Culicoides *midges (Diptera: Ceratopogonidae) as vectors of orbiviruses in Slovakia*

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Summary
In recent years, rapid spread of *Culicoides*-borne pathogens such as bluetongue (BT) and Schmallenberg viruses have been reported in Europe. In this study we examined the *Culicoides* populations in farms with wild and domestic ruminants in Eastern Slovakia with the aim to confirm the presence of biting midges serving as potential vectors of important pathogens. The main vector complexes were the Obsoletus complex (54%; n=4,209) and the Pulicaris complex (23%; n=1,796). To estimate the relative abundance of the cryptic species of the Obsoletus complex (*Culicoides obsoletus*, *Culicoides scoticus* and *Culicoides montanus*), we performed the multiplex polymerase chain reaction (PCR) based on ITS-2 and ITS-1 segments, on 125 midges randomly sampled. The relative abundance of *C. obsoletus* ranged from 5.26% in the farm with wild ruminants to 85.71% in another farm with cattle and sheep. A total of 112 pools of parous and gravid females belonging to the Obsoletus and Pulicaris complexes were tested for virus detection by the real-time reverse transcription polymerase chain reaction (RT-PCR) for BT virus, as well as for the Epizootic Hemorrhagic Disease Virus (EHDV), with negative results.

Studio sui Culicoides *(Diptera: Ceratopogonidae) possibili vettori di orbivirus in Slovacchia*

Parole chiave
Bluetongue,
Culicoides,
ITS-1,
ITS-2,
Malattia emorragica epizootica,
Slovacchia.

Riassunto
Recentemente in Europa si è verificata una rapida diffusione di malattie trasmesse da *Culicoides* come la Bluetongue (BT) e l'infezione determinata da Schmallenberg virus (SBV). In questo lavoro sono state studiate le popolazioni di *Culicoides* in allevamenti di ruminanti di specie domestiche e selvatiche nella Slovacchia orientale con l'obiettivo di verificare la presenza di potenziali specie vettori di virus. Quelle più abbondanti sono risultate le specie di *Culicoides* appartenenti all'Obsoletus e Pulicaris Complexes (rispettivamente 54%; n=4.209 e 23%; n=1.796). Per valutare l'abbondanza relativa delle singole specie dell'Obsoletus Complex (*Culicoides obsoletus*, *Culicoides scoticus* e *Culicoides montanus*) sono stati identificati 125 moscerini mediante PCR multiplex basata sui segmenti ITS-2 e ITS-1. L'abbondanza relativa di *C. obsoletus* è risultata compresa tra 5,26% in un allevamento con ruminanti selvatici e 85,71% in un allevamento con bovini e ovini. In totale sono stati analizzati, con real-time RT-PCR per BT e RT-PCR per malattia emorragica epizootica, 112 femmine adulte, pluripare e gravide di specie appartenenti a Obsoletus e Pulicaris Complexes, tutte risultate negative.

Introduction

Vector-borne pathogens, such as bluetongue and Schmallenberg viruses, have recently emerged and spread in Europe. Another vector borne pathogen, such as the Epizootic Hemorrhagic Disease (EHD) Virus, has lately been reported in countries bordering the European Union. All these viruses affected mainly ruminants and are transmitted by biting midges of the *Culicoides* genus.

Bluetongue is an infectious, non-contagious disease of wild and domestic ruminants caused by the Bluetongue virus (BTV) of the *Orbivirus* genus in the *Reoviridae* family. In August 2006, the first cases of the BTV serotype 8 infections were detected in Western Europe: Netherlands, Belgium, and Germany (Wilson and Mellor 2009). In 2007, a massive geographic spread of the disease and a dramatic increase in the number of affected farms and infected animals were observed. In 2007 and 2008, the outbreaks of bluetongue were reported in countries bordering the Slovak Republic, such as Czech Republic, Hungary, and Austria (Carpenter *et al.* 2009). In 2012, the BTV-14 appeared in Poland and other Baltic states[1]. In countries where the main Afro-Asian vector *Culicoides imicola* is not present, the BTV transmission among hosts occurs through the indigenous species of *Avaritia* and *Culicoides* subgenera. The bluetongue virus has been detected in the Obsoletus complex (De Liberato *et al.* 2005, Mehlhorn *et al.* 2007, Mellor and Pitzolis 1979, Savini *et al.* 2004, Savini *et al.* 2005), *Culicoides obsoletus* (Hoffmann *et al.* 2009), *Culicoides dewulfi* (Meiswinkel *et al.* 2007), *Culicoides chiopterus* (Dijkstra *et al.* 2008), and also *Culicoides pulicaris* (Caracappa *et al.* 2003, Vanbinst *et al.* 2009) and *Culicoides lupicaris* (Romón *et al.* 2012).

