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DNA Identification and Diversity of the Vector Mosquitoes *Culex pipiens* s.s. and *Culex torrentium* in Belgium (Diptera: Culicidae)

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Citation: Vanderheyden, A.; Smitz, N.; De Wolf, K.; Deblauwe, I.; Dekoninck, W.; Meganck, K.; Gombeer, S.; Vanslembrouck, A.; De Witte, J.; Schneider, A.; et al. DNA Identification and Diversity of the Vector Mosquitoes *Culex pipiens* s.s. and *Culex torrentium* in Belgium (Diptera: Culicidae). *Diversity* **2022**, *14*, 486. https://doi.org/10.3390/d14060486

Academic Editors: Carlos Barceló and Ignacio Ruiz-Arrondo

Received: 16 May 2022
Accepted: 13 June 2022
Published: 15 June 2022

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Abstract: This survey reports on the DNA identification and occurrence of *Culex torrentium* and *Cx. pipiens* s.s. in Belgium. These native disease-vector mosquito species are morphologically difficult to separate, and the biotypes of *Cx. pipiens* s.s. are morphologically indistinguishable. *Culex torrentium* and *Cx. pipiens* s.s. were identified using the COI and ACE2 loci. We recorded 1248 *Cx. pipiens* s.s. and 401 *Cx. torrentium* specimens from 24 locations in Belgium (collected between 2017 and 2019). *Culex pipiens* biotypes *pipiens* and *molestus*, and their hybrids, were differentiated using fragment-size analysis of the CQ11 locus (956 *pipiens* and 227 *molestus* biotype specimens, 29 hybrids). Hybrids were observed at 13 out of 16 sympatric sites. These results confirm that both species are widespread in Belgium, but while *Cx. torrentium* revealed many COI haplotypes, *Cx. pipiens* s.s. showed only one abundant haplotype. This latter observation may either reflect a recent population-wide demographic or range expansion, or a recent bottleneck, possibly linked to a *Wolbachia* infection. Finally, new evidence is provided for the asymmetric but limited introgression of the *molestus* biotype into the *pipiens* biotype.

Keywords: *Culex pipiens* biotypes *pipiens*/*molestus*; hybrids; disease vectors; DNA-based identification; cytochrome c oxidase I (COI); fragment-size analyses (ACE2; CQ11)

1. Introduction

Culex pipiens s.l. is a complex of three species, viz. *Cx. australicus* Dobrotworsky & Drummond, 1953; *Cx. pipiens* s.s. Linnaeus, 1758; and *Cx. quinquefasciatus* Say, 1823. The latter species is common in (sub)tropical regions with no known established populations in Europe [1], but has been introduced in the Netherlands with airplane traffic [2]. In contrast, *Culex australicus* is endemic to Australia. In Europe, hybrids between *Cx. quinquefasciatus*

and *Cx. pipiens* s.s. were characterized in southern coastal regions and the Mediterranean region by applying DNA methods [3]. Despite morphological similarities with *Cx. pipiens* s.s. [4], *Culex torrentium* Martini, 1925 is no longer considered as belonging to the *Cx. pipiens* species complex [5,6]. Nevertheless, both species occur throughout Europe; *Cx. torrentium* is more common in northern Europe and at high elevations further south, whereas *Cx. pipiens* s.s. is more common in the south, but the exact species distribution limits are still unclear [7]. The two species occur in sympatry and are native in Belgium, where *Cx. pipiens* s.s. appears to be more abundant and widespread [8–13]. Within *Cx. pipiens* s.s., two biotypes are recognized, viz. *Cx. pipiens* biotype *pipiens* Linnaeus, 1758, and *Cx. pipiens* biotype *molestus* Forskål, 1775 [14].

