Parasites & Vectors

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Molecular characterisation of common *Culicoides* biting midges (Diptera: Ceratopogonidae) in Ireland

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Abstract

Background Biting midges of the genus *Culicoides* (Diptera: Ceratopogonidae) act as vectors for several arboviruses, including bluetongue virus (BTV) and Schmallenberg virus (SBV), which affect livestock health and productivity. In Ireland, limited genetic data are available regarding the diversity of *Culicoides* species. This study represents the first attempt to characterise *Culicoides* in this region using molecular techniques.

Methods Adult *Culicoides* samples were captured using Onderstepoort Veterinary Institute (OVI) traps across six locations in Ireland. Subsequent molecular analyses involved polymerase chain reaction (PCR) and sequencing of the cytochrome oxidase subunit 1 (*CO1*) and the internal transcriber spacer (ITS) barcoding regions to obtain species identities. In addition, using both markers, we inferred the population genetic structure and potential colonisation pathways of *Culicoides obsoletus* sensu stricto (s. str.), the major vector species in Ireland.

Results DNA barcoding facilitated identification of 177 specimens. Eight common *Culicoides* species were identified through DNA barcoding of *CO1* and ITS gene regions. The presence of putative vectors of bluetongue virus (BTV) and Schmallenberg virus (SBV) were also confirmed, including species in the subgenus *Avaritia* (*C. obsoletus* s. str., *C. scoticus*, *C. chiopterus*, and *C. dewulfi*) and subgenus *Culicoides* s. str. (*C. pulicaris* and *C. punctatus*). Phylogenetic analysis confirmed the relationship between these vector species and facilitated the placement of *Culicoides* spp. that could not be identified to species level through DNA barcoding. Haplotype network analysis of *C. obsoletus* showed that some haplotypes of these species are shared between Continental Europe, the UK, and Ireland, suggesting a possible incursion pathway for this vector.

Conclusions DNA barcoding employing a combination of two barcodes, *CO1* and ITS, proved effective in identifying *Culicoides*, especially species within the *obsoletus* complex, which are difficult to morphologically distinguish. Our findings also suggest that investigation of the population genetic structure of *Culicoides* spp. could be used to model the potential introduction routes of midge-borne pathogens into the country.

Keywords *Culicoides* biting midges, DNA barcoding, Vector surveillance

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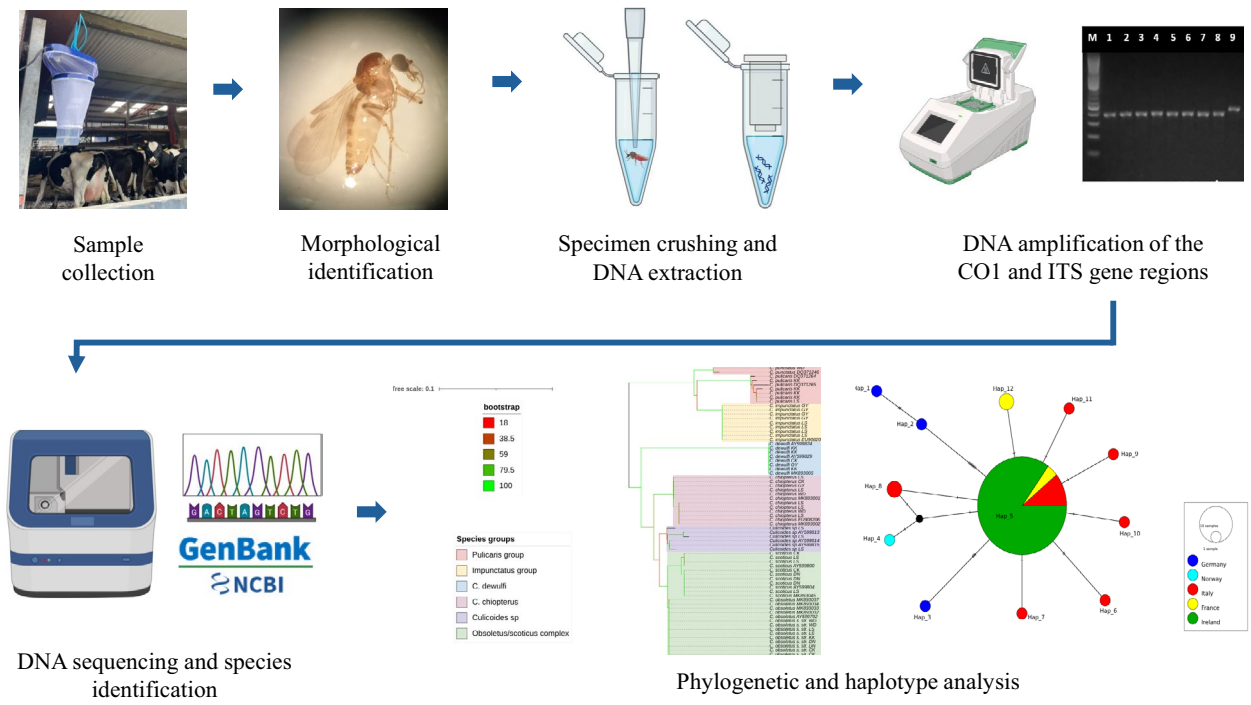
Graphical Abstract



Background

There are over 1400 species of biting midges in the genus *Culicoides* Latreille, 1809 (Diptera: Ceratopogonidae) and they are found worldwide in all landmasses except Antarctica [1]. Their broad distribution is attributed to various factors, such as the availability of different ecological niches, breeding sites and hosts [2]. Identification of biting midges has become increasingly important because they play a central role in the transmission of several pathogens to humans and animals, including arboviruses, bacteria, protozoa and parasitic nematodes [3, 4]. In Europe, they are known to transmit bluetongue virus (BTV), Schmallenberg virus (SBV), Epizootic Haemorrhagic Disease Virus (EHDV) [5] and African Horse Sickness virus (AHSV) [1, 6, 7].

BTV and SBV have significant economic and animal health implications in Europe. In the early 2000s, the number of BTV cases in Europe increased dramatically, with a particularly severe outbreak that occurred between 2006 and 2008. During this period, BTV serotype 8 (BTV-8) caused economic losses owing to decreased production, mortality, and costs related to control and management measures [8, 9]. In the case of SBV, following its discovery in Germany in 2011, it spread across Europe, causing fever, reduced milk yield, abortions, stillbirths and congenital abnormalities in ruminants [10–13]. While BTV is absent from Ireland, SBV was first detected in 2012 causing a major outbreak in 2016 and 2017, and is now considered endemic in Ireland [14–17].

Following the BTV-8 outbreak from 2006 to 2008 and the SBV outbreak from 2011 to 2012 in Northern Europe, surveillance programmes were established as part of contingency plans to control arboviruses transmitted by biting midges. Various species within different subgenera, including *Culicoides chiopterus* Meigen, 1830, *Culicoides dewulfi* Goetghebuer, 1936, *Culicoides obsoletus* Meigen, 1818, *Culicoides scoticus* Downes & Kettle, 1952 (subgenus *Avaritia* Fox, 1955), *Culicoides lupicaris* Downes & Kettle, 1952, *Culicoides punctatus* Meigen, 1804, and *Culicoides pulicaris* Linnaeus, 1758 (subgenus *Culicoides* s. str.), have been identified as potential vectors of BTV [18, 19].