Epizootic Haemorrhagic Disease is an infectious non-contagious viral disease of wild ungulates and rarely cattle. The causative agent, the Epizootic Haemorrhagic Disease Virus (EHDV), also belongs to the *Reoviridae* family, *Orbivirus* genus. The disease was observed in North America, Australia, Asia, and Africa; while, the EHDV has never been reported in Europe (Savini *et al.* 2011). In recent years, the disease has been expanding in countries surrounding the Mediterranean basin, including Morocco, Algeria, Tunisia (Efsa 2009), Israel (Wilson and Mellor 2009), and Turkey (Temizel *et al.* 2009). The EHDV can share common vectors with the BTV in South Africa (Paweska *et al.* 2002, Venter *et al.* 1998) and it is likely that the species of *Culicoides* that could transmit the EHDV in Europe are similar, if not identical to those transmitting the BTV (Savini *et al.* 2011).

Within the *Avaritia* subgenus there are several species involved in the arbovirus transmission in Europe: *C. imicola*, the Obsoletus complex, *C. dewulfi* and *C. chiopterus*. The Obsoletus complex includes cryptic species (*C. Obsoletus sensu stricto*, *C. scoticus* and *C. montanus*) which are very difficult to identify by means of morphology (Meiswinkel *et al.* 2004). The males of *C. obsoletus* and *C. scoticus* can be distinguished according to the shape of genitalia; while the diagnostic characters of females overlap.

Several molecular tools have been developed to identify the *Culicoides* species or to study their phylogenetic relationships. Many of them have focused on the internal transcribed spacer 1 (ITS-1) (Cêtre-Sossah *et al.* 2004, Mathieu *et al.* 2007) and ITS-2 region of the ribosomal DNA (Gomulski *et al.* 2005), or on mitochondrial cytochrome oxidase subunit I (COI) DNA (Augot *et al.* 2010, Lehmann *et al.* 2012, Nolan *et al.* 2007, Pagès *et al.* 2009, Pagès and Sarto 2005, Schwenkenbecher *et al.* 2009). In addition to these qualitative PCR assays, the quantitative real-time PCR has recently been developed (Mathieu *et al.* 2011) to estimate simultaneously the relative abundance of *C. obsoletus* and *C. scoticus* in large samples.

In this study we examined the *Culicoides* populations in farms with ruminants in Eastern Slovakia with the aim to identify the presence and profusion of the species of *Culicoides* in the region and to assess the presence of biting midges that could act as potential vectors of important pathogens such as BTV and EHDV. To identify the species of the Obsoletus complex (*C. obsoletus*, *C. scoticus* and *C. montanus*) and to estimate their relative abundance, we performed the multiplex PCR based on ITS-2 and ITS-1 segments.

Material and methods

Insect collections

A total of 6 *Culicoides* captures were collected between May and June 2011 from 3 farms in Eastern Slovakia: a cattle farm (Tulcik, 1 collection), a farm with cattle and sheep (Michalany, 1 collection), and a farm with fallow deer and mouflons (Rozhanovce, 4 collections). Midges were collected by miniature blacklight traps model 1212. The traps were situated in close proximity to the cattle in the first 2 livestock farms, while in the farm with wild animals, where more than 200 fallow deer and 70 mouflons are reared on the 470 ha area, the trap was hung on a tree near a water pond on the border between the forest and the meadow.

[1] PRO/AH/EDR. 2012. Bluetongue - Europe (10): Estonia, Latvia, Lithuania, Poland, BTV-14, susp., archive number: 20121127.1426885. 2012. http://www.geostrategicforecasting.com/proahedr-bluetongue-europe-10estonia-latvia-lithuania-poland-btv/.

