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Phylogenetic analysis indicates that *Culicoides dewulfi* should not be considered part of the *Culicoides obsoletus* complex

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Abstract

Analysis of DNA sequence data has proven invaluable for defining the relationships among taxa, as well as resolving their evolutionary histories. Here, we analyzed DNA sequence variation of one mitochondrial gene (COI) and two nuclear regions (ITSI and II) to clarify the phylogenetic position of *Culicoides dewulfi*, a midge species widely spread in Europe and a suspected vector for bluetongue virus. Various authors have described *C. dewulfi* either as part of the *Culicoides obsoletus sensu lato* complex or as a separate taxonomic group. A maximum likelihood phylogeny, based upon an optimal model of sequence evolution, placed *C. dewulfi* outwith the *C. obsoletus s.l.* complex. Shimodaira-Hasegawa test highlighted that this topology was significantly more likely than any topology that placed *C. dewulfi* anywhere else in the phylogeny. As such, *C. dewulfi* should not be considered part of the *C. obsoletus s.l.* complex and instead be treated as a separate group, phylogenetically close to the classical Old World vector *C. imicola*.

Keywords: *Culicoides dewulfi*, *C. obsoletus s.l.* complex, genetic barcoding, phylogeny

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Introduction

Phylogenetic analysis of DNA sequence data, in conjunction with associated approaches such as identification of species based on DNA sequences (barcoding), has facilitated both the identification of robust taxonomic groupings and also helped to clarify their systematic interrelationships (Besansky *et al*., 2003; Hajibabaei *et al*., 2007). Such approaches have proven most useful in highly speciose taxa, especially where morphological similarity precludes identification of species using traditional cladistic approaches (Hebert *et al*., 2004; Smith *et al*., 2006).

DNA-derived phylogenies have proven extremely useful in defining the biodiversity and systematics of midges of the genus *Culicoides* Latreille 1809. *Culicoides* midges are the major vectors of bluetongue virus (BTV), African horse sickness virus (AHSV) and epizootic hemorrhagic disease virus (EHDV) of the family *Reoviridae*. BTV infection can cause severe clinical disease, primarily in sheep, leading to high levels of mortality, with symptoms including oedema in the pharyngeal cavity and nasal discharge. Cattle and wild ruminants can develop a viraemia, but show fewer signs of disease. The recent incursion and spread of bluetongue disease in Europe has been linked to climate change, with warmer winters and higher precipitation providing conditions which promote the survival of midge vectors (Purse

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et al., 2005). The classical vector, *Culicoides imicola*, has been found in areas where it has not previously been present. Between 1998 and 2005, outbreaks of bluetongue affected 12 countries in the Mediterranean and central European regions about 800 km north of the expected geographic range. Moreover, expansion and propagation of BTV in these areas has been facilitated by endemic palaearctic species. Two widespread and abundant species groups are the *Culicoides obsoletus sensu lato* group and the *Culicoides pulicaris sensu lato* group. Members of both species groups have been shown to be able to transmit BTV (Carpenter *et al.*, 2006), and the overlapping geographical expansion of *C. imicola* and palaearctic species facilitated a crossover of the infection. In 2006, outbreaks of bluetongue serotype 8 were observed for the first time in The Netherlands, Belgium, Germany, France and Luxembourg (Meiswinkel *et al.*, 2007; Wilson *et al.*, 2007); and, in 2007, the first cases of BTV-8 were observed in southern England.

