Infection, Genetics and Evolution 10 (2010) 629–637

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Discrimination of *Culicoides obsoletus* and *Culicoides scoticus*, potential bluetongue vectors, by morphometrical and mitochondrial cytochrome oxidase subunit I analysis

D. Augot [a,*], F. Sauvage [b], D. Jouet [a], E. Simphal [a], M. Veuille [c], A. Couloux [d], M.L. Kaltenbach [e], J. Depaquit [a]

[a] USC Vecpar, AFSSA LERPAZ, JE2533, Université de Reims Champagne-Ardenne, UFR Pharmacie, 51 rue Cognacq-Jay, 51100 Reims, France
[b] Université de Lyon, F-69000, Lyon; Université Lyon 1; CNRS, UMR5558, Laboratoire de Biométrie et Biologie Evolutive, F-69622, Villeurbanne, France
[c] Unité de recherche 5202 CNRS, MNHN, Ecole pratique des hautes études, 16 rue Buffon, 75005 Paris, France
[d] Genoscope, 91000 Evry, France
[e] Laboratoire de Pharmacologie, Université de Reims Champagne-Ardenne, UFR Pharmacie, 51 rue Cognacq-Jay, 51100 Reims, France

ARTICLE INFO

Article history:
Received 14 January 2010
Received in revised form 29 March 2010
Accepted 29 March 2010
Available online 8 April 2010

Keywords:
Culicoides obsoletus
Culicoides scoticus
Cytochrome C oxidase I
Barcode
Morphometric analyse
France

ABSTRACT

Biting midges of the *Culicoides obsoletus* Meigen species complex (Diptera: Ceratopogonidae) are increasingly suspected as vectors of the recent emergence of bluetongue virus in Europe. Within this complex, identification of the *C. obsoletus* and *Culicoides scoticus* females is considered as difficult or sometimes not possible while the identification of males is easy, based on genitalia observation. Nolan et al. (2007) concluded that the distinction of *C. obsoletus* and *C. scoticus* females is not possible according to morphology but require molecular analyses. In 2010, the identification of biting midges is done under a stereomicroscope without specific identification within the *C. obsoletus* species complex. However, such a specific identification distinguishing *C. obsoletus* s. str. and *C. scoticus* s. str. is crucial to identify the European competent vectors of the virus, their relative abundances and then accurately assess the risk.

We performed morphometric analyses of head, genitalia and thorax of females combined with sequencing of the cytochrome oxidase I barcode fragment of mitochondrial DNA on 88 specimens in order to have a molecular identification of our sampled species. As we knew the actual species of individuals thanks to molecular results, we explored the discriminant power of 15 morphometric variables to distinguish the females according to their species. Multivariate analyses were performed on the morphometric measurements to identify and validate a combination of variables leading to an accurate species identification. It appears that females of *C. obsoletus* and *C. scoticus* can be accurately distinguished based on only four variables: width between chitinous plates, length and width of spermathecae1 and length of spermatheca2. This approach should improve the accuracy of morphologically-based species identification.

1. Introduction

Arbovirus diseases are an increasing threat for public health, food supply and economy. This fact makes important the identification of arthropod vectors and their distinction with close species. Bluetongue virus (BTV) can infect all domestic and wild ruminants but severe infection is observed mainly in sheep and deer, and to a lesser extent in cattle (Linden et al., 2008; Fernández-Pacheco et al., 2008; Ruiz-Fons et al., 2008; Schwartz-Cornil et al., 2008). The etiologic agent is a double-stranded RNA virus. Like other members of the *Orbivirus* genus, the virus is transmitted between ruminants by insect vectors, which in the case of BTV belong to the *Culicoides* genus (Mellor and Boorman, 1995). Initially, the virus was endemic in South Africa. It spread to other regions by the introduction of infected animals, especially in the Mediterranean area, including Southern Europe countries like Spain and Portugal (Mehlhorn et al., 2007). Before 1998, BTV was considered as an exotic virus in Europe with just a few sporadic cases. From 1998 through 2005, BTV was continuously present in the Mediterranean Basin. Since August 2006, BTV-8 has caused a severe epizootic of bluetongue in northern Europe (Saegerman et al., 2008).

In Africa and Southern Europe, *Culicoides imicola* has been considered for a long time as the only competent BTV vector (Meiswinkel et al., 2008). Recently, several authors have demonstrated the potential role of the other *Culicoides* species as BTV

* Corresponding author: Tel.: +33 3 26 91 37 23; fax: +33 3 26 91 35 97.
E-mail address: d.augot@afssa.fr (D. Augot).

1567-1348/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.meegid.2010.03.016

vectors: (i) *Culicoides pulicaris* (Caracappa et al., 2003); (ii) *Culicoides obsoletus* (Savini et al., 2005); (iii) *Culicoides scoticus* (Savini et al., 2005); (iv) *Culicoides dewulfi* (Meiswinkel et al., 2007); (v) *C. obsoletus* complex (Mehlhorn et al., 2007); (vi) *Culicoides chiopterus* (Dijkstra et al., 2008). *C. chiopterus* was also mentioned as a potential vector in France (French Ministry of Agriculture, 2008). The role of both *obsoletus* and *pulicaris* species complexes in the transmission of BTV is of real concern because they are common and widespread across central and northern Europe (Baldet et al., 2008).

Thirty *Culicoides* species are involved in the transmission of orbiviral diseases (Schwenkenbecker et al., 2009). To date, the internal taxonomy of the genus *Culicoides* has relied mainly on morphological identification—e.g. pigmentation pattern of the wings, length and shape of the antennal segments, characteristics of the genitalia in males, distribution of the sensillae on the antennae, and the number and size of the spermathecae in females (Campbell and Pelham-Clinton, 1960; Wirth and Hubert, 1989; Delécolle, 1985; Boorman, 1993; Rawlings, 1996; Boorman and Hagan, 2007).

In the *obsoletus* complex – *C. obsoletus* s.s., *C. scoticus*, *C. dewulfi* and *C. chiopterus* (Campbell and Pelham-Clinton, 1960) – a specific diagnostic is often difficult or impossible between closely related species. For example, only males can be discriminated between *C. obsoletus* s.s. and *C. scoticus* s.s. according to genitalia characteristics. For females, the measurement of the size of the spermathecae is not sufficient for precise identification of specimens from Spain (Pagès and Sarto i Monteys, 2005). Since these species can only be identified after an intense work from well-trained scientists according to morphological criteria (Balczun et al., 2009) and even then, not without mistakes (Pagès and Sarto i Monteys, 2005), new criteria or other methodologies are required. Identification is also a great issue in discrimination between vertebrate subspecies and their hybrids (e.g. Krüger et al., 2009 for the example of domestic and wild cats).