The *Culicoides* captures were analysed and morphologically identified according to Delécolle (Delécolle 1985), Campbell and Pelham-Clinton (Campbell and Pelham-Clinton 1960) and Goffredo and Meiswinkel (Goffredo and Meiswinkel 2004). The males were identified on the basis of the shape of the genitalia (Delécolle 1985). Subsequently, the females were age-graded according to the abdomen pigmentation as nulliparous, parous, gravid and freshly engorged (Dyce 1969) and stored in 70 % ethanol.

To estimate the relative abundance of the species belonging to the Obsoletus complex, a total of 125 nulliparous females were randomly sorted out (at least 21 midges per location) and identified individually by the multiplex PCR. Of these, 110 were identified by using the ITS-2 ribosomal DNA segment (Gomulski *et al.* 2005) and 15 by using the ITS-1 segment (Mathieu *et al.* 2011). The multiplex PCR based on the ITS-1 segment was also used to confirm the identification of 4 midges morphologically suspected to belong to *C. chiopterus*.

The parous and gravid females belonging to the Obsoletus and to the Pulicaris complexes were divided into pools and tested for the presence of EHDV and BTV RNA.

DNA extraction

The DNA for molecular analysis was extracted from randomly selected 125 individuals morphologically ascribed on the basis of wing pattern to the Obsoletus complex and 4 females suspected as *C. chiopterus*. Extraction was carried out using the automated Maxwell 16 system (Promega, Madison, Wisconsin, USA) with the DNA IQ Casework Sample kit (Promega, Madison, Wisconsin, USA) according to manufacturer´s instructions.

PCR amplification of the ITS-2 segment for identification of species belonging to the Obsoletus Complex

The ITS-2 segment of ribosomal DNA was amplified using the primers 5.8 SF, 28 SR, Scoticus-194R, MOU-316F, and Montanus-227R (Gomulski *et al.* 2005). The reaction volume was 25 µl, consisting of 2.5 µl 10x buffer, 2 mM of MgCl2, 0.2 mM of dNTPs (Promega, Madison, Wisconsine, USA), 1 µM of primer 5.8SF, 0.8 µM of primer 28 SR, 0.4 µM of Scoticus-194R, 0.2 µm of primer MOU-316F, 1 µM of primer Montanus-227R, 0.2 µl of Ampli Taq Gold (Applied Biosystems, Carlsbad, California, USA), 16.1 µl of H2O, and 2 µl of DNA. The thermal profile consisted of an initial denaturation step at 94°C for 10′, followed by 40 cycles of denaturation at 94°C for 30″, annealing at 56°C for 30″, elongation at 72°C for 30″ and ended with the final elongation at 72°C for 7′. The PCR products were separated on the E-gel 4% Agarose (Invitrogen, Carlsbad, California, USA).

PCR amplification of the ITS-1 segment for identification of species belonging to the Obsoletus Complex, *C. chiopterus* and *C. dewulfi*

Multiplex PCR based on ITS-1 sequences (Mathieu *et al.* 2007) were used to identify 15 individuals from the Obsoletus complex and 4 specimens morphologically described as *C. chiopterus*, as well to categorise the species of Obsoletus complex and the morphologically related species *C. chiopterus* and *C. dewulfi*. The midges DNA was amplified with the primers PanCulF, Obs-sl-R, Obs-ss-R, Dewulfi-R, Montanus-R, and Chiopterus-R (Mathieu *et al.* 2007). Reactions were performed in a total volume of 25 µl consisting of 10 x PCR reaction buffer; 1.5 mM of MgCl2; 250M of each dATP, dCTP, dGTP, and dTTP; 20 pmol of the primers Obs-ss-R, Obs-sl-R, Dewulfi-R, and Chiopterus-R; 40 pmol of Montanus-R; 60 pmol of Pan CulF; and 2.5 U of TaqDNA polymerase and 1 µl of DNA. The PCR reaction was carried out under the following cycling conditions: an initial denaturation stage at 94°C for 5′ and then 30 cycles at 94°C, 1′; 61°C, 1′; 72°C, 1′, and the final extension phase at 72 °C for 10′. PCR products were examined by electrophoresis in the 2.5% agarose gel.