There are 30 *Culicoides* species involved in the transmission of orbiviral diseases. The internal taxonomy of the genus *Culicoides* has traditionally relied mainly on morphological identification based upon variation in wing pattern and male genitalia (Boorman, 1986). Therefore, in many cases, only males can be differentiated, even by specialists; and such traits are used without any recourse to potential phenotypic plasticity or geographic variation among individuals or populations. Moreover, this morphological variation cannot be used to gauge evolutionary distance and, thus, does not allow relative placement of the individual species in a broader *Culicoides* phylogeny (Edwards, 1939; Downes & Kettle, 1952; Khalaf, 1954; Fox, 1955). Application of DNA-based phylogenetic analysis has facilitated reliable species identification within the species complexes. Sequence analyses based on variations of the mitochondrial cytochrome oxidase I (COI) gene (Linton *et al.*, 2002; Dallas *et al.*, 2003; Nolan *et al.*, 2007) and the internal transcribed spacer regions I (ITSI) (Perrin *et al.*, 2006; Mathieu *et al.*, 2007) and II (ITSII) (Gomulski *et al.*, 2006) have been used to delimit species, primarily to develop diagnostic DNA-barcoding assays for the rapid and economical identification of individuals of unknown species provenance.

Less effort has been focussed on phylogenetic relationships among the species. In the context of BTV transmission, related species may express similar abilities to transmit the virus, which is the main motivation to resolve the exact phylogeny of the genus *Culicoides*. A major unresolved issue is the phylogenetic position of *C. dewulfi*, which is found from Britain to Eastern Europe. The importance of *C. dewulfi* as a bluetongue vector results from its breeding behaviour in cow dung. As a reservoir host for BTV, cattle can become viraemic without developing clinical signs of bluetongue disease characteristic of sheep, which facilitates the silent spread of the disease and also represents a possible overwintering mechanism. The role of *C. dewulfi* as a vector is further underlined by its affinity with species of the *C. obsoletus s.l.* complex. Insect trapping in central Europe revealed that the main vector for BTV in this climate zone is *C. obsoletus* due to dominant presence (Mehlhorn *et al.*, 2007). *C. dewulfi* has, however, also been trapped in bluetongue-affected areas along with *C. obsoletus sensu strictu* and *C. scoticus* (Meiswinkel *et al.*, 2007).

Several publications have discussed the phylogenetic position of *C. dewulfi*. It belongs to the subgenus *Avaritia* along with *C. imicola* and the *C. obsoletus s.l.* complex. Some consider it a member of the *C. obsoletus s.l.* complex (Savini *et al.*, 2005; Mathieu *et al.*, 2007; Nolan *et al.*, 2007), while other sources have preferred to keep the species as a separate entity outwith the *C. obsoletus s.l.* complex (Conte *et al.*, 2007; Meiswinkel *et al.*, 2007). All phylogenetic studies so far have used only a small region of a single gene to resolve variation among *Culicoides* taxa, which precludes robust phylogenetic placement of *C. dewulfi*, and also introduces the potential that the phylogenies produced are actually gene trees and not true representations of species relationships (Gomulski *et al.*, 2006; Perrin *et al.*, 2006; Nolan *et al.*, 2007). Here, we analyse the phylogenetic relationship among the *Culicoides* species, based on DNA sequence variation from three concatenated genetic markers, to produce a more robust phylogeny based upon combined nuclear and mitochondrial variation. We show that *C. dewulfi* indeed is a phylogenetically distinct clade positioned between the *C. obsoletus s.l.* complex and *C. imicola*.

Material and methods

Data mining

The majority of sequences were retrieved by database search. COI sequences were obtained for *C. obsoletus* (AM236652), *C. scoticus* (AM236625), *C. dewulfi* (AM236672), *C. chiopterus* (AM236751), *C. pulicaris* (AM236708), *C. punctatus* (AM236733), *C. impunctatus* (AM236717), *C. newstaedi* (AM236738), *C. imicola* (EU189056), and *Aedes aegypti* (DQ792578). ITSI sequences were obtained for *C. obsoletus* (AY861152), *C. scoticus* (AY861160), *C. dewulfi* (DQ408045), *C. chiopterus* (DQ408543), *C. pulicaris* (AY861156), *C. punctatus* (AY861157), *C. impunctatus* (AJ417986), *C. newsteadi* (AY861151), *C. imicola* (AY861144), and *A. aegypti* (M95126). ITSII sequences were obtained for *C. obsoletus* (AY599780), *C. scoticus* (AY599796), *C. dewulfi* (AY599818), *C. pulicaris* (DQ371264), *C. punctatus* (DQ371246), *C. newsteadi* (DQ371257), *C. imicola* (AY599832), and *A. aegypti* (M95126).