Different molecular methods are available and supply unambiguous results: sequencing of the nuclear internal transcribed spacer (Cêtre-Sossah et al., 2004) or of the mitochondrial cytochrome oxidase subunit I (Nolan et al., 2007). In the present study, females of the *C. obsoletus* complex (*C. scoticus* and *C. obsoletus*) were characterized using the traditional morphometric approach and a molecular approach using cytochrome oxidase subunit I (COI). Here, we propose an additional molecular primer for this group, routinely used with other insects (Hajibabaei et al., 2006). However, the molecular approach implies time, accurate materials and conditions in labs.

Based on the molecular results that allow to validate with certainty the species of the individuals, we aimed in this study to identify the combination of the lowest number of morphological characters needed to distinguish between female specimens from the *C. obsoletus* complexes with a high level of confidence. We considered potential morphological differences between species in shape and size, with all available variables at first. Then, we looked for the best morphological discriminant compromise to decrease the amount of work to get the right identification for most insects and spare the molecular method for the litigious cases.

2. Materials and methods

2.1. Sample collection and preliminary identification

2.1.1. Sample collection

Insects were collected from July to September 2008 in two locations in France. The first site, Montigny-la-Cour (49°59′69″N, 4°01′45″E), was a farm in Northern France that was BTV-infected in 2007. Midges were caught by an ultraviolet CDC trap (John W. Hock Company, Gainesville, FL, USA) indoor and outdoor. For this study, we dissected a part of collected *Culicoides*. All considered specimens caught at this location (2 males and 79 females) have a processing code beginning with the letter P. The second site was Sumène, a village located in Southern France (43°97′97″N, 3°71′47″). Midges were caught by standard CDC miniature light traps (John W. Hock Company) outdoor. All dissected specimens caught here (13 females) have a processing code beginning by the letter D. Traps were set approximately 1 h before sunset until 1 h after sunrise under favorable climatic conditions (absence of heavy rain and/or wind). Midges were stored in 96% ethanol before morphological and molecular analyses.

2.1.2. Specimen identification

All *Ceratopogonidae* (including *Culicoides*) were separated from other insects and *Culicoides* identified according to wing characters (Mellor et al., 2000) using a binocular microscope in the laboratory. The head, wings and genitalia of individual biting midges were cut off within a drop of ethanol, cleared in boiling Marc–André solution, and mounted between slide and cover slide. The thorax related to each specimen was stored in a vial at −20 °C before DNA extraction.

Culicoides biting midges were identified at the species level according to their morphological characters (Delécolle, 1985; Kremer and Rebholtz, 1977) using a microscope. Among the population of collected *Culicoides* in two sites, 94 *Culicoides* individuals from the *obsoletus* complex were dissected (92 females and 2 males). Females were processed similarly to males, and identifed as *C. scoticus*, *C. obsoletus* or *C. dewulfi* on the basis of *spermathecae* size and chitinous plates surrounding the genital opening. Molecular analyses were performed on 88 of these females to assess their species. *C. obsoletus*, *C. scoticus* and *C. dewulfi* males are easily distinguished on the basis of their genitalia. Therefore, their genetic variability could be studied and used later to distinguish the females of the three species.

2.2. DNA extraction, amplification (polymerase chain reaction), sequencing, and molecular analyses

All specimens sequenced in the present studies were females except two males: P4C20 (*C. obsoletus*) and P7C5 (*C. scoticus*). *C. obsoletus*, *C. scoticus* and *C. dewulfi* females and males, previously identified morphologically and stored at −20 °C, were individually examined for DNA extraction.

2.2.1. DNA extraction

After crushing using a piston pellet (Treff, Switzerland), Genomic DNA from the thorax was extracted using the QIAmp DNA Mini Kit (Qiagen, GmbH, Hilden, Germany), following manufacturer's instructions.

2.2.2. Amplification

Polymerase chain reactions (PCR) of COI domain were performed in a 50 μl volume using 5 μl of extracted DNA solution and 50 pmol of the primers LepF (5′-ATT CAA CCA ATC ATA AAG ATA TTG G-3′) and LepR (5′-TAA ACT TCT GGA TGT CCA AAA AAT CA-3′) (Hajibabaei et al., 2006). These primers were never used for *Culicoides* but used daily in the laboratory on Phlebotomine sandflies (Diptera: Psychodidae) (Bounamous et al., 2008). Amplification conditions (Costa et al., 2007) were as follows: after an initial denaturation step at 94 °C for 3 min, 5 cycles of denaturation at 94 °C for 30 s, annealing at 45 °C for 90 s, and extension at 68 °C for 60 s were followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 51 °C for 90 s, and extension at 68 °C for 60 s and a final extension at 68 °C for 10 min by using *Taq* polymerase (5′, Germany). Amplicons were analysed

by electrophoresis in 1.5% agarose gel containing ethidium bromide.

2.2.3. Sequencing

Direct sequencing in both directions was performed using the primers used for DNA amplification. The correction of sequences was done using Pregap and Gap included in the Staden Package software (Bonfield and Staden, 1996).

2.2.4. Molecular analyses

Sequence alignment was performed using the ClustalW routine included in the MEGA version 3.1 software (Kumar et al., 2004), and checked by eye. Neighbor-joining (NJ) analyses were performed using MEGA 3.1 software. Maximum likelihood (ML) analysis was performed in PHYML online (Guindon et al., 2005). For NJ and ML analyses, node support was assessed by bootstrapping over 500 replications.

COI haplotypes of *Culicoides* were analysed together. In order to assess a molecular ID of our samples, we used two males from our sampling, P4C20 (*C. obsoletus*) and P7C5 (*C. scoticus*), easily identified. Moreover, we also included some COI sequences of European specimens previously sequenced by Nolan et al. (2007) and Pagès and Sarto i Monteys (2005): *C. obsoletus* s.s. (accession numbers DQ162808 from Spain; AM236652 from United Kingdom), *C. scoticus* (accession numbers DQ162804 from Spain; AM236625 from United Kingdom), and *C. dewulfi* (accession number AM236672 from United Kingdom).

2.3. Morphological protocol

Specimens were observed on a BX50 microscope (Olympus, Japan). Measurements were performed using the Perfect Image software (Aries Company, Chatillon, France) by means of a video camera connected to the microscope. Females to be dissected were chosen arbitrarily from the collection traps. Morphometric measurements were taken on the head, wing and genitalia of individual *Culicoides* females.

