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Surveillance and screening of Stomoxyinae flies from Mallorca Island (Spain) reveal the absence of selected pathogens but confirm the presence of the endosymbiotic bacterium *Wolbachia pipientis*

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ABSTRACT

Adult brachycera biting flies can significantly impact livestock through both direct effects (reduction of food intake, disturbance, painful bites, and blood loss) and indirect effects (pathogen transmission), leading to substantial economic losses and production damage. This study aimed to assess the presence of blood-sucking flies in six mixed-animal farm environments on the island of Mallorca (Balearic Islands, Spain) by employing multiple trapping methods. Additionally, distribution maps of brachycera biting fly species recorded in Spain were created, based on data extracted thorough review of scientific literature and citizen digital databases. Investigation of several pathogens, including equine infectious anemia virus (EIAV), Anaplasmataceae bacteria, and piroplasm protozoa, was carried out using different PCR targets (18S rRNA, 16S rRNA, *groESL*, and *tat* genes). Citizen science databases and literature review corroborated the consistent distribution trend for two Stomoxyinae species, underscoring the importance of citizen collaboration as a complement to traditional entomological surveillance. Our study confirmed the presence of two biting Stomoxyinae species: the prevalent stable fly *Stomoxys calcitrans* across all sampled farms, and the horn fly *Haematobia irritans*, which turned out to be less abundant. DNA barcoding techniques validated the identification of the two species. Neither EIAV nor bacterial/protozoan pathogens were detected using the selected PCR targets in either fly species. However, *Wolbachia pipientis* (clustered in the supergroup A together with the only sequence of *W. pipientis* from the USA) was identified through PCR targeting 16S rRNA, *groESL* and *wsp* genes in all pools of *H. irritans* ($n = 13$) collected from two of the examined farms. This study represents the first attempt to investigate pathogens in Stomoxyinae biting flies in Spain. The discovery of the endosymbiotic *Wolbachia* organism in *H. irritans* represents the first record in Spain and the second from Europe. This finding holds significant implications for future research on the applications of this bacterium in biocontrol programs.

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1. Introduction

Haematophagous fly infestations pose significant health and economic challenges in livestock farming. Together with tabanids (Diptera: Tabanidae) and hippoboscids (Diptera: Hippoboscidae), Stomoxyinae flies (Diptera: Muscidae) are parasitic flies of medical and veterinary importance, causing major disturbance in livestock (Baker, 1967; Halos et al., 2004). In Spain, the vast family Muscidae contains the subfamily Stomoxyinae, which comprises three genera (*Haematobia*, *Haematobosca* and *Stomoxys*) with four living species. Both sexes of these flies are obligate haematophagous of mammals since they have developed unique mouth parts adapted for blood feeding (Peris and Llorente, 1963). The genus *Haematobia* comprises two small size species (3.5–4.5 mm): *Haematobia irritans* and *Haematobia titillans*. These species typically remain on or near cattle throughout their entire life cycle, leaving the host only to lay eggs in newly laid cow pats before promptly returning to the host (Brewer et al., 2021). *Haematobia irritans* (horn fly) is a widespread and relatively abundant species, while *H. titillans* is apparently only found in the Canary Islands (Spain) (Pont and Báez, 2002). The genus *Haematobosca* includes a rare medium-size (4–7 mm) monospecific fly (*Haematobosca stimulans*) found on the back or flanks of cattle (Hillerton et al., 1984). The genus *Stomoxys* only contains the species *Stomoxys calcitrans* (stable fly), a synanthropic fly with a worldwide distribution. This species is highly active, with fly movement occurring between host animals and resting sites to feed and mate, particularly at on-farm locations where herbivorous livestock are regularly gathered (Patra et al., 2018).

The direct effects of these biting pests on hosts might cause disturbance, skin lesions, reduction of food intake, stress, blood loss and alterations in immunosuppressive system, particularly in livestock (Zumpt, 1973; Baldacchino et al., 2013). Substantial economic losses are more commonly reported in America and Australia than in Europe (Brewer et al., 2021). Information about the abundance and distribution of biting flies in Mediterranean Europe is scarce. Regarding *Haematobia,* the only published study noted *H. irritans* infestations in fighting bulls in Spain (Muñoz and Serrano, 2007). Conversely, in South Europe, *S. calcitrans* is linked with the so-called "fly strike dermatitis" in dogs (Castilla-Castaño et al., 2019), and their recurrent biting activity may led to severe skin lesions in equines (González et al., 2022a). Large populations of *S. calcitrans*, *H. irritans*, and *Tabanus* spp. have resulted in significant economic losses to the livestock industry worldwide (Hornok et al., 2020; Koonyosying et al., 2022). In addition, stable flies can also attack various domestic mammals such as cows, sheep, horses, goats, dogs, camels and many wild mammals (Baldacchino et al., 2013).

These neglected haematophagous insects also have indirect effects as mechanical vectors of pathogens. Pathogenic agents associated with *Stomoxys* are much more evident and numerous than those of *Haematobosca* and *Haematobia,* including a wide range of viruses, bacteria, and parasites such as protozoa, and helminths (Torres et al., 2012; Baldacchino et al., 2013). Numerous viruses can be mechanically transmitted by *Stomoxys* spp. and *Haematobia* spp. such as equine infectious anemia virus (EIAV), African swine fever virus (ASFV), West Nile virus (WNV), Rift Valley fever virus (RVFV) and lumpy skin disease virus (LSDV), among others (Baldacchino et al., 2013; Cook et al., 2013). Mechanical transmission is thought to be important in spreading diseases within and across herds. EIAV is a notifiable disease in Spain, and sporadic circulation has been spotted in Mediterranean countries of Europe and some regions of central and inland Spain for a few years (Cappelli et al., 2017; Camino and Cruz, 2017; Gaudaire et al., 2018; Lupulovic et al., 2021).

Moreover, while ticks are recognized for their significant role in transmitting bovine haemoparasitic diseases like babesiosis (*Babesia* spp.), theileriosis (*Theileria* spp.), and anaplasmosis (*Anaplasma* spp.), numerous haematophagous Diptera are also associated as mechanical vectors (Baldacchino et al., 2013, 2014). *Anaplasma* spp., *Ehrlichia* spp., and *Rickettsia* spp. pathogens have been detected in livestock farms in Menorca (Balearic Islands, Spain). Furthermore, a notable prevalence of piroplasms has been documented, specially *Theileria* within the dairy farms on the island, where it is endemic (Almeria et al., 2001; Ros-García et al., 2012; Monerris, 2016).

