Medical and Veterinary Entomology

Medical and Veterinary Entomology (2017) **31**, 333–339 doi: 10.1111/mve.12247

EDITOR'S CHOICE

First molecular identification of the vertebrate hosts of *Culicoides imicola* in Europe and a review of its blood-feeding patterns worldwide: implications for the transmission of bluetongue disease and African horse sickness

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Abstract. *Culicoides* (Diptera: Ceratopogonidae) are vectors of pathogens that affect wildlife, livestock and, occasionally, humans. *Culicoides imicola* (Kieffer, 1913) is considered to be the main vector of the pathogens that cause bluetongue disease (BT) and African horse sickness (AHS) in southern Europe. The study of blood-feeding patterns in *Culicoides* is an essential step towards understanding the epidemiology of these pathogens. Molecular tools that increase the accuracy and sensitivity of traditional methods have been developed to identify the hosts of potential insect vectors. However, to the present group's knowledge, molecular studies that identify the hosts of *C. imicola* in Europe are lacking. The present study genetically characterizes the barcoding region of *C. imicola* trapped on farms in southern Spain and identifies its vertebrate hosts in the area. The report also reviews available information on the blood-feeding patterns of *C. imicola* worldwide. *Culicoides imicola* from Spain feed on blood of six mammals that include species known to be hosts of the BT and AHS viruses. This study provides evidence of the importance of livestock as sources of bloodmeals for *C. imicola* and the relevance of this species in the transmission of BT and AHS viruses in Europe.

Key words. Biting midges, blood-sucking insects, cattle, diseases, DNA barcoding, feeding preferences, pathogens.

Introduction

Biting midges of the genus *Culicoides* are blood-sucking insects and vectors of pathogens that affect wildlife, livestock [e.g. *Orbivirus* and *Orthobunyavirus* (Mellor *et al.*, 2000; Carpenter *et al.*, 2013)] and humans (Carpenter *et al.*, 2013; Seblova *et al.*, 2015). *Culicoides imicola* (Kieffer, 1913) is currently found in Africa, southern Europe and Southeast Asia, but recent models predict that this species will expand into northern Europe and China (Guichard *et al.*, 2014), a process that could increase frequencies of the transmission of diseases such as bluetongue (BT) and African horse sickness (AHS) in these areas. In addition, a Faustovirus-like virus, a giant virus, has recently been isolated from *C. imicola* and may be present in rodents, cows and humans (Temmam *et al.*, 2015). Since 1998, *C. imicola* (along with *Culicoides obsoletus* and *Culicoides pulicaris*) has been associated with BT outbreaks in Europe in Cyprus, Greece, Italy, Portugal, Turkey and Spain (Saegerman *et al.*, 2008). Moreover,

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long-distance wind dispersal of infected *Culicoides* has been reported as an important factor in the range expansion of BT virus (Ducheyne *et al.*, 2007; de Diego *et al.*, 2012; Jacquet *et al.*, 2016).