RNA extraction for BTV and EHDV detection

Individual pools containing the maximum of 50 parous and gravid females were homogenized with the pellet pestle motor (Kontes, Vineland, New Jersey, USA) before extraction in 2 ml tubes filled with 300 µl of PBS. The RNA was extracted using the High Pure Viral Nucleic Acid Kit (Roche, Mannheim, Germany) according to the manufacturer´s instructions.

Real-Time RT-PCR for BTV detection

The BTV detection (Hofmann *et al.* 2008) was carried out using the real time RT-PCR Kit SuperScript III Platinum® One-Step Quantitative RT-PCR System (Invitrogen, Carlsbad, California, USA) and primers and the TaqMan probe were used for the region NS1 of the Bluetongue virus and for the NS5-2 region of the West Nile virus (Table I). The West Nile virus was used as the internal positive control. In total, 5 µl of viral RNA with 5 µl of the denaturation mix composed of 0.9 µM primers BTNS1-F and BTNS1-N and water was heated at 95 °C for 5 minutes and then submerged in ice for 5 minutes. Subsequently, 10 µl of denatured RNA was added to the amplification mix consisting of 12.5 µl 2x Reaction Mix, 0.5 µl

of ROX Reference Dye, 0.2 µM of primer NS5-2 F and NS5-2 R, 0.15 µM of probe NS5-2 P, 0.08 µM of probe WNEnv-P, 0.99 µl of nuclease free water, 1µl of armored WND (NY 1999) (dilution 1:100), and 0.5 µl of SuperScript III Platinum Taq Mix. The thermal cycle condition was: retrotranscription at 50°C for 15 minutes, activation 95°C for 2 minutes, 45 cycles of denaturation at 95°C for 15 seconds, annealing at 60 °C for 30 seconds.

RT-PCR for EHDV detection

The EHDV detection (Clavijo *et al.* 2010) was performed in one step RT-PCR with primers E1 (5'-TCG AAG AGG TGA TGA ATC GC-3') and E4 (5'-TCA TCT ACT GCA TCT GGC TG-3'). The mixture of 0.8 µM of primers E1-E4 and 3 µl of RNA in 5 µl of water was denatured at 95°C for 5 seconds and submerged in ice also for 5 seconds. It was followed by the addition of 40 µl of Master Mix (10µl of 5x Buffer, 10 µl of Q-solution, 2 µl of 10mM dNTP, 2 µl of RT-PCR enzyme, 16 µl of water), while respecting the One-Step RT-PCR kit (Qiagen, Hilden, Germany) instructions. The thermal cycler conditions were: 48°C for 30 minutes, 95°C for 10 minutes, 40 cycles at 95°C for 1 minutes, 55 °C for 30 seconds, 72 °C for 30 seconds, and 72°C for 10 minutes. The PCR products were examined by electrophoresis in a 1.5% agarose gel.

Table I. *Primers and probe TaqMan for the region NS1 of BTV and for the NS5-2 region of WNV (IPC).*

Primers and probe TaqMan for the region NS1 of BTV	
BTNS1 probe TaqMan	5'-FAM-CGC TTT TTG AGA AAA TAC AAC ATC AGT GGG GAT-TAMRA-3'
Primer BTNS1-F	5'-TGG CAA CCA CCA AAC ATG G-3'
Primer BTNS1-N	5'-CCA AAA AAG TCC TCG TGG CA -3'
Primers and probe TaqMan for the NS5-2 region of WNV	
NS5-2 probe TaqMan	5'-VIC-CCA ACG CCA TTT GCT CCG CTG – TAMRA-3'
Primer NS5-2-F	5'-GAA GAG ACC TGC GGC TCA TG -3'
Primer NS5-2-R	5'-CGG TAG GGA CCC AAT TCA CA -3'

Results

A total of 7,773 midges were collected and morphologically identified in 6 collections from the 3 localities, 1,888 from Rozhanovce, 3,940 from Michalany and 1,945 from Tulcik. The Obsoletus complex represented 54 % (n=4209) of total midges, followed by the Pulicaris complex (23%; n= 1,796). On 2 farms, species belonging to the Nubeculosus complex were also captured, constituting 6.5% of the collected midges at Michalany (n=255) and 0.1% in Tulcik (n=2) (Table II).

