

The 'Culicoides obsoletus group' in Italy: relative abundance, geographic range, and role as vector for Bluetongue virus

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Obsoletus complex,
Vector competence.

Summary

As Bluetongue virus (BTV) spread in Italy following its first incursion in 2000, it soon became apparent that, besides *Culicoides imicola*, additional species of the subgenus *Avaritia* were involved as vectors, namely one or more of the species that belong to the so-called 'Culicoides obsoletus group', which comprises *C. dewulfi*, *C. chiopterus*, *C. obsoletus sensu stricto*, *C. scoticus* and *C. montanus*; the three last named species are considered generally as forming the Obsoletus complex. This study presents the findings made over the last decade and more, within the Italian entomological surveillance program for Bluetongue. It describes the integrated morphological and molecular approach used to identify the species of the 'C. obsoletus group', maps in detail their relative abundances and geographic ranges in Italy, clarifies the hitherto unknown comparative seasonal abundances of *C. obsoletus s.s.* and *C. scoticus* in a site in Central Italy, and provides further details on the potential vector status of five species of the 'C. obsoletus group', with emphasis on *C. obsoletus s.s.*, *C. scoticus* and *C. montanus*. Unlike the situation in Northern Europe, *Culicoides dewulfi* and *C. chiopterus* are uncommon to rare in Italy. In contrast, the Obsoletus complex occurs abundantly throughout Italy, with *C. obsoletus s.s.* being the most prevalent and ecologically adaptive of the three species making up the complex. A longitudinal study conducted at a site in Central Italy revealed that: (i) species of the Obsoletus complex prefer horses to sheep; (ii) their parity rates range from 10% (March) to 56% (November); (iii) throughout the year *C. scoticus* is consistently more abundant than *C. obsoletus s.s.*; (iv) abundances in both, *C. obsoletus s.s.* and *C. scoticus*, peak in May-June, with the peak of the latter species being more evident. Bluetongue virus was first isolated from wild caught midges of the Obsoletus complex in 2002. Thereafter, pools of selected parous midges collected across Italy, and during multiple outbreaks of BT, have been found consistently PCR-positive for the virus. More recently, viral RNA has been detected in field specimens of *C. dewulfi*, *C. obsoletus s.s.*, *C. scoticus* and *C. montanus*.

Il "Culicoides obsoletus group" in Italia: abbondanza, distribuzione geografica e ruolo vettoriale

Parole chiave

Avaritia,
Competenza vettoriale,
Culicoides chiopterus,
Culicoides dewulfi,
Obsoletus complex.

Riassunto

Sin dalle prime incursioni del virus della Bluetongue (BTV) in Italia a partire dal 2000, è risultato evidente che altre specie di vettori del sottogenere *Avaritia* fossero coinvolte, oltre a *Culicoides imicola*, in particolare quelle del cosiddetto "Culicoides obsoletus group", che comprende *C. dewulfi*, *C. chiopterus*, *C. obsoletus sensu stricto*, *C. scoticus* e *C. montanus*; le ultime tre specie formano nell'insieme l'Obsoletus complex. Questo lavoro riassume i risultati ottenuti in oltre dieci anni di sorveglianza entomologica per la BT. Descrive il metodo di identificazione di specie, basato su un approccio integrato morfologico e molecolare, riporta le mappe di distribuzione e abbondanza delle singole specie, nonché l'abbondanza relativa stagionale di *C. obsoletus s.s.* e *C. scoticus* in un sito di cattura permanente nell'Italia

Centrale, e fornisce informazioni sul possibile ruolo vettoriale in Italia di cinque specie del "C. obsoletus group". Al contrario di quanto avviene in Nord Europa, *Culicoides dewulfi* e *C. chiopterus* non sono specie comuni in Italia. Invece l'Obsoletus complex è abbondante in tutta la penisola, soprattutto *C. obsoletus s.s.*, che sembra infatti essere la specie più adattabile. Uno studio longitudinale, condotto in un sito permanente di cattura nell'Italia centrale, ha evidenziato che: (i) le specie dell'Obsoletus complex preferiscono i cavalli alle pecore; (ii) la percentuale dei parous nella popolazione varia dal 10% (marzo) al 56% (novembre); (iii); *C. scoticus* è costantemente più abbondante di *C. obsoletus s.s.*, durante tutto l'anno; (iv); entrambi *C. obsoletus s.s.* e *C. scoticus* mostrano un picco di abbondanza in maggio-giugno, il secondo con un picco più marcato. Il BTV è stato isolato per la prima volta in Italia dall'Obsoletus complex nel 2002, mentre successivamente vari sierotipi sono stati rilevati tramite PCR, nel corso delle diverse epidemie di BT, recentemente, RNA virale è stato riscontrato in campo, per la prima volta in Italia, da *C. dewulfi*, e inoltre da pool di *C. obsoletus s.s.*, *C. scoticus* e *C. montanus*, identificati a livello specifico.