Fifteen variables (Fig. 1) were recorded in *Culicoides* adult females: (1) length (Fig. 1a1: LengthWing) and (2) width of wing (Fig. 1a2: WidthWing); (3) length of costa (Fig. 1a3: LengthCosta); (4) length of the joint between both eyes (Fig. 1b1: LengthEye) and (5) the area of the triangle defined by the 3 sensilla up the eyes (Fig. 1b2: SurfaceSensilla); (6) length (Fig. 1c1: Length3SegmentPalpus) and (7) width of the third segment of the palp (Fig. 1c2: Width3SegmentPalpus); (8) length of flagellomere 10 (Fig. 1d1: LengthFlagellomere10) and (9) length of flagellomere 11 (Fig. 1d2: LengthFlagellomere11); (10) length (Fig. 1e1: LengthSpermatheca1) and (11) width of the first spermathecae (Fig. 1e2: WidthSpermatheca1), (12) length (Fig. 1e3: LengthSpermatheca2) and (13) width of the second spermathecae (Fig. 1e4: WidthSpermatheca2), (14) length between both chitinous plates surrounding the genital opening (Fig. 1f1: LengthChitinousPlates), and (15) width between these two plates (Fig. 1f2: WidthChitinousPlates). Six additional variables were calculated as ratios of these measures: LengthWing/WidthWing, LengthWing/LengthCosta, LengthSpermatheca/WidthSpermatheca, Length3SegmentPalpus/Width3SegmentPalpus, LenghtFlagellomere11/LenghtFlagellomere10, and LengthChitinousPlates/WidthChitinousPlates.

2.3.1. Statistical analyses

All statistical analyses were performed using R 2.10.0 (R Development Core Team, 2009) and differences were considered statistically significant when $p < 0.05$. Principal component analyses (PCA) used to explore the correlation structure between variables, and linear discriminant analyses (LDA) used to predict the individual species based on variable values were performed using the ADE4 package (Dray et al., 2007). Actually, as PCA maximizes the preserved variance originally present in the data, the selected factorial plan from the previous analysis was not necessarily the best to discriminate species. This is where linear discriminant analyses can help.

To describe variables, we used the genetically determined species since they were available.

Measurements of each variable were evaluated using the coefficient of variation ($CV = SD/\bar{X} \times 100\%$, expressing the standard deviation SD as a percentage of the mean $\bar{X}$) and the coefficient of difference (defined for a given variable measured in two groups A and B of individuals as $CD = |\bar{X}_A - \bar{X}_B|/(SD_A + SD_B)$). CV supplies information about data homogeneity whereas CD is linked to the overlap or the degree of separation between two distributions, e.g. the measures of a given character among

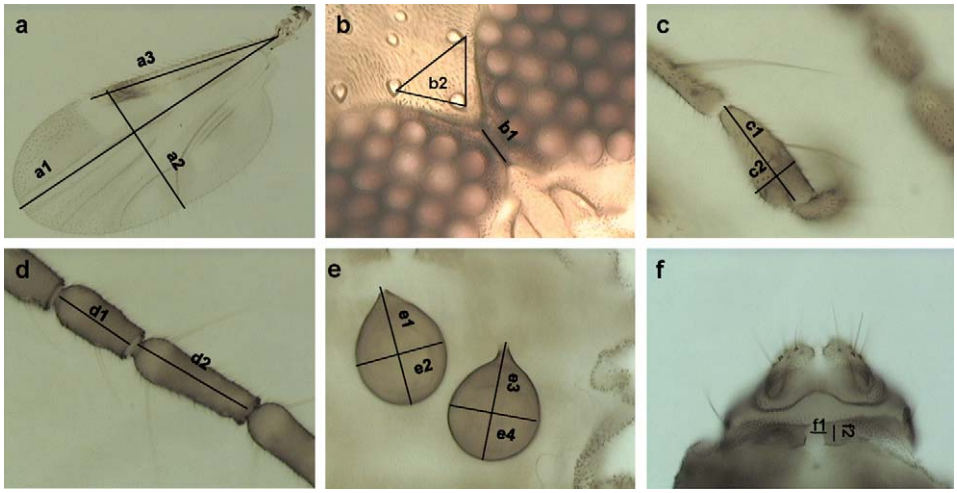

Fig. 1. Morphometrical characters used to characterize *Culicoides obsoletus* complex females. Wing (a): length (a1: LengthWing), width (a2: WidthWing); costa (a3: LengthCosta). Head (b): length of joint between the 2 eyes (b1: LengthEye), the triangle defined by the 3 sensilla up the eyes (b2: SurfaceSensilla). Palpus (c): length (c1: Length3SegmentPalpus), width of third segment of palp (c2: Width3SegmentPalpus). Antenna (d): length of flagellomere 10 (d1: LengthFlagellomere10), length of flagellomere 11 (d2: LengthFlagellomere11). Spermathecae (e): length (e1: LengthSpermatheca), width of the first spermathecae (e2: WidthSpermatheca), length (e3: LengthSpermatheca), width of the second spermathecae (e4: WidthSpermatheca). Chitinous plates surrounding the genital opening (f) length (f1: LengthChitinousPlates), width (f2: WidthChitinousPlates).

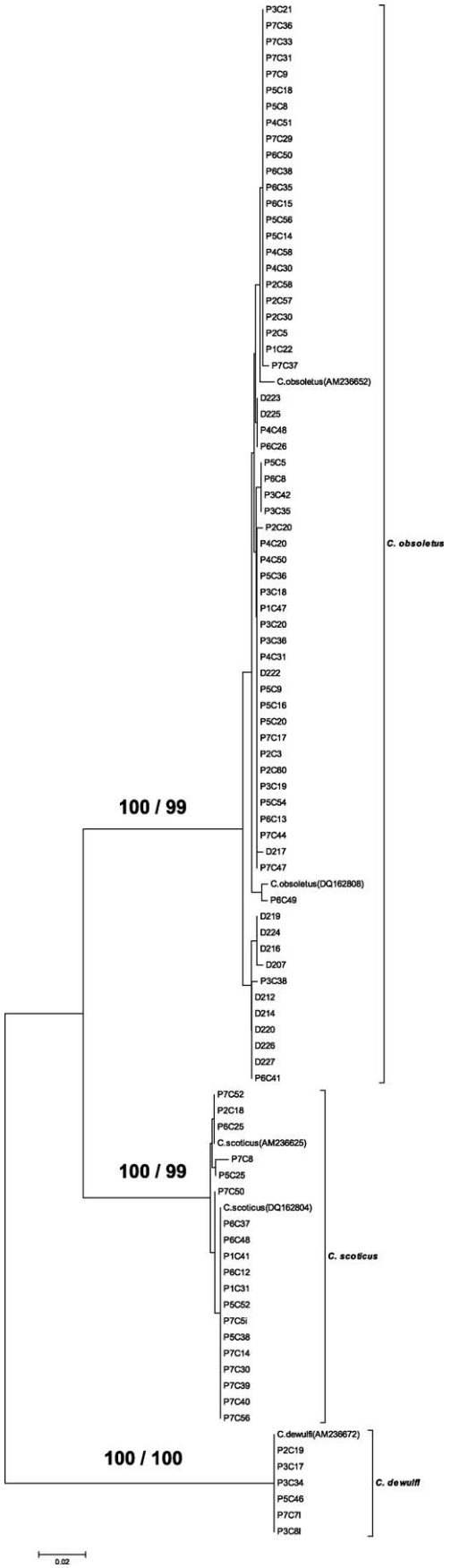

Fig. 2. Neighbor-joining tree based on COI haplotypes of 95 (88 females, 2 males and 5 database entries) midges of *Culicoides obsoletus*, *C. scoticus* and *C. dewulfi*. A similar topology (not shown) was obtained after a maximum likelihood analysis. Specimens were caught at Montigny-la-Cour (P) and at Sumène (D). Males: P4C20 (*C. obsoletus*) and P7C5 (*C. scoticus*).

