Vector species of Culicoides *midges implicated in the 2012-2014 Bluetongue epidemics in Italy*

Maria Goffredo*, Monica Catalani, Valentina Federici, Ottavio Portanti, Valeria Marini, Giuseppe Mancini, Michela Quaglia, Adriana Santilli, Liana Teodori & Giovanni Savini

Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale', Campo Boario, 64100 Teramo, Italy.

** Corresponding author at: Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale', Campo Boario, 64100 Teramo, Italy.*
Tel.: + 39 0861 332416, Fax: +39 0861 332251, e-mail: m.goffredo@izs.it.

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Keywords
Bluetongue,
Culicoides,
C. newsteadi,
C. punctatus,
Vector competence.

Summary
In 2012, serotypes 1 and 4 of bluetongue virus (BTV) entered and co-circulated in Sardinia. The following year, BTV-1 spread all over Sardinia and invaded Sicily and the Italian Tyrrenian coast. In 2014, this strain spread extensively in mainland Italy, causing severe outbreaks. In late 2014, BTV-4 was detected in Southern Italy (Apulia region). This study reports the detection of BTV in species of *Culicoides* (Diptera: Ceratopogonidae) collected in Italy during the epidemics between 2012 and 2014. A total of 2,925 pools (83,102 midges), sorted from 651 collections made on 339 affected farms of 12 Italian regions, were tested for the presence of BTV by real time polymerase chain reaction (RT-PCR). The study clearly shows that *Culicoides imicola* and Obsoletus complex have played a crucial role in the bluetongue (BT) epidemics in Italy in 2012-2014. Nevertheless, it also shows that other species may have played a role in transmitting BTV during these outbreaks. *Culicoides dewulfi* and at least 3 species of the Pulicaris complex, namely *Culicoides pulicaris, Culicoides newsteadi* and *Culicoides punctatus*, were found positive to BTV. Serotype 1 was detected in all species tested, whereas the BTV-4 was detected in the Obsoletus complex, *C. imicola*, and *C. newsteadi*.

Specie di Culicoides *coinvolte nell'epidemia di Bluetongue 2012-2014 in Italia*

Parole chiave
Bluetongue,
Culicoides,
C. newsteadi,
C. punctatus,
Competenza vettoriale.

Riassunto
Nel 2012 i sierotipi 1 e 4 del virus della bluetongue (BTV) sono entrati ed hanno circolato in Sardegna. L'anno seguente il BTV-1 si è diffuso in tutta l'isola, in Sicilia e nelle coste tirreniche italiane mentre, nel 2014, ha invaso gran parte della penisola italiana causando numerosi focolai e casi clinici. Infine, verso la fine dello stesso anno, il BTV-4 è stato nuovamente rilevato nell'Italia meridionale, in Puglia. Questo studio descrive le specie di *Culicoides* (Diptera: Ceratopogonidae) in cui è stato possibile rilevare il BTV nelle epidemie 2012-2014. Gli insetti sono stati catturati in 12 regioni italiane, in 339 allevamenti colpiti dalla bluetongue (BT). In totale 2.925 pool (composti da 83.102 *Culicoides*), selezionati da 651 catture, sono stati analizzati per BTV tramite real time RT-PCR. Questo studio mostra chiaramente il ruolo cruciale giocato da *Culicoides imicola* e Obsoletus complex in Italia durante le epidemie di BT occorse tra il 2012 e il 2014. Evidenzia inoltre come altre specie possono aver giocato un ruolo nella trasmissione del virus. *Culicoides dewulfi* e almeno 3 specie del Pulicaris complex, precisamente *Culicoides pulicaris, Culicoides newsteadi* e *Culicoides punctatus*, sono infatti risultate positive al BTV. Il sierotipo BTV-1 è stato riscontrato in tutte le specie testate, mentre l'Obsoletus complex, *C. imicola e C. newsteadi* sono risultati positivi anche al BTV-4.