Characterization of pathogens in blood-sucking ectoparasites is important to understand its biology and potential role in pathogen transmission. Positive results of pathogen screening in arthropods need to be carefully evaluated in the context of vector capacity. Since a positive result in a PCR test for the detection of a pathogen does not necessarily imply that the arthropod is a competent vector. In fact, the vector competence of an arthropod is defined as the ability of an arthropod to transmit an infectious agent following exposure to that agent. Since detailed data on stable and horn fly vectors and the pathogens they harbour in Spain are lacking, the objectives of this study were to assess the distribution and abundance of biting flies in various farm environments on Mallorca (Balearic Islands, Spain), and to screen for pathogens of major veterinary interest using different PCR targets. Additionally, we reviewed the recent and historic distribution of Stomoxyinae flies in Spain.

2. Material and methods

The study was conducted in six locations distributed along animal farm environments and equine centers on the island of Mallorca (Balearic Islands, Spain) (Table 1; Figure Supplementary material A). These locations were chosen based on criteria to encompass different eco-geographical locations across the island, ensuring easy accessibility to sites and a high abundance and diversity of enclosed animal species. Three of these locations were situated near natural wetlands where the circulation of some pathogens could be important due to the presence of wild and domestic animals including potential vectors (Defaye et al., 2022). The Balearic Islands, located in the western Mediterranean Sea, typically experience a Mediterranean climate characterized by hot, dry summers and mild, rainy winters.

2.1. Entomological field trapping of Stomoxyinae flies

Three different sampling methods were employed to capture Stomoxyinae adults: sticky traps, suction traps, and hand netting. The study was conducted from September 15th to November 30th, 2021, aligning with the period of increased rainfall and favourable temperatures.

2.1.1. Sticky traps

The traps consisted of commercially available white sticky traps 400 × 250 mm (Econex®, Spain) attached to a 2-mm twin-walled polypropylene black sheet of 420 × 297 mm. This configuration resulted in a white central target (1000 cm^2) surrounded by a 20 and 47 mm-wide black backdrop frame. The black border aimed to increase *Stomoxys* collections on white sticky traps (Murchie et al., 2018).

Three sticky traps were positioned approximately 1 m above ground level at each sampling site near the animal enclosures, and they were attached to trees, metal fences, walls, and/or wooden stakes. The three traps were separated approx. 30 m each other, collected weekly, with sticky cardboards replaced as needed, and transported to the laboratory for further analysis.

2.1.2. Suction traps

As part of a project focused on the collection of nematoceran blood-feeding arthropods, we employed four different suction traps: i) CDC trap without light baited with CO_2 (Miniature Light Trap 512, John W. Hock, UK John Cook®, USA), ii) home-made trap with incandescent light, iii) UV trap (Onderstepoort model; OVI-ARC, Onderstepoort, South Africa), and iv) BG-Sentinel trap (Biogents®, Germany). The four traps were deployed on each of the six sampling sites previously mentioned. The traps were separated from each other by a minimum distance of 15 m. Trapping was performed weekly, and insects were collected in 96% ethanol (OVI-ARC) or kept dry (the other three traps).

Table 1
Profile of the six farm environments selected for sampling Stomoxyinae species in Mallorca (Balearic Islands, Spain) in 2021.

Sampling site	n°	Altitude (m.a.s.l.)[1]	Type of farm	Geographical coordinates		Animals (ca. number)
Formatges Burguera	1	3	Dairy farm	39.366579	3.02172891	Cattle (> 50), pigs (3) and dogs (2)
Son Ajaume nou	2	89	Rural farm	39.6448596	2.65217317	Cattle (10), sheep, goat, pigs, dogs, horses, and birds
Can Cosme	3	80	Rural farm	39.5222862	3.10583271	Cattle (15), sheep, pigs, and horses
Son Simó vell	4	14	Rural farm	39.8173189	3.05957789	Horses (> 20) and sheep (> 100)
Ranxo Ses Roques	5	3	Equine center	39.8331397	3.10518693	Horses (30), cows (2), goat (6), and pigs
Centre Hipic Son Reus	6	76	Equine center	39.6377295	2.66639607	Horses (> 30) and donkeys (<10)

[1] m.a.s.l.: meters above the sea level.

2.1.3. Hand netting

Flies were collected for 15–30 min in the morning (9:30–11:00 a.m.) and/or in the afternoon (18:30–20:00 p.m.) using a polyester mesh long-handled net (diameter: 38 cm, mesh size: 0.8 mm; BioQuip Products Inc., Rancho Dominguez, USA). Flies were netted directly from animals, fences, and walls in and around some animal enclosures in the same dates and sampling sites. However, the aggressive nature and mistrust of certain animals prevented uniform sampling. Flies were carefully extracted from the mesh using a mechanical aspirator (Fulton® MX 991, USA) into plastic cylinders containing a mesh on both ends, then transported to the laboratory inside polyester cages. Hand netting collections also provided fresh field-collected specimens for the screening of selected pathogens.

2.2. Morphological identification of Stomoxyinae flies

The Stomoxyinae flies captured were identified to species level and separated by sex (except in sticky traps) under a binocular microscope (SMZ 645; Nikon®, Japan). Flies captured in the hand netting for molecular pathogen detection were determined on a chill table (BioQuip Products Inc., Rancho Dominguez, USA). Additionally, any other blood-sucking Diptera (Muscidae, Hippoboscidae, Culicidae, Psychodidae) captured were also noted.

The flies under study, mainly *S. calcitrans* and *H. irritans,* can be easily determined attending to the size and proboscis characteristics (Peris and Llorente, 1963). In *S. calcitrans,* the maxillary palpi are small and short, and the proboscis is rigid, slender, and long, projecting forward from the head, whereas in *H. irritans,* maxillary palpi are nearly as long as its proboscis and are clapper-like, with moderate subapical expansion. These characteristics also allow their differentiation from *Haematobosca* (Peris and Llorente, 1963).

2.3. Distribution data and mapping of Stomoxyinae flies

Based on the results obtained from sampling, we focused on creating a comprehensive dataset to map the distribution of *S. calcitrans* and *H. irritans* in Spain. We assembled information from extensive literature reviews (141 records) and digital databases (325 records). When georeferenced information was unavailable, we added the centroid of the specified locality. Records with obviously incorrect georeferencing, such as points located in the sea or with inverted latitude/longitude coordinates, were excluded from our dataset. The easily distinguishable morphological features of both species allow for visual species identification through pictures, reducing the risk of errors.