In Ireland, the Department of Agriculture, Food and the Marine (DAFM) carried out the first surveillance of *Culicoides* from 2007 to 2009 as part of the National BTV Vector Surveillance Programme. This study was prompted by the outbreak of BTV in Europe and aimed to identify potential vector species. Several suspected vector species were identified, with the most abundant species including *C. obsoletus* sensu lato (*s. lat.*),

C. dewulfi, *C. chiopterus*, *C. pulicaris* and *C. punctatus*, collectively accounting for 80–90% of all identified *Culicoides* [20].

In response to the 2012/2013 SBV epidemic in Ireland [14, 15], a sentinel herd surveillance study was initiated across 26 livestock farms in the south of Ireland to monitor the post-epizootic circulation of the virus [16]. *Culicoides* surveillance was later conducted on ten of these farms, and it was reported that the most abundant species identified were *C. obsoletus*/*C. scoticus* (38%), *C. dewulfi* (36%), *C. chiopterus* (5%), *C. pulicaris* (9%) and *C. punctatus* (5%), collectively accounting for 93% of all *Culicoides* collected. In this study, 20 species of *Culicoides* biting midges were identified, including 1 species not previously known from Ireland, *C. cameroni* Campbell and Pelham-Clinton, 1960, which raised Ireland's species list to 31 species [21], belonging to eight subgenera. As these previous studies predominantly relied on morphological examinations for species identification, there is a lack of genetic information regarding *Culicoides* in Ireland, which has inhibited the development of rapid molecular identification techniques.

Regarding the introduction of SBV into Ireland in 2012, several transmission routes have been suggested, including the importation of infected animals and windborne dispersal of infected midges. Surveillance in 2012 and 2013 identified a high concentration of SBV cases in the southeast of the island [16], suggesting that SBV may have been introduced via winds carrying infected midges from the UK or continental Europe. Moreover, McGrath et al. [22] utilised serological analysis of archived bovine sera to identify potential dispersal windows and atmospheric dispersion modelling (ADM) to evaluate environmental conditions conducive to the transportation of *Culicoides* into Ireland. Atmospheric dispersion modelling pointed to favourable conditions for midge dispersal from southern England in August 2012, though long-range transportation events were rare, with only one significant instance identified during the 2012 vector season. These studies and hypotheses highlight the need for further research to reveal potential routes for the spread of midges and midge-borne viruses into Ireland.

Morphological identification of *Culicoides* species is time-consuming and requires highly trained personnel. Moreover, they are a difficult to identify cryptic species, which can result in misidentifications. DNA barcoding of a common gene region has enabled the accurate and reliable molecular identification and phylogenetic placement of *Culicoides* species [23–27] and has helped to catalogue previously hidden diversity within this taxonomic group in various countries [27–32]. Molecular data have also proven invaluable in population genetics studies, particularly for tracing the colonisation history of biting midges. These studies indicate that, while native populations exhibit strong genetic structuring, recently colonised regions often show low genetic differentiation. It has been suggested that this pattern which has been observed in important vector species, such as *C. obsoletus* sensu lato, *C. imicola* Kieffer, 1913, *C. brevitarsis* Kieffer, 1917 and *C. mahasarakhamense*, could potentially be used to investigate the incursion and spread of pathogens that they transmit [33–37].

The present study aimed to conduct the first comprehensive molecular characterisation of common biting midge species found in Ireland. In addition, we undertook a population genetic analysis of the vector species, *C. obsoletus* s. str., to determine the genetic diversity and relationship of Irish specimens to populations characterised elsewhere; potentially revealing migration patterns and sources of incursion. To achieve this, we employed two molecular markers: the mitochondrial cytochrome c oxidase subunit I (*CO1*) and the nuclear ribosomal internal transcribed spacer (ITS) gene regions. The *CO1* gene region has been widely used for species identification, offering high resolution and effectively identifying most *Culicoides* species owing to its conserved nature and the advantage of a large existing database [2, 23, 28–32]. The ITS marker, known for its rapid evolution and high variability, was included to differentiate closely related species and provide additional species resolution [6, 24–27]. This study addresses a critical gap in biodiversity knowledge by establishing a genetic database for *Culicoides* species in Ireland, providing insights into species diversity, evolutionary relationships and population dynamics. Genetic data on vector species will also aid in *Culicoides* vector and vector-borne disease monitoring while supporting biodiversity preservation in Ireland.

Methods

Sample collection

As part of the Network of Insect Vectors (NetVec) Ireland project https://www.ucd.ie/netvecireland/, ultraviolet (UV) light suction traps from the Onderstepoort Veterinary Institute (OVI) were used to collect biting midges biweekly overnight (1 hour before dusk to 1 hour after sunrise). A subset of samples from six study sites (Fig. 1), with only one collection considered per site, sampled either in June or July 2022, were selected for characterisation using molecular methods. The study sites were distributed across various regions, with sites 1 (Dublin), 2 (Laois), 3 (Cork) and 6 (Kilkenny) situated inside farms where cattle rearing was the predominant farming practice. Sites 4 (Galway) and 5 (Waterford) were located in close proximity to farms where typical farming practices included cattle and horse rearing.

Fig. 1 Location of sample collection sites in six counties in Ireland during the midge surveillance activities conducted in June and July 2022. The map was generated in QGIS 3.38.1 with shape files provided by Natural Earth (http://www.naturalearthdata.com/)

DNA extraction, amplification and sequencing

Whole individual biting midges were air-dried for 1 hour to remove ethanol, and subsequently crushed individually in 1.5 mL microcentrifuge tubes. DNA was extracted from each crushed midge using the NucleoSpin DNA Extraction Kit (Macherey–Nagel), following the manufacturer's protocol and eluted in 100 µL of elution buffer. The purity and concentration of the extracted DNA were measured using a NanoDrop™ 8000 Spectrophotometer (Thermo Scientific).

Culicoides DNA samples were amplified in the *CO1* region with LCO1490/HCO2198 primers [39], as well as ITS regions using the ITSA/ITS2B primers [40] and PanCul [6]. PCR reactions were performed in a final volume of 10 µl, consisting of 5 µL GoTaq™ Hot Start Polymerase: Green Master Mix 2× (Promega), 3 µL nuclease-free water, 1 µl of a primer mix containing 5 µM each of the forward and reverse primers, and 1 µL of the template. Negative controls contained 1 µl of nuclease-free water in lieu of template DNA. The PCR cycling conditions used for *CO1* included an initial denaturation at 95 °C for 15 min, followed by 40 cycles of 95 °C for 40 s, 46 °C for 40 s, 72 °C for 40 s, and a final extension at 72 °C for 7 min and final hold at 4 °C. For ITS2 and PanCul the PCR cycling conditions were as follows: an initial denaturation at 95 °C for 3 min; followed by 35 cycles of 95 °C for 1 min; 54 °C for 1 min; 72 °C for 1 min; and a final extension at 72 °C for 20 min and a final hold at 4 °C. Following the amplification of DNA, agarose gel electrophoresis was used to visualise the PCR products on a 1.6% agarose gel.