The identification of *Cx. torrentium* and *Cx. pipiens* s.s. is difficult as the two species differ by a few subtle morphological characteristics only [4,15]. The biotypes of *Cx. pipiens* s.s. are morphologically indistinguishable [14], but they show four key behavioural differences [16–19]. Females of *Cx. pipiens* biotype *pipiens* need a bloodmeal to produce their first batch of viable eggs, prefer feeding on birds, breed in open spaces, and overwinter in a state of diapause. In contrast, females of *Cx. pipiens* biotype *molestus* can produce a first batch of viable eggs without a bloodmeal, prefer feeding on mammals, can breed in confined mating spaces, and do not overwinter in a state of diapause. In temperate regions of Europe, including Belgium, both biotypes co-occur in open aboveground spaces, but *Cx. pipiens* biotype *molestus* has a preference for confined spaces such as cellars, cesspits, human-made basements, or subways, where these mosquitoes mate and remain active throughout the year [20,21]. Hybrids between biotypes have been reported [13,19,22], displaying a combination of the behavioural traits of both biotypes [20,23]. However, since hybrids are less frequent than expected under random mating, the biotypes may show some degree of reproductive isolation [24,25]. Across the Mediterranean basin, populations in open spaces are genetically more homogenous, with individuals displaying mixed biotype ancestry and a mix of the four key behavioural traits [20]. Thus, the genetic differentiation between biotypes decreases gradually from north to south across the western Palearctic. This may be linked to less severe winters, allowing the non-diapausing *molestus* biotype to survive in open-space environments and admix [20].

In Europe, *Culex pipiens* s.s. is the principal vector for West Nile Virus (WNV), and several other arboviruses [26]. *Culex pipiens* s.s. biotype hybrids with an opportunistic feeding behaviour seem to transmit WNV between birds and humans more easily than non-hybrid *Cx. pipiens* biotypes [23,27–29]. *Culex torrentium*, in turn, is an important vector for Sindbis virus (SINV) in Sweden [30]. However, *Cx. torrentium* also has a high potential to transmit WNV [31,32]. In view of the recent outbreaks of WNV infections in Germany and the Netherlands [33], it is important to closely monitor competent *Culex* vectors.

The distinction between *Cx. torrentium*, *Cx. pipiens* s.s., and *Cx. pipiens* biotypes, has not been investigated systematically. Hence, the distribution and identity of these taxa is still poorly known in most European countries [34]. However, this information is essential to establish reliable risk projection and control programmes, particularly for the early detection of WNV vectors and their potential spread in Europe [35,36]. Therefore, the present paper reports on the DNA-based identification, occurrence, and diversity of *Cx. torrentium* and *Cx. pipiens* s.s., as well as *Cx. pipiens* biotypes *molestus* and *pipiens* and their hybrids, in Belgium.

2. Materials and Methods

2.1. Sampling

Adult and larval mosquitoes were collected in 2017 (August–November), 2018, and 2019 (both April–November) in the framework of the MEMO project (Monitoring of Exotic MOsquito species in Belgium [11]). Thirty-one potential points of entry (PoEs) for exotic mosquitoes in Belgium were surveyed using different sampling and trapping methods [11]. The PoEs included ports and airports, used tire and lucky bamboo import companies, parking lots along highways, wholesale markets, a flower auction, an allotment garden, an

industrial area, and cemeteries along the border with Germany. Specimens were morphologically identified as *Cx. pipiens* s.l./*Cx. torrentium* using the keys of [4,37]. A random subset of 1689 *Cx. pipiens* s.l./*Cx. torrentium* specimens were selected for DNA-based identification (Table S1), using the sample_frac function of the dplyr package in R v4.03 [38].