Introduction

In the late 1990s, when bluetongue (BT) re-appeared in the Mediterranean basin, knowledge about the vectors in this area was scanty. Apart from the major renowned vector *Culicoides imicola*, 2 additional species were listed as potential vectors of bluetongue virus (BTV) in Europe, namely *Culicoides obsoletus* and *Culicoides pulicaris*. *Culicoides obsoletus* was incriminated as a BTV vector in Cyprus in 1979 and it was believed to sustain the outbreaks in Bulgaria in 1998. *Culicoides pulicaris* was included as a potential vector of BTV after the African horse sickness virus

was isolated from a mixed pool of *C. obsoletus* and *C. pulicaris* in Spain in 1989 (Mellor and Pitzolis 1979, Mellor *et al.* 1990).

During the last decade, following numerous incursions of various BTV serotypes in Europe, the knowledge on the capability of several species of *Culicoides* to transmit orbiviruses in general, and BTV in particular, greatly improved. Thanks to morphologic and phylogenetic studies, the taxonomy of the potential Palearctic vector species *C. obsoletus* and *C. pulicaris* 'groups' was further clarified, even though it can be still considered an 'unfinished business' (Meiswinkel *et al.* 2004, Harrup *et al.* 2015, Nielsen and Kristensen 2015).

Culicoides pulicaris belongs to the subgenus *Culicoides* (*Culicoides*). At present, this subgenus includes about 50 species that should probably be divided into at least 4 subgenera. The subgenus *Culicoides sensu stricto* includes, among others, some species commonly found in Italy such as *Culicoides pulicaris* Linné, 1758; *Culicoides punctatus* Meigen, 1818; *Culicoides newsteadi* Austen, 1921; and *Culicoides lupicaris* Downes and Kettle, 1952. Moreover, the subgenus *Culicoides* could be divided into at least 3 species complexes: the Pulicaris, Newsteadi, and Impunctatus complexes and, finally, more than one taxon probably refer to *C. pulicaris* and *C. newsteadi* (Meiswinkel *et al.* 2004, Gomulski *et al.* 2006, EFSA 2008, Harrup *et al.* 2015). In this study, for convenience, the species belonging to the subgenus *Culicoides* were referred to as the 'Pulicaris complex'.

To date, in Europe, BTV was isolated from *C. imicola*, *C. obsoletus/scoticus*, and *C. pulicaris*. The genome of BTV was also detected from parous females of *C. dewulfi*, *C. chiopterus, C. lupicaris*, and *C. obsoletus* (Caracappa *et al.* 2003, Savini *et al.* 2005, Vanbinst *et al.* 2009, Romón *et al.* 2012).

Within the Pulicaris complex, *C. punctatus* and *C. newsteadi* are known to be widespread and abundant in Europe, but have never been found infected with orbiviruses in the field (EFSA 2008, Meiswinkel *et al.* 2008).

In 2012, serotypes 1 and 4 of BTV entered and co-circulated in Sardinia. The following year, in absence of vaccination, BTV-1 spread throughout Sardinia and invaded Sicily and the Italian Tyrrenian coast. In 2014, the same strain spread extensively in mainland Italy, causing severe outbreaks. In late 2014, serotype 4 of BTV was detected in Southern Italy (Apulia region) (Figure 1).

The National Entomological Surveillance Plan for Bluetongue, which is in place in Italy since 2001. includes *Culicoides* collections on BTV affected farms with the goal of identifying and evaluating the vector species involved in virus transmission. This study reports on the species of *Culicoides* collected in Italy during the epidemics between 2012 and 2014 and in which BTV could be detected, with a particular focus on the midges belonging to the Pulicaris complex (*C. newsteadi*, *C. pulicaris*, and *C. punctatus*).

Materials and methods

Culicoides light trap collections were performed according to the protocol of the National Reference Center for Exotic Diseases (Goffredo and Meiswinkel 2004). *Culicoides* were collected on farms where BTV circulation was demonstrated by seroconversions of sentinel animals and/or clinical outbreaks.

The *Culicoides* collected were identified (Campbell and Pelham-Clinton 1960, Delécolle 1985, Goffredo and Meiswinkel 2004) and age-graded (Dyce 1969). Particularly, *C. pulicaris*, *C. punctatus*, and *C. newsteadi* were identified according to the wing morphology as described by Delécolle (Delécolle 1985). When it was not possible to assign a midge to any of these 3 taxa based on these criteria, the species was classified as belonging to the Pulicaris complex.