The Pulicaris complex was represented by *C. pulicaris*, *C. punctatus*, *C. newsteadi*, and *C. lupicaris* (Table III). Only 1 male of *C. pulicaris* was captured (Table IV). *Culicoides imicola* and C. *dewulfi* resulted absent in all collection sites. Only 2 out of 4 females suspected of *C. chiopterus* were confirmed by the PCR; the remaining 2 were identified as *C. scoticus*.

Within the 125 randomly selected females of the

Table II. Culicoides *collected on three farms in Eastern Slovakia (2011).*

Locality	Date	Total *Culicoides*	Pulicaris Complex (%)	Obsoletus Complex (%)	Nubeculosus Complex (%)	*Culicoides* spp. (%)
Rozhanovce	13/05/2011	619	38 (6.14)	562 (90.79)	0	19 (3.07)
Rozhanovce	26/05/2011	125	22 (17.6)	81 (64.8)	0	22 (17.6)
Rozhanovce	02/06/2011	415	82 (19.76)	170 (40.96)	0	163 (39.28)
Rozhanovce	23/06/2011	729	130 (17.83)	438 (60.1)	0	161 (22.09)
Michalany	25/05/2011	3940	1149 (29.16)	1517(38.5)	255 (6.47)	1019 (25.87)
Tulcik	25/05/2011	1945	375 (19.28)	1441 (74.09)	2 (0.11)	124 (6.38)

Table III. *Species composition of Pulicaris complex.*

Locality	Date	*C. pulicaris*	*C. punctatus*	*C. lupicaris*	*C. newsteadi*	Total
Rozhanovce	13/05/2011	32 (84.21)	6 (15.79)	0	0	38
Rozhanovce	26/05/2011	6 (27.27)	16 (72.73)	0	0	22
Rozhanovce	02/06/2011	74 (90.24)	8 (9.76)	0	0	82
Rozhanovce	23/06/2011	40 (30.77)	90 (69.23)	0	0	130
Michalany	25/05/2011	246 (21.41)	854 (74.33)	2 (0.17)	47 (4.09)	1149
Tulcik	25/05/2011	350 (93.34)	14 ((3.73)	0	11 (2.93)	375

Table IV. *Age grading of Obsoletus and Pulicaris complexes.*

Locality	Obsoletus complex							Pulicaris complex					
	N (%)	P (%)	G (%)	E (%)	*C. scoticus* M (%)	*C. obsoletus* M (%)	Total	N (%)	P (%)	G (%)	E (%)	M	Total
Rozhanovce	485 (86.3)	62 (11.03)	0	10 (1.78)	0	5 (0.89)	562	17 (44.74)	20 (52.63)	0	1 (2.63)	0	38
Rozhanovce	19 (23.46)	61 (75.31)	0	1 (1.23)	0	0	81	6 (27.27)	16 (72.73)	0	0	0	22
Rozhanovce	80 (47.06)	77 (45.29)	1 (0.59)	4 (2.35)	4 (2.35)	4 (2.35)	170	44 (53.66)	36 (43.9)	0	1 (1.22)	1 (1.22)	82
Rozhanovce	347 (79.22)	85 (19.41)	0	6 (1.37)	0	0	438	72 (55.38)	41 (31.54)	0	17 (13.08)	0	130
Michalany	297 (19.58)	531 (35.0)	687 (45.29)	0	0	2 (0.13)	1517	513 (44.65)	624 (54.31)	12 (1.04)	0	0	1149
Tulcik	697 (48.37)	600 (41.64)	130 (9.02)	11 (0.76)	0	3 (0.21)	1441	149 (39.73)	224 (59.73)	1 (0.27)	1 (0.27)	0	375

N = nulliparous; P = parous; G = gravid; E = engorged; M = males.