PCR

ITSII sequences for *C. chiopterus* (EU8206) and *C. impunctatus* (EU908207) were amplified by PCR using the primers 5′gatgaagaccgcagcaaact3′ and 5′atttgggggtagtcacacat3′ (Gomulski *et al.*, 2006) in a volume of 50 µl using the HotStarTaq Mastermix Kit (Qiagen, UK) with 0.4 µM of each primer and approximately 20 ng of DNA. Amplifications were done by an initial denaturation at 94°C for 15 min, then 32 cycles of denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s, primer extension at 72°C for 1 min and, then, a final extension phase at 72°C for 6 min. PCR products were purified with QIAquick columns (QIAGEN, UK). Sequencing was performed at Eurofins MWG Operon (Martinsried, Germany).

COI, ITSI and ITSII sequences for each species were concatenated.

Phylogenetic analysis

The sequences were aligned in Bioedit version 5.0.9 (www.mbio.ncsu.edu/BioEdit). The phylogenetic relationships among taxa were resolved using a maximum likelihood approach in PAUP v4b10 (Swofford, 1998), with the resultant topology rooted using *A. aegypti*. The maximum

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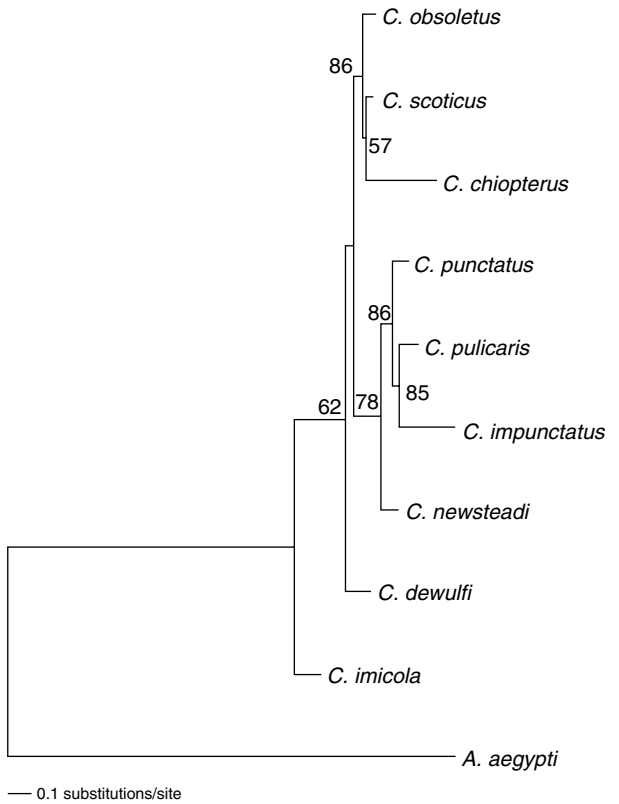

Fig. 1. Phylogenetic relationships among COI, ITSI and ITSII sequences of nine *Culicoides* species and the outgroup species *Aedes aegypti*, based on a maximum likelihood analysis. The values at the nodes indicate the percentage of times the clade to the right was retained when the tree was redrawn (1000 times) using randomly sampled data with replacement from the original dataset (bootstrapping).