The parous females were divided in pools of not more than 50 midges. The pools were tested for BTV by real time polymerase chain reaction (RT-PCR)

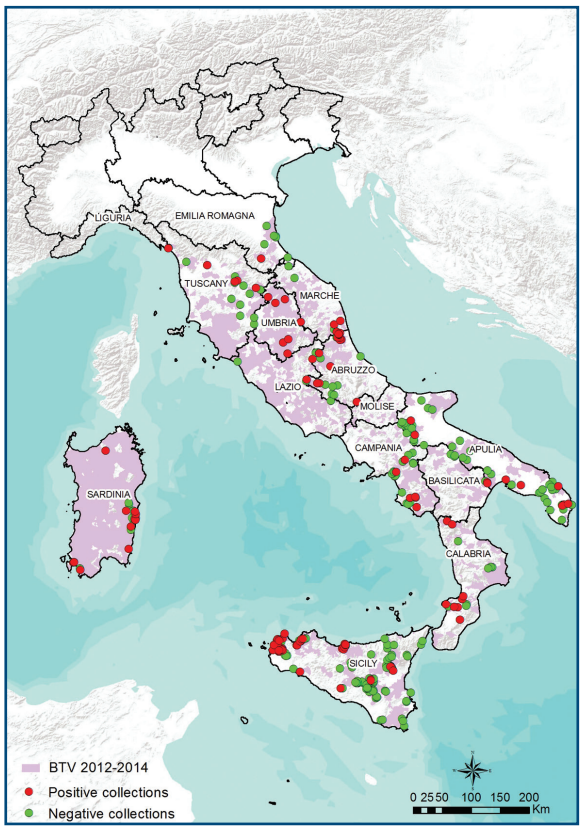

Figure 1. Culicoides *collection sites and bluetongue circulation in Italy (seroconversions and/or clinical outbreaks) during the epidemics 2012-2014.*

(BTV_{Hof}, Hofmann *et al.* 2008) or processed with a commercial real time PCR kit able to recognize all known BTV serotypes (BTV_{LSI}, LSI - Laboratoire Service International Lissieu, France - VetMAX™ Bluetongue Virus NS3 Real-Time PCR Kit, all genotypes). Threshold cycle (Ct) values of less than 50 or 40, respectively, were considered positive. Serotyping was performed on all BTV positive samples by using the TaqVet European BTV Typing kit (BTV_{sero}, LSI - Laboratoire Service International Lissieu, France).

If necessary midges belonging to the Obsoletus complex were identified with a multiplex PCR based on internal transcribed spacer 2 ribosomal DNA sequences (ITS2) (Gomulski *et al.* 2005).

Table I. *Adult parous females of* Culicoides*, tested during the 2012-2014 bluetongue epidemics in Italy (from 651 collections made on 339 affected farms).*