2.3.1. Literature review

We conducted an extensive literature review including indexed references and grey literature from 1881 to 2023 in both Spanish and English. Bibliographic databases researched were PubMed, Scopus, Web of Science, Google Scholar, and digital repositories such as ResearchGate, Digital CISC, TESEO, REBIUN, RUO, Dehesa, and Dialnet. Searches were performed using specific combinations of keywords such as 'Biting flies', 'Muscidae' 'Stomoxini', 'Stomoxyiini', '*Stomoxys*', '*calcitrans*', 'Lyperosia', '*Haematobia*', '*irritans*', or in conjunction with 'presence' or 'distribution' and 'Iberian Peninsula', or 'Spain'. The bibliographic references associated with each species recorded in Spain are available in Supplementary Material B.

2.3.2. Digital databases and citizen science

We downloaded 62 research-grade observations from the iNaturalist database (Nugent, 2018). Observations on the iNaturalist platform meet research-grade criteria when they are verifiable (including images), contain the date and location of observation, and receive agreement on species identification from at least two-thirds of the community users. Annotated observation records can be found in Supplementary material B. Most processed records, which are licensed under CC-BY-NC, are also accessible on GBIF.org (https://doi.org/10.15468/dl.z7fhbp) making a total of 75 records available through both platforms (last access 06/12/2023).

We reviewed 218 records from the biodiversity web database platform Biodiversidad Virtual (https://www.biodiversidadvirtual.org/). All uploaded pictures undergo expert verification for species identification. Therefore, we considered only those presences that were previously confirmed, associating each record with its corresponding 10 × 10 km UTM grid (last access 06/12/2023).

For the Canary Islands, data from the Biodiversity Data Bank of the Canary Islands (BDBC) were obtained. All registered data in the BDBC are supported by validated documents ($n = 21$) overseen by scientific supervisors. The extracted data from the BDBC belongs to *S. calcitrans* citations after 1969 (https://www.biodiversidadcanarias.es/biota) (last access 06/12/2023).

All collected data together with new collected records obtained from this research project were integrated into a georeferenced database. Distribution maps of both species *S. calcitrans* and *H. irritans* have been made at province level (NUTS3) using the software QGIS Geographic Information System, version 3.30 s- Hertogenbosch (QGIS Development Team 2023). The reference coordinate system established in the work was EPSG: 4258-ETRS89 (QGIS Association, http://www.qgis.org).

2.4. Molecular laboratory assays

2.4.1. Molecular identification of Stomoxyinae flies

DNA barcoding PCR targeting a 658 bp fragment of the mitochondrial cytochrome c oxidase subunit I (*COI*) gene (Hernández-Triana et al., 2015; Ruiz-Arrondo et al., 2018) (Table Supplementary material C) was carried out in six Stomoxyinae flies to confirm the morphological identification.

2.4.2. Sample preparation and nucleic acid extraction

Only Stomoxyinae flies collected by hand netting were used for pathogen screening. Flies with blood visible on the abdomen were not included in the analyses to avoid possible detection of host pathogens in the blood and compromise the study of their role as vectors. Pools of five specimens of the same species, sex, date, and farm were stored in screw cap vials at −80 °C until further analysis. The total fresh fly collections was categorized into two groups: group one ($n = 157$) for DNA extraction and investigation of selected protozoa and bacteria, and group two ($n = 240$) for RNA extraction to investigate EIAV, and DNA extraction for screening of protozoa and bacteria.

Specimens from group one were washed individually in petri dishes

with 70% ethanol (5 min), rinsed twice in pure water (1 min), and air-dried on filter paper (1 min). Flies were then dissected into head and thorax-abdomen (Rezende Araújo et al., 2021) by a sterilized scalpel, and each part was transferred to 1,5 ml tubes in pools of five. The fly body parts were gently grinded manually with sterile-disposable lancets and used for DNA extraction using a commercially available kit (DNeasy kit, Qiagen, Germany), following the manufacturer's protocol.

For specimens in group two, washing and dissection steps were conducted on a portable chill-table under the stereomicroscope. Following the homogenization of flies in Dulbecco's Modified Eagle Medium (DMEM) using lancets, the supernatant was used for RNA extraction, and the pellet (rest of flies) was used for DNA extraction as explained above. In this case, RNA extraction and reverse transcription were performed by the RNeasy kit and the Omniscript Reverse Transcription kit (Qiagen, Germany), respectively.

2.4.3. Pathogen detection

For EIAV detection, a PCR assay targeting a highly conserved region located in the trans-activator (*tat*) gene was conducted. The primers used are detailed in Table Supplementary Material C. In the case of EIAV, DNA extracted from the antigen provided in the serological AGID EIA test (IDEXX Laboratories, Maine, USA) was used as positive control. For Anaplasmataceae detection, we performed two PCRs targeting a fragment of the 16S rRNA gene and the *groESL* heat shock operon gene (Inokuma et al., 2000; Liz et al., 2002). After detecting *Wolbachia* positive specimens for both 16S rRNA and *groESL* genes through PCR and Sanger sequencing, we conducted a *Wolbachia* surface protein (*wsp*) gene-based PCR (Braig et al., 1998; Zhou et al., 1998) for further confirmation and phylogenetic analysis.

A fragment of the V4 hyper-variable region of the 18S rRNA gene was analysed to investigate piroplasms (*Babesia* spp. and *Theileria* spp.) (Gubbels et al., 1999). Negative controls containing distilled water were included in all PCR assays. DNA of *Anaplasma phagocytophilum, Babesia microti,* and *Wolbachia* spp. were used as positive controls in their corresponding PCR assays. Amplification products were sequenced in both directions using the BigDye R Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Forest City, CA, USA) at the Sequencing Unit, Center for Biomedical Research of La Rioja (CIBIR), Spain. Subsequently, nucleotide sequences were edited in BioEdit 7.2 software to generate a consensus sequence and compared with sequences deposited in GenBank using BLASTn (MegaBlast option; available at https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 10 June 2023).

