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FIRST RECORD OF *MUSCA AUTUMNALIS* DE GEER, 1776 (DIPTERA, MUSCIDAE) IN ASSOCIATION WITH MYIASIS IN CATTLE IN THE BASQUE COUNTRY (IBERIAN PENINSULA)

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Abstract: Myiasis, understood as the infestation of living vertebrate tissues by dipteran larvae, is usually considered to be linked with tropical and subtropical areas. Nevertheless, it may occur all over the world, causing serious damage to the welfare and the economy of livestock and wild fauna. In Europe it is commonly caused by species of the families Oestridae, Sarcophagidae, Calliphoridae and Muscidae (Diptera), among others. This is the first recorded case of myiasis by *Musca autumnalis* De Geer, 1776 in cattle in the north of the Iberian Peninsula. An immature specimen was extracted from a cow and identified at the species level, using the mitochondrial gene cytochrome c oxidase subunit I barcode region (COI, 658 bp), as *M. autumnalis*. Additionally, the specimen confirms the presence of this species in the south of the Basque Country, extending its known distribution area from the Atlantic watershed, with an oceanic climate, to the transition zone, with a sub-mediterranean climate.
Keywords: Diptera, Muscidae, *Musca autumnalis*, DNA, COI barcode, myiasis, Iberian Peninsula.

Primer registro de *Musca autumnalis* De Geer, 1776 (Diptera, Muscidae) asociada a un caso de miasis, en ganado del País Vasco (Península Ibérica)
Resumen: Las miasis, consideradas como infestaciones por larvas de dípteros de tejidos vivos de animales vertebrados, se asocian comúnmente a áreas tropicales y subtropicales. No obstante, pueden estar presentes en todo el mundo, provocando serios daños económicos y veterinarios en el ganado y fauna salvaje. En Europa, generalmente, son causadas por las familias Oestridae, Sarcophagidae, Calliphoridae y Muscidae (Diptera), entre otras. Este es el primer caso registrado de miasis por *Musca autumnalis* De Geer, 1776 en ganado vacuno en el norte de la Península Ibérica. Un espécimen inmaduro fue extraído de una vaca e identificado a nivel de especie, utilizando la región *barcode* del gen mitocondrial citocromo oxidasa subunidad I (COI, 658 pb). Adicionalmente, el espécimen confirma la presencia de esta especie en el sur del País Vasco, ampliándose el área de distribución conocida desde la costa atlántica, de clima oceánico, a la zona de transición, de clima sub-mediterráneo.
Palabras clave: Diptera, Muscidae, *Musca autumnalis*, DNA, COI *barcode*, miasis, Península Ibérica.

Introduction

Myiasis refers to vertebrate infestation by dipterans larvae, which feed, at least for a period of time, on the necrotic or alive tissues, liquids or foods ingested by the host (Zumpt, 1965). This serious pest causes a considerable impact on production economy, principally, of the livestock industry all over the world, causing serious health and welfare problems (Petney, 1997; Catts & Mullen, 2002). However, it may affect not only to animals (domestic or wild) but also to humans (children and elderly), being the period of insect activity (PIA) an important estimation to detect mistreatments situations and occupational risks (Benecke & Lessing, 2001; Amendt *et al.*, 2007; Charabidze, 2013).

In the Palaearctic region, obligate myiasis species are mainly related to four genera of Oestridae family, *Oestrus* (*O. ovis* and *O. picta*), commonly found in *Ovis*, *Capreolus* and *Cervus*, *Hypoderma* (*H. bovis* and *H. lineatum*), that primary affect to bovids, *Gasterophilus* (*G. intestinalis* and *G. nasalis*), which mostly infest equids, and *Cephenemyia* (*C. stimulator*), that is frequently found in wild cervids; one of Sarcophagidae (*Wohlfahrtia*: *W. magnifica*), that may infest a great number of mammals (bovids, ovids, equids, etc); and one of Calliphoridae (*Lucilia*: *L. bufonivora*), specialized in amphibians (Ruíz-Martínez *et al.*, 1992a-b; Ruíz-Martínez & Palomares, 1993; Soler-Cruz, 2000; Alcaide *et al.*, 2003; Anderson, 2005; Otranto *et al.*, 2005; Gosá *et al.*, 2009; Weigl *et al.*, 2010; Giangaspero *et al.*, 2011; Calero-Bernal & Habela, 2013; GilArriortua *et al.*, 2014). Meanwhile, the facultative or accidental myiasis in Europe, may affect to a wide range of vertebrates and is commonly caused by species of the Calliphoridae (*Calliphora*: *C. vicina* and *C. vomitoria; Lucilia: L. sericata, L. cuprina, L. ampullacea, L. caesar* and *L. illustris; Chrysomya*: *C. albiceps; Phormia: P. regina*), Sarcophagidae (*Sarcophaga*: *S. carnaria* and *S. haemorroidalis*) or Muscidae (*Musca*: *M. domestica* and *M. sorbens*; and *Muscina*: *M. stabulans*) families (Rognes, 1994; Hall & Wall, 1995; Stevens & Wall, 1997; Soler-Cruz, 2000; Bolek & Coggins, 2002;Stevens, 2003; Shivekar *et al.*, 2008; Derraik *et al.*, 2010; Salvetti *et al.*, 2012; Clarke, 2013).