individuals of different species (Mayr et al., 1953). The level considered as the CD critical threshold by Mayr et al. (1953) to distinguish subspecies is 1.28. CV values were calculated by genetically determined species, but CD values were for *C. obsoletus* and *C. scoticus* exclusively as only 6 specimens are *C. dewulfi*. For most descriptive statistics, we nevertheless gave the results for *C. dewulfi* as well, as additional information and we considered them in multivariate analysis, in case a pattern appeared. Of course, the small number of specimen prevents any definitive conclusion in *C. dewulfi* case.

We were unable to measure all characters in all individuals because of alterations in some appendices. Among the 88 individuals, 36 presented no missing values for any of the 15 variables In order to keep the maximum of the available information to perform the multivariate analyses, missing values were replaced by the average of the genetically determined species. Another means to complete missing values is the use of the median (Krüger et al., 2009) but as the averages and medians were always close together in our study, and as the maximum likelihood estimators for genetically identified individuals' missing values are the species' average, we prefered to use this parameter.

Genetic characterization and measures for 15 morphological variables are available for 88 specimens, leading to a total of 1320 entries for the 15 raw variables and 122 missing values (9.24%) among them (97 for *C. obsoletus*, 17 for *C. scoticus* and 8 for *C. dewulfi*).

3. Results

3.1. Molecular analysis

Partial COI sequences were obtained from 95 specimens belonging to *C. obsoletus*, *C. scoticus* and *C. dewulfi* species. They have been deposited into GenBank under the following accession numbers: *C. obsoletus* (HM022792–HM022856) *C. scoticus* (HM022857–HM022875) and *Culicoides dewulfi* (HM022876–HM022881). A length of 420 bp was used for maximum likelihood and neighbor-joining analyses. Similar topologies were obtained with both methods (Fig. 2). The membership of each sample in the various branches was strongly supported by bootstrap values. Distance pairwise computed between groups means showed 77.9% and 75.9% homology between *C. dewulfi* and the closely related species *C. scoticus* and *C. obsoletus*, respectively. However, being morphologically close, *C. scoticus* and *C. obsoletus* are molecularly strongly separated. Their genetic distance pairwise shows 86% homology, while distance pairwise computed within species showed 99.8% homology within *C. scoticus* and 99.4% within *C. obsoletus*.

4. Morphometrical analysis

4.1. Descriptive statistics

Among the 88 PCR that provided an unambiguous result, 64 *C. obsoletus*, 18 *C. scoticus* and 6 *C. dewulfi* were identified. Distributions for *C. dewulfi* were not tested since only 6 specimens were available. However, preliminary results could be given. Concerning *C. obsoletus* and *C. scoticus*, only 3 specific distributions appeared significantly as non-gaussian (Shapiro–Wilk's test p-value <0.01): eye's area, WidthChitinousPlates and LenghtFlagellomere11, all for *C. obsoletus*.

Table 1
Descriptive statistics of morphometrical characters in three members of the *Culicoides obsoletus* species complex (*C. scoticus*, *C. obsoletus*, and *C. dewulfi*). Units are expressed in μm excepted the Surface Sensilla expressed in μm^2.

Parameter (maximum measurements)	Mean (±CI)	SD	Median
	C. scoticus (17 missing values)		
LengthWing	1284.87 [1245.46–1324.28]	79.23	1288.12
WidthWing	544.66 [526.79–562.53]	35.93	544.41
LengthCosta	821.8 [796.21–847.39]	51.48	815.67
LengthSpermatheca1	61.08 [59.18–62.98]	3.84	61.13
WidthSpermatheca1	41.43 [40.08–42.78]	2.65	41.92
LengthSpermatheca2	61.19 [58.72–63.66]	4.66	60.71
WidthSpermatheca2	40.3 [36.46–44.14]	6.31	42.75
LengthChitinousPlates	22.41 [19.71–25.11]	5.45	23.83
WidthChitinousPlates	20.6 [18.22–22.98]	4.64	20.35
Length3SegmentPalpus	49.77 [47.3–52.24]	4.84	48.17
Width3SegmentPalpus	18.93 [17.85–20.01]	2.09	18.63
LengthEye	27.8 [25.69–29.91]	4.14	27.61
SurfaceSensilla	267.37 [241.97–292.77]	45.04	255.58
LengthFlagellomere10	33.95 [32.56–35.34]	2.8	34.18
LengthFlagellomere11	49.45 [47.78–51.12]	3.37	50.15
	C. obsoletus (97 missing values)		
LengthWing	1140.41 [1120.59–1160.23]	75.58	1145.87
WidthWing	478.81 [469.38–488.24]	35.98	481.49
LengthCosta	730.23 [716.82–743.64]	51.10	734.18
LengthSpermatheca1	47.79 [46.77–48.81]	4.10	47.12
WidthSpermatheca1	33.3 [32.58–34.02]	2.87	33.52
LengthSpermatheca2	46.82 [45.58–48.06]	4.18	46.78
WidthSpermatheca2	32.92 [31.54–34.3]	4.28	32.62
LengthChitinousPlates	11.77 [11.19–12.35]	2.32	11.91
WidthChitinousPlates	18.63 [18.07–19.19]	2.28	18.93
Length3SegmentPalpus	47.62 [46.6–48.64]	4.01	47.51
Width3SegmentPalpus	20.42 [19.5–21.34]	3.58	20.50
LengthEye	22.47 [21.27–23.67]	4.55	22.96
SurfaceSensilla	303.04 [283.84–322.24]	65.21	306.23
LengthFlagellomere10	32.67 [31.81–33.53]	3.35	32.53
LengthFlagellomere11	43.65 [42.43–44.87]	4.74	43.27
	C. dewulfi (8 missing values)		
LengthWing	1180.94 [1096.91–1264.97]	80.07	1170.71
WidthWing	510.23 [472.65–547.81]	35.81	501.04
LengthCosta	750.53 [688.76–812.3]	58.85	750.44
LengthSpermatheca1	49.13 [40.78–57.48]	7.95	52.28
WidthSpermatheca1	39.31 [31.55–47.07]	7.39	42.13
LengthSpermatheca2	39.69 [34.52–44.86]	2.84	39.69
WidthSpermatheca2	31.48 [12.17–50.79]	10.62	31.48
LengthChitinousPlates	10.38 [5.91–14.85]	4.26	12.07
WidthChitinousPlates	16.06 [13.44–18.68]	2.51	16.25
Length3SegmentPalpus	52.26 [46.76–57.76]	5.23	50.00
Width3SegmentPalpus	19.51 [16.45–22.57]	2.90	19.74
LengthEye	13.77 [10.38–17.16]	3.23	12.80
SurfaceSensilla	308.42 [247.99–368.85]	57.6	282.50
LengthFlagellomere10	30.96 [28.57–33.35]	2.29	30.14
LengthFlagellomere11	43.05 [40.09–46.01]	2.81	42.17