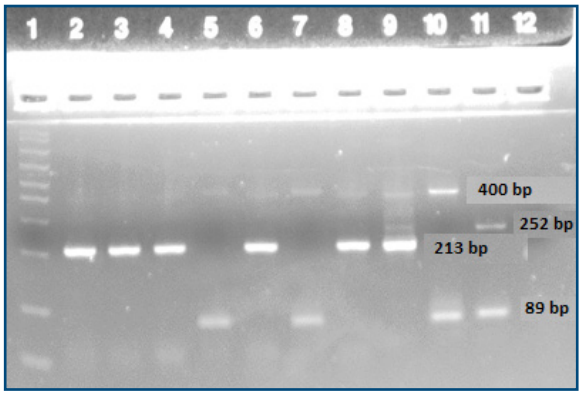

Figure 1. *Gel electrophoresis of amplification products of multiplex PCR assay for ITS-2.*

Lines 2-4, 6, 8 - *C. scoticus;* lines 5, 7 - *C. obsoletus;* line 9 - *C. scoticus* (positive control); line 10 - *C. obsoletus* (positive control); line 11 - *C. montanus* (positive control).
(*C. obsoletus* - 400 and 89 bp; *C. scoticus* - 400 and 213 bp; *C. montanus* - 400, 252 and 89 bp).

Obsoletus complex identified by the multiplex PCR, *C. obsoletus sensu stricto* and *C. scoticus* were confirmed, while *C. montanus* resulted absent in the samples (Figures 1 and 2). Their relative abundance is shown in Table V. At the locality of Rozhanovce, *C. scoticus* resulted as the most abundant species of the complex (59.04%). At the other 2 sites of Michalany and Tulcik, *C. obsoletus* resulted largely as the most abundant in the samples, 85.71% and 80.95%, respectively. The males of *C. obsoletus* were caught in all locations and *C. scoticus* only in Rozhanovce (Table IV).

Within the Obsoletus and Pulicaris complexes, the parous/gravid rate observed in the 6 collections ranged between 11%-80% and 31.5%-73%, respectively (Table IV).

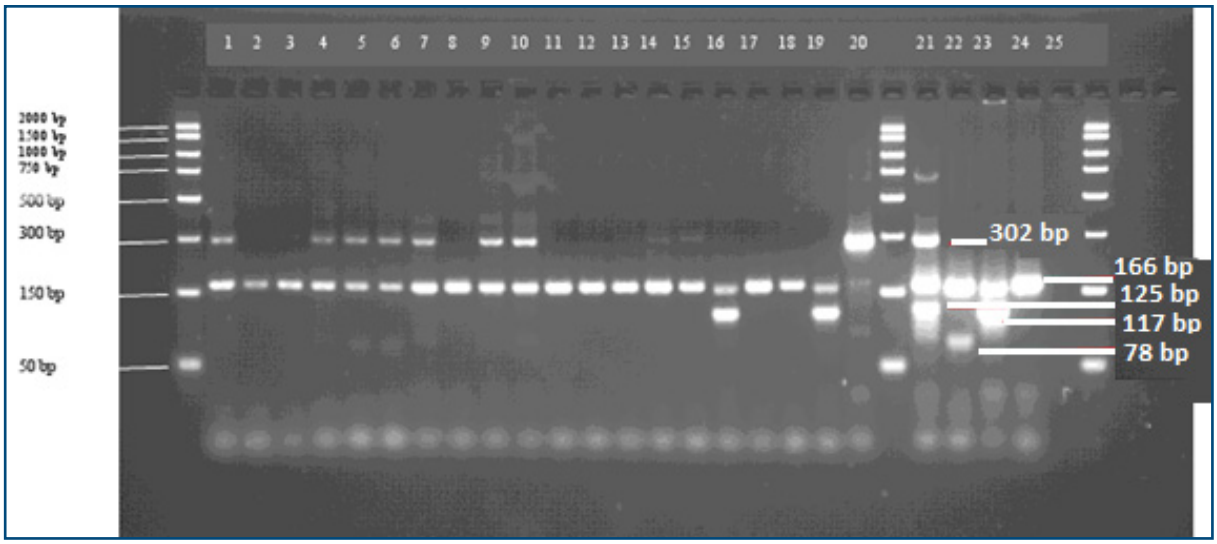

Figure 2. *Gel electrophoresis of amplification products of the multiplex PCR assay for ITS-1.*

Lines 1, 4-7, 9, 10 - *C. obsoletus*; lines 2, 3, 8, 11-15, 17, 18 - *C. scoticus*; lines 16, 19 - *C. chiopterus*; line 20 - *C. obsoletus* (positive control); line 21 - *C. montanus* (positive control); line 22 - *C. dewulfi* (positive control); line 23 - *C. chiopterus* (positive control); line 24 - *C. scoticus* (positive control).
(*C. obsoletus* - 302 bp and 166 bp; *C. scoticus* - 166 bp; *C. chiopterus* - 166 bp and 117 bp; *C. montanus* - 302 bp, 166 bp and 125 bp; *C. dewulfi* - 166 bp and 78 bp).