Until the date, as we know, myiasic species of Muscidae family recorded, all over the world, in documented cases have been exclusively referred to *Musca domestica* Linnaeus, 1758, *Musca sorbens*, Wiedemann, 1830 and *Muscina stabulans* (Fallén, 1817) (Shivekar *et al.*, 2008; Soler-Cruz, 2000; Clarke, 2013). Only Gállego Berenguer (2007) mentioned *Musca autumnalis* De Geer, 1776, together with *M. domestica*, just as possible cause of accidental myiasis, but without reporting any specific case. These last two species show substantial morphological similarities, being *M. autumnalis* originally Palaearctic, with an Old World distribution (Savage & Vockeroth, 2010). Nevertheless, *M. autumnalis* currently has a cosmopolite distribution, and its presence as part of the livestock insect fauna has been recently confirmed in the north of the Basque Country (Province: Bizkaia, Spain) (Valbuena & Saloña, 2011). Additionally, it is worth to mention that in this species, adults feed on livestock secretions (eyes, nose and mouth) and excretions (mature), even on blood or wounds exudates (Matthew & Dobson, 1960), being mechanic vectors of infection agents (virus, bacteria, parasites), and causing the lost of weight and milk production (Bowman, 2004). Whereas immature, essentially coprophagous, develop on any kind of mature or faeces, although could be associated to other substrates (Skidmore, 1985).

Finally, it should be considered that traditional identification of immature specimens is usually hampered by species morphological similarities and could be delayed by the need to wait until the adult emergence, which may be a difficult task especially for host dependent species (Wallman & Donnellan, 2001). Moreover, sometimes the specimens can be collected in bad conditions or fragmented, making unsuccessful the identifications, even for experts (GilArriortua *et al.*, 2013; Rolo *et al.*, 2013). In these situations, molecular methods represent a useful alternative for fast and accurate species identification at any developmental stages, even for which show subtle differences in the taxonomic diagnostic characters. For this task, the analysis of the mitochondrial DNA cytochrome oxidase c subunit I barcode region (COI barcode, 658 bp) could be the most suitable option, since it is widely used for metazoan species molecular taxonomy and, nowadays, is considered the standard locus for insect identification (Herbert *et al.*, 2003; Nelson *et al.*, 2012). Unfortunately, the applicability of molecular diagnosis depends on the representativeness and reliability of the database reference sequences (Sonet *et al.*, 2013). This issue makes it necessary to analyze the database information with special care.

This paper reports for the first time the capability of *M. autumnalis* to operate as a myiasic species associated to an accidental infestation of a cow from the south of the Basque Country (Spain). Additionally, the present work proposes DNA-based molecular approach to perform reliable and unambiguous myiasis causing Diptera species identification as a valuable alternative to morphological methods, since these kinds of caseworks involves, almost exclusively, immature stages.

Materials and methods

In june 2011, during a yearly study conducted by the University of the Basque Country (UPV/EHU) about the incidence of miasis in the Basque Country livestock, an approximately six-years-old female crossbred cow of about 550 kg was found with a foot infection on the right hind limb, in the Ozaeta village (42º92'north, 2º30' west) at the Araba province in southern Basque Country (north Spain). The wound exploration showed a severe infection on the right hind limb phalange with tissue necrosis and inflammation, exudation and purulent secretions. Immature specimen was manually removed after washing the lesion with physiologic saline solution (NaCl 0.9%), collecting only one larva. The infested autochthonous cow was in a grassland area located about 70 km far away from the Cantabrian sea coast at 578 m altitude, and it is characterized by a sub-mediterranean climate. The larva recovered from the animal was placed in a closed vial with small holes for air entry, and transferred to the Forensic Entomology Laboratory, Faculty of Science and Technology, UPV/EHU, Spain. Additionally, it was elaborated a document where to record all the information associated to myiasis cases (Entomological evidences collection log sheets, Annexe A), following European standards which allow to standardize the information to perform comparative studies in the future (Amendt *et al.*, 2007).

Morphological identification

It was impossible to raise the specimen until the imago emergence because it did not arrive alive to the laboratory. Therefore, the larva was processed by boiling it in hot water (approximately 80ºC) during few minutes, preserved in ethanol 80% (Amendt *et al.*, 2007) and stored at -80ºC up to the time of DNA extraction (GilArriortua *et al.*, 2013). Morphological identification was carried out under stereomicroscope (Motic microscopes SMZ 168, Motic, Barcelona, Spain) following specific taxonomic keys (Skidmore, 1985; Smith, 1986).