2.4.4. Phylogenetic analysis

Phylogenetic analyses were performed by constructing multiple alignments of nucleotide sequences, including 5 high-quality amplicon-length sequences with 1380–1400 bp for *groESL* gene and 563 bp for *wsp* gene. A phylogenetic analysis based on the 16S rRNA gene was not performed because when comparing our sequences with those deposited in GenBank there were no differences. Sequences of *groESL* and *wsp* from *Wolbachia* endosymbionts isolated in *H. irritans*, as well as other insects obtained from GenBank, were incorporated into the phylogenetic analyses. In addition, a sequence of *Erlichia canis* (GenBank accession n°: MG953295) was included as the outgroup for *groESL* gene. These analyses were constructed using MAFFT vs. 7 (https://mafft.cbrc.jp/alignment/server/, accessed on 15 July 2023) and subsequently edited with GBlocks (http://molevol. cmima.csic.es/castresana/Gblocks_server.html, accessed on 15 July 2023). The phylogenetic tree was built using the maximum likelihood (ML) method in IQ-tree v.2.2.0 (http://www.iqtree.org, accessed on 15 July 2023). The best-fitting evolutionary model was TPM3u + F + I for *groESL* gene and GTR + F + G4 for *wsp* gene. Intraspecific and interspecific genetic divergences were calculated based on the Tamura–Nei model in MEGA X (Kumar et al., 2018). Nucleotide sequences were analysed and compared with those available in the NCBI database using BLASTn (MegaBlast option; https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 10 July 2023).

2.4.5. Statistical analysis

The number of flies collected by sticky traps in different farm environments was compared using non-parametric Kruskal-Wallis (K-W) tests. In case of significant differences between farms, Duncan's post hoc test was carried out. A p value <0.05 was considered statistically significant. All statistical analysis were conducted in IBM SPSS Statistics for Windows, Version 29.0 (Armonk, NY: IBM Corp.).

3. Results

3.1. Entomological field trapping of Stomoxyinae species

A total of 5201 *S. calcitrans* flies (60.1 ± 52.8, mean ± SD) and 10 *H. irritans* flies (0.1 ± 0.5) were recorded from sticky traps during a 45-day period (Table 2). The stable fly *S. calcitrans* was recorded in high numbers at all the sampling sites, whereas *H. irritans* was less frequent and found in three of the six sites (Table 2). The abundance of *S. calcitrans* and *H. irritans* was significantly different among farms (p-value = 0.029). Suction traps (Onderstepoort traps and CDC traps baited with CO_2) only collected seven *S. calcitrans* (0.2 ± 0.5) and 24 *H. irritans* (0.6 ± 0.2). No flies were collected in home-made suction traps with incandescent light and BG-Sentinel traps. Hand netting contributed with 397 *S. calcitrans* (females = 200, males = 197) and 69 *H. irritans* (females = 37, males = 32). Three farm environments (n° 3–5, Table 1) yielded the vast number of flies in windless and mild temperature mornings. Other interesting blood-sucking Diptera species were also recorded in sticky traps: 103 mosquitoes (11 blood fed females), 12 sand flies and one specimen of *Hippobosca equina*. By hand netting we collected 37 mosquitoes, 121 *Musca domestica* and flies of several families including Syrphidae, Drosophilidae, and Sphaeroceridae, among others. Data on sand flies, mosquitoes and biting midges caught in suctions traps are not shown.

3.2. Distribution data and mapping of Stomoxyinae flies

The stable fly showed to be a widespread species across Spain, with a higher concentration of records in the coastal regions of Andalusia, Catalonia, and the Cantabrian zone. It is also reported in the seven main islands of the Canary Islands. However, inland communities such as Castilla-La Mancha and Castilla y León have fewer documented observations (Fig. 1). *Haematobia irritans* received considerably less attention, as it has been infrequently documented, with records in only 12 out of the 50 provinces of Spain (Fig. 2). It is worth noting that we made the first-ever recorded observation of this species in the Balearic Islands, but it can be said that its presence was assumed but unpublished.

3.3. Molecular laboratory assays

3.3.1. Molecular identification of Stomoxyinae flies

Stomoxys calcitrans specimens (n = 3) were molecularly confirmed showing a homology of 100% to specimens from Poland (KU932147) and UK (HE614024). *Haematobia irritans* (n = 3) showed a homology of 100% with specimens from USA (KM669714). Detailed specimen records and sequence information of flies were submitted to the GenBank public database under the following accession numbers: OR742412-OR742417.

3.3.2. Pathogen detection

All fly specimens (n = 466) tested were negative for piroplasmid infection and EIAV. However, all oral and thorax-abdomen pools from *H. irritans* (n = 13, 8 females and 5 males) showed positive results for the Anaplasmataceae PCR *assays* (16S rRNA and *groESL* genes). These results were further genetically characterized by the amplification of *wsp* gene. The nucleotide sequence of all pools of *H. irritans* analysed using the 16S rRNA, *groESL* and *wsp* genes showed 100% identity with *Wolbachia pipientis* (CP037426) isolated in *H. irritans*.

Table 2
Counts of *Stomoxys calcitrans* and *Haematobia irritans* collected by sticky traps, hand netting, and suction traps in six sampling sites from Mallorca (Balearic Islands, Spain) in 2021.

N°	Sticky traps		Hand netting [1]			Suction traps	
	S. calcitrans	*H. irritans*	*S. calcitrans*		*H. irritans*	*S. calcitrans*	*H. irritans*
	Mean ± SD		DNA	RNA	DNA	Mean ± SD	
1	53.9 ± 50.5[a]	0 ± 0[a]	10 (2)	–	–	0.1 ± 0.3	0 ± 0
2	63.3 ± 45.7[a]	0 ± 0[a]	25 (5)	–	–	0.3 ± 0.8	0 ± 0
3	121.8 ± 53.7[b]	0.6 ± 1.1[b]	5 (1)	113 (20)	52 (10) *	0 ± 0	0.6 ± 0.2
4	29.5 ± 23.1[a]	0.2 ± 0.6[ab]	47 (10)	37 (8)	–	0.1 ± 0.3	0 ± 0
5	69.1 ± 59.6[a]	0 ± 0[a]	60 (12)	82 (12)	–	0.2 ± 0.6	0 ± 0
6	62.2 ± 55.4[a]	0.1 ± 0.3[a]	10 (2)	8 (2)	17 (3) *	0 ± 0	0 ± 0
Total	60.1 ± 52.8	0.1 ± 0.5	157 (32)	240 (42)	69 (13)	0.2 ± 0.5	0.6 ± 0.2

N° = Farm number based on Table 1. [1]Total numbers (n° of pools) analysed for DNA pathogens (Anaplasmataceae and piroplasms) and RNA pathogens (EIAV). (*) denote positive pools for *Wolbachia pipientis* in the head and thorax-abdomen pools. Different superscripts for each farm indicate significant differences according to Duncan's post hoc test ($p < 0.05$). SD: Standard deviation.