Region and species	Number of positive/tested pools; Number of tested midges (Minimum Infection Rate %)			
	2012	2013	2014	Total
ABRUZZO		**0/31; 502**	**94/338; 7,764 (1.2)**	**94/369; 8,266 (1.1)**
C. dewulfi			2/18; 40 (5)	2/18; 40 (5)
C. newsteadi			0/1; 1	0/1; 1
C. pulicaris		0/3; 7	7/44; 204 (3.4)	7/47; 211 (3.3)
C. punctatus		0/2; 5	5/20; 263 (1.9)	5/22; 268 (1.9)
Nubeculosus complex			1/4; 12 (8.3)	1/4; 12 (8.3)
Obsoletus complex		0/26; 490	76/211; 7,079 (1.1)	76/237; 7,569 (1)
Pulicaris complex			3/40; 165 (1.8)	3/40; 165 (1.8)
APULIA		**0/17; 337**	**45/190; 3,262 (1.4)**	**45/207; 3,599 (1.3)**
C. dewulfi			0/2; 2	0/2; 2
C. imicola			4/5; 218 (1.8)	4/5; 218 (1.8)
C. newsteadi		0/5; 35	1/15; 35 (2.9)	1/20; 70 (1.4)
C. pulicaris		0/2; 99	3/20; 350 (0.9)	3/22; 449 (0.7)
C. punctatus		0/1; 1	1/7; 9 (11.1)	1/8; 10 (10)
Obsoletus complex		0/9; 202	10/75; 1,097 (0.9)	10/84; 1,299 (0.8)
Pulicaris complex			26/66; 1,551 (1.7)	26/66; 1,551 (1.7)
CALABRIA			**106/179; 6,425 (1.6)**	**106/179; 6,425 (1.6)**
C. dewulfi			0/1; 2	0/1; 2
C. newsteadi			2/7; 70 (2.9)	2/7; 70 (2.9)
C. pulicaris			1/7; 78 (1.3)	1/7; 78 (1.3)
C. punctatus			0/5; 7	0/5; 7
Obsoletus complex			102/149; 6,226 (1.6)	102/149; 6,226 (1.6)
Pulicaris complex			1/10; 42 (2.4)	1/10; 42 (2.4)
CAMPANIA		**0/46; 1,172**	**42/322; 7,426 (0.6)**	**42/368; 8,598 (0.5)**
C. dewulfi			0/24; 265	0/24; 265
C. imicola			0/3; 9	0/3; 9
C. newsteadi		0/9; 220	0/11; 19	0/20; 239
C. pulicaris		0/6; 30	0/20; 182	0/26; 212
C. punctatus		0/8; 170	0/23; 163	0/31; 333
Nubeculosus complex		0/3; 3		0/3; 3
Obsoletus complex		0/20; 749	41/179; 6,490 (0.6)	41/199; 7,239 (0.6)
Pulicaris complex			1/62; 298 (0.3)	1/62; 298 (0.3)
EMILIA ROMAGNA			**1/21; 334 (0.3)**	**1/21; 334 (0.3)**
C. dewulfi			0/1; 1	0/1; 1
C. pulicaris			0/1; 1	0/1; 1
C. punctatus			0/1; 1	0/1; 1
Obsoletus complex			1/15; 323 (0.3)	1/15; 323 (0.3)
Pulicaris complex			0/3; 8	0/3; 8
LAZIO		**0/3; 5**		**0/3; 5**
C. newsteadi		0/1; 3		0/1; 3
C. pulicaris		0/1; 1		0/1; 1
Obsoletus complex		0/1; 1		0/1; 1

continued

The Minimum Infection Rate (MIR) was calculated by dividing the number of positive pools by the number of midges tested. The MIR is calculated on the assumption that a positive pool contains only 1 infected midge, an assumption that may have underestimated high infection rates.

Results

A total of 651 collections were assayed for the presence of BTV between 2012 and 2014. The collections were made on 339 affected farms in 12 Italian regions including Southern Italy, Central

Table I. *Adult parous females of* Culicoides*, tested during the 2012-2014 bluetongue epidemics in Italy (from 651 collections made on 339 affected farms).—cont'd*