Descriptive statistics, i.e. means, SD and 95% confidence intervals (CI), are presented in Table 1. Table 2 presents CD and CV values for each character, in descending order according to CD values. CD values ranged from about 0.21 (LengthFlagellomere10) to 1.70 (LengthSpermatheca1). Four variables (LengthSpermatheca1 and LengthSpermatheca2, WidthSpermatheca1, and LengthChitinousPlates) exhibited CD values over 1.28.

Such values indicated the absence of overlap between the distributions of the four variables between both species; LengthSpermatheca1, LengthSpermatheca2, WidthSpermatheca1 and LengthChitinousPlates would certainly be the most reliable characters to differentiate between *C. scoticus* and *C. obsoletus*.

CV values ranged from 5.06 (LengthSpermatheca2 in *C. dewulfi*) to 37.42 (LengthChitinousPlates in *C. dewulfi*) with a weighted average of 11.779. Such values are above those commonly observed in mammals (Krüger et al., 2009) and suggest a lower homogeneity of length-related traits in studied insects. Despite a lot of moderate CD values for the studied variables, Table 1 reveals that 95% confidence intervals for the means in *C. scoticus* are not overlapping with those in other species for most of studied variables.

Clear differences in averages of LengthSpermatheca1 and LengthSpermatheca2, WidthSpermatheca1 and LengthChitinousPlates were noted between *C. scoticus* on one side and *C. obsoletus* and *C. dewulfi* on the other side (see Table 1), variables that also exhibited the highest CD values. The highest values of LengthSpermatheca1, LengthSpermatheca2, WidthSpermatheca1 and LengthChitinousPlates in both *C. obsoletus* and *C. dewulfi* were not much higher than the shortest one measured among *C. scoticus*.

4.1.1. Correlation between traits

Pearson's *r* correlation matrix (see Table 3) of the 15 raw characters, used for the main multivariate analysis of the 88 genetically identified specimens, exhibited moderate strength in the relationships for most coefficients of distinct variables (7 absolute values (6.67%) above 0.80, 19 (18.10%) under 0.10). Most variables displayed a positive correlation that certainly rises from the overall body-size. The highest positive correlations were

Table 2
Characterization of 3 *Culicoides* species (*C. scoticus*, *C. obsoletus*, *C. dewulfi*) using the results of values of the coefficients of variation (CV) and of Mayr's coefficients of difference (CD) on 15 morphometrical parameters.

Parameter (maximum measurements)	Coefficients of variation (CV)			
	C. scoticus	*C. obsoletus*	*C. dewulfi*	CD
LengthSpermatheca1	6.11	8.52	14.77	1.70
LengthSpermatheca2	7.38	8.82	5.06	1.66
WidthSpermatheca1	6.21	8.55	17.17	1.50
LengthChitinousPlates	23.62	19.59	37.42	1.40
LengthWing	5.99	6.57	6.19	0.95
WidthWing	6.41	7.45	6.41	0.93
LengthCosta	6.09	6.94	7.16	0.91
LengthFlagellomere11	6.62	10.76	5.95	0.73
WidthSpermatheca2	15	12.84	23.86	0.72
LengthEye	14.43	20.06	21.42	0.63
Surface Sensilla	16.23	21.28	17.05	0.33
WidthChitinousPlates	21.86	12.13	14.25	0.29
Width3SegmentPalpus	10.68	17.4	13.59	0.27
Length3SegmentPalpus	9.43	8.35	9.14	0.25
LengthFlagellomere10	8.01	10.16	6.74	0.21

between wing's length and width, LengthCosta and antennae ($r > 0.80$).

4.2. Principal component analyses

4.2.1. PCA on size variables

Firstly, we studied size differences through a normed PCA on raw data. *C. scoticus* could be clearly separated from both other species (Fig. 3a). The first 3 axes accounted for 43.2%, 14.3% and 8.1% of the total variance. Hence, we focused on the first two axes that explained more variance than expected by chance. The scatterplot separated unambiguously, without overlap, *C. scoticus* on the one hand and *C. obsoletus*–*C. dewulfi* on the other hand (Fig. 3).

The first axis, called PC1 hereafter, was highly negatively correlated with wing's length and width, with LengthCosta ($r \approx -0.9$), with Spermatheca1 and Spermatheca2 length, with antenna 11 length ($r \approx -0.8$), and to a lesser extent with LengthChitinousPlates, Spermatheca1 and Spermatheca2 width ($r \approx -0.70$) (Table 4). These variables certainly characterized the best the overall body-size. The second axis PC2 appeared to be correlated with palpi and eye characters ($|r|_{max} \approx 0.60$) and also with Spermatheca2's length and LengthChitinousPlates but Pearson's r are lower than with PC1 for both latter traits.

4.2.2. PCA on shape variables

Secondly, we looked at differences in shape between species of the *C. obsoletus* complex. With that objective, we performed a first normed PCA using the 6 ratio variables. The six axes accounted for 27%, 22%, 18%, 14%, 10% and 9% of the total variance, which suggest a weak structuration of the data. This was confirmed by a scatterplot of PCA axes 1 and 2 that was unable to separate the 3 genetic species (results not shown).

A discrimination according to the size of the species is then supported and some measures characterizing body-size could allow better and faster identification of *Culicoides* from the *obsoletus* complex. With that objective in mind, we performed linear discriminant analyses on the same dataset. On the contrary, shape thus appears to be similar between species and a discrimination by shape cannot be supported.

4.3. Discriminant analyses

A first LDA with the 15 raw characters allowed a clear separation between the three species (results not shown but very

Table 3
Correlation matrix of the 15 morphometrical characters of *Culicoides obsoletus*, *C. scoticus*, and *C. dewulfi*.