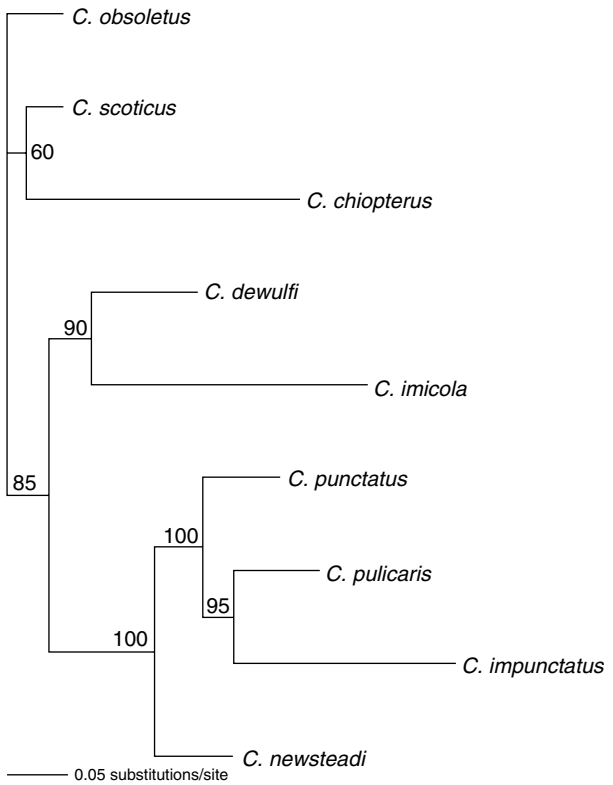

Fig. 2. Phylogenetic tree based on a maximum likelihood analysis using COI, ITSI and ITSII sequences of nine *Culicoides* species without an outgroup species.

likelihood method was preferred to other methods because it allows phylogenetic analysis based on probabilistic models of molecular evolution.

The program MODELTEST v3.06 (Posada & Crandall, 1998) was used to determine the most suitable model of DNA substitution. This analysis was done without the *A. aegypti* DNA sequence data included to optimise the phylogenetic relationship of *Culicoides* species. The model of evolution identified by the Akaike Information Criterion (AIC) had base frequencies of A = 0.3132, C = 0.1598, G = 0.1730, T = 0.3540; a proportion of invariable sites of 0; and a Gamma distribution shape parameter of 0.6538. The rate matrix parameters were A to C = 1.4169, A to G = 2.2331, A to T = 1.6856, C to G = 0.8305, C to T = 3.4798 and T to G = 1. These parameters were used in a heuristic search, and the reliability of the resulting tree topology was ascertained by bootstrapping (1000 replicates). Insertion and deletion mutations were removed from the analysis.

To test the hypothesis that *C. dewulfi* groups outside the *C. obsoletus s.l.* complex, the position of *C. dewulfi* was exchanged with other species within the phylogeny such that the number of different constrained trees in the file, each containing a different *C. dewulfi* position, equalled the number of species. Shimodaira-Hasegawa (SH) tests (Shimodaira & Hasegawa, 1999) under full maximum likelihood optimisation were used to examine whether the optimal tree was significantly more likely than any of the constrained trees under the same model of sequence evolution. The SH test provides a likelihood-based statistical test of competing evolutionary hypotheses that have not been specified *a priori*, but instead when one of the topologies is the maximum likelihood topology for the dataset that was analysed; as such, the SH test details whether the resolved optimal topology is more likely than any topology constrained to satisfy an alternative hypothesis (Goldman *et al*., 2000).

Results

A maximum likelihood phylogenetic analysis, derived from an alignment of COI, ITSI and ITSII sequences of the nine Culicoides species and *A. aegypti* as an outgroup, resulted in a tree with clades that were well supported by bootstrapping (fig. 1). The species *C. obsoletus*, *C. scoticus* and *C. chiopterus* formed one clade. The species *C. pulicaris*, *C. punctatus*, *C. impunctatus* and *C. newsteadi* formed a second clade. *C. dewulfi* grouped well outside these two clades, as did *C. imicola*. The topology of the tree was consistent with those derived from maximum parsimony and distance-based neighbour-joining analyses (data not shown).

Removal of the *A. aegypti* outgroup did not change the topology of the *C. obsoletus* and *C. pulicaris* complexes nor their high level of bootstrap support as reciprocally monophyletic clades. However, *C. dewulfi* grouped with *C. imicola*,

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Table 1. Relative maximum likelihood scores for resolved phylogeny and phylogenies constrained to place *C. dewulfi* elsewhere within the topology. Probability values, *P*, that the constrained topology is less likely as the resolved topology are provided (Shimodaira-Hasegawa test).