Region and species	Number of positive/tested pools; Number of tested midges (Minimum Infection Rate %)			
	2012	2013	2014	Total
LIGURIA		**2/13; 192 (1)**		**2/13; 192 (1)**
C. pulicaris		0/1; 1		0/1; 1
C. punctatus		0/1; 1		0/1; 1
Obsoletus complex		2/11; 190 (1.1)		2/11; 190 (1.1)
MARCHE			**25/162; 6,185 (0.4)**	**25/162; 6,185 (0.4)**
C. imicola			0/1; 1	0/1; 1
C. pulicaris			1/16; 280 (0.4)	1/16; 280 (0.4)
C. punctatus			4/33; 1,097 (0.4)	4/33; 1,097 (0.4)
Obsoletus complex			20/107; 4,792 (0.4)	20/107; 4,792 (0.4)
Pulicaris complex			0/5; 15	0/5; 15
SARDINIA	**417/584; 23,538 (1.8)**	**39/121; 4,252 (0.9)**		**456/705; 27,790 (1.6)**
C. imicola	387/454; 22,032 (1.8)	20/45; 2,002 (1)		407/499; 24,034 (1.7)
C. newsteadi	18/42; 1,364 (1.3)	5/46; 1,721 (0.3)		23/88; 3,085 (0.7)
C. pulicaris	1/10; 62 (1.6)	3/9; 89 (3.4)		4/19; 151 (2.6)
C. punctatus	0/4; 6	4/7; 12 (33.3)		4/11; 18 (22.2)
Obsoletus complex	11/74; 74 (14.9)	7/14; 428 (1.6)		18/88; 502 (3.6)
SICILY	**0/55; 683**	**181/348; 6,618 (2.7)**	**4/76; 495 (0.8)**	**185/479; 7,796 (2.4)**
C. imicola	0/3; 51	123/160; 4,838 (2.5)	4/12; 130 (3.1)	127/175; 5,019 (2.5)
C. newsteadi	0/28; 546	46/111; 1,152 (4)	0/12; 41	46/151; 1,739 (2.6)
C. pulicaris	0/7; 47	3/11; 71 (4.2)	0/1; 3	3/19; 121 (2.5)
C. punctatus	0/5; 13	0/5; 9	0/1; 1	0/11; 23
Nubeculosus complex		0/1; 2	0/1; 1	0/2; 3
Obsoletus complex	0/12; 26	7/49; 417 (1.7)	0/19; 159	7/80; 602 (1.2)
Pulicaris complex		2/11; 129 (1.6)	0/30; 160	2/41; 289 (0.7)
TUSCANY		**0/29; 691**	**31/136; 4,851 (0.6)**	**31/165; 5,542 (0.6)**
C. dewulfi			0/5; 15	0/5; 15
C. imicola		0/1; 1		0/1; 1
C. newsteadi		0/3; 10		0/3; 10
C. pulicaris		0/4; 8	2/12; 118 (1.7)	2/16; 126 (1.6)
C. punctatus		0/5; 71	0/3; 3	0/8; 74
Nubeculosus complex		0/1; 4		0/1; 4
Obsoletus complex		0/14; 592	28/98; 4,578 (0.6)	28/112; 5,170 (0.5)
Pulicaris complex		0/1; 5	1/18; 137 (0.7)	1/19; 142 (0.7)
UMBRIA			**120/254; 8,370 (1.4)**	**120/254; 8,370 (1.4)**
C. dewulfi			0/4; 4	0/4; 4
C. imicola			0/1; 1	0/1; 1
C. newsteadi			0/1; 1	0/1; 1
C. pulicaris			3/23; 185 (1.6)	3/23; 185 (1.6)
C. punctatus			3/17; 167 (1.8)	3/17; 167 (1.8)
Obsoletus complex			111/180; 7,798 (1.4)	111/180; 7,798 (1.4)
Pulicaris complex			3/28; 214 (1.4)	3/28; 214 (1.4)
Total	**417/639; 24,221 (1.7)**	**222/608; 13,769 (1.6)**	**468/1,678; 45,112 (1)**	**1,107/2,925; 83,102 (1.3)**

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Table II. *Threshold cycle (Ct) values of real time RT-PCR tests for BTV (total positive pools 1,107).*

Species	Number of positive pools	Ct values								
		BTV_{Hof} (positive <50)			BTV_{LSI} (positive < 40)			BTV_{sero} (positive < 45)		
		Min	Max	Mean	Min	Max	Mean	Min	Max	Mean
C. dewulfi	2	-	-	-	-	-	-	27	38	32.5
C. imicola	538	20	46	32.1	18	38	25.5	18	41	27.9
C. newsteadi	72	30	43	37.3	22	34	30.2	25	43	34.3
C. pulicaris	24	33	42	36.3	32	32	32	35	40	37.4
C. punctatus	17	36	44	39	35	35	35	36	41	38.2
Nubeculosus complex	1	-	-	-	-	-	-	38	38	38
Obsoletus complex	416	22	44	37.5	26	38	34.8	21	42	35.7
Pulicaris complex	37	33	37	35	-	-	-	32	39	35.7

BTV_{Hof} = real time RT-PCR (Hofmann *et al.* 2008); BTV_{LSI} = commercial real time PCR kit (LSI - Laboratoire Service International Lissieu, France - VetMAX™ Bluetongue Virus NS3 Real-Time PCR Kit, all genotypes); BTV_{sero} = TaqVet European BTV Typing kit (LSI - Laboratoire Service International Lissieu, France).