Knowledge of the bloodmeal sources of blood-sucking insects is essential to understanding the epidemiology of vector-borne pathogens (Simpson *et al.*, 2012). Frequencies of contact between insect vectors and competent and non-competent vertebrate hosts are closely related to pathogen amplification and the risk for transmission to different vertebrates (Kilpatrick *et al.*, 2006; Muñoz *et al.*, 2012). In recent decades, researchers have used a number of methods to identify the main hosts of *Culicoides*, including identification of the midge species attracted to particular hosts (e.g. Gerry *et al.*, 2009; Martínez-de la Puente *et al.*, 2009) and analyses of bloodmeal origins using techniques such as precipitin tests (e.g. Braverman *et al.*, 1971), indirect enzyme-linked immunosorbent assays (ELISAs) (e.g. Blackwell *et al.*, 1994) and molecular approaches (e.g. Bartsch *et al.*, 2009; Martínez-de la Puente *et al.*, 2012, 2015). Molecular tools are especially useful for identifying hosts to species level and for increasing the sensitivity and specificity of the results of immunological assays (Alcaide *et al.*, 2009; Kent, 2009). However, the success of molecular identification depends on both the gene marker used to discriminate between closely related species and the availability of previous genetic characterizations of vertebrate species (Hadj-Henni *et al.*, 2015). Identification of the host species of *C. imicola* as a means of quantifying the potential role of this vector in the transmission of pathogens to livestock and between livestock and wildlife is essential to understanding the epidemiology of BT disease in Mediterranean Europe (Ruiz-Fons *et al.*, 2014). Despite their importance in pathogen amplification, very little is known about the vertebrate hosts of *C. imicola* in Europe (Martínez-de la Puente *et al.*, 2015; Ruiz-Fons *et al.*, 2014). To the present authors' knowledge, extensive molecular studies have been conducted only in Africa, specifically in Tunisia, where *C. imicola* feeds mainly on humans [93% of 96 bloodmeals identified based on the amplification of the cythocrome *b* (cyt *b*) and PNOC genes (Slama *et al.*, 2015)] and in Senegal, where 96% of 26 identified bloodmeals derived from horses [based on cyt *b* amplifications (Bakhoum *et al.*, 2016)].

The present study was designed to identify the vertebrate hosts of *C. imicola* on farms in southern Spain using molecular methods. In addition, a fragment of the cytochrome oxidase I (COI) gene (barcoding region) of *Culicoides* females was amplified and sequenced to confirm the morphology-based identification and to provide additional information on the genetic variability of the species in southern Spain. This region has the highest abundance of *C. imicola* in the country and outbreaks of BT disease and AHS have occurred in recent decades (Rodríguez *et al.*, 1992; de Diego *et al.*, 2014). In addition, this report includes a review of information on the vertebrate hosts of *C. imicola* published in previous studies.

Materials and methods

As part of a BT surveillance programme, biting midges of the genus *Culicoides* were collected in 2011 and 2012 on farms in the provinces of Huelva (Almonte), Cadiz (Tarifa), Cordoba (Cañete de las Torres), Granada (Orgiva), Malaga (Coin) and Seville (Lora del Rio, Castilblanco de los Arroyos) in Andalusia (southern Spain) (Table 1). Centers for Disease Control (CDC) traps baited with ultraviolet (UV) light were placed 1.5–2.5 m above ground level close to livestock and were used to sample insects during 24-h sessions. Insect trapping was carried out in line with the necessary permits issued by the Junta de Andalucía. Entomological surveys and sampling on private land were conducted with the necessary permits and consent, and in the presence of landowners. This study did not affect any endangered or protected species. *Culicoides imicola* females were identified by their wing spot patterns using a stereomicroscope (Nikon SMZ645; Nikon Corp., Tokyo, Japan) and published morphological keys (González & Goldarazena, 2011). The biting midges collected were conserved at room temperature in 70% ethanol until further molecular analysis.

In the laboratory, fully or partially recently engorged *C. imicola* females preserved in good condition were selected for the molecular identification of their bloodmeals [Bartsch *et al.* (2009) describe a similar procedure]. The head-thoraces of *Culicoides* were separated from the abdomen using sterile tips. Genomic DNA in the abdomens of engorged *C. imicola* females was extracted using the DNeasy® Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany). Although it is more expensive than other DNA isolation methods, this commercial kit increases the success of host identification (Martínez-de la Puente *et al.*, 2013) and generates high-quality sequences for the barcoding characterization of insects (Gutiérrez-López *et al.*, 2015). The barcoding region of 16 *C. imicola* was amplified using the primers LCO1490 (5′-GGTCAACAAAT CATAAAGATATTG G-3′) and HCO2198 (5′-TAAACTTCAGG GTGACCAAAAAATCA-3′) (Folmer *et al.*, 1994) following the procedures described by Gutiérrez-López *et al.* (2015). The vertebrate hosts of 46 *C. imicola* were tested according to Alcaide *et al.* (2009). Amplicons were sequenced by a third-party sequencing service (Macrogen Europe, Inc., Amsterdam, the Netherlands) and sequences were edited using SEQUENCHER Version 4.9 (Gene Codes Corp., Ann Arbor, MI, U.S.A.). The COI sequences of biting midges and vertebrate hosts were identified to species level by comparing the present sequences with *Culicoides* sequences deposited in the GenBank [National Center for Biotechnology Information (BLAST)] and/or the Barcode of Life Data (BoLD) systems. *Culicoides imicola* sequences corresponding to each of the haplotypes identified were deposited in the GenBank database and vouchers corresponding to these individuals were deposited in the Museo Nacional de Ciencias Naturales (MNCN), Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain.