Introduction

Historically, the year 2000 marks the first incursion of Bluetongue (BT) into Italy. The disease emerged in sheep and spread through the principal Afro-Asian vector *Culicoides imicola* Kieffer on Sardinia island. In 2001, Bluetongue reached the mainland and soon began to spread into areas where *C. imicola* was absent; in time this led to the discovery that also other biting midge species act as vectors for Bluetongue virus (BTV), namely *Culicoides obsoletus* (Meigen) and *Culicoides scoticus* Downes and Kettle (Savini et al. 2005). In the decade since its arrival in the Mediterranean region, BTV has continued to incur and spread ever northwards, eventually reaching 58°N, more than 1,000 km beyond its traditional limit of 45°N. Simultaneously, other biting midge species have been added to the growing vector list and now includes also *Culicoides dewulfi* Goetghebuer, *Culicoides chiopterus* (Meigen) and *Culicoides montanus* Shakirzjanova.

All six of the abovementioned species belong to the subgenus *Avaritia* Fox; excepting for *C. imicola*, most researchers consider the five remaining species to form the 'C. obsoletus group'. Phylogenetically speaking, this group does not form a natural (monophyletic) group so, cladistically speaking, is artificial. Based on adult morphology, only *C. obsoletus*, *C. scoticus* and *C. montanus* form a natural group, the Obsoletus complex (not to be confused with the 'C. obsoletus group'); this narrow interpretation has the effect of leaving *C. chiopterus* and *C. dewulfi* as two separate, free-floating monotypic entities within *Avaritia*. To a great extent, this fragmented arrangement is supported molecularly, but it remains unclear as to whether *C. chiopterus* should be included (Schwenkenbacher et al. 2009, Schulz et al. 2015) or excluded (Meiswinkel et al. 2004, Gomulski et al. 2005, Ander et al. 2013) from the Obsoletus complex. Ultimately,

the phylogeny of the subgenus *Avaritia* will be resolved only once the entire Holarctic fauna (of approximately 30 species) is treated as a whole.

In the interim, we will continue to use the paraphyletic taxon 'C. obsoletus group', but retain it in double quotes to signpost its cladistic artificiality. In Europe today, the 'C. obsoletus group' (excluding *C. imicola*) comprises seven species; the actual number is probably closer to ten. As noted above, five species of the 'C. obsoletus group' occur in Italy, namely *C. obsoletus sensu stricto*, *C. scoticus*, *C. montanus*, *C. dewulfi* and *C. chiopterus*. Integrated morphological and molecular studies indicate the occurrence of a fourth species of the Obsoletus complex in Italy and referred to provisionally as '*Culicoides* sp. unidentified' (Gomulski et al. 2005); it does not form part of this study.

Below, with reference to the 'C. obsoletus group', we present a brief five-part review of the entomological findings made in Italy since 2000 and obtained under the aegis of the Italian entomological surveillance program for Bluetongue:

- i. brief overview (above) of the unresolved taxonomy of the 'C. obsoletus group';
- ii. description of the integrated morphological and molecular approach used within the Italian entomological surveillance program of Bluetongue to identify species of the 'C. obsoletus group';
- iii. mapping of the relative abundances and geographic ranges of each species in Italy;
- iv. clarifying the hitherto unknown comparative seasonal abundances of *C. obsoletus s.s.* and *C. scoticus* based on a longitudinal study conducted at a site in Central Italy, and
- v. the potential vector status of each of the five species of the 'C. obsoletus group' is detailed,

with emphasis on *C. obsoletus s.s.*, *C. scoticus* and *C. montanus*.