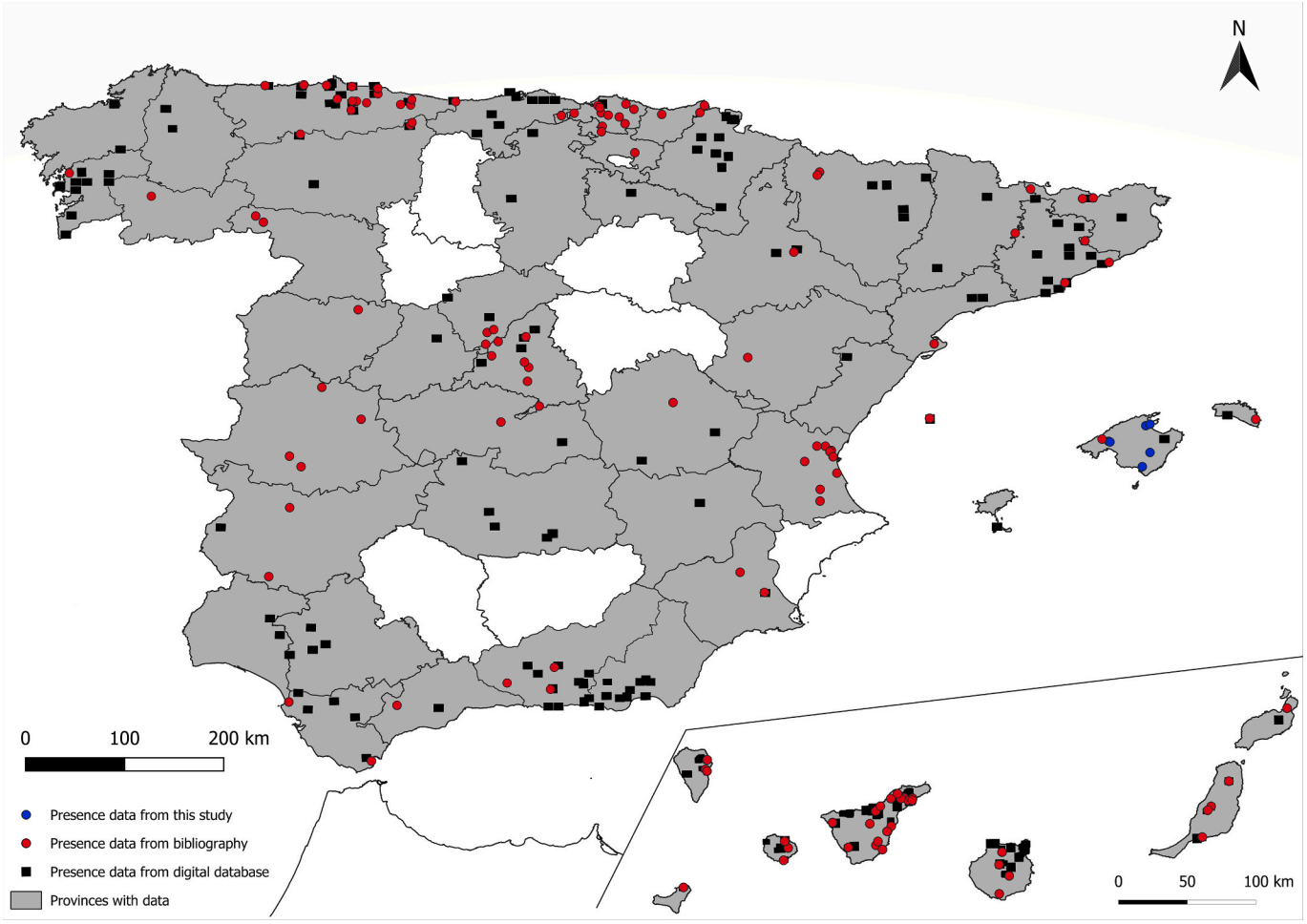

Fig. 1. Distribution of *Stomoxys calcitrans* (Diptera: Muscidae) in Spain based on historical bibliographic information and observations available in citizen/science digital databases.

3.3.3. Phylogenetic analysis

Phylogenetic analysis based on *groESL* gene clustered our *Wolbachia* sequences in a subclade together with the only sequence of *W. pipientis* for *H. irritans* (CP037426, USA) available in GenBank and other Diptera such as *Merzomyia wetermanni* (OX366375), *Epistrophe grosularia* (OX366363), *Gymnosoma rotundatum* (OX366347), or the lepidopterans *Epapoge grotina* (OX366381) and *Eupithecia tripunctaria* (OX366369). *Wolbachia* sequences identified in other haematophagous dipterans such as the mosquitoes *Aedes albopictus* and *Aedes aegypti* were grouped in different subclades (Fig. 3).

Phylogenetic analysis based on the *wsp* gene grouped our *Wolbachia* sequences in the same clade within supergroup A, along with the other *Wolbachia* sequences from *H. irritans* available in GenBank from countries such as Australia, Canada, USA, and Mexico (Fig. 4). This clade also includes *Wolbachia* isolated from other insects. The sequence divergence was 0% for *Wolbachia* of *H. irritans*, including the sequences available from other countries, based on the three genes studied. The interspecific divergence based on *groESL* gene was 0.6% between *H. irritans* from *Ae.*

