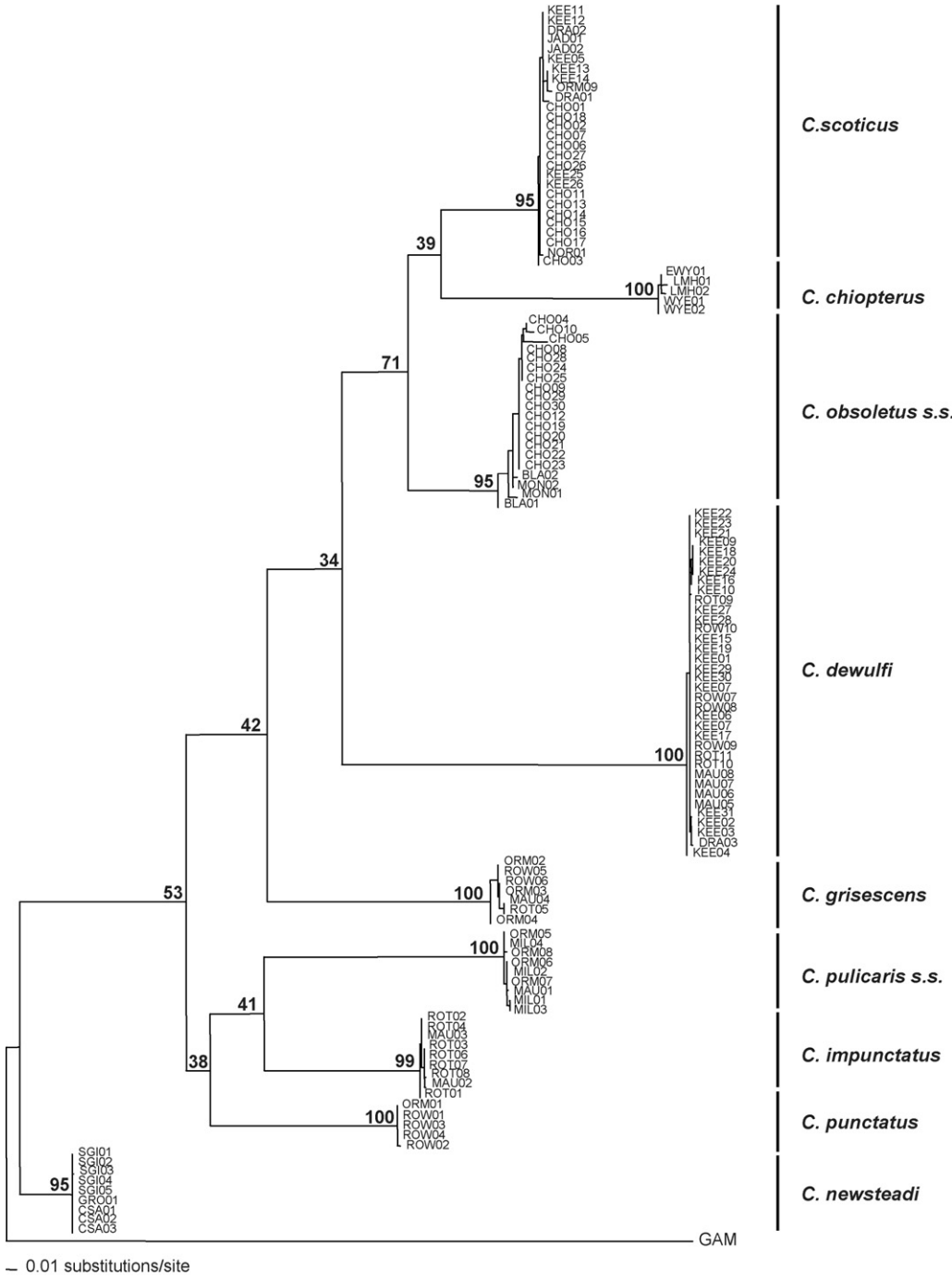

Fig. 2. Phylogenetic relationships among COI haplotypes of 127 midges of the *Culicoides obsoletus* and *Culicoides pulicaris* species complexes. Maximum likelihood tree constructed using a TrN substitution model with a proportion of invariable rates and a γ-distributed rate. Numbers on the nodes represent 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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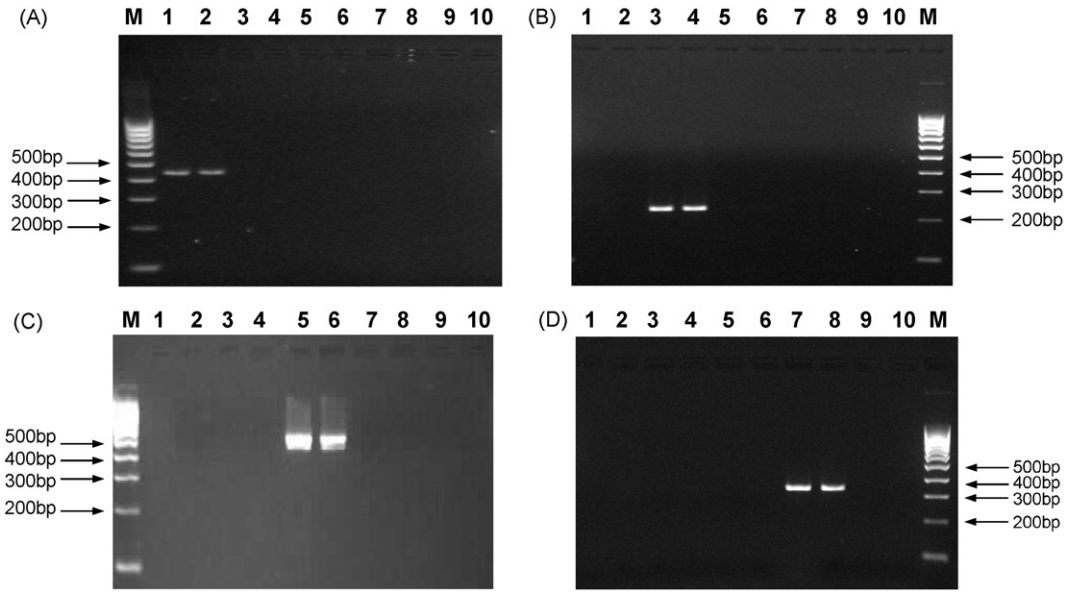

Fig. 3. Validation of diagnostic PCR primers for identifying members of the *C. obsoletus* species complex. M is hyperladder IV (Bioline), Lanes 1 and 2, *Culicoides chiopterus*, 3 and 4, *Culicoides scoticus*, 5 and 6, *C. dewulfi*, 7 and 8, *C. obsoletus* s.s., 9, *Culicoides imicola*, and 10, no-template 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