PCR products of the expected size were purified using microCLEAN (Microzone) following the manufacturer's protocol and sequenced in the forward direction on an Applied Biosystems 3500 Genetic Analyser using the BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems). The quality of sequences generated was evaluated on Chromas (version: 2.6.6) before performing the standard nucleotide basic local alignment search tool (BLASTn) on GenBank. Sample identity was verified on the basis of DNA sequence similarity with reference sequences from the GenBank public repository, using

default search parameters. BLAST hits with the highest query cover score and percentage identity values were considered candidate species (Additional file 1).

For specimens with lower sequencing quality, amplicons were cloned into the pDrive Cloning Vector and transformed into competent Top Ten cells using the Qiagen PCR Cloning Kit, following the manufacturer's protocol, as described in previous studies. [25, 26, 32, 41]. Direct colony PCR was performed to confirm the presence of inserts in transformants using M13 primers (Table 1). The PCR reaction mixture for each reaction comprised 5 µL GoTaq™ Hot Start Polymerase: Green Master Mix, 2× (Promega), 3 µL nuclease-free water, 5 µM of the forward and reverse M13 primers, and 1 µL template. Negative controls contained 1 µl of nuclease-free water in lieu of template DNA. The PCR conditions included an initial step at 95 °C for 10 min, followed by 30 cycles at 95 °C for 30 s, 57 °C for 30 s, 72 °C for 1 min and 72 °C for 10 min. One microlitre of each PCR product was visualised using 1% agarose gel electrophoresis. Positive PCR products were purified and sequenced as previously described.

Culicoides species identification

To visualize the occurrence of common *Culicoides* species of the subgenus *Avaritia* and *Culicoides* trapped in the six study sites, a chord diagram was created using the "circlize" package in RStudio [42]. Its interconnections illustrating the relationship between species occurrence and sampling sites [43].

Phylogenetic analysis

To examine the genetic relationships among species in this study, multiple sequence alignments of *CO1* and the ITS sequences were performed using the online versions of MAFFT [44] and Molecular Evolutionary Genetics Analysis (MEGA) software version 11 [45]. In addition to the sequences generated in this study, *CO1* and ITS sequences of the same species obtained from GenBank (Additional File 2) were incorporated into the alignments to facilitate a broader comparison. The *CO1* alignment was truncated to 507 base pairs (bp), while the ITS alignment was trimmed to 218 bp, with gaps excluded from the ITS alignment using MEGA. The ITS dataset included a total of 69 sequences, encompassing both newly generated and previously published sequences and similarly, the *CO1* dataset comprised 59 sequences. Neighbour-joining phylogenetic trees based on both the ITS and *CO1* sequences were constructed, incorporating reference sequences obtained from GenBank. To assess the robustness of the phylogenetic trees, the bootstrapping method with 1000 bootstrap replications was utilised. The resulting trees were visualised using the online tool Interactive Tree of Life (iTOL, available at https://itol.embl.de/).

Haplotype analysis of *Culicoides obsoletus*

In this study, 53 *CO1* and 62 ITS gene sequences of *Culicoides obsoletus* were aligned using the CLUSTAL W method in MEGA. These sequences were compared with reference sequences from GenBank, representing different *C. obsoletus* haplotypes (Additional file 3). For the *CO1* alignment, reference sequences were sourced from *C. obsoletus* populations in the UK ($n=9$) as well as from multiple countries across Continental Europe and neighbouring regions, including France ($n=2$), Switzerland ($n=2$), Italy ($n=2$), Germany ($n=3$), the Netherlands ($n=3$), Spain ($n=3$), Norway ($n=3$), Greece ($n=1$), Denmark ($n=3$), Poland ($n=3$), Serbia ($n=3$), Bulgaria ($n=3$), Macedonia ($n=3$), Morocco ($n=4$) and Turkey ($n=2$). For the ITS locus, fewer reference sequences were available and the following sequences were included: UK ($n=9$), Italy ($n=15$), France ($n=5$), Norway and Germany ($n=3$). A total of 102 *CO1* and 86 ITS2 sequences were truncated to 528 bp and 246 bp, respectively. Both alignments were exported as NEXUS files into Population Analysis with Reticulate Trees (PopART; http://popart.otago.ac.nz/), and a Median-joining network diagram

Table 1 Primers used in DNA amplification and sequencing

Gene	Primer name	Sequence 5′–3′	Fragment length	References
CO1	LCO1490	GGTCAACAAATCATAAAGATATTGG	~710 bp	[39]
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA		
ITS	ITS2A	TGTGAACTGCAGGACACAT	~350 bp	[40]
	ITS2B	TATGCTTAAATTCAGGGGGT		
	PanCul F	GTAGGTGAACCTGCGGAAGG	~785 bp	[6]
	28S R	ATTTGGGGGTAGTCACACAT		
	M13 F	GTAAAACGACGGCCAGT		
	M13 R	AAACAGCTATGACCATG		

illustrating haplotype diversity, was constructed using the median algorithm with default settings [46].

Results

Genetic identification of common *Culicoides* spp.

A total of 177 specimens were analysed, with morphological identification assigning them to the Obsoletus complex, *C. pulicaris*/*C. lupicaris*, *C. impunctatus* or as "unknown". Species-level resolution was achieved using simultaneous sequencing of the *CO1* and ITS gene regions, which enabled accurate genetic identification and clarification of species diversity. Specimens initially identified as belonging to the Obsoletus complex were genetically resolved into four distinct species: *C. obsoletus*, *C. scoticus*, *C. chiopterus* and *C. dewulfi*, and *Culicoides* spp. Similarly, specimens morphologically classified as *C. pulicaris*/*C. lupicaris* were genetically identified as either *C. pulicaris* or *C. punctatus*. For those identified as *C. impunctatus* on the basis of morphological features, genetic resolution confirmed them as *C. impunctatus* (Additional file 1).

Both barcodes were successful in determining species identities, with the sequencing results from each barcode region complementing the other. Of the 177 specimens, 139 (78.5%) were identified through their *CO1* region, and 166 (93.2%) were identified by the ITS region. Overall, eight species were identified. *Culicoides obsoletus* s. str. represented the most dominant species in the sample set with 34.4% of specimens identified (62 specimens), followed by *C. impunctatus* at 20.6% (36 specimens), *C. scoticus* at 16.1% (27 specimens), *C. chiopterus* at 11.1% (20 specimens) and *C. dewulfi* at 9.4% (17 specimens). The least common species were *C. pulicaris* (3.9%, seven specimens), *C. punctatus* (2.8%, five specimens) and an unidentified *Culicoides* sp. (1.7%, three specimens). In most cases, the ITS2 barcode was more effective, particularly in identifying the closely related species *C. scoticus* and *C. obsoletus* s. str. within the Obsoletus complex.