LengthWing	1														
WidthWing	0.95	1													
LengthCosta	0.99	0.94	1												
LengthSpermatheca1	0.60	0.57	0.58	1											
WidthSpermatheca1	0.57	0.60	0.52	0.74	1										
LengthSpermatheca2	0.42	0.41	0.40	0.74	0.51	1									
WidthSpermatheca2	0.45	0.45	0.46	0.60	0.56	0.55	1								
LengthChitinousPlates	0.61	0.53	0.58	0.64	0.60	0.65	0.65	1							
WidthChitinousPlates	0.20	0.15	0.19	−0.07	0.05	0.12	0.24	0.26	1						
Length3SegmentPalpus	0.63	0.63	0.66	0.35	0.33	0.06	0.17	0.30	0.13	1					
Width3SegmentPalpus	0.30	0.30	0.33	−0.10	−0.02	−0.18	0.11	−0.09	0.09	0.47	1				
LengthEye	0.19	0.16	0.22	0.16	0.06	0.33	0.46	0.35	0.15	0.01	0.01	1			
SurfaceSensilla	0.12	0.13	0.16	−0.22	0.02	−0.42	−0.11	−0.21	−0.15	0.15	0.43	−0.30	1		
LengthFlagellomere10	0.77	0.75	0.77	0.35	0.37	0.26	0.41	0.32	0.26	0.65	0.52	0.30	0.18	1	
LengthFlagellomere11	0.83	0.80	0.81	0.44	0.45	0.31	0.48	0.49	0.18	0.65	0.45	0.34	0.09	0.89	1
	Length Wing	Width Wing	Length Costa	Width Spermatheca1	Length Spermatheca1	Width Spermatheca2	Length Spermatheca2	Width Chitinous Plates	Length Chitinous Plates	Length3 Segment Palpus	Width3 Segment Palpus	Length Eye	Surface Sensilla	Length Flagellomere10	Length Flagellomere11

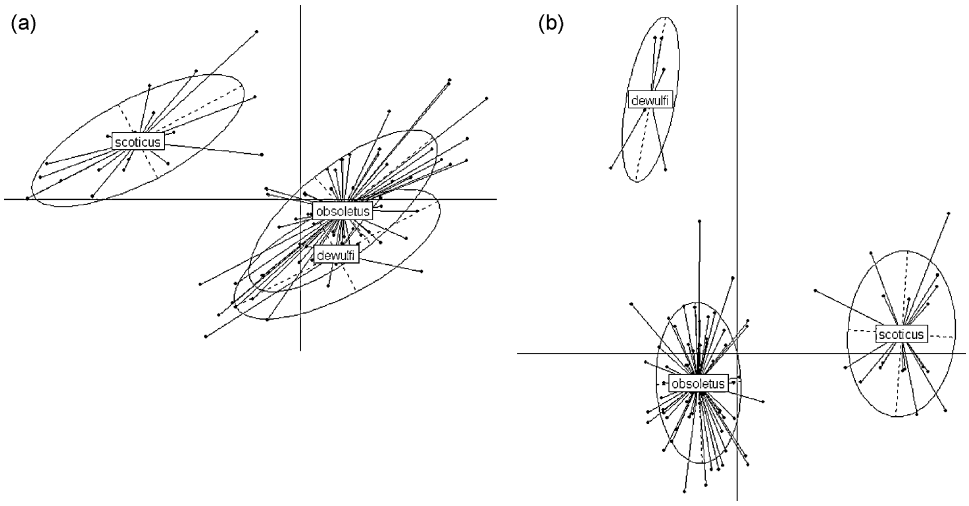

Fig. 3. Factorial analyses of morphological *Culicoides* data. Results of principal component analysis (a) and linear discriminant analysis (b).

similar to Fig. 3b). The interclass variances over the two axes were, respectively, of 0.89 and 0.64 (Wilk's Lambda = 0.0393, $F = 19.13$, $p < 10^{-4}$). According to the loadings (Table 4), the main contributions to the first axis were LengthSpermatheca2 and LengthChitinousPlates, which are also the variables presenting the highest CD values. The main contributions to the second axis were LengthEye and WidthSpermatheca1. The first axis separated *C. scoticus* from *C. obsoletus* and *C. dewulfi*, an observation which was expected as the main contributors were the same traits as in the previous PCA, but the second axis allowed to distinguish *C. dewulfi* from the two other species (see Fig. 3b for the second LDA performed on these 4 variables only). Although only six *C. dewulfi* were available, slight variations in shape can be used to discriminate between *C. obsoletus* and *C. dewulfi* ($t = -4.55$, df = 62, $p < 10^{-4}$).

LengthWing, LengthCosta, Width3SegmentPalpus and Surface Sensilla did not present real discriminant value and may thus not be routinely measured without significant loss with regards to these three species classification.

Table 4
Characterization of *C. scoticus*, *C. obsoletus*, and *C. dewulfi* species using the loadings of principal component analyses (PCA) and linear discriminant analyses (LDA) on 15 morphometrical parameters.

Parameter (maximum measurements)	Principal component analyses		Linear discriminant analyses	
	PCA1*	PCA2*	LDA1*	LDA2*
LengthWing	−0.359	−0.179	0.285	−0.391
WidthWing	−0.349	−0.185	−0.057	0.829
LengthCosta	−0.354	−0.184	−0.016	0.351
LengthSpermatheca1	−0.305	0.230	0.077	0.099
WidthSpermatheca1	−0.261	0.184	0.080	0.674
LengthSpermatheca2	−0.281	0.345	0.392	−0.621
WidthSpermatheca2	−0.254	0.192	0.161	−0.133
LengthChitinousPlates	−0.292	0.262	0.238	0.034
WidthChitinousPlates	−0.148	0.054	−0.009	−0.062
Length3SegmentPalpus	−0.211	−0.315	−0.138	0.168
Width3SegmentPalpus	−0.053	−0.384	−0.061	−0.174
LengthEye	−0.174	0.285	0.061	−0.400
SurfaceSensilla	−0.016	−0.376	−0.168	−0.288
LengthFlagellomere10	−0.200	−0.298	−0.017	−0.242
LengthFlagellomere11	−0.308	−0.168	0.033	−0.367

Note: (PCA1*, PCA2*) and (LDA1*, LDA2*) are two linear combinations of parameters.

5. Discussion

The diagnostic value of 15 morphometrical variables in females of *C. obsoletus, C. scoticus* and *C. dewulfi* for which specific identification was confirmed by molecular typing using the COI gene sequence was investigated. Among females of these three species, traditional specific diagnostic is often difficult since shapes are very similar. Our results show that the variables (LengthSpermatheca, WidthSpermatheca and LengthChitinousPlates) would certainly be the most reliable characters in differentiating *C. scoticus* and *C. obsoletus* with absence of overlap between the distributions of theses variables considered all together. Although only six specimens of *C. dewulfi* were available, the variable LengthEye is the only one statistically different between *C. dewulfi* and *C. obsoletus*. Futher work will however be needed to confirm this character in *C. dewulfi* female. On the other hand, variables like LengthWing, LengthCosta, Width3SegmentPalpus or Surface Sensilla do not seem to present a really discriminant value and can be ignored for a quick identification.