Results

A comparison with sequences deposited in the BoLD system revealed that the sequences of the barcoding region of the 16 biting midges analysed in the present study corresponded to *C. imicola* (similarity of $> 99.7\%$ in all cases). Overall, seven different genetic haplotypes were identified. The haplotype

Table 1. Localities of farms sampled in this study, main livestock species and hosts identified on each farm.

Province	Locality	Coordinates	Most common mammal on farm	Hosts identified (*n*)
Cadiz	Tarifa	36°4′46.80″ N, 5°37′38.31″ E	Goat	Goat (10), cow (1)
Cordoba	Cañete de las Torres	37°51′58.09″ N, 4°19′35.72″ E	Sheep, goat	Goat (10), sheep (2), horse (1)
Granada	Orgiva	36°53′54.90″ N, 3°28′17.28″ E	Cow, goat	Cow (1), dog (1)
Huelva	Almonte	37°8′31.05″ N, 6°27′52.56″ E	Horse	Horse (1), human (1)
Malaga	Coin	36°39′26.17″N, 4°45′1.82″ E	Cow	Horse (1)
Seville	Castilblanco de los Arroyos	37°44′34.83″N, 6°0′45.30″ E	Bull	Human (1)
	Lora del Rio	37°39′59.39″ N, 5°30′23.43″ E	Sheep, goat	Sheep (9), cow (2), horse (2), goat (1), human (1)

CULIMI01 (GenBank accession no. KX641483) was identified in nine individuals captured in four different provinces (Cordoba, Malaga, Seville and Cadiz), whereas the haplotype CULIMI02 (KX641484) was identified in two individuals captured in Cadiz and Seville. The remaining five haplotypes [CULIMI03–CULIMI07 (KX641485–KX641489)] were each identified in a single biting midge. In order to confirm these identifications, the haplotypes derived from a single individual were sequenced twice in both directions in two independent polymerase chain reactions, which gave identical results. The remaining head thoraces of the biting midges corresponding to each of the seven haplotypes identified in this study were deposited in the collection of the MNCN-CSIC (accession nos. MNCN/ADN: 91207–91213).

The origins of the bloodmeals of 45 *C. imicola* females were identified to species level. Six different host species were identified, including: goat (*Capra hircus*), $n = 21$; sheep (*Ovis aries*), $n = 11$; horse (*Equus caballus*), $n = 5$; cow (*Bos taurus*), $n = 4$; human (*Homo sapiens*), $n = 3$, and dog (*Canis lupus familiaris*), $n = 1$ (Fig. 1, Table 2). None of the bloodmeals were of avian origin.