The chord diagram (Fig. 2) shows the distribution of the eight species across the six sampling sites. *Culicoides obsoletus* s. str. was detected at all six sites, while the other species, *C. scoticus*, *C. punctatus*, *C. impunctatus*, *C. dewulfi* and *C. chiopterus*, were only detected at some sites. Two *Culicoides* samples from site 3 (Laois) (Fig. 2) amplified and sequenced using ITS2 primers only, yielded short sequences of approximately 108 base pairs, which hindered analysis. Following cloning and resequencing, approximately 380 base pair sequences were produced, enabling species identification. Nucleotide BLAST analysis revealed 99% and 98% similarity to an unidentified *Culicoides* sp. reported by Gomulski et al. [25] (GenBank accessions AY599813 and AY599814, respectively). A third sample from the same site, displaying similar characteristics, was also identified as a *Culicoides* sp. with approximately 98% similarity to the sequence deposited under GenBank accession number AY599815.

Phylogenetic analysis of the *Culicoides* species

Phylogenetic trees representing the two gene regions were generated, with branches of the tree depicting the evolutionary relationships among *Culicoides* species (Figs. 3 and 4). On the basis of the *CO1* and ITS2 sequences, two subgenera, *Avaritia* and *Culicoides* s. str. were identified. Bootstrap values are shown on a gradient from red to green, indicating the reliability of these inferred relationships, with red representing lower confidence (18/19%) and green representing higher confidence (100%).

The analysis of the *CO1* and ITS2 phylogenetic trees revealed distinct groupings. According to the *CO1* phylogenetic tree, three species groups were identified: the *pulicaris* group, the *impunctatus* group and the Obsoletus/Scoticus complex, as well as the species *C. dewulfi* and *C. chiopterus*. In contrast, the ITS2 phylogenetic tree distinguished between three species groups: the *pulicaris* group, the *impunctatus* group and the Obsoletus/Scoticus complex, and in addition: an unidentified *Culicoides* species, *C. dewulfi* and *C. chiopterus*. In both trees, most of the branches within clades had high bootstrap support, indicating strong confidence in these evolutionary relationships. However, some branches that connect the major groups with lower bootstrap values, reflect a degree of uncertainty.

One of the most notable findings was the well-supported clade of the Obsoletus/Scoticus complex. Both, the *CO1* tree and the ITS tree indicate a very close genetic relationship between *C. obsoletus* s. str. and *C. scoticus* (Figs. 3 and 4) most likely reflecting this species complex. In contrast, *C. dewulfi* forms a distinct branch, separate from the other groups in both trees, representing a separate evolutionary lineage. The tight clustering of *C. dewulfi* sequences within this branch suggests a clear genetic identity, distinct from the other species. *C. chiopterus* forms a clade closely related to the Obsoletus/Scoticus complex in the *CO1* phylogenetic tree but is more distantly separated in the ITS tree. The *impunctatus* group also forms a distinct clade that is separate from, but closely related to, the *pulicaris* group, suggesting a similar evolutionary lineage. The clustering of multiple sequences of *C. impunctatus* (Figs. 3 and 4) within this clade indicates internal diversification within the species. Interestingly, the unidentified *Culicoides* species formed a distinct branch positioned between the Obsoletus/Scoticus complex and *C. chiopterus*.

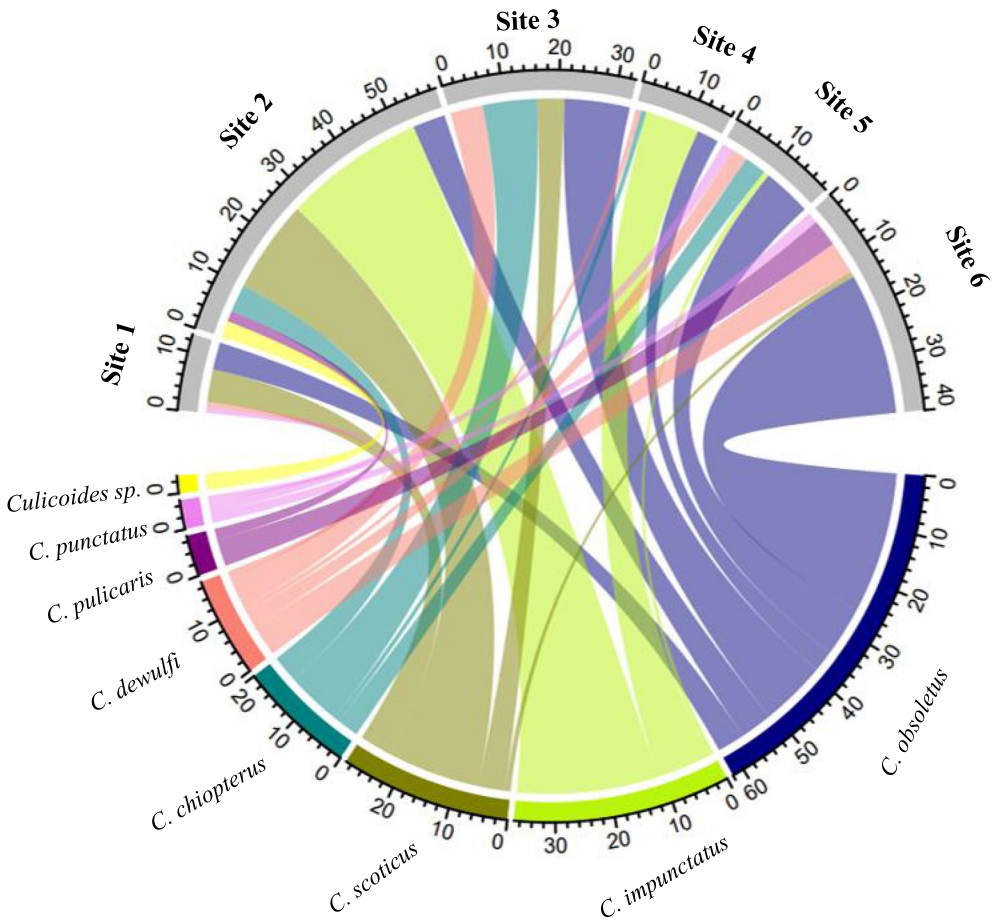


Fig. 2 Species occurrence across the six sample sites: sites 1 (Dublin Site), site 2 (Laois), site 3 (Cork), site 4 (Galway), site 5 (Waterford) and site 6 (Kilkenny)

Population genetic structure of Irish *Culicoides obsoletus*

Analysis of the mtDNA (*CO1*) sequences revealed 17 haplotypes, with 6 haplotypes: Hap_1, Hap_2, Hap_3, Hap_4, Hap_5 and Hap_6, being the predominant haplotypes in the Irish population of *C. obsoletus* s. str. (Fig. 5). Hap_1, which was the central and most prevalent haplotype in this network, was found in all counties considered in the analysis. Hap_6, Hap_3 and Hap_4 were also significant nodes connected to several other haplotypes, indicating that they are also important intermediaries in the network. Peripheral haplotypes (Hap_5, Hap _ 8, Hap _ 9, Hap _ 10, Hap _ 11, Hap _ 12 and Hap _ 14) were more isolated and showed more specific geographic distributions.