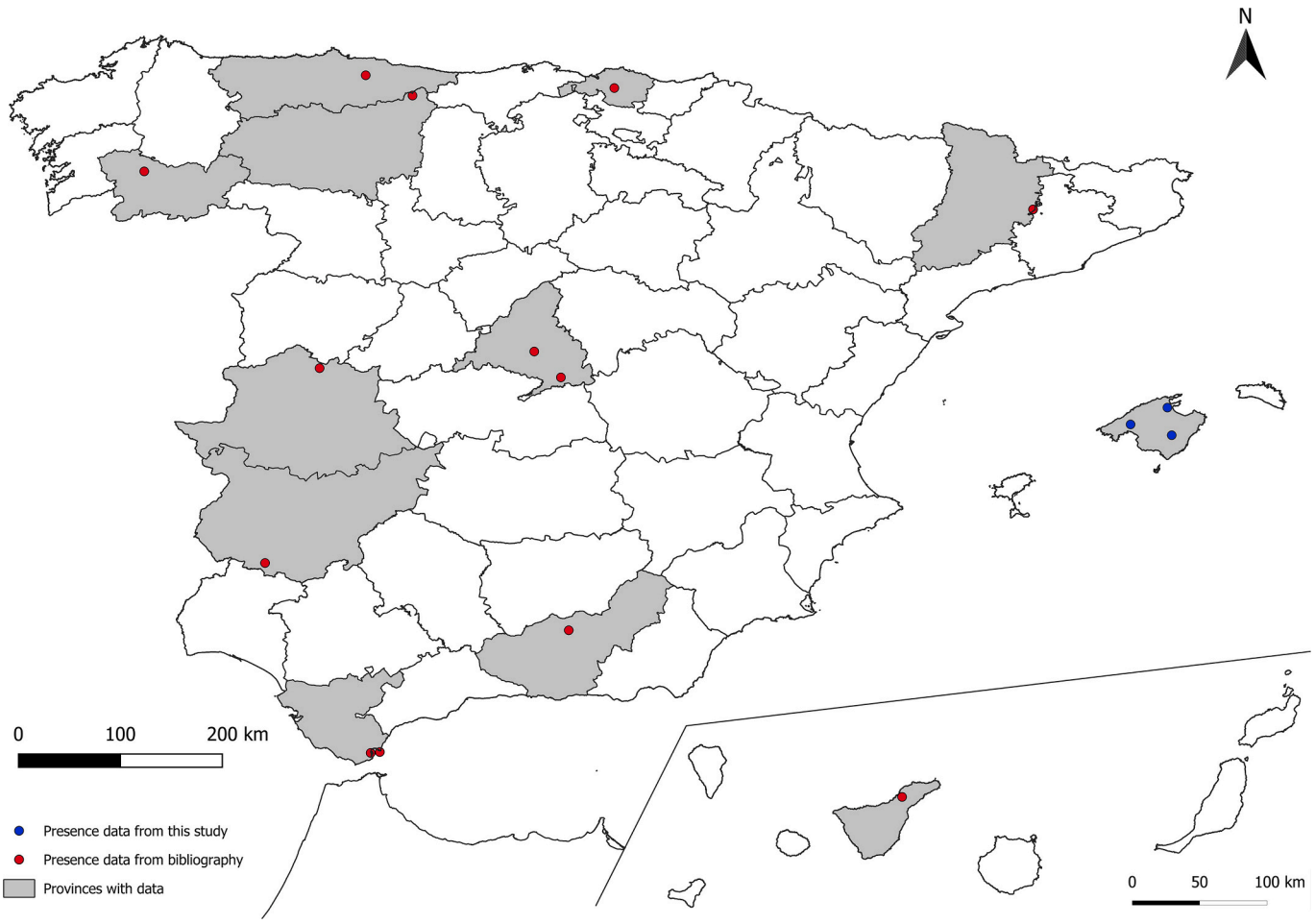

Fig. 2. Distribution of *Haematobia irritans* (Diptera: Muscidae) in Spain based on historical bibliographic information and observations available in citizen/science digital databases.

aegypti and *Drosophila melanogaster*. The interspecific divergence based on *wsp* gene was 24.4% between supergroup A and supergroup B.

4. Discussion

Our study confirms through comprehensive trapping methods and analysis, the presence of two Stomoxyinae species, notably *S. calcitrans* and *H. irritans* in Mallorca farms. This study provides the first comprehensive investigation into haematophagous flies in the Balearic Islands and the first pathogen screening in Spain. Despite the absence of certain pathogens such as EIAV and specific bacterial/protozoan strains in these flies, our discovery of *W. pipientis* within *H. irritans* stands as a significant revelation. This finding marks the first record of this endosymbiotic microorganism in *H. irritans* in Spain, suggesting potential implications for future biocontrol programs and urging further research into its applications within this context. Additionally, our study highlights the valuable role of citizen collaboration in augmenting traditional entomological surveillance methods. It offers insights into distribution trends and emphasizes its significance in broader scientific endeavours.

The presence of *S. calcitrans* was evident not only in farms housing cattle, as extensively reported in the literature, but also in equine farms (horses and donkeys) in line with other studies conducted in Europe and USA (Machtinger et al., 2016; Parravani et al., 2019; González et al., 2022a). In the current study, several defensive behavioural responses to repel stomoxyinae flies were observed on Vietnamese pigs, cows, horses, and donkeys (Mullens et al., 2006) but physical damage due to recurrent biting was only observed in the latter. Horn flies were only present in specific sampling sites and were exclusively collected through hand netting in cows, which are recognized as one of the primary hosts of this species (Brown et al., 1994). The three methods employed to collect these blood-sucking vectors yielded data but at varying densities, likely related to different trapping effectiveness, availability of hosts, suitability of habitat for breeding and/or the location of trap (Semelbauer et al., 2018). Various traps have been widely used for surveying and controlling stable fly populations (Baldacchino et al., 2013), with sticky traps emerging as a practical and cost-effective option for monitoring these flies (Taylor et al., 2020). Hand netting collections, although often difficult to perform, allow the collection of fresh material for the investigation of pathogens as well as obtaining accurate information on the host animal species. Unfortunately, no reliable methods are available to collect horn flies since they typically remain on their host, and control or monitoring methods often involve sophisticated structures (Denning et al., 2014). Suction light traps designed for different vectors (e.g., mosquitoes, biting midges, and sandflies) yielded limited collections in our study, aligning with prior findings by other researchers (González et al., 2022a, 2022b).

Based on literature data and digital observations by citizens and scientists, we have mapped for the first time, the historic and recent reports of these two biting fly species in Spain. From now on, *S. calcitrans* can be considered a widespread species in most of the geographical Spanish territory, while information regarding *Haematobia* flies remains scarce, with very few studies with records in the country. This lack of citizen information seems reasonable, as this fly is not easily photographed except when resting on hosts. Citizen science platforms are valuable tools for improving records on insect species distribution, including first detections, and serve as a complement to traditional