Primer	Sequences (5′–3′)	T_m (°C)	Annealing temperature[a] (°C)	Product length[b] (bp)
C. obsoletus s.s.				
UOAobsF	TGCAGGAGCTTCTGTAGATTTG	59	61	335
C. scoticus				
UOAscoF	ACCGGCATAACTTTTGATCG	60	61	229
C. chiopterus				
UOAchiF	TACCGCCCTCTATCACCCTA	59	61	435
C. dewulfi				
UOAdewF	ATACTAGGAGCGCCCGACAT	61	61	493
C. pulicaris s.s.				
UOApulF	CATCCGTAGACTTGGCCATT	60	62	327
C. punctatus				
UOApunF	CTCTTTCGGCCAATGTATCC	60	62	357
C. impunctatus				
UOAimpF	GGAGCATCAGTCGATCTAGCA	61	64	331
C. grisescens				
UOAgriF	CCCAGTCTTAGCAGGAGCCATT	61	62	150
C. newsteadi				
UOAnewF	CCCCCTCTTTCAGCAAATATC	60	64	361
C1-N-2191[c]	CAGGTAAAATTAAAATATAAACTTCTGG			

[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).

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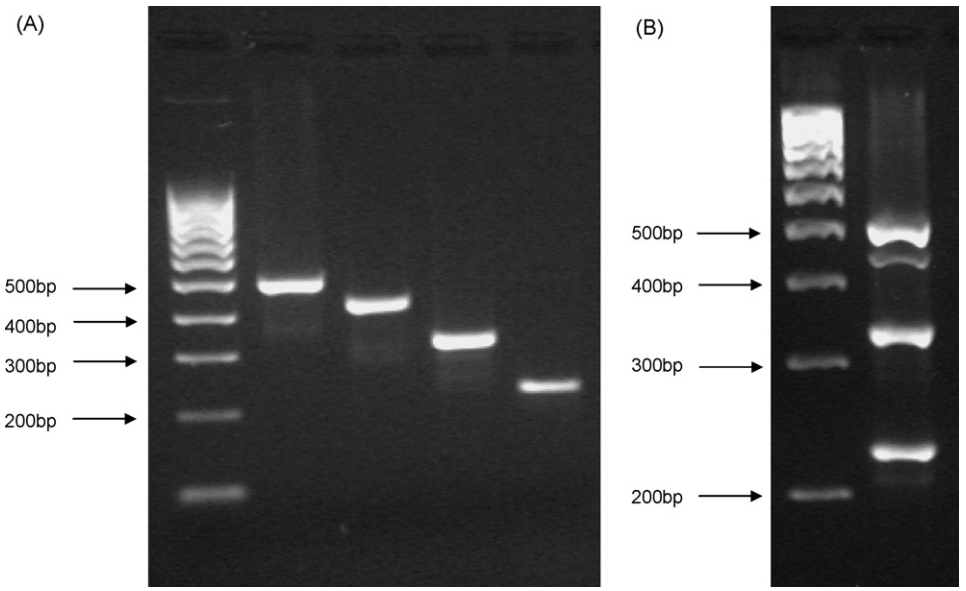