Analysis of the ITS2 sequences revealed just 1 haplotype (Hap_5) in Ireland, compared with 12 haplotypes reported elsewhere (Fig. 6). The haplotype Hap_5 was exclusively found in Ireland and has also been detected in Continental Europe, including France and Italy. It is closely related to other haplotypes (Hap_6, Hap_8, Hap_9, Hap_10, Hap_11 and Hap_12). In contrast, Hap_1, Hap_2 and Hap_3 found in Germany, had more mutation steps. In both network diagrams, the black node can be visualised, indicating a potential connection between the haplotypes.

Discussion

Culicoides species, such as *C. obsoletus* s. str., *C. scoticus*, *C. dewulfi*, *C. chiopterus*, *C. pulicaris*, *C. punctatus* and *C. impunctatus* have an extensive distribution across the Palearctic region, rendering them among the most widely dispersed species within this geographical area. The accurate delimitation of the status of these vector species, along with an in-depth understanding of their population dynamics and evolutionary genetics, is essential for biosecurity measures and the mitigation of *Culicoides*-borne viruses. Different molecular markers, including nuclear ribosomal DNA (16 s rDNA, 28 s rDNA, ITS1 and ITS2) and mitochondrial DNA [*CO1* and cytochrome b oxidase (Cyt b)], have been utilised to distinguish *Culicoides* species [2, 6, 23–32]. The *CO1* barcode is the most widely used barcode for identifying *Culicoides* species [47–51].

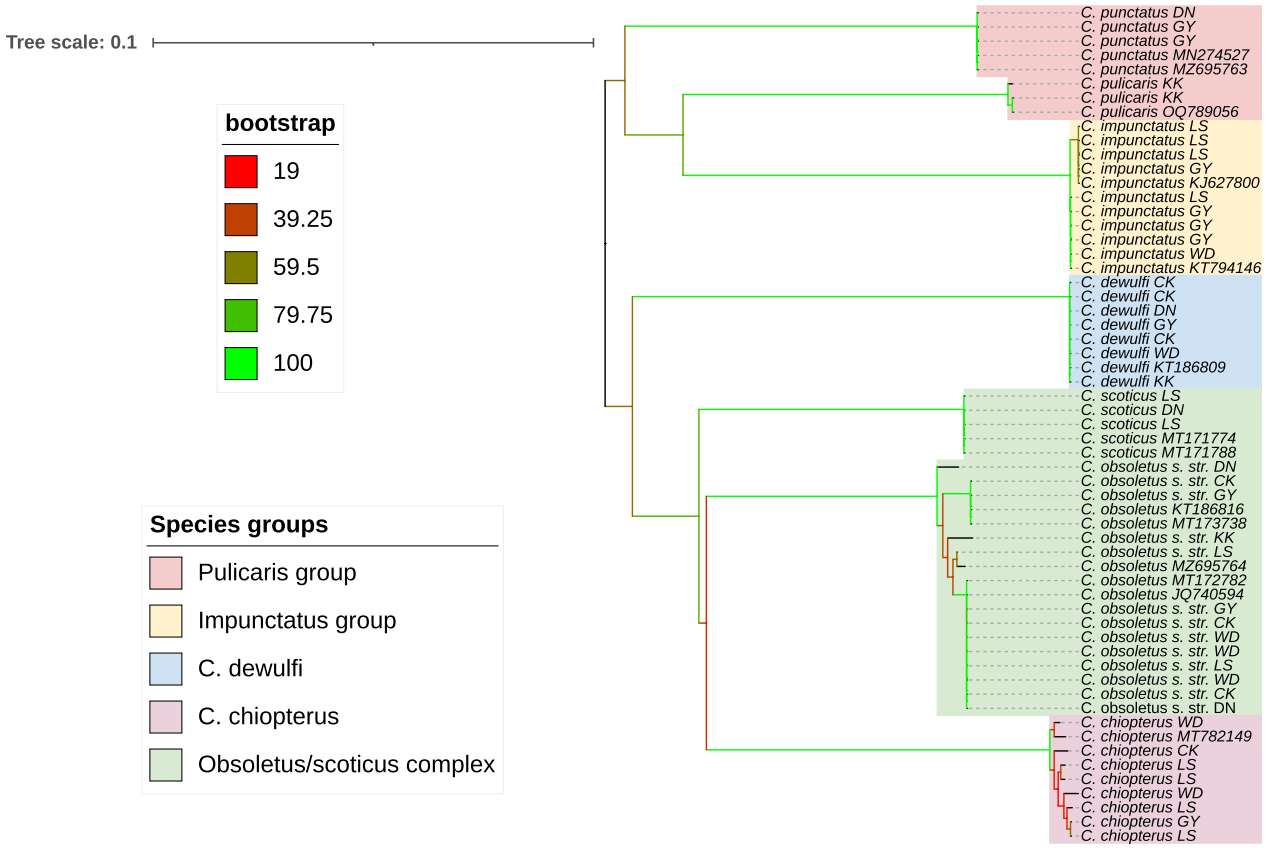


Fig. 3 Neighbour-joining phylogenetic tree generated from an alignment of identified Irish *CO1 Culicoides* sequences and reference sequences obtained from GenBank truncated to a final sequence length of 507 bp. Labels on the right side indicate the species name and county code (*WD* Waterford, *KK* Kilkenny, *CK* Cork, *DN* Dublin, *LS* Laois and *GY* Galway)

However, the use of this barcode can be challenging [52, 53] because of high intraspecific and low interspecific variability, leading to similar genetic distance and low genetic differentiation between species [54]. Therefore, multi-locus approaches are often recommended to enhance species detection and provide accurate species composition data [2, 24]. The ITS regions flanking the 5.8S ribosomal RNA gene evolve rapidly and are a reliable tool for species identification because sequence similarity in these regions is greater within species than between species.

In this study, both barcoding regions were used to identify common *Culicoides* species in Ireland, including species within the subgenus *Avaritia* (*C. obsoletus* s. str., *C. scoticus*, *C. chiopterus* and *C. dewulfi*) and the subgenus *Culicoides* s. str. (*C. impunctatus*, *C. pulicaris* and *C. punctatus*). On the basis of our results, ITS2 barcode sequences were more reliable than *CO1* sequences for species identification. This was particularly evident for closely related species within the *C. obsoletus* complex, where ITS2 provided clearer differentiation between *C. scoticus* and *C. obsoletus* s. str. Similar findings have been reported in studies on species complexes in the mosquito genera *Culex* and *Anopheles* [55–57]. The integration of genetic species resolution reaffirmed the validity of morphological groupings, but also highlighted the importance of molecular techniques in differentiating cryptic species. This combined approach emphasises the value of genetic tools in improving the accuracy of species identification and advancing our understanding of species diversity in the genus *Culicoides*. The occurrence of significant numbers of *C. obsoletus* s. str. at all trapping sites in significant numbers suggests that it is widely distributed, potentially due to a generalist habitat preference or adaptability to diverse environmental conditions. The other species are known to exhibit more specialized ecological niches [29, 58–64]. It is important to note that the small sample size and lack of longitudinal genetic data limits conclusions about these species occurrence and abundance. Also, this study period was confined to species active during peak midge season and does not include those active in other seasons. Therefore, our findings focus on the occurrence of common *Culicoides* species rather than their distribution, abundance