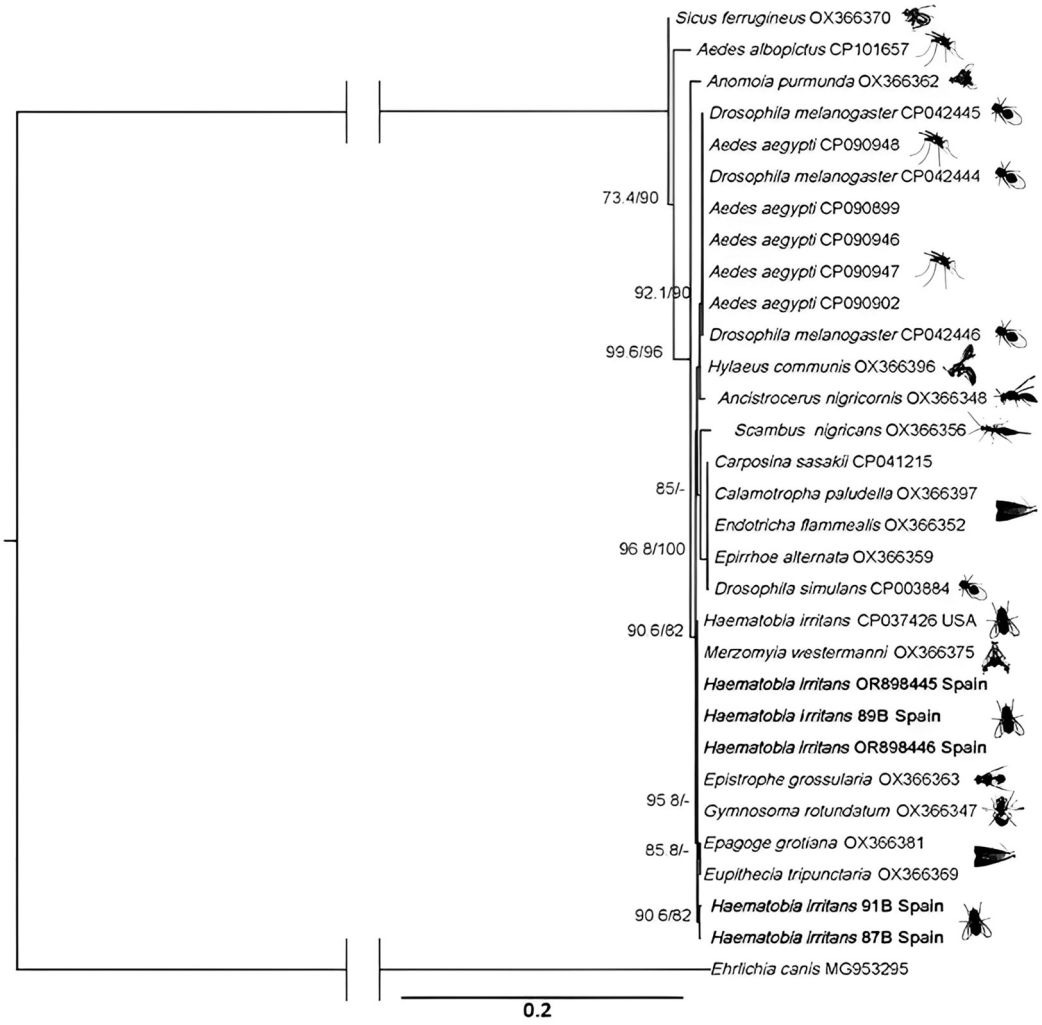

Fig. 3. Maximum likelihood (ML) phylogenetic tree based on *groESL* heat shock operon sequences in *Wolbachia* endosymbionts. At specific branches, the first and second values separated by "/" indicate the topological branch support for the ML analysis (aLRT/bootstrap), with values >75% defining high stability. For each record, the name of the dipteran species from which *Wolbachia* was isolated is shown first, followed by the GenBank accession number. Small silhouettes of insects on the right were added together with accession numbers.

entomological research (Callaghan et al., 2022).

While haematophagous flies are a concern in many regions worldwide, they may not have received as much attention as other blood-sucking vectors in Europe. Financial constraints can limit monitoring and control effort for these flies, especially when they cause only transient or periodic problems. Furthermore, their role as disease vectors may be considered less significant than that of nematoceran dipterans, primarily because they are typically regarded as mechanical vectors (Baldacchino et al., 2013). Therefore, in an attempt to elucidate the potential role of *S. calcitrans* and *H. irritans* flies as mechanical vectors of various pathogens and to explore possible transmission routes, this study aimed to detect the presence of EIAV, Anaplasmataceae, and piroplasms in haematophagous flies of Mallorca Island. Notably, this research represents the first entomological investigation into pathogens screening in these flies carried out in Spain. Flies were dissected to separate into two body parts, as some parasites could be classified as potentially transmissible whether they were found in the mouthparts or the thorax-abdomens (Odeniran et al., 2019). The absence of Anaplasmataceae among horn flies is consistent with other studies that have pointed out that these flies are not significant vectors of *Anaplasma* spp. (Hornok et al., 2008). In contrast, *Stomoxys* flies have been extensively documented as carrying RNA and DNA of various pathogens (Baldacchino et al., 2013). One potential explanation for the absence of pathogen detection in Stomoxyinae flies in the current study is that previous investigations reporting positive results were primarily conducted in endemic regions, during or with a history of disease outbreaks (Machado et al., 2015; Hornok et al., 2020; Makhahlela et al., 2022). Consequently, it is expected that these studies would have a higher success rate in detecting such pathogens. It is noted that pathogens such as EIAV are notifiable animal diseases, and no cases were reported in the Balearic Islands. Another plausible explanation is that most of flies collected were either unfed or recently emerged, and/or the study areas chosen were free of them.

Demonstrating the role of these flies as mechanical vectors may pose more significant challenges compared to biological vectors, as the transmission of pathogens by haematophagous flies may be less apparent or direct (Baldacchino et al., 2013). However, it is crucial to recognize the potential for these flies to transmit pathogens and to implement appropriate measures to control their populations and minimize the risk of disease spreading. In recent years, there has been growing interest in the study of these fly groups, especially following the outbreaks of LSDV and ASFV in Europe (Sohier et al., 2019; Paslaru et al., 2021; Anwar et al., 2022; Balmoș et al., 2023).