2.2. DNA Extraction and COI Amplification

DNA was extracted from legs (adults) or abdomen (larvae) using the NucleoSpin® Tissue DNA extraction kit (Macherey-Nagel), following the manufacturer's protocol, but with an elution volume of 70 µL. Remaining parts of the specimens and dried DNA extracts are stored at the Royal Belgian Institute of Natural Sciences (Collection Identifier: IG34179). The universal primers LCO1490 and HCO2198 [39] were used to amplify the mitochondrial cytochrome c oxidase subunit I (COI) barcode region (658 bp) [40]. If this was unsuccessful, the C1N-2191 and C1J-1718 primer combination [41] was used to amplify a 472 bp fragment of the COI barcode region. All PCR mixtures, cycling conditions, purification, and sequencing details are as described by [42] (Table S2). Raw sequences were trimmed, corrected, translated into amino acids, and assembled using Geneious® v.10.0.4 (Biomatters Ltd., Auckland, New Zealand). A consensus sequence was generated for each specimen.

2.3. Fragment-Size Analyses

To distinguish between *Cx. pipiens* s.s. and *Cx. torrentium*, a fragment of the Acetylcholinesterase-2 locus (ACE2) was amplified in a 10 µL PCR reaction volume as described by [43] (Table S2). This method also allows for detection of the eventual presence of introduced exotic *Cx. quinquefasciatus*. Using the forward primer B1246s and the reverse primers ACEpip, ACEquin, and ACEtorr, species-specific fragment sizes were produced, viz. 610 bp for *Cx. pipiens* s.s., 416 bp for *Cx. torrentium*, and 274 bp for *Cx. quinquefasciatus* [43]. PCR products were checked on a 2.5% agarose gel (45 min; 90 V).

To identify the two *Cx. pipiens* s.s. biotypes and their hybrids, the CQ11 microsatellite locus was amplified using the forward primer CQ11F2 and the reverse primers pipCQ11R and molCQ11R, following [44] (Table S2). PCR products were checked on a 2.5% agarose gel (45 min; 90V), with a band at 200 bp for *Cx. pipiens* biotype *pipiens*, and at 250 bp for *Cx. pipiens* biotype *molestus*. Hybrids showed both bands. Such hybrids were subsequently re-extracted and re-amplified for the CQ11 locus to exclude possible DNA contamination and confirm their status by visualisation of the two bands.

2.4. COI Data Analyses

The species identification engine of BOLD was used (www.boldsystems.org, accessed on 24 February 2020) with the species-level barcode records option to find the closest matching reference sequence. A Neighbour-Joining (NJ) tree was constructed to examine the clustering support of each *Culex* species occurring in Belgium [12,45] (Geneious® v10.0.4. (Biomatters Ltd., Auckland, New Zealand), Tamura-Nei distance model, 1000 bootstrap replicates). To do so, all publicly available COI sequences (http://www.boldsystems.org/index.php/databases, 16 March 2020) for these *Culex* species were aligned, using ClustalW in Geneious® v10.0.4 (Biomatters Ltd., Auckland, New Zealand), with the newly generated COI sequences in this study. COI sequences of four species of the genus *Coquillettidia* Dyar, 1905 were included as outgroup (GenBank accession numbers: GQ165785, GQ165801, GQ165802, and GQ165803). The alignment was checked for stop codons and trimmed to retain 658 bp. Sequences of less than 300 bp and conspecific identical sequences were discarded.

Pairwise differences in COI nucleotide frequencies between species, biotypes, and biotype hybrids were evaluated using Wright's *F*-statistics in Arlequin v3.5 (1000 random permutations for significance, with subsequent standard Bonferroni correction) [46]. Haplotype frequencies, mean number of pairwise nucleotide differences (*k*) and nucleotide diversity (*Pi*) were also estimated with Arlequin v3.5, excluding sequences with ambiguous sites.

2.5. Habitat Characterization: Land-Cover Classes

The percentage of Corine Land-Cover (CLC) classes (© European Union, Copernicus Land Monitoring Service 2021, European Environment Agency (EEA)) was calculated in a 2.5 km buffer zone around each sampling location. The latest raster file (CLC 2018) was used, and calculation was performed in Q-GIS and R v4.03. The levels were grouped into five main CLC classes, i.e., artificial or urban areas, agricultural areas, forest and seminatural areas, wetlands, and water bodies.