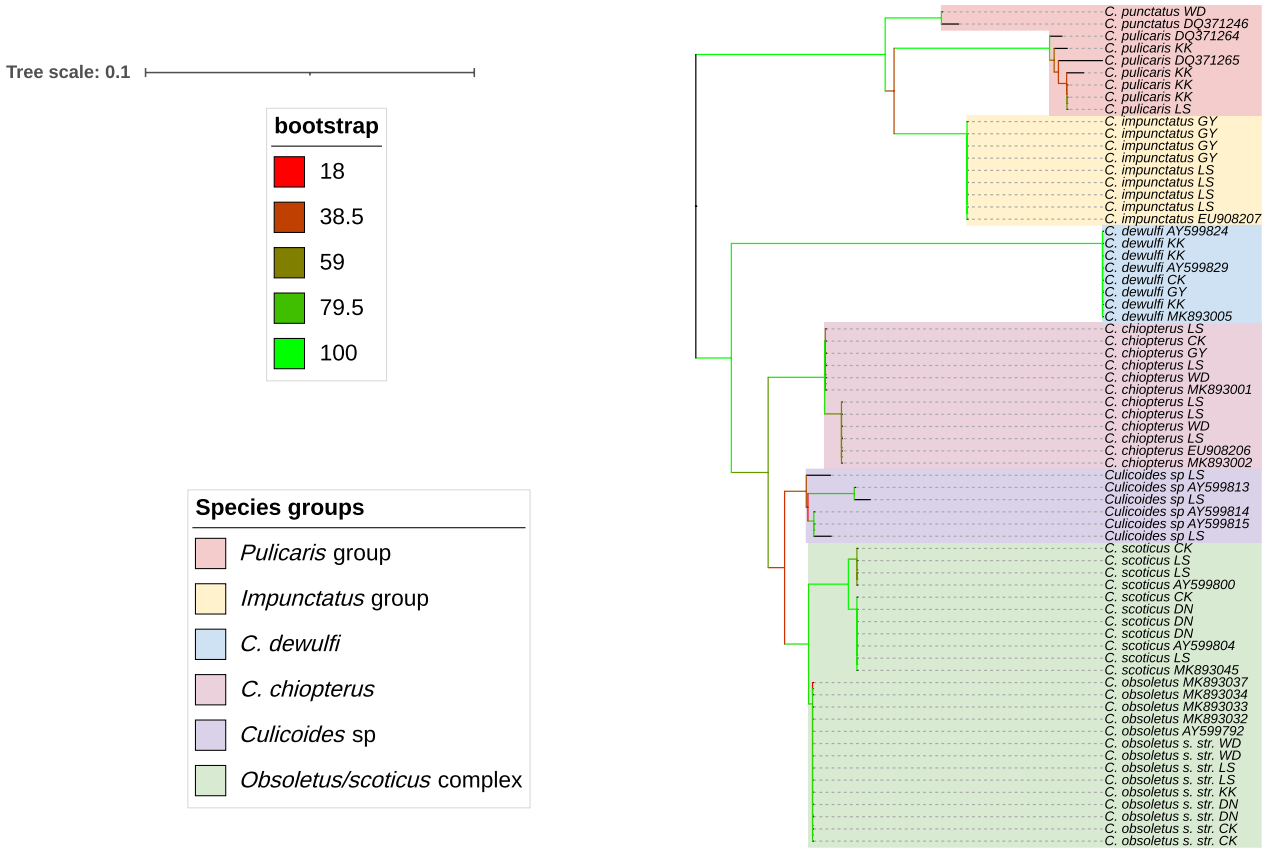


Fig. 4 Neighbour-joining phylogenetic tree generated from an alignment of identified Irish ITS2 *Culicoides* sequences and reference sequences obtained from GenBank truncated to a final sequence length of 218 bp. Labels on the right side indicate the species name and county code (*WD* Waterford, *KK* Kilkenny, *CK* Cork, *DN* Dublin, *LS* Laois and *GY* Galway)

and overall diversity. Additional seasonal sampling would be necessary for a more comprehensive molecular characterisation.

Phylogenetic analysis

Phylogenetic analysis based on *CO1* is considered more effective for detecting ancient hybridization in molecular phylogeny, while ITS is more reliable for resolving evolutionary issues in recently diverged taxa or cryptic species. Although the mtDNA *CO1* region has been extensively used to confirm relationships within *Culicoides* [23], some studies have found it insufficient for analysing the closely related species within the subgenus *Avaritia*. In these cases, the use of ITS alone or in combination with *CO1* has successfully clarified species relationships within this subgenus [24]. In our study, phylogenetic analysis of both the *CO1* and ITS gene regions enabled the classification of *Culicoides* species. Previous studies have shown that trimming ITS1 and ITS2 sequence alignments in *Culicoides* can result in sequences of varying lengths due to numerous indels (nucleotide insertions/deletions) in these regions, as has also been reported in mosquitoes [55, 65]. Matsumoto et al. [66] observed that indel regions within ITS1 sequences divide some *Culicoides* species into two types: those with long or short ITS1 regions. This issue was overcome by analysing short ITS1 and ITS2 fragments separately after excluding indels, then concatenating them to create phylogenetic trees [24]. This approach was also employed in the present study resulting in the successful classification of the different *Culicoides* species and species groups.

Although the different species in the Obsoletus/Scoticus complex can be reliably identified on the basis of examination of their genitalia [21, 38], adult females are challenging to distinguish morphologically owing to their poorly defined wing patterns. Recent molecular characterisation studies have identified nine species in the *obsoletus* group, including *C. obsoletus* s. str., *C. scoticus*, *C. montanus* Shakirzyanova, 1962, *C. gornostavae* Mirzaeva, 1984, *C. abchazicus* Dzhafarov, 1964 and *C. filicinus* Gornostaeva & Gachegova, 1972 in the Palearctic region; and *C. alachua* Jamnback & Wirth, 1963 and *C. sanguisuga* Coquillett, 1901 in the Nearctic region, and reserved the term "Obsoletus complex" primarily for *C.*