Integrated morphological and molecular approach used to identify species of the 'C. obsoletus group'

Various approaches are used to identify individual species of the *C. obsoletus* group. These include traditional morphology, advanced morphometrics, mass spectrometry, various polymerase chain reaction (PCR) tests, microarrays, and the molecular sequencing of specific gene regions, in particular CO1 (barcoding), ITS1 and ITS2. Within the Italian surveillance programme for Bluetongue, the laboratory analysis of an entomological field sample is treated as rigorously as other kinds of field samples are (and involving either blood, serum, or animal tissue). Furthermore, vector species identification, which requires the screening of hundreds of samples monthly, needs to be achieved rapidly and accurately. For these reasons, a standardized protocol, integrating both morphological and molecular methods, has been developed, not only to differentiate between cryptic species such as *C. obsoletus* and *C. scoticus*, but also to confirm the identity of rarer species (such as *C. dewulfi* and *C. chiopterus*) (Figure 1).

The first step, conducted under the dissecting microscope, involves the morphological separation of specimens of the 'C. obsoletus group' from other

Culicoides species, and utilizes in combination four wing pattern characters (Goffredo and Meiswinkel, 2004), which must be applied strictly:

- distal part of second radial wing cell (r2) partially pale;
- wing macrotrichia sparse, restricted to distal half or third of wing;
- pale wing spots mostly diffuse, wing bisected into a paler basal half and a darker distal half; pale spot in the apex of r5 poorly defined, round or square, and
- absence of a central dark spot in the cubital wing cell.

The females of *C. chiopterus* and *C. dewulfi* can be distinguished from each other, and from species of the *Obsoletus* complex, based on morphology (Campbell and Pelham-Clinton 1960, Delécolle 1985). However, it is far more difficult to identify the females of the three (or more) sibling species of the *Obsoletus* complex and is the reason why species-specific molecular assays have been developed over the last decade and are being used increasingly.

Morphologically, *C. dewulfi* is the only species of *Avaritia* in Europe in which the female has > 8 long setae projecting laterally from the first abdominal segment; all the remaining species possess fewer setae, usually in the range of 2-5. Furthermore, in *C. dewulfi*, the two spermathecae differ significantly in size, while the wing has a more rounded shape;