Number	Change	−lnL	Diff	*P*
1	original	6889.9132		
2	*C. obsoletus*	6915.4287	25.5155	0.777
3	*C. scoticus*	6919.4247	29.5115	0.817
4	*C. chiopterus*	6917.7854	27.8721	0.796
5	*C. pulicaris*	7056.5050	166.5918	1.000
6	*C. punctatus*	7041.8760	151.9627	1.000
8	*C. impunctatus*	7057.7414	167.8281	1.000
9	*C. newsteadi*	6917.7854	27.8721	0.999
10	*C. imicola*	6889.9132	0.0000	0.000

and there was a strong bootstrap support for this shared clade (fig. 2).

The SH analysis clearly indicated that the resolved maximum likelihood tree was significantly more likely than any other topology where *C. dewulfi* was placed elsewhere (table 1).

Discussion

This study has highlighted that *C. dewulfi* should not be considered part of the *C. obsoletus s.l.* complex, and instead should be defined as a separate grouping more closely related to *C. imicola*. Previous phylogenetic analyses have placed *C. dewulfi* close to the *C. obsoletus s.l.* complex, but these were based either upon single gene sequences (Nolan *et al*., 2007) or were compromised by including too few species (Gomulski *et al*., 2006; Mathieu *et al*., 2007). A species tree, resulting from the analysis of several genes combined, is expected to yield a more accurate phylogeny than single gene trees, which can be biased by different mutation rates, recombination events, effective population sizes, etc. Moreover, as phylogenetic analysis of closely related species should include independent sequences with high rates of mutation (Slade *et al*., 1994), our approach, combining a mitochondrial gene and two nuclear sequences in a single analysis, should produce a robust phylogeny.

The so-called species-complexes of the genus *Culicoides* are essentially arbitrary groupings based upon the similarity of constituent species in wing morphology and male genitalia. *C. dewulfi* was placed in the *C. obsoletus s.l.* complex because it most closely resembles *C. chiopterus* in these traits. *C. dewulfi* has a similar geographic distribution to members of the *C. obsoletus s.l.* complex, as evidenced from light-trapping in bluetongue-affected areas (Savini *et al*., 2005; Meiswinkel *et al*., 2007). It also breeds in unadulterated dung heaps, as does *C. chiopterus* (Kettle, 1977). It is clear, however, that morphological and behavioural similarities do not reflect evolutionary history and genetic similarity; and, as such, there are no grounds for grouping *C. dewulfi* with *C. chiopterus* in the *C. obsoletus s.l.* complex.

Based on our data, we would consider *C. chiopterus* to be part of the *obsoletus* complex as our analyses group *C. obsoletus* and *C. scoticus* in a well-supported clade. If *C. obsoletus sensu strictu* and *C. scoticus* are usually considered as one complex (Mellor & Wittmann, 2002), then DNA sequence analysis strongly supports *C. chiopterus* to be a member of this complex. Much less controversy has occurred about the so called *C. pulicaris s.l.* complex, which includes five species as indicated in a study by Nolan *et al*. (2007), and this complex is well bootstrap-supported by our results.

The phylogeny of the tree indicates that, during evolution, *C. imicola* branched off from a common ancestor first, followed by *C. dewulfi* and then the ancestors of the two complexes. If the level of vector-competence is a feature of closely related species, then the close relationship of *C. dewulfi* with the classical Old World vector *C. imicola* recommends a close monitoring of potential hosts in areas at risk of bluetongue outbreaks where this species is abundant. However, the different grouping of vector-competent species suggests that this feature has evolved on more than one occasion. This highlights (i) the need for the vector competence of all *Culicoides* species to be ascertained and (ii) that the assessment of disease risk for BTV should not be based upon the presence of *C. obsoletus s.l.* group vectors but, potentially, all *Culicoides* midge species.

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