In our study, LengthSpermatheca1 and LengthChitinousPlates (Table 5) reveal small overlaps; two specimens of *C. scoticus* present a LengthChitinousPlates <16.1 μm and three others present a LengthSpermatheca1 <56.61 μm. One specimen of *C. obsoletus* exhibited a LengthSpermatheca2 >53.61 μm. Three specimens of *C. scoticus* displayed a WidthSpermatheca1 <38.18 μm. If we consider, the independent variables and not a multivariate analysis, then the data show an overlapping zone; here, three specimens at the maximum would belong to another

Table 5
Characterization of the main morphometrical parameters used to distinguish *C. obsoletus* from *C. scoticus*.

Variable	Mean (μm)	Min (μm)	Max (μm)
C. scoticus			
LengthSpermatheca1	61.08	53.87	67.43
LengthSpermatheca2	61.19	53.61	70.517
WidthSpermatheca1	41.43	36.32	45.90
LengthChitinousPlates	22.41	9.63	29.81
C. obsoletus			
LengthSpermatheca1	47.79	37.67	56.61
LengthSpermatheca2	46.82	35.48	60.59
WidthSpermatheca1	33.30	24.41	38.18
LengthChitinousPlates	11.77	5.372	16.10

class. However, with multivariate analyses, the distributions of the four variables between both species (LengthSpermatheca1, LengthSpermatheca2, WidthSpermatheca1 and LengthChitinousPlates) would certainly be the most reliable characters to differentiate between *C. scoticus* and *C. obsoletus*, without overlapping zone. Delécolle (1985) identified *C. obsoletus* and *C. scoticus* females by the measurement of the length of the biggest spermatheca. Pagès and Sarto i Monteys (2005) showed for this character the existence of an important overlapping zone, with a bimodal distribution, and their data revealed that the shape of the chitinous plate (convergent or parallel) cannot be used to distinguish *C. scoticus* and *C. obsoletus*.

In all cases, specimens from different geographical sources were grouped together within their species in the COI tree. The barcode fragment of the mtDNA COI sequence data can thus be used as a tool to identify unambiguously different species of *Culicoides* (Nolan et al., 2007; Pagès and Sarto i Monteys, 2005; Pagès et al., 2009), especially *C. scoticus* and *C. obsoletus*. There was no evidence of intraspecific differences within *C. obsoletus* specimens as based on our COI database. Among the 88 specimens analysed, 13 *C. obsoletus* came from Southern France, whereas all other insects came from the north of the country. No difference appeared in the factorial plan between *C. obsoletus* according to the location (results not shown). These results indicate that size differences distinguish mainly the species whereas shape disparity plays a minor role. Such results were also obtained in *Acari* (Pfingstl et al., 2009).

Our observations on 88 specimens are coherent with all these findings but they also highlight that distinguishing the females of both species on the basis of a few measured morphological variables is possible for most individuals. However, direct COI sequencing may still be required to confirm the identification of doubtful specimens, but a lot of time and resources would be spared if an accurate LDA factorial plan would be defined once for all from a large dataset. Actually, 5 specimens whose DNA extraction result was initially unclear were projected on the factorial plan (Linear Discriminant Analysis) and their species inferred from their position using variables with CD $>$ 1.28 (data not shown). Our results showed that these specimens S1, S2, S3, S4 were certainly *C. scoticus*, what confirmed new PCR. Statistical analysis on morphometrical data could then identify the females in each species.

Acknowledgments

The authors would like to thank Agathe, Hubert, Pierre, Mireille and Bob Killick-Kendrick for providing *Culicoides* specimens in Gard department. We are grateful to D. Pontier for helpful comments on the earlier manuscript. The sequencing of the barcode fragment was carried out at the CNS Genoscope (Evry, France), under the "Barcoding insect for identification" grant to Michel Veuille.

References

Balczun, C., Vorsprach, B., Meiser, C.K., Schaub, G.A., 2009. Changes of the abundance of *Culicoides obsoletus* s.s. and *Culicoides scoticus* in Southwest Germany identified by a PCR-based differentiation. Parasitol. Res. 105, 345–349.

Baldet, T., Delécolle, J.C., Cêtre-Sossah, C., Mathieu, B., Meiswinkel, R., Gerbier, G., 2008. Indoor activity of *Culicoides* associated with livestock in the bluetongue virus (BTV) affected region of northern France during autumn 2006. Prev. Vet. Med. 87, 84–97.

Bonfield, J.K., Staden, R., 1996. Experiment files and their application during large-scale sequencing projects. DNA Seq. 6, 109–117.

Boorman, J., 1993. Biting midges (Ceratopogonidae). In: Lane, R.P., Crosskey, R.W. (Eds.), Medical Insects and Arachnids. Chapman & Hall, London, pp. 289–309.

Boorman, J.P.T., Hagan, D.V., 2007. A Name List of World Culicoides (Diptera: Ceratopogonidae). http://campus.belmont.edu/cienews/culicoides_worldlist.html.

Bounamous, A., Boudabous, R., Jouet, D., Augot, D., Ferte, H., Babba, H., Berchi, S., Depaquit, J., 2008. Molecular and morphological characterisation of two closely related species belonging to the belonging to the subgenus Paraphlebotomus Phlebotomus chabaudi Croset, Abonnenc & Rioux, 1970 and P. riouxi Depaquit, Killick-kendrick & Leger, 1998 (Dipera, Psychodidae). Parasite 15, 565–571.

Campbell, J.A., Pelham-Clinton, E.C., 1960. A taxonomic review of the British species of Culicoides Latreille (Diptera: Ceratopogonidae). Proc. R. Soc. Edinb. (B) 67, 181–302.

Caracappa, S., Torina, A., Guercio, A., Vitale, F., Calabro, A., Purpari, G., Ferrantelli, V., Vitale, M., Mellor, P.S., 2003. Identification of a novel bluetongue virus vector species of *Culicoides* in Sicily. Vet. Rec. 153, 71–74.

Cêtre-Sossah, C., Baldet, T., Delécolle, J.C., Mathieu, B., Perrin, A., Grillet, C., Albina, E., 2004. Molecular detection of *Culicoides* spp. and *Culicoides imicola*, the principal vector of bluetongue (BT) and African horse sickness (AHS) in Africa and Europe. Vet. Res. 35, 325–337.

Costa, F.O., Dewaard, J.R., Boutillier, J., Ratnasingham, S., Dooh, R.T., Hajibabaei, M., Hebert, P.D.N., 2007. Biological identifications through DNA barcodes: the case of the Crustacea. Can. J. Fish. Aquat. Sci. 64, 272–295.

Delécolle, J.C., 1985. Nouvelle contribution à l'étude systématique et iconographique des espèces du genre *Culicoides* (Diptera: Ceratopogonidae) du Nord-Est de la France. Thèse d'Université. Université Louis Pasteur de Strasbourg, UER Sciences Vie et Terre.