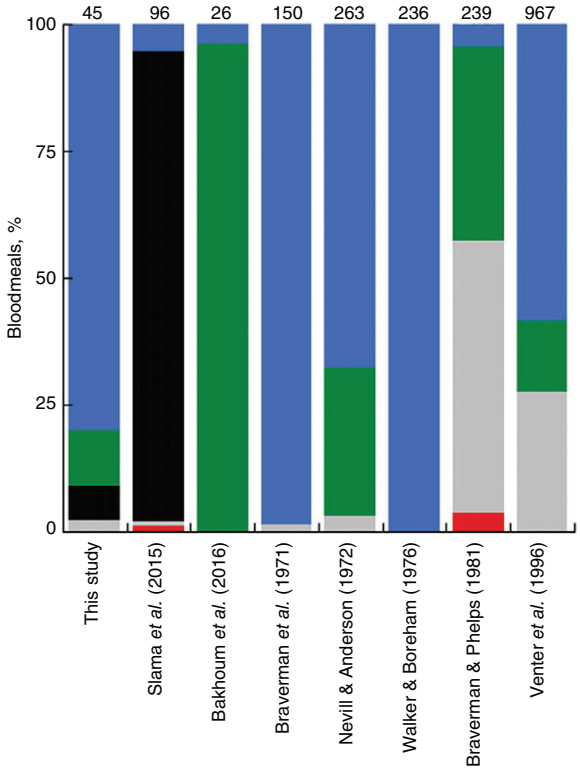

Fig. 1. Percentages of hosts identified in previous studies using molecular approaches or the precipitin test including only studies in which over 15 bloodmeals were identified (Table 1). Hosts are grouped according to their relevance in the epidemiology of bluetongue disease and African horse sickness: bovids (i.e. goats, sheep, cows and other members of the Bovidae) (blue); horses (green); humans (black); other mammals (i.e. dogs) (grey), and birds (red). Numbers above columns indicate numbers of bloodmeals identified. [Colour figure can be viewed at wileyonlinelibrary.com].

Discussion

Traditionally, *Culicoides* species have been identified on the basis of the morphological characteristics of specimens. However, this task is laborious due, above all, to the minute size of individuals and the huge number of species included in this genus. Thus, the present results provide support for the feasibility of the COI marker as a barcoding region for use in the identification of *C. imicola* (Harrup *et al.*, 2015). Along with other genetic markers, study of the genetic diversity of the COI region of *C. imicola* has been used to examine the current distribution and colonization routes of this species in Europe (Jacquet *et al.*, 2015, 2016). On the basis of sequences from 225 specimens collected in Europe and Africa, Jacquet *et al.* (2015) reported that COI sequences of *C. imicola* from southern Spain cluster together with those from western Mediterranean populations from Africa (Morocco, Algeria and Tunisia) and Europe (France and Italy). The present findings add valuable information on the genetic diversity of this vector species in southern Europe.

To the best of the present authors' knowledge, this is the first study in Europe to use molecular tools to identify the vertebrate hosts of the BT and AHS virus vector *C. imicola*. The study identified goats, sheep, cows and horses as the most frequent hosts of *C. imicola*. Species other than animals common on farms may also represent hosts of *C. imicola* (Table 1). Further studies combining animal censuses with insect captures and bloodmeal identification to quantify the preferences of insects for a particular species over other available animals in the area are required. Ruminants are considered to be the

Table 2. Vertebrate hosts of the bluetongue virus and African horse sickness virus vector *Culicoides imicola* according to the different methods of host identification used and countries studied.