Fig. 4. Validation of a multiplex PCR assay for identifying members of the *C. obsoletus* species complex. Lane 1, *C. dewulfi*, 2, *C. chiopterus*, 3, *C. obsoletus* s.s., 4, *C. scoticus*. (A) Individual adult specimens and (B) mixture of DNA of all four species. M is hyperladder IV (Bioline). The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

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 intraspecfic 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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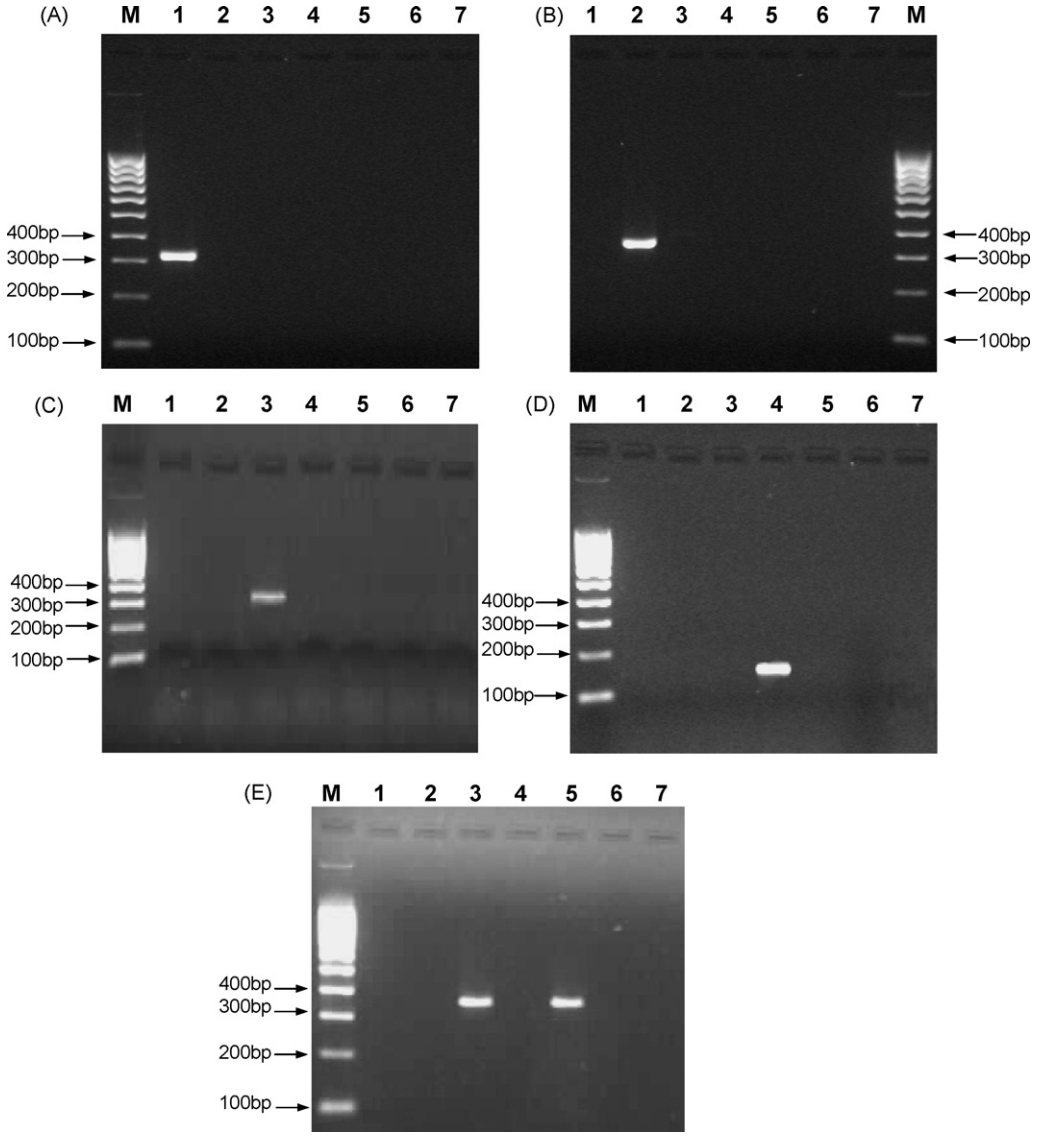

Fig. 5. Validation of diagnostic PCR primers for identifying members of the *C. pulicaris* complex. M is hyperladder IV (Bioline), Lane 1, *C. pulicaris*, 2, *Culicoides punctatus*, 3, *C. impunctatus*, 4, *C. grisescens*, 5, *Culicoides newsteadi*, 6, *C. imicola*, and 7, no-template negative control. 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.

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

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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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