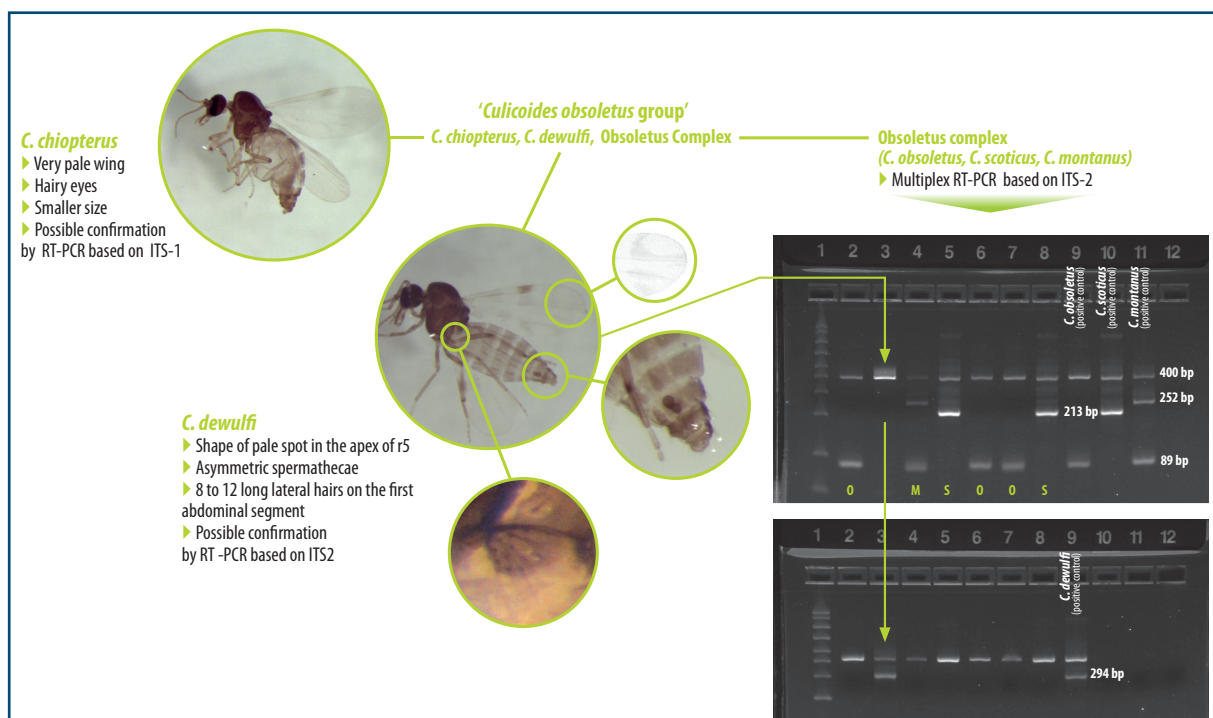


Figure 1. Integrated morphological and molecular approach to identify the species of the 'C. obsoletus group'.

unfortunately, both characters are very difficult to observe and can be quantified only under a dissecting microscope with very high resolution. *Culicoides chiopterus* is one of the smallest European *Culicoides* species, however size is not definitive as larger specimens do exist and will form part of any collection made. Uniquely, the wing in *C. chiopterus* is yellowish rather than greyish; furthermore, the pale spots are very poorly defined and difficult to observe, whilst on the wing the macrotrichia are sparse and restricted to the distal third. However, the near lack of a well-defined wing pattern means that *C. chiopterus* may be misidentified as a plain-wing species. However, the 10 plain-winged species that make up the European fauna, all differ from *C. chiopterus* in having the wing noticeably more 'hairy', i.e. the entire wing is covered densely with macrotrichia and, in most species, are dark and easy to observe.

A practiced eye is however needed to identify accurately species of *Avaritia* based on morphology alone, especially in the female, the sex that forms the bulk (> 95%) of any light-trap collection made. Especially time-consuming, is to morphologically differentiate between *C. obsoletus* s.s. and *C. scoticus* and which occur widely and abundantly across Italy. Consequently, while assaying *Culicoides* for the presence of virus, it is becoming the norm to identify them simultaneously to species molecularly.

Within the protocol used for the Italian BT entomological surveillance, an in-house multiplex real time polymerase chain reaction (RT-PCR) has been developed to differentiate between

C. obsoletus s.s., *C. scoticus* and *C. montanus*; it is based on the internal transcribed spacer 2 (ITS2) (Gomulski et al. 2005). An additional species-specific RT-PCR, based also on ITS2, was developed to confirm the identification of *C. dewulfi*. Control plasmids are used as positive reaction controls. These PCRs are based on field samples of *C. obsoletus* s.s. (male), *C. scoticus* (male), *C. dewulfi* and *C. montanus*, their identities based on homologous sequences available in GenBank; to confirm the identification of *C. chiopterus*, an RT-PCR based on ITS1, is used (Mathieu et al. 2007).

It is important to conclude by saying that within the Holarctic region, the taxonomy of the 'C. obsoletus group' is not resolved. At least three cryptic species – related closely to the five listed above and not forming part of current molecular assays – are known to exist (Gomulski et al. 2005, Meiswinkel et al. 2004, Meiswinkel et al. 2015, Kirkeby et al. 2014, Ander et al. 2012).

Distribution and abundance

Based on > 6,000 light-trap collections made across Italy, the geographic range and seasonal abundances of the species that comprise the 'C. obsoletus group' have been clarified; the three species that comprise the Obsoletus complex (*C. obsoletus* s.s., *C. scoticus* and *C. montanus*) are mapped separately, based on 3,020 individual midges – selected randomly and identified molecularly – from 87 sites (Figure 2).