Method of identification	Country	*C. imicola* bloodmeals identified	Vertebrate hosts (*n*)	Reference
Molecular identification (COI)	Spain	45	Goat (21) Sheep (11) Horse (5) Cow (4) Human (3) Dog (1)	This study
Molecular identification (cyt b and PNOC)	Tunisia	96	Human (89) Goat (3) Sheep (2) Dog (1) Southern grey shrike (1)	Slama *et al.* (2015)
Molecular identification (cyt b)	Senegal	26	Horse (25) Cow (1)	Bakhoum *et al.* (2016)
Precipitin tests	Israel∗	150	Cow (62) Sheep/goat (48) Bovid (38) Mammal (2)	Braverman *et al.* (1971)
	South Africa∗	263	Cow/sheep (95) Cow (82) Horse (77) Unidentified mammal (8) Sheep (1)	Nevill & Anderson (1972)
	Kenya∗	236	Bovid (192)† Sheep/goat (44)‡	Walker & Boreham (1976)
	Israel	3	Bird (3)	Braverman *et al.* (1977)
	Zimbabwe	239	Mammal (126) Horse (91) Bovid (11) Bird (9) Pig (2)	Braverman & Phelps (1981)
	South Africa	967	Sheep (411) Pig (266) Cow (154) Horse (136)	Venter *et al.* (1996)
Enzyme-linked immunosorbent assay	South Africa	Unknown	Horse Cow Sheep	Logan *et al.* (2010)
Agar immunodiffusion	Spain	3	Sheep/goat‡(3)	Mullens *et al.* (2010)
Host-baited traps	Senegal		Horse Sheep	Fall *et al.* (2015a, 2015b)
Direct aspiration of engorged females on hosts	Israel		Cattle Horse	Braverman (1988, 1992)
	Spain		Sheep	Gerry *et al.* (2009)
	South Africa		Horse	Scheffer *et al.* (2012)

∗*Culicoides imicola* referred as *Culicoides pallidipennis*.
†Unidentified species of the family Bovidae other than sheep or goats ($n = 149$) or any Bovidae species ($n = 43$).
‡Reacted with sheep and goat antisera at roughly equal intensities.

principal competent hosts of BT virus, although the impact of infection differs among species (Sperlova & Zendulkova, 2011; Jiménez-Clavero, 2012). Bluetongue disease usually causes significant clinical signs in sheep, whereas infections in goats and cows are usually asymptomatic (Jiménez-Clavero, 2012). However, the latter two species may play key roles as virus reservoirs (Jiménez-Clavero, 2012). *Culicoides imicola* is also involved in the transmission of AHS virus to horses. Outbreaks of both BT disease and AHS have occurred in recent decades in southern Europe, including in the study area (Rodríguez *et al.*, 1992; Saegerman *et al.*, 2008; de Diego *et al.*, 2014), and *C. imicola* is suspected or has been identified as a vector (Saegerman *et al.*, 2008). In addition to the transmission of pathogens, *C. imicola* bites may provoke the development of skin lesions and allergic dermatitis in animal species (Yeruham *et al.*, 2004; Corrêa *et al.*, 2007).

In the case of *C. imicola*, animals found within 200 m of traps were the most common sources of bloodmeals for midges (Bakhoum *et al.*, 2016), which may explain why the present study failed to identify any wild species as a host of *C. imicola* in the study area. Wild ruminants are considered to represent common hosts of *C. imicola* in southern Spain in view of the relatively high prevalence of *C. imicola*-borne pathogens in these vertebrate species (Ruiz-Fons *et al.*, 2014). However, to date most studies have been performed either on or close to farms and hence further information on *C. imicola* feeding patterns in the wild is lacking. Such knowledge will be especially relevant to identifying the roles of wildlife species as reservoirs and disseminators of *C. imicola*-borne pathogens and to describing the risk for spillover between wildlife and livestock.