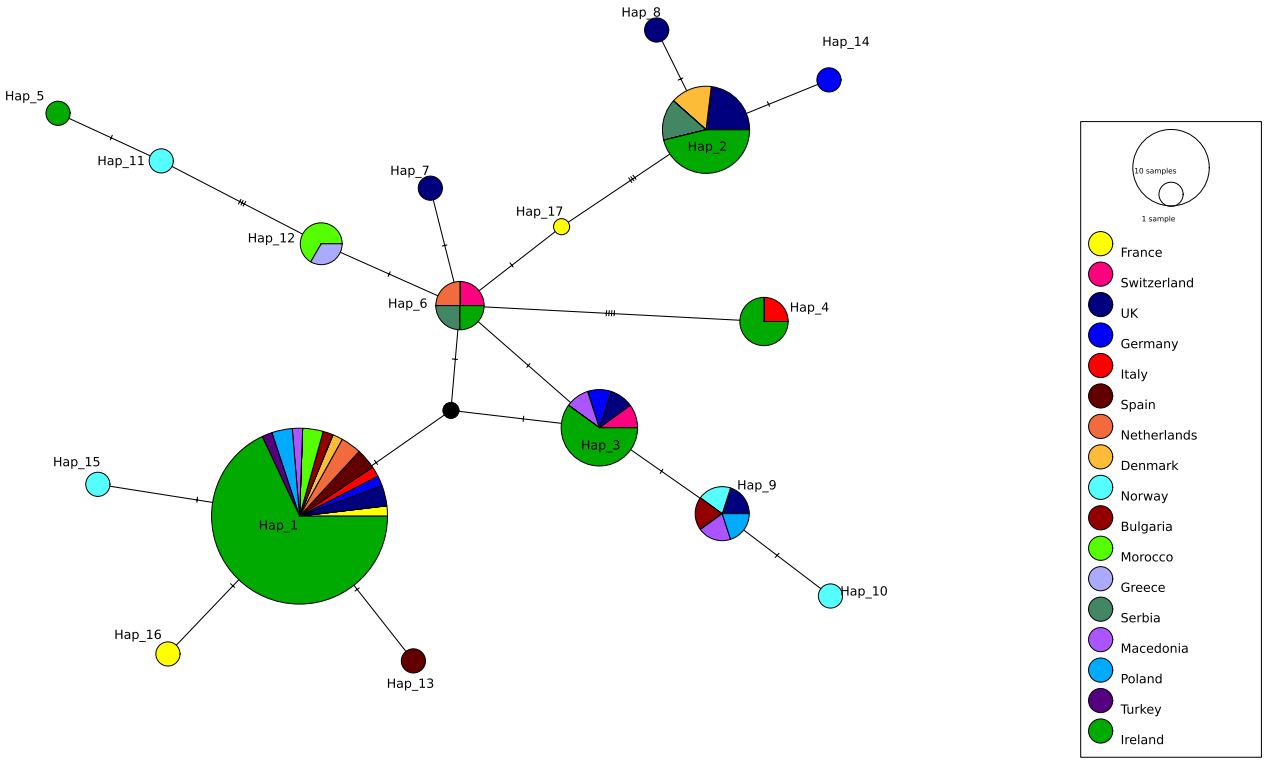


Fig. 5 Median-joining network diagram of *C. obsoletus* s. str. *CO1* haplotypes from Ireland generated in this study, compared with reference *CO1* sequences from other countries obtained from GenBank, truncated to 528 bp

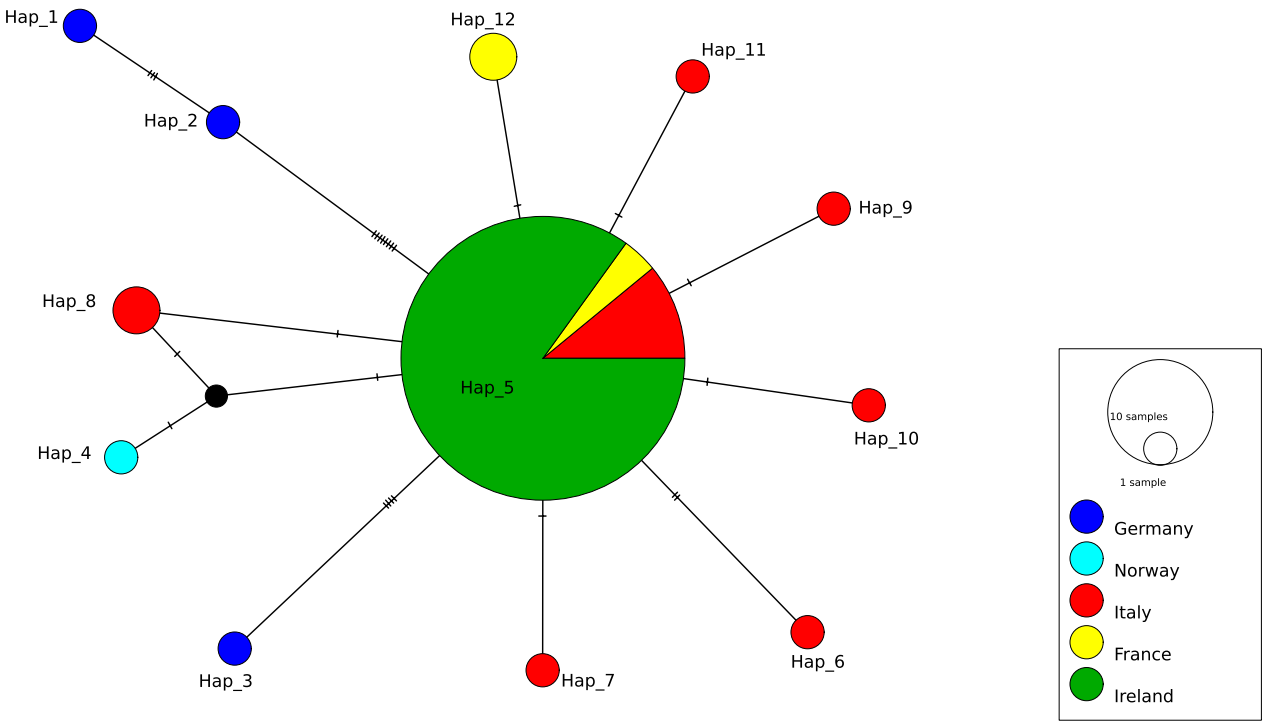


Fig. 6 Median-joining network diagram of *C. obsoletus* s. str. ITS2 haplotypes from Ireland generated in this study compared with reference ITS2 sequences from other countries obtained from GenBank, truncated to 246 bp

obsoletus, *C. scoticus* and *C. montanus* on the basis of adult morphology and molecular phylogenetic analysis [2, 23, 24, 67]. During the earlier Irish surveys which were chiefly based on morphological identification, McCarthy et al. [20] used the term "Obsoletus complex" to refer to members of the subgenus *Avaritia*, including *C. dewulfi*, *C. obsoletus s. lat.* and *C. chiopterus*. In addition, owing to insufficient morphological data to distinguish female *C. obsoletus* from *C. scoticus*, these species were grouped together [21]. Using molecular tools in the present study it was possible to distinguish between *C. obsoletus* s. str. and *C. scoticus*, which grouped tightly together in the phylogenetic tree (Figs. 3 and 4). This close relationship suggests a high likelihood of recent divergence or ongoing gene flow between these species, resulting from potential incomplete speciation or hybridisation. '*Culicoides* sp', which closely resembled specimens reported in Italy [25], form a monophyletic clade with *C. obsoletus* and *C. scoticus* (Fig. 4). Their phylogenetic placement suggests that these unidentified *Culicoides* species may represent new or less-studied lineages, or variants of the Obsoletus/Scoticus complex or *C. chiopterus*. However, at present we cannot make a definitive identification/classification. Additional molecular data from sequencing other gene markers or whole-genome sequencing, along with detailed morphological descriptions, are needed to accurately identify/classify these specimens. The close clustering of *C. chiopterus* with the Obsoletus/Scoticus complex in the *CO1* tree, compared with the ITS tree, suggests that the ITS region provides stronger evolutionary differentiation between *C. chiopterus* and related species. In contrast, the *CO1* gene indicates a closer evolutionary relationship between *C. chiopterus* and the Obsoletus/Scoticus complex. Phylogenetic analysis of both the *CO1* and ITS2 gene regions also indicated that *C. dewulfi*, previously classified within the Obsoletus group, actually forms a distinct, monophyletic clade. This finding aligns with other studies suggesting that *C. dewulfi* only shows 86% similarity with *C. scoticus* and *C. obsoletus* s. str., and should be regarded as a distinct, monotypic entity within the subgenus *Avaritia*; excluding this species from the Obsoletus/Scoticus complex [25, 27, 47, 68].