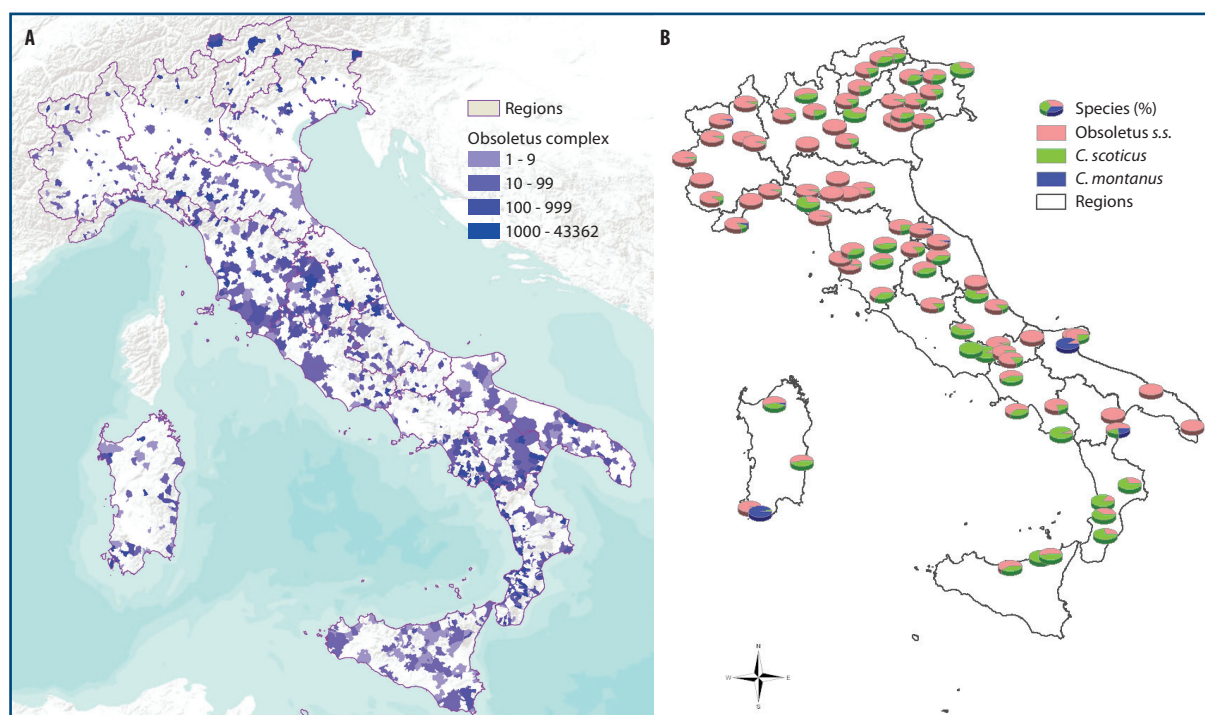


Figure 2. Abundance and distribution map of the Obsoletus complex (A), and relative abundance of *C. obsoletus*, *C. scoticus* and *C. montanus* in Italy (B).

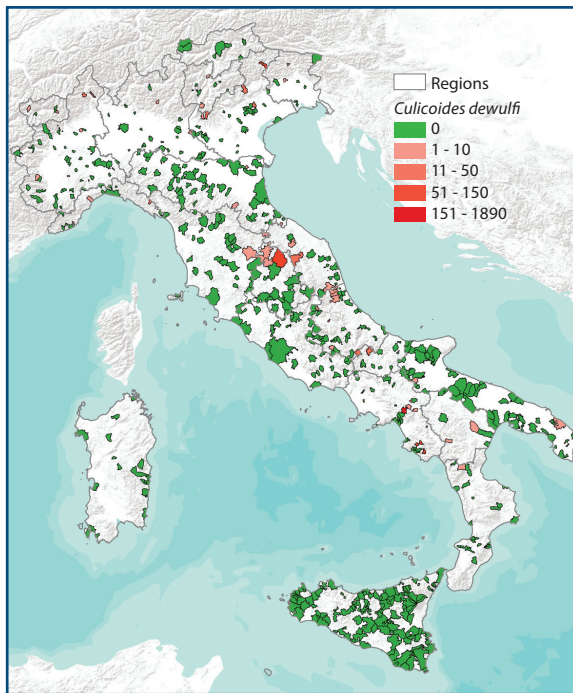


Figure 3. Abundance and distribution map of *C. dewulfi* in Italy.

Culicoides chiopterus is rarely captured in Italy, with *C. dewulfi* uncommon. Although *C. dewulfi* occurs throughout the length of the mainland, it rarely exceeds 3% of any light-trap collection made near livestock; the record catch is 1,890 specimens (relative abundance 21,7%) (Figures 3 and 4). In contrast, the Obsoletus complex, in particular *C. obsoletus s.s.* and *C. scoticus*, occurs abundantly around livestock and throughout the whole of Italy. In Northern Italy, *C. obsoletus s.s.* is the most prevalent, by far. In sharp contrast, *C. montanus* – the third species of the Obsoletus complex – is very rare (Goffredo et al. 2013). Compared to *C. imicola*, the Obsoletus Complex favours more densely vegetated habitats with increased green leaf density (Conte et al. 2007). Within the Obsoletus complex, although both *C. obsoletus s.s.* and *C. scoticus* are usually present at a collection site, the former is the most prevalent and ecologically adaptive of the three species that make up the Obsoletus complex.