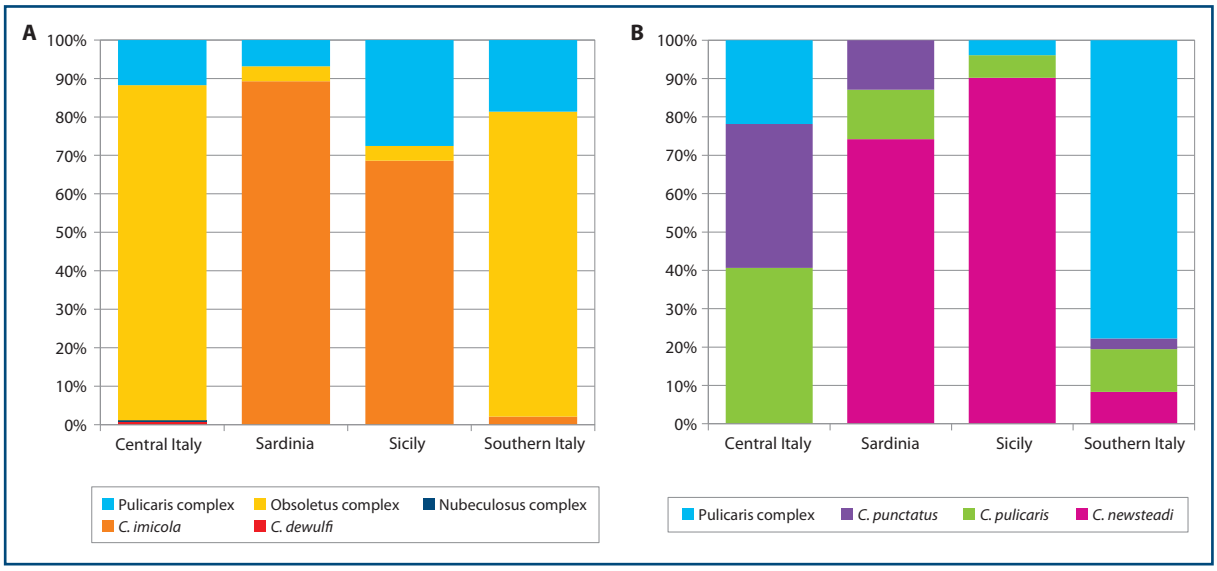

Figure 2. *Species composition of the Bluetongue virus positive pools (total pools 1,107) in Sardinia, Sicily, Southern Italy (Calabria, Apulia, Campania regions) and Central Italy (Abruzzo, Marche, Umbria, Tuscany, Southern parts of Emilia Romagna and Liguria regions).* The species of the Pulicaris complex are shown as complex (**A**) and in detail (**B**).

Italy, and the main islands of Sicily and Sardinia (Figure 1).

In this study, 2,925 pools (83,102 midges) were sorted and tested for BTV. They were composed by Obsoletus complex (43.2%), *C. imicola* (23.4%), *C. newsteadi* (10%), Pulicaris complex (9.4%), *C. pulicaris* (6.8%), *C. punctatus* (5%), *C. dewulfi* (1.9%), and Nubeculosus complex (0.3%). BTV was detected in 11 Italian regions, with the MIR ranging from 0.3% to 2.4%, in Emilia Romagna and Sicily, respectively (Table I). Overall 1,107 pools were positive for BTV resulting in a MIR of over 1%. The minimum, maximum, and mean Ct values are reported in Table II. Figure 2 shows the species composition of the positive pools according to regions.

All the taxa tested resulted positive to BTV, at least once. In particular, *C. imicola*, *C. newsteadi*, *C. pulicaris*, and the Obsoletus complex were found positive during the 3 epidemics 2012-2014. The MIR of these 4 taxa can be seen in Figure 3.

BTV-1 was detected in all species tested, whereas the BTV-4 was detected in the Obsoletus complex collected in Apulia in 2014. In addition, BTV-1 and BTV-4 were simultaneously found in 14 pools of *C. imicola* and in 1 pool of *C. newsteadi*, collected in Sardinia in 2012.