Species of the subgenus *Avaritia*, which includes the BT vectors *C. imicola* and *C. obsoletus*, use mammals as their preferred hosts, although the host species used and the frequencies of their use vary among studies (Table 2) (Martínez-de la Puente *et al.*, 2015). For instance, in Senegal, most *C. imicola* were found to feed on horses, but a percentage of the identified midges also fed on cows (Bakhoum *et al.*, 2016). By contrast, humans were by far the most common hosts of *C. imicola* in Tunisia, although midges of this species also fed on goats, sheep, dogs and a single bird species (Slama *et al.*, 2015). Using a crossover electrophoresis precipitin test, Venter *et al.* (1996) identified cows, horses, sheep and pigs as hosts of *C. imicola* females trapped near livestock. Further information on the vertebrate hosts of *C. imicola* has been derived from additional studies using other methods. For instance, Gerry *et al.* (2009) collected an engorged *C. imicola* by direct aspiration from a sheep in northeast Spain and females of this species have been collected frequently from calves and/or horses in Israel (Braverman, 1988, 1992) and South Africa (Scheffer *et al.*, 2012). Studies in Senegal have captured blood-engorged *C. imicola* using traps baited with horses or sheep, which suggests that this species feeds on these mammals (Fall *et al.*, 2015a, 2015b). A global proteomic analysis of engorged *C. imicola* identified peptides related to those found in bovids, rodents and humans (Temmam *et al.*, 2015). Overall, these results reveal the ability of *C. imicola* to feed on a relatively broad range of hosts, which reflects the general pattern found in *Culicoides* species (Martínez-de la Puente *et al.*, 2015). However, further knowledge of the differential attractiveness of vertebrate species to *C. imicola* is required as this information is currently lacking in most cases. Differences in host availability between areas may explain, at least in part, this midge's distinct feeding patterns in different countries and hence future studies should consider including information on the vertebrate species present in study areas. Interspecific differences in attractiveness between vertebrates may affect the transmission of pathogens, such as in the case of cows and sheep stabled in close proximity, and may reduce the incidence of BT in the latter animal (Nevill, 1978; Randolph & Dobson, 2012).

Bird-derived bloodmeals have only occasionally been identified in *C. imicola* (Braverman *et al.*, 1977; Slama *et al.*, 2015). Despite the development of methods to identify avian-derived bloodmeals, bird blood has not been found in previous studies (Nevill & Anderson, 1972; Walker & Boreham, 1976) or in the present study. In addition, traps located close to an aviary in a zoo captured far fewer *C. imicola* females than traps located near large mammals such as elephants, buffalos, rhinoceros and impalas (Labuschagne *et al.*, 2007). Likewise, although insect captures using UV light traps on an ostrich farm in Botswana were dominated by *C. imicola*, the total number of midges captured was considered to be low and to probably reflect the absence of mammal livestock in the surrounding area (Mushi *et al.*, 1999).

In conclusion, mammals, especially ruminants, are the most common sources of bloodmeals for *C. imicola* worldwide. The finding that goats, sheep, cows and horses are the main hosts of this vector species in southern Spain provides evidence of these mammals' relevance in the transmission of BT and AHS viruses in southern Europe.

Acknowledgements

Special thanks are extended to Laura Gómez and Isabel Martín, Departamento de Ecología de Humedales, Estación Biológica de Doñana (EBD-CSIC), Seville, Spain, for their invaluable help with molecular analyses. Beatriz Alvarez, Colección de tejidos y ADN, Museo Nacional de Ciencias Naturales, Madrid, Spain, helped with the deposition of samples in the Museo Nacional de Ciencias Naturales, Consejo Superior de Investigaciones Científicas (MNCN-CSIC). Yehuda Braverman, retiree from the Department of Parasitology, Kimron Veterinary Institute, Bet Dagan, Israel, kindly provided key references. This study was partially funded by projects CGL2015-65055-P from the Spanish Ministry of Economy and Competitiveness, P11-RNM-7038, from the Junta de Andalucía and a *Microproyecto* funded by the internal Estación Biológica de Doñana proposal financed by the Spanish Ministry of Economy and Competitiveness through the Severo Ochoa Centres of Excellence in Research, Development and Innovation programme (SEV-2012-0262). Funds were also partially provided by EU grant HEALTH.2010.2.3.3-3 project 261391 EuroWestNile. JM-dlP and MF were funded by a Juan de la Cierva contract and a Formación de Profesorado Universitario grant, respectively. Comments by two anonymous reviewers improved a previous version of the manuscript. The English text was revised by Michael Lockwood.

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Accepted 20 March 2017
First published online 27 July 2017