Molecular identification: DNA extraction, amplification and sequencing

The immature specimen was washed with a 20% bleach solution to remove all external contamination (GilArriortua *et al.*, 2013; GilArriortua *et al.*, 2014). DNA was extracted with the Qiagen DNeasy®Tissue Kit (Qiagen, Valencia, Spain) following the manufacturer instructions for animal tissues.

Molecular identification was performed amplifying the barcode region of the mitochondrial gen cytochrome c oxidase subunit I (COI barcode, 658 pb), using the adapted primers LCO1490 5´-TTT CAA CTA ATC ATA AAG ATA TTG G-3´and HCO2198 5´-TAA ACT TCA GGA TGA CCA AAG AAT CA-3´ modified from Folmer *et al.*, 1994; via PCR in an iCycler thermocycler (Bio-Rad, Madrid, Spain), using approximately 10 ng of DNA in a final volume of 25 µl: 0.2 mM dNTP (Bioline, Berlin, Germany), 1.5 mM $MgCl_2$ (Bioline, Berlin, Germany), 0.2 µM of each primer, 1.6 10^{-4} mg/µl BSA (Bioline, Berlin, Germany), 1 unit of Taq polymerase (Biotaq, Bioline, Berlin, Germany) and 1X Buffer (pH: 8.8, Bioline, Berlin, Germany), under the following thermocycling conditions: 95ºC for 10 min, 40 cycles 94ºC → 30 s, 55ºC → 30 s and 72ºC → 1 min, followed by final extension at 72ºC during 5 minutes (GilArriortua *et al.*, 2014).

Electrophoretic separation and sequencing reaction product detection were handled in an ABI 3130 genetic analyzer (Applied Biosysytems, Foster City, CA, USA). The forward and reverse sequences obtained were edited with BioEdit (Hall, 1999), the absence of stop codons was confirmed in MEGA6 (Tamura *et al.*, 2013) and the homology search was

Table I. COI barcode sequence (658 bp) obtained from *M. autumnalis* sample. Nucleotide notation follow IUB code and sequence begin at the 3´ end of the forward primer. // **Tabla I.** Secuencia de COI *barcode* (658 pb) obtenida de la muestra de *M. autumnalis*. La notación nucleotídica sigue el código IUB y la secuencia comienza en el extremo 3´del primer *forward*.

1	T A C C T T A T A T T T T A T C T T T G G A G C A T G A T C T G G T A T A A T T G G A A C T T C C T T A	52
53	A G A A T T T T A A T T C G A G C T G A A T T A G G G C A C C C T G G T G C A C T A A T T G G T G A T G	104
105	A C C A A A T T T A T A A T G T T A T T G T A A C A G C T C A T G C T T T T A T T A T A A T T T T C T T	156
157	T A T A G T T A T A C C T A T T A T A A T T G G A G G A T T T G G A A A T T G A T T A G T T C C T T T A	208
209	A T A C T A G G A G C T C C T G A T A T A G C A T T C C C T C G A A T A A A T A A T A T A A G T T T C T	260
261	G A C T T T T A C C T C C T G C T T T A A C C T T A T T A T T A G T T A G A A G C A T A G T A G A A A A	312
313	G G G A G C T G G G A C A G G A T G A A C T G T A T A C C C A C C T T T A T C T T C A A T T A T T G C T	364
365	C A T G G A G G A G C T T C T G T T G A T T T A G C T A T T T T T T C A T T A C A T T T A G C T G G A A	416
417	T T T C T T C A A T T T T A G G A G C A G T A A A T T T T A T T A C A A C C G T A A T T A A T A T G C G	468
469	G G C T A C T G G G A T T A C A T T T G A T C G A A T A C C T T T A T T C G T T T G A T C C G T T G T A	520
521	A T T A C T G C T C T A T T A C T T T T A C T T T C T C T T C C A G T T T T A G C C G G A G C T A T C A	572
573	C T A T A T T A T T A A C G G A T C G A A A T T T A A A T A C T T C A T T C T T T G A C C C T G C G G G	624
625	A G G A G G A G A C C C A A T T C T T T A C C A A C A T T T A T T T	658

done using the BLAST option of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) and the BOLD-IDS tool of the Barcode of Life Database (BOLD) (http://www.boldsystems.org/index.php/IDSOpenIdEngine).