Among the Obsoletus complex collected in Sardinia, and positive to BTV-1, 42 individuals were identified at species level: 35 were *C. scoticus* (min Ct value 26, BTV_{LSI}), 4 *C. montanus* (min Ct value 35, BTV_{LSI}), and 3 *C. obsoletus* (min Ct value 34, BTV_{LSI}).

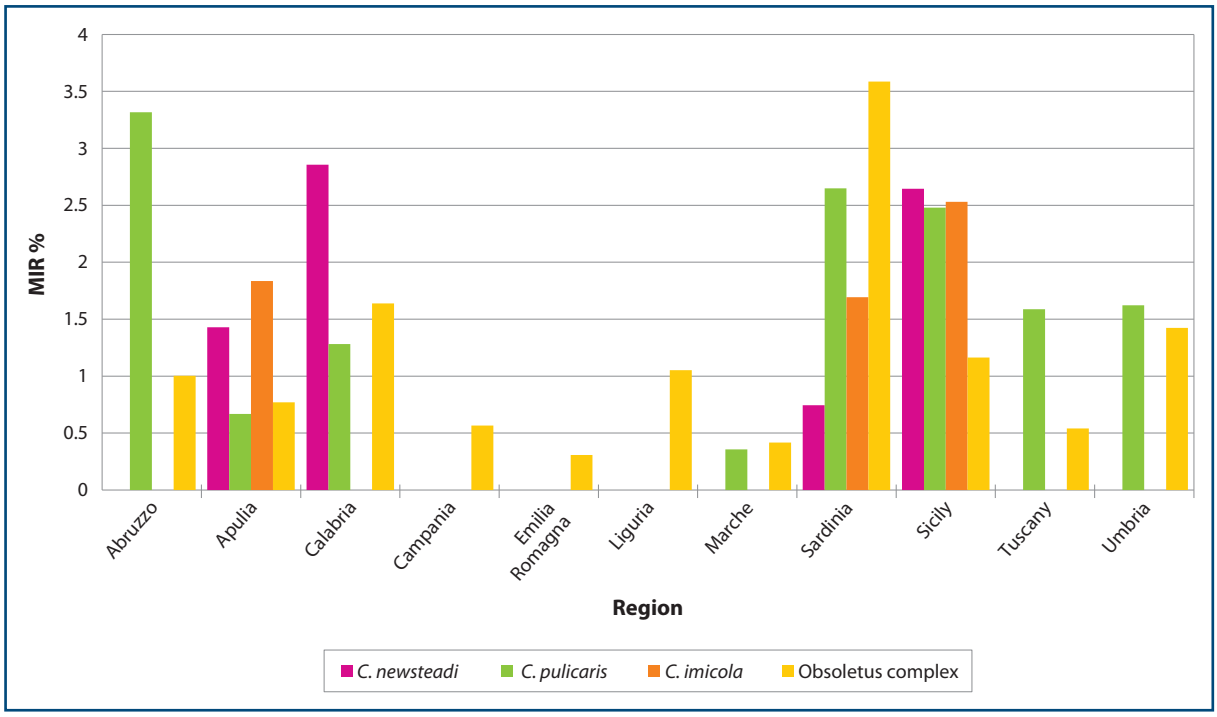

Figure 3. *Minimum Infection Rate (MIR %) in 4* Culicoides *taxa, found positive for Bluetongue virus during 3 epidemics in Italy (2012-2014).*

Discussion

The results of this study clearly show that *C. imicola* and Obsoletus complex have played a crucial role in the BT epidemics in Italy in 2012-2014. However, it also become evident that other species could have played a role in transmitting BTV during these outbreaks. *Culicoides dewulfi* and at least 3 species of the Pulicaris complex, namely *C. pulicaris*, *C. newsteadi*, and *C. punctatus*, were found positive to BTV. The Nubeculosus complex resulted positive as well, but only once, in Abruzzo region in 2014. Furthermore, within the Obsoletus complex, 3 species were found positive for BTV in the field: *C. scoticus*, *C. obsoletus* and, for the first time, *C. montanus* in Sardinia.

BTV-1 and BTV-4 were detected in *C. imicola* and in species of the Obsoletus complex collected during these 3 years of epidemics. When examining the species composition of the positive pools, *C. imicola* was particularly relevant in Sardinia and Sicily, while the Obsoletus complex was dominant in Southern and Central Italy (Figure 2 A).