Within the subgenus *Culicoides* s. str., the *impunctatus* and *pulicaris* groups are well-supported clades, each characterised by high-confidence bootstrap values, indicating strong statistical support for their evolutionary relationships. The *pulicaris* group in our study consists of *C. pulicaris* and *C. punctatus*, while the *impunctatus* group includes *C. impunctatus*, a classification also observed in Northern Ireland [60]. However, some studies group *C. pulicaris*, *C. impunctatus* and *C. punctatus* together within the *pulicaris* group [51]. Our findings on *Culicoides* occurrence and phylogenetic relationships in Ireland are crucial for understanding the region's genetic biodiversity and confirming the presence of vector species.

Culicoides obsoletus network analysis

Culicoides obsoletus s. str., the main vector species responsible for transmitting BTV and SBV to wild and domestic ruminants in the western Palearctic region [3, 69–72], was the most abundant species identified in this study, accounting for more than half of the *Culicoides* specimens analysed. The dominance of this species has also been reported in other studies in Europe and is attributed to its generalist nature, i.e. its ability to tolerate diverse eco-climatic conditions, and its capacity to breed in a wide range of habitats including manure in indoor locations [73, 74]. The latter is a likely reason for its significant role as a vector of livestock pathogens. Therefore, accurate delineation and understanding of its population genetic structure are important for mitigating vector-borne diseases.

The population genetic analysis of *C. obsoletus* s. str. revealed greater intraspecific variation in the *CO1* region, with six haplotypes (Hap_1, Hap_2, Hap_3, Hap_4, Hap_5 and Hap_6) observed within the same *C. obsoletus* s. str. population, underscoring a wider barcoding gap in mtDNA. Conversely, just one ITS haplotype of *C. obsoletus* s. str. was revealed in Ireland, and a relatively small number elsewhere, suggesting that the locus serves as a more reliable barcoding region. This has also been observed in mosquitoes, where the barcoding gap for ITS ranged from 0.042 to 0.193, while for mtDNA *COII*, it ranged from 0.033 to 0.047, suggesting that ITS provides a more accurate molecular identification of *Anopheles* species [57].

Haplotypes characterised in the present study were compared with published sequences reported from the UK, Continental Europe, Morocco and Turkey. The central position and widespread distribution of *CO1* Hap_1 (Fig. 5) and ITS Hap_5 (Fig. 6) in the median-joining network diagram suggest that these may represent key ancestral haplotypes, with the other haplotypes reflecting various branches of genetic divergence. Adult *Culicoides* typically have relatively low self-propelled flight speeds compared with other insect vectors, generally covering distances of 200–300 m during their lifetime [75]. However, they can travel several kilometres through passive wind dispersal, which significantly aids their colonisation and spread across countries [1]. Shared haplotypes with Ireland indicate potential incursion pathways for this vector species into Ireland via passive wind dispersal. These findings support the hypothesis that previous SBV cases in Ireland [15, 22] were caused by infected

Culicoides being carried by easterly winds, leading to their colonisation and expansion in Ireland. Haplotype diversity of *C. obsoletus* in Ireland, compared with neighbouring regions, enhances our understanding of potential vector movement and serves to inform biosecurity measures. With SBV already circulating in Ireland, and BTV at risk of introduction, information on potential incursion pathways strengthens the county's preparedness for the introduction of new vectors and the pathogens they can carry. In addition, this information supports the agricultural industry by addressing risks related to BTV, SBV and other exotic arbovirus outbreaks. However, our study is limited by its restricted geographical scope and reliance on data from a single time point during peak midge activity. The lack of longitudinal data limits our ability to assess temporal shifts in haplotype diversity, which may change over time due to population dynamics, genetic drift, or environmental adaptation, potentially affecting pathogen spread.

Conclusions

This study represents the most comprehensive molecular characterisation of common *Culicoides* biting midges in Ireland, in contrast to previous surveys which chiefly relied on morphological data for identification. DNA barcoding revealed common biting midge species within the subgenera *Avaritia* and *Culicoides*, and also confirmed the presence of potential vectors of BTV and SBV in Ireland previously reported from morphological characterisation. Given the importance of the livestock sector to the economy, this is valuable information for understanding the potential spread of future *Culicoides*-borne pathogens. Despite their significance, the historical molecular evolutionary pathways of *Culicoides* species in Ireland remain largely unknown. Our haplotype analysis of *C. obsoletus* elucidates potential gene flow patterns, suggesting the likely introduction of midges from Europe, and possibly from North Africa and West Asia. However, to better understand potential incursion pathways, a concerted effort across Europe may be necessary to comprehensively sample *Culicoides*. In addition to the markers used here, incorporating microsatellite or single nucleotide polymorphism (SNP) data could provide more robust insights for investigating incursion pathways through a widescale phylogeographic approach.

Abbreviations

BTV	Bluetongue virus
SBV	Schmallenberg virus
EHDV	Epizootic haemorrhagic disease virus
AHS	African horse sickness virus
CO1	Cytochrome c oxidase subunit 1
ITS	Internal transcriber spacer
OVI	Onderstepoort veterinary institute
UV	Ultraviolet
NetVec Ireland	Network of insect vectors Ireland
BLAST	Basic local alignment search tool
MEGA	Molecular evolutionary genetics analysis
iTOL	Interactive tree of life
PopART	Population analysis with reticulate trees

Supplementary Information

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Additional file 1

Additional file 2

Additional file 3

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Author contributions

E.I. conducted the molecular characterisation of the biting midge specimens and prepared the original manuscript draft and subsequent revisions. A.V.O. was responsible for specimen collection, sorting and the initial morphological examination. T.C. and A.O.H. contributed to manuscript revisions. D.O.N. provided molecular biology technical expertise and guidance, and was involved in project supervision. T.d.W. and G.B. also participated in project supervision and contributed to funding acquisition. J.O.S. carried out midge trapping, collection and storage, and also contributed to manuscript revisions. N.K.M., A.V., A.J., A.J., D.B. and S.C. participated in midge trapping, collection and storage. A.Z. provided overall project supervision and contributed to manuscript revisions, as well as leading funding acquisition, project concept and design. D.O.M. also provided overall project supervision and contributed to manuscript revisions, project concept and design, as well as midge trapping and collection. All authors reviewed and approved the final manuscript.

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Availability of data and materials

The data supporting the conclusions of this article are included in the manuscript and supplementary files, with additional sequence data accessible through GenBank (http://www.ncbi.nlm.nih.gov/). The raw data used in this study can be obtained from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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