A statistical Bayesian approach (Beta distribution) was used to assign the sites to a prevalent species and resulted in 52 sites for *C. obsoletus s.s.*, eight for *C. scoticus* and three for *C. montanus*. Landscape and remote sensing analysis, applied to these sites (Ippoliti et al. 2016) showed that compared to *C. obsoletus s.s.*, which is often encountered within urban and cultivated areas, *C. scoticus* is more habitat selective, favouring naturally vegetated areas, including forests. Finally, *C. montanus* is by far the rarest of the three species and is found mainly at lower altitudes in the drier southern half of Italy.

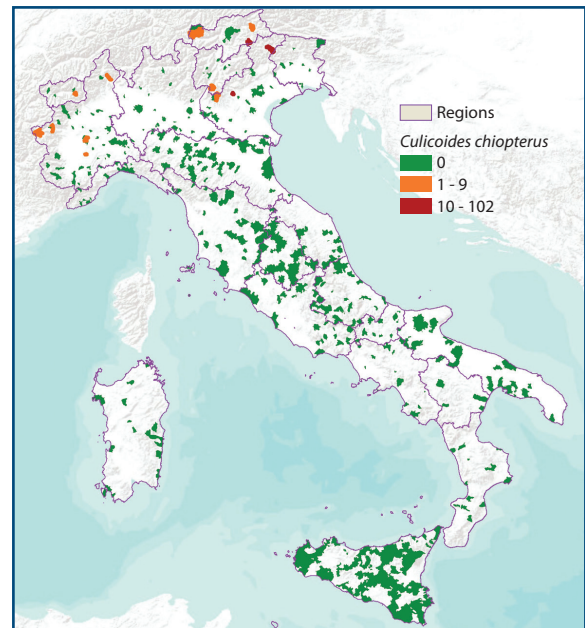


Figure 4. Abundance and distribution map of *C. chiopterus* in Italy.

A longitudinal light-trap study conducted at a permanent collection site in Central Italy revealed that:

- species of the Obsoletus complex prefer horses to sheep (100 paired collections, U-Mann Whitney test = 43.47, $p < 0.001$);
- based on 341 collections made near to horses between April 2003 and March 2007, adult females were active throughout the year, their parity rates ranging from 10% (March) to 56% (November) (Figure 5);
- based on at least 20 nulliparous females (identified by multiplex PCR) extracted at random from the 15 largest collections made monthly between January 2006-March 2007, it was determined that the relative abundances

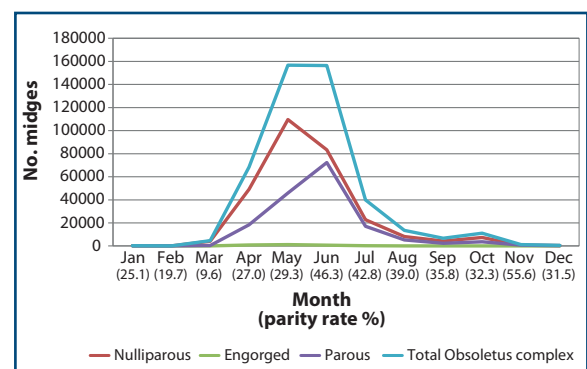


Figure 5. Age grading and parity rate of the Obsoletus complex in a permanent collection site in Central Italy. Total number of midges per month has been considered, from 341 collections near horses, between April 2003 and March 2007.