Table V. *Relative abundance of the sibling species of the Obsoletus complex.*

Locality	Date	N. of PCR analysed F	*C. obsoletus* (%)	*C. scoticus* (%)
Rozhanovce	13/05/2011	21	13 (61.9)	8 (38.1)
Rozhanovce	26/05/2011	19	1 (5.26)	18 (94.74)
Rozhanovce	02/06/2011	22	4 (18.18)	18 (81.82)
Rozhanovce	23/06/2011	21	16 (76.19)	5 (23.81)
Michalany	25/05/2011	21	18 (85.71)	3 (14.29)
Tulcik	25/05/2011	21	17 (80.95)	4 (19.05)

F = females.

A total of 112 pools of parous and gravid females were prepared; 79 from the Obsoletus complex (n=2,233) and 33 from the Pulicaris complex (n=978). When tested for BTV and EHDV, all pools resulted negative.

Discussion

The lack of information about the *Culicoides* abundance and biology in the Slovak Republic and current situation in *Culicoides*-borne diseases in Europe are the reasons why we performed this entomological survey. The last entomological survey focused on *Culicoides* abundance in Slovakia was carried out in 1993-1994 in Western Slovakia (Mráz and Országh 1998). The most abundant species found in this study was *C. obsoletus* (87.94%-88.33%) and the second one was *C. punctatus* (4.72%-7.36%). The species included in the Obsoletus complex (including *C. obsoletus* and *C. scoticus*) were similarly the predominant species captured at each trapping site, representing 40.96%-90.79% at Rozhanovce, 38.5% at Michalany, and 74.09% at Tulcik (Table II). The distribution of the Obsoletus complex is in accordance with the results observed by several authors (Ander *et al.* 2012, Balenghien *et al.* 2011, Mehlhorn *et al.* 2007, Purse *et al.* 2006, Romón *et al.* 2012) meaning that *C. obsoletus*/*C. scoticus* represents the dominant species of *Culicoides* recorded from throughout the Palaearctic region. The data from the neighbouring countries (Ukraine, Poland, Hungary) are not available, however the results from Austria confirm that the majority of *Culicoides* specimens belong to the *Avaritia* subgenus (89.3%), followed by the *Culicoides* subgenus (5.8%) and the *Monoculicoides* subgenus (0.8%) (Anderle *et al.* 2008). The relative abundances of these 3 subgenera in our study are comparable: the abundance of the Pulicaris complex (subgenus *Culicoides*) ranged from 6.14% to 19.76% at Rozhanovce, 29.16% at Michalany and 19.28% at Tulcik (Table II); the Nubeculosus complex (subgenus *Monoculicoides*) resulted absent at the forested site of Rozhanovce, very low abundant north in Tulcik (0.11%) and presented in higher abundance only in south-eastern Slovakia, in Michalany (6.47%) (Table II).

As we expected, *Culicoides imicola*, the most important *Culicoides* vector species in the Mediterranean basin, was not found in our sampling.

Since wild ruminants may serve as a reservoir for the BTV virus (Linden *et al.* 2008, Niedbalski and Kesy 2008, Ruiz-Fons *et al.* 2008), we included a farm with wild ruminants in this entomological survey. The aim was to find out the presence of potential vectors in the nature close to wild ruminants and compare the abundance of vectors with the abundance on the farms with domestic animals. The abundance of midges on the farms with domestic animals was 2.7-fold higher in Tulcik and 5.4-fold higher in Michalany, in comparison to the most abundant collection in Rozhanovce. This could be related to the proximity of the traps to the cattle, whereas the wild animals in Rozhanovce are not close to the traps.