Interestingly, our study marks the second recorded of *W. pipientis* infecting *H. irritans* in Europe, following a prior detection of a *Wolbachia*-like bacterium in Hungary (Hornok et al., 2008). A highly robust

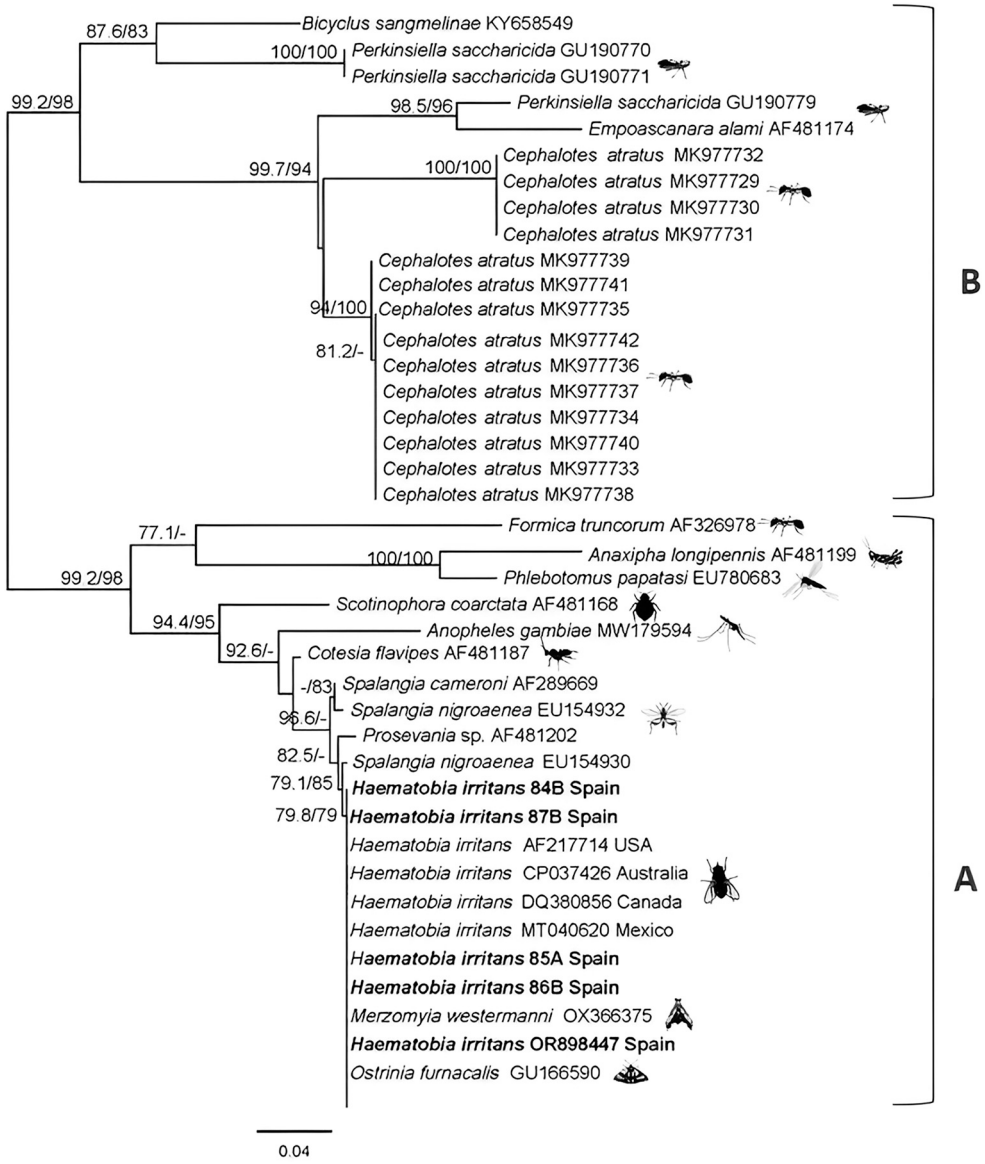

Fig. 4. Maximum likelihood (ML) phylogenetic tree based on *Wolbachia* surface protein (*wsp*) sequences in *Wolbachia* endosymbionts. At specific branches, the first and second values separated by "/" indicate the topological branch support for the ML analysis (aLRT/bootstrap), with values >75% defining high stability. For each record, the name of the dipteran species from which *Wolbachia* was isolated is shown first, followed by the GenBank accession number. Small silhouettes of insects on the right were added together with accession numbers. A = Supergroup A; B = Supergroup B.

signal for *Wolbachia* was also reported in wild-caught samples of *H. irritans* from Canada (Zhang et al., 2009), the USA (Jeyaprakash, 2000; Palavesam et al., 2012), and Mexico (Torres et al., 2012). *Wolbachia* is a genus of intracellular bacteria that infects mainly arthropod species and has garnered attention due to its potential biological effects on the hosts, such as reproduction, cytoplasmic incompatibility, feminization, male killing, and influencing vector transmission.

The phylogeny of the *Wolbachia* genus has shown the existence of 12 major clades (A–Q), which, in the absence of a formal species designations, have been termed as 'supergroups' (Lo et al., 2007). Supergroups A and B encompass the majority of parasitic *Wolbachia* discovered in arthropods (Werren et al., 1995) whereas Supergroups C and D comprise the majority of *Wolbachia* discovered in filarial nematodes (Bandi et al., 1998). Our sequences revealed that *Wolbachia* from *H. irritans* belongs to the well-supported supergroup A lineage (Fig. 4, *wsp* gene), which is the most common one of the three supergroups that occurs in insects (O'Neill et al., 1997; Madhav et al., 2020).

The growing development of insecticide resistance is reducing the effectiveness of chemical control measures in arthropod populations. This has increased the interest of potential use of *Wolbachia* in biocontrol programs against vector-borne diseases (Jeyaprakash, 2000; Floate et al., 2006; Jeffries and Walker, 2016). However, there is a dearth of studies examining the biological effects induced by *Wolbachia* for the control of horn flies, with the exception of a computational study (Madhav et al., 2020).

5. Conclusions

Our research significantly contributes by providing new insights into the distribution and abundance of *S. calcitrans* and *H. irritans*. Although no pathogens have been detected, the present study represents the first attempt to detect EIAV and *Ehrlichia* spp., *Anaplasma* spp., *Theileria* spp. and *Babesia* spp. in potential mechanical vector species in Spain, and therefore is of great importance from a medical and veterinary perspective. The detection of *Wolbachia* in *H. irritans* flies underscores the imperative need for innovative and sustainable management

approaches.

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CRediT authorship contribution statement

Mikel A. González: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Ignacio Ruiz-Arrondo:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Daniel Bravo-Barriga:** Writing – review & editing, Writing – original draft, Investigation, Data curation. **Cristina Cervera-Acedo:** Writing – review & editing, Methodology, Investigation. **Paula Santibáñez:** Writing – review & editing, Methodology, Investigation. **José A. Oteo:** Writing – review & editing, Resources, Funding acquisition. **Miguel Á. Miranda:** Writing – review & editing, Resources. **Carlos Barceló:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rvsc.2024.105206.

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