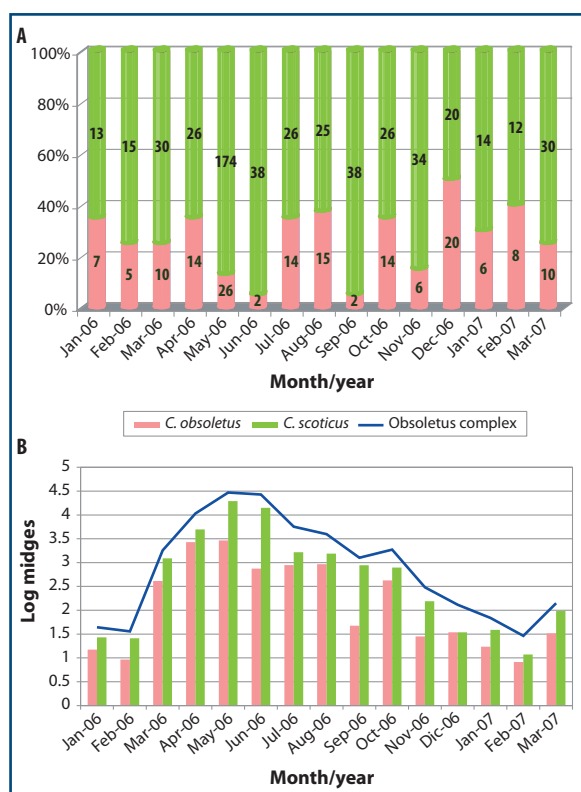


Figure 6. Seasonal abundance of *C. obsoletus* and *C. scoticus* in a permanent collection site in Central Italy, basing on 15 biggest collection/month (January 2006 - March 2007). From each collection at least 20 random nulliparous were identified by multiplex PCR (A). The species abundance has been extrapolated to the total Obsoletus complex of the collections (B).

of the two species (*C. obsoletus* s.s. and *C. scoticus*) do not change across the year. *Culicoides scoticus* was consistently the most abundant of the two species, its relative abundances in collections ranging from 50% in December to 95% in September (average 76.6%); for both species, abundances peaked in May-June, but differed in that the peak in *C. scoticus* was more pronounced (Figure 6).

Vector status

In Table 1 the vector status for each of the five species of the 'C. obsoletus group' in Italy is shown, based on field infection rates involving BTV, and laboratory infection rates for related *Orbivirus* (Goffredo et al. 2015, Federici et al. 2016). Of the five species, only *C. chiopterus* has not been implicated in BT outbreaks; BTV-1 was detected in parous *C. dewulfi* for the first time in Italy in 2014.

Table 1. Vector status of the 'C. obsoletus group' in Italy, for Bluetongue virus (BTV) and related orbiviruses (African horse sickness virus, AHSV, and Epizootic haemorrhagic disease virus, EHDV).

	Association with outbreaks	Field		Laboratory conditions
		'Virus detected by PCR'	Virus isolation	Oral infection
<i>C. chiopterus</i>	none	-	-	-
<i>C. dewulfi</i>	rare	BTV-1	-	-
Obsoletus complex	frequent	BTV (various serotypes)	BTV-2; BTV-9	AHSV-4; AHSV-9; EHDV-6
<i>C. obsoletus</i>	frequent	BTV-1	-	AHSV-4; AHSV-9; EHDV-6
<i>C. scoticus</i>	frequent	BTV-1	-	AHSV-4; AHSV-9; EHDV-6
<i>C. montanus</i>	rare	BTV-1	-	-

So far, the species of the Obsoletus complex have been involved in Italy in the transmission of BTV and of Schmallenberg virus (Goffredo et al. 2013, Balenghien et al. 2014, Goffredo et al. 2015).

In Italy, the Obsoletus complex has repeatedly been found PCR-positive to BTV genome and representing multiple serotypes (BTV-1, BTV-2, BTV-4, BTV-8, BTV-9, BTV-16); furthermore, two serotypes, namely BTV-2 and BTV-9, were isolated successfully from this vector complex (Savini et al. 2005). Subsequently, single midge specimens, identified molecularly to species and representing all three species of the Obsoletus complex, were found PCR-positive for BTV (Goffredo et al. 2015).

Finally, piecemeal observations (mostly unpublished) made on the Obsoletus complex and of potential relevance to vector capacity and the overwintering of viruses in adult females, were made: (i) midges are active during daylight hours, (ii) feed on livestock inside stables, and (iii) in winter can be found flying at temperatures below 5°C. In addition, midges maintained under laboratory conditions without a blood meal, but fed a sucrose solution, can survive for up to 104 days at 17-25°C. Furthermore, refrigerated midges maintained without sustenance and light for 10 days at 4°C were able to recover fully and suddenly thereafter (Goffredo et al. 2004).

These findings prove that BTV might overwinter in *C. scoticus* and *C. obsoletus* adult females and the same can happen for related *Orbivirus*, such as African horse sickness or Epizootic haemorrhagic disease viruses.

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