Due to uncertainty of morphological characters of females, *C. obsoletus* and *C. scoticus* are usually determined as Obsoletus complex, but the vector competence for virus transmission is not identical. Carpenter and colleagues (Carpenter *et al.* 2008) demonstrated in experimental infections using the BTV-8 and BTV-9 that *C. scoticus* was infected with 3 log10 higher virus titers than *C. obsoletus*. Similarly, Elbers and colleagues (Elbers *et al.* 2013) confirmed that in field caught Obsoletus complex, the rate of *C. scoticus* SBV positive females was higher than SBV positive *C. obsoletus* females (Elbers *et al.* 2013). On the other hand, in Belgium, the SBV was not detected in *C. scoticus* but only in *C. obsoletus* (De Regge *et al.* 2012) and in Italy, *C. obsoletus* resulted as the most abundant species of the Obsoletus complex in the area where the SBV circulated, and was positive to SBV (Goffredo, *pers. obs.*)

Results of the multiplex PCR identification based on the ITS-1 and the ITS-2 segments gave an estimation of the relative abundance of *C. obsoletus* and *C. scoticus* species in the three sampling sites in Slovakia. The two species were present in all sites, representing overall also the most abundant potential vectors of *Culicoides*-borne diseases in the study area. On the two farms with domestic animals, Tulcik and Michalany, *C. obsoletus* resulted largely as the most abundant species of the complex being identified in the 85.71 % and 80.95 % of the captured *Culicoides*, respectively. On the contrary, in Rozhanovce, *C. scoticus* was found more abundant (94.74%) in the same period (25th-26th May); however, its abundance varied during individual captures from 23.81% to 94.74% (Table V).

The analysis of the reasons causing such variations

can only be speculative; they could be ascribed to the different ecology of the Rozhanovce area, to the different animal species in the surrounding of the traps, or to different climatic conditions during various collection nights. More studies are needed for better understanding of these data, representing the first survey based on molecular methods in Slovakia.

The BT and the EHD are diseases caused by closely related viruses of the *Orbivirus* genus. Although no autochthonous outbreak of bluetongue was confirmed in the Slovak Republic, the presence of bluetongue antibodies was observed in Holstein heifers imported from France in August 2008 (Lacková *et al.* 2012). The occurrence of BT outbreaks in the Czech Republic and Hungary caused that a part of the Slovak area was lying in the restricted zones in 2008-2010. Serological testing in 10 sentinel animals on 100 farms has been carried out every month since April 2008, and entomological monitoring on 8 farms performed by the State Veterinary and Food Administration[2]. All 3 farms selected for this study were lying in restricted zones.

The EHD has not been reported in Europe yet, but it is unknown whether the virus is present in Europe causing subclinical infection or not (Savini *et al.* 2011). After experimental infection of the European Holstein cattle with EHDV-7 and EHDV-6 the cattle was productively infected, but caused no clinical signs (Batten *et al.* 2011, Eschbaumer *et al.* 2012). The EHDV has been associated with the disease in wild cervids (Kastard *et al.* 1961). In recent years, however, clinical cases due to EHDV-6 or EHDV-7 infections were reported in cattle in Israel in the autumn of 2006 (Yadin *et al.* 2008), and in Turkey in 2007 (Temizel *et al.* 2009). No clinical signs were observed in wild and domestic small ruminants (Kedmi *et al.* 2011). Since the infection of wild ruminants with the BTV is frequently asymptomatic (Falconi *et al.* 2011) and similarly the EHDV infection may be asymptomatic, 3,211 parous and gravid females of Obsoletus and Pulicaris complexes collected in this study were tested for BTV and EHDV. No BTV or EHDV RNA was detected in any of the tested samples.

Conclusions

Considering the current situation of *Culicoides*-borne diseases in Europe and the scanty information about *Culicoides* in Slovakia, further research is required to understand the ecology of the midges and the potential spread of pathogens.

The study highlights the presence of *Culicoides* vectors in Slovakia and the abundance of competent species, such as *C. obsoletus* and *C. scoticus*, in proximity of wild and domestic ruminants. These vectors are able to transmit viruses presently circulating in Europe, such as BTV and SBV.

However, the abundance of midges can change in space and time, and subsequently the risk of *Culicoides*-borne diseases spread changes; therefore a survey with longer duration should be implemented in Slovakia.

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