3. Results

In total, 34,401 specimens from 27 out of 31 PoEs were morphologically identified as *Cx. pipiens* s.l./*Cx. torrentium* of which 1689 specimens from 24 sites were selected for DNA-based verification. Of these, 573 were adults and 1113 were larvae. Adults were collected using a Mosquito Magnet trap (N = 242; 42.2%), Frommer Updraft Gravid trap (N = 59; 10.3%) and BG-Sentinel trap (N = 272; 47.5%). The four PoEs where these species were not collected were only surveyed using oviposition traps. Based on the BOLD similarity percentages, the COI NJ-tree (Figure S1), and the ACE2 fragment sizes (Figure S2), 401 specimens were identified as *Cx. torrentium* (N_{adult} = 40; N_{larva} = 361—Table S1), and 1248 as *Cx. pipiens* s.s. Thirty-seven specimens did not provide ACE2 results and were therefore considered as *Cx. pipiens* s.l./*Cx. torrentium*. Three sequences were of too low quality for identification. The ACE2 fragment-size analysis provided no evidence of *Cx. quinquefasciatus*. In the NJ-tree, *Cx. torrentium* forms a cluster with 74.9% bootstrap support inside the *Cx. pipiens* s.s./*Cx. torrentium* group (Figure S1). The *Cx. torrentium* cluster includes all generated and downloaded (BOLD) COI sequences.

Based on the CQ11 fragment-size analysis 956 specimens were assigned to *Cx. pipiens* biotype *pipiens* (N_{adult} = 315 (33%); N_{larva} = 641 (67%)) and 227 specimens to *Cx. pipiens* biotype *molestus* (N_{adult} = 187 (82%); N_{larva} = 40 (18%)) (Figure S3, Table S1). More adults of the *molestus* biotype were collected than larvae, and the *pipiens* biotype. Additionally, 29 specimens were identified as hybrids between both biotypes (N_{adult} = 8 (28%); N_{larva} = 21 (72%)), while the biotypes of 36 *Cx. pipiens* s.s. sequences were not determined due to missing CQ11 results. These sequences, together with those identified as *Cx. pipiens* s.l./*Cx. torrentium* (N = 37), were excluded from further analyses. The abundance of each taxon at each sampling site is shown in Figure 1. In most sites where both biotypes co-occur (N = 16), crossbreeding was identified, with hybrids detected at 13 sampling locations (Figure 1, Table S3). Sites where biotypes co-occurred included environments dominated by urban (Kallo, Charleroi, Zeebrugge and Zaventem), agricultural (Villers-Le-Bouillet, Vrasene, Frameries, Aische-en-Refail, Büllingen and Natoye), and forest and seminatural (Eupen, Dilsen-Stokkem, Houyet and Maasmechelen) areas (Figure 2). At these sites, larval stages of both biotypes were collected on the same days in the same types of larval habitats, viz. used tires, drainage holes, plastic containers, and cemetery flower vases, on multiple occasions. Once they were also found together in a large artificial pond. *Culex pipiens* s.s. and *Cx. torrentium* were collected on the same days in the following same types of larval habitats: used tires, drainage holes, cemetery flower vases, plastic sheets, and metal and plastic containers. The new COI sequences were deposited in GenBank (accession numbers: *Cx. torrentium*—OM749168-OM749568; *Cx. pipiens* biotype *pipiens*—OM748139-OM749094; *Cx. pipiens* biotype *molestus*—OM747912-OM748138; *Cx. pipiens* biotype *pipiens* X *Cx. pipiens* biotype *molestus*—OM747883-OM747911; *Cx. pipiens* s.s.: OM749132-OM749167; *Cx. pipiens* s.l./*Cx. torrentium*—OM749095-OM749131).