Results and discussion

For the collected specimen, the morphological identification based on the taxonomic characters was nearly impossible, due to the bad conditions. Nevertheless, it was possible to identify the larva morphologically as Muscidae family and *Musca* genus, while the species diagnosis based on external characters was not conclusive enough. Afterwards, to overcome this problem, we performed the molecular analysis of the COI barcode molecular marker which allowed the specimen unequivocal classification as *M. autumnalis*. This final result was achieved after applying BLAST and BOLD-IDS search tools of GenBank and BOLD databases, with respective results of a 99% of maximum identity and a 99.81-99.49% of similarity to *M. autumnalis*. In GenBank, BLAST obtains only 3 highly similar best matches of 99%, with coverage of 99-100% for the species *M. autumnalis*. However, in BOLD, IDS returns a list of 20 best matches with a closest species under the 1% divergence, in all cases *M. autumnalis* species. Additionally, we ensure the reliability of the database sequences record considering if the works are published and the authors' expertise in Diptera identification. In this regard, it is word to highlight that some sequences are part of international works published by Kutty *et al.*, 2008 or Sonet *et al.*, 2013, where collaborate highly qualified taxonomy specialist. The new COI barcode (658 bp) sequence has been deposited in GenBank as accession number KF751383 (Table I).

It is well known that other *Musca* species, such as *M. domestica* and *M. sorbens* could cause accidental myiasis in animals, or even in humans, (Dogra & Mahajan, 2010; Clarke, 2013), while *M. autumnalis*, also called face fly, has not been previously reported related to myiasis. However, it is noteworthy that these species have common nutritional habits for immature stages, which development is associated to animal excrements or faeces. Furthermore, *M. autumnalis* and *M. domestica* larvae show an extremely high morphological similarity with plenty of taxonomic characters shared, which difficult considerably the unequivocal diagnosis through morphological methods (Matthew & Dobson, 1960; Skidmore, 1985) unless maggots are properly reared into adult for specific identification. Probably, this complexity to differentiate the external features between these species may be related to the lack of myiasis case reported for *M. autumnalis*. All these confluence of biological points, suggest the reasonable possibility of associating *M. autumnalis* to accidental myiasis, since its adults, as those of the other two species, are attracted to soiling or mature odors, which may be sometimes similar to necrotic animal flesh. In fact, *M. autumnalis* has recently been associated to cadaveric decomposing processes in Portugal (Rolo *et al.*, 2013).

In conclusion, this study records for the first time *M. autumnalis* species as an accidental parasite that may be involved in traumatic myiasic cases. Additionally, the findings here reported confirm the presence of *M. autumnalis* in the Araba region, extending it to the south of the Basque Country (north Spain). As an added value, our study contributes with new nucleotidic information which is of great interest for accurate species identification. Moreover, this work demonstrates the need to complement the traditional taxonomy methodology with alternative nowadays methods, such as DNA-based approach, which allow performing reliable identifications even when specimens are degraded or lack of diagnostic characters.

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Annexe A

Entomological Evidences Collection Log Sheets

Case number: Case 3 Collected by: Private information
Location: Ozaeta (Araba) Data: 06/10/2011 Time: 14:35 h

Specifications

Type of animal: Wild (deer, wild board, etc.) ☐ Domestic (goat, sheep, cow, etc.) ☒
Specify: Bovine Species/variety: Crossbred Origin: Basque Country
Estimation about: Age: 6 years Weight: 550 kg
Remarks: _____
Sex: Male ☐ Female ☒
Position of dipterans on the animal**: Wound on the heel of the right hind limb
Evidence of wounds*: Yes ☒ No ☐
Kind of wound: Podal infection
Infection degree: Severe infection with swelling and purulence
Remarks: Located in the phalange

Collection Environment

Outdoor: Forest ☐ Field ☐ Grassland ☒
Others/Remarks: _____
Indoors: Stable ☐ Livestock pavilion ☐
Climate-control/Heated: Yes ☐ No ☐
Open access to outside: Yes ☐ No ☐
Others/Remarks: _____

Temperature and Climate

Environment temperature: 17 ºC Season: Springtime
Regional climate: sub-mediterranean climate

* **Wounds (W):** please mark the position on the final drawing.
** **Egg (E), Larvae (L), Pupa (P), Adult (A) or sample location (S1, S2, etc.):** please mark the position on the final drawing.

Case number: C3 Collected by: Private information
Location: Ozaeta (Araba) Data: 06/10/2011 Time: 14:35 h

Sample	Specimens number	Sample type	Preserved/Alive	Animal location
1	1	☐ Egg ☒ Larva ☐ Pupa ☐ Adult	Alive	Right hind limb heel
2		☐ Egg ☐ Larva ☐ Pupa ☐ Adult		
3		☐ Egg ☐ Larva ☐ Pupa ☐ Adult		
4		☐ Egg ☐ Larva ☐ Pupa ☐ Adult		
5		☐ Egg ☐ Larva ☐ Pupa ☐ Adult		
6		☐ Egg ☐ Larva ☐ Pupa ☐ Adult		

Please make an schematic drawing of the animal