According to this survey, species of Pulicaris complex, although to a lesser extent, also seem to have played a role in all the affected areas (Figure 2 A). Regarding the species of the complex involved, as expected BTV-1 was found in *C. pulicaris*. Bluetongue virus was previously isolated from this species in Italy (Caracappa *et al.* 2003). In the present study, positive pools of *C. pulicaris* were collected from 8 regions during the 3 epidemics occurred between 2012 and 2014, confirming the potential role of *C. pulicaris* in the epidemiology of BTV. Unexpectedly, BTV was also detected in *C. punctatus* and *C. newsteadi* which, to the best of our knowledge, had never been identified as BTV vectors before (Meiswinkel *et al.* 2007, Meiswinkel *et al.* 2008, EFSA 2008).

According to WHO (WHO 1967), to assess the vector competence of a *Culicoides* species for viruses such as BTV, 4 criteria should be satisfied: i) the species should be associated to the disease in the field; ii) the virus should be recovered from field collected adult females, which do not have a fresh blood meal in the abdomen; iii) the species should be able to become infected after oral infection; iv) the species should be able to biologically transmit the infection.

Very few of the *Culicoides* species, considered as potential vectors, have satisfied all 4 requirements (*i.e. C. imicola*), and, actually, the detection of viral genome by PCR in field collected *Culicoides*, has been recently used to impeach 'new' European vectors, *i.e. C. dewulfi* and *C. chiopterus* (Meiswinkel *et al.* 2007, Dijkstra *et al.* 2008).

The Ct values could give further indications on vector competence of species found positive to real time RT-PCR in the field (Veronesi *et al.* 2013). Low Ct values, indicating high amounts of viral RNA, may not always be sufficient to demonstrate that viable virus are present. On the contrary, it is indeed possible to isolate viable virus from pools with high Ct values. Nevertheless, in this study, among the 'new' potential BTV vector species, *C. newsteadi*

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showed low Ct values (min. 22, when utilizing a cut-off value of 40) comparable to those of 'known' vectors, such as *C. imicola*, *C. pulicaris*, Obsoletus complex, and *C. dewulfi* (Table II). The RNA of BTV was repeatedly found in parous females of *C. newsteadi* and *C. punctatus* collected in areas where BTV was circulating, as demonstrated by seroconversions in sentinel animals or presence of clinical outbreaks.

Within the Pulicaris complex, *C. newsteadi* represented more than 70% of the positive pools in Sicily and Sardinia (Figure 2 B). Positive pools were, however, also found in Apulia and Calabria during the 2014 epidemic (Table I). In addition, BTV-1 and BTV-4 were simultaneously detected in a single pool of *C. newsteadi*.

Culicoides punctatus resulted positive to BTV-1 in 5 regions (Sardinia, Southern and Central Italy) in 2 BTV seasons (Table I; Figure 2 B). This species was recently also found positive for Schmallenberg virus in Poland (Larska *et al.* 2013) and it was previously reported in literature as a potential vector of epizootic haemorrhagic disease virus (EHDV), an *Orbivirus* closely related to BTV (Yanase *et al.* 2005).

Culicoides dewulfi has been listed as a BTV vector since 2006, after it was found positive to BTV, during the BTV-8 outbreak in Northern Europe (Meiswinkel *et al.* 2007). In Italy, BTV has never been detected in this species and the finding of this survey represents the first record.

In conclusion, the results of this study implicate multiple vectors in the recent epidemics of BTV-1 and, to a lesser extent, of BTV-4. Although further field and laboratory studies are needed to proof the vector status of these species, this survey also indicates that *C. newsteadi* and *C. punctatus* might act as BTV vectors. Further studies are also required to better know the possible role as vector played by species of Nubeculosus complex (including in Italy at least *C. nubeculosus, C. puncticollis,* and *C. riethi*), and by *C. montanus*, a species belonging to the Obsoletus complex. Other significant information provided by this survey is that, as demonstrated in other European countries, *C. dewulfi* may also be responsible of BTV transmission in Italy. However, because of its low relative abundance, it probable does not play a pivotal role.

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