Dijkstra, E., Van der Ven, I.J.K., Meiswinkel, R., Hölzel, D.R., Van Rijn, P.A., Meiswinkel, R., 2008. *Culicoides chiopterus* as a potential vector of bluetongue virus in Europe. Vet. Rec. 162, 422.

Dray, S., Dufour, A.B., Chessel, D., 2007. The ade4 package-II: two-table and K-table methods. R. News. 7 (2), 47–52.

Fernández-Pacheco, P., Fernández-Pinero, J., Agüero, M., Jiménez-Clavero, M.A., 2008. Bluetongue virus serotype 1 in wild mouflons in Spain. Vet. Rec. 162, 659–660.

French Ministry of Agriculture (2008): *Culicoides chiopterus*: confirmation of its status as potential vector of bluetongue virus in Europe. Promed. Archive No. 20080403.1222. Available at http://www.promedmail.org (accessed 20 4 2009).

Guindon, S., Lethiec, F., Duroux, P., Gascuel, O., 2005. PHYML online–a web server for fast maximum likelihood-based phylogenetic inference. Nucleic Acids Res. 33, 557–559.

Hajibabaei, M., Janzen, D.H., Burns, J.M., Hallwachs, W., Hebert, P.D.N., 2006. DNA barcodes distinguish species of tropical Lepidoptera. Proc. Natl. Acad. Sci. U.S.A. 103, 968–971.

Kremer, M., Rebholtz, C., 1977. Systematics of the *obsoletus* group of *Culicoides* (subgenus *Avaritia)* in the Palaearctic Region, with remarks on some types. Mosq. News 37, 278.

Krüger, M., Hertwig, S.T., Jetschke, G., Fischer, M.S., 2009. Evaluation of anatomical characters and the question of hybridization with domestic cats in the wildcat population of Thuringia, Germany. J. Zool. Syst. Evol. Res. 47 (3), 268–282.

Kumar, S., Tamura, K., Nei, M., 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief. Bioinform. 5, 150–163.

Linden, A., Mousset, B., Grégoire, F., Hanrez, D., Vandenbussche, F., Vandemeulebroucke, E., Vanbinst, T., Verheyden, B., De Clerck, K., 2008. Bluetongue virus antibodies in wild red deer in southern Belgium. Vet. Rec. 162, 459.

Mayr, E., Linsley, E.G., Usinger, R.L. (Eds.), 1953. Methods and Principles of Systematic Zoology. McGraw-Hill, New York, 328 pp.

Mehlhorn, H., Walldorf, V., Klimpel, S., Jahn, B., Jaeger, F., Eschweiler, J., Hoffmann, B., Beer, M., 2007. First occurrence of *Culicoides obsoletus*-transmitted Bluetongue virus epidemic in Central Europe. Parasitol. Res. 101, 219–228.

Meiswinkel, R., Van Rijn, P., Leijs, P., Goffredo, M., 2007. Potential new *Culicoides* vector in northern Europe. Vet. Rec. 161, 564–565.

Meiswinkel, R., Baldet, T., De Deken, R., Takken, W., Delécolle, J.C., Mellor, P.S., 2008. The 2006 outbreak of bluetongue in northern North-western Europe—the entomological perspective. Prev. Vet. Med. 87, 55–63.

Mellor, P.S., Boorman, J., Baylis, M., 2000. *Culicoides* biting midges: their role as arbovirus vector. Annu. Rev. Entomol. 45, 307–340.

Mellor, P.S., Boorman, J., 1995. The transmission and geographical spread of African horse sickness and bluetongue viruses. Ann. Trop. Med. Parasitol. 89, 1–15.

Nolan, D.V., Carpenter, S., Barber, J., Mellor, P.S., Dallas, J.F., Mordue Luntz, A.J., Piertney, S.B., 2007. Rapid diagnostic PCR assays for members of the *Culicoides obsoletus* and *Culicoides pulicaris* species complexes, implicated vectors of bluetongue virus in Europe. Vet. Microbiol. 124, 82–94.

Pagès, N., Sarto i Monteys, V., 2005. Differentiation of *Culicoides obsoletus* and *Culicoides scoticus* (Diptera: Ceratopogonidae) based on mitochondrial cytochrome oxidase subunit I. J. Med. Entomol. 42, 1026–1034.

Pagès, N., Muñoz-Muñoz, F., Talavera, S., Sarto, V., Lorca, C., Núñez, N.I., 2009. Identification of cryptic species of *Culicoides* (Diptera: Ceratopogonidae) in the subgenus *Culicoides* and development of species-specific PCR assays based on barcode regions. Vet. Parasitol. 165, 298–310.

Pfingstl, T., Schäffer, S., Ebermann, E., Krisper, G., 2009. Differentiation between two epilittoral species. Scutovertex arenocolus spec. nov. and Scutovertex pilosetosus Polderman (Acari: Oribatida) from different European coasts. Zootaxa 2153, 35–54.

R Development Core Team, 2009. R: A Language and Environment for Statistical Computing. R Foundation Statistical Computing. http://www.R-project.org.

Rawlings, P., 1996. A key, based on wing patterns of biting midges (genus *Culicoides* Latreille-Diptera: Ceratopogonidae) in the Iberian Peninsula, for use in epidemiological studies. Graellsia 52, 57–71.

Ruiz-Fons, F., Álvaro, R., Reyes-García, A.R., Alcaide, V., Gortázar, C., 2008. Spatial and temporal evolution of buletongue virus in wild ruminants, Spain. Emerg. Infect. Dis. 14, 951–953.

Saegerman, C., Berkvens, D., Mellor, P.S., 2008. Bluetongue epidemiology in the European Union. Emerg. Infect. Dis. 14, 539–544.

Savini, G., Goffredo, M., Monaco, F., Di Gennaro, A., Cafiero, M.A., Baldi, L., De Santis, P., Meiswinkel, R., Caporale, V., 2005. Bluetongue virus isolations from midges belonging to the *obsoletus complex* (*Culicoides*, Diptera: Ceratopogonidae) in Italy. Vet. Rec. 157, 133–139.

Schwartz-Cornil, I., Mertens, P.P., Contreras, V., Hemati, B., Pascale, F., Bréard, E., Mellor, P.S., Maclachlan, N.J., Zientara, S., 2008. Bluetongue virus: virology, pathogenesis and immunity. Vet. Res. 39, 46.

Schwenkenbecker, J.M., Mordue (Luntz), J., Switek, K., Piertney, S.B., 2009. Discrimination of *Culicoides* midge larvae using multiplex polymerase chain reaction assays based on DNA sequence variation at the mitochondrial cytochrome C oxidase I gene. J. Med. Entomol. 46, 610–614.

Wirth, W.W., Hubert, A.A., 1989. The *Culicoides* of Southeast Asia (Diptera: Ceratopogonidae). Mem. Am. Entomol. Inst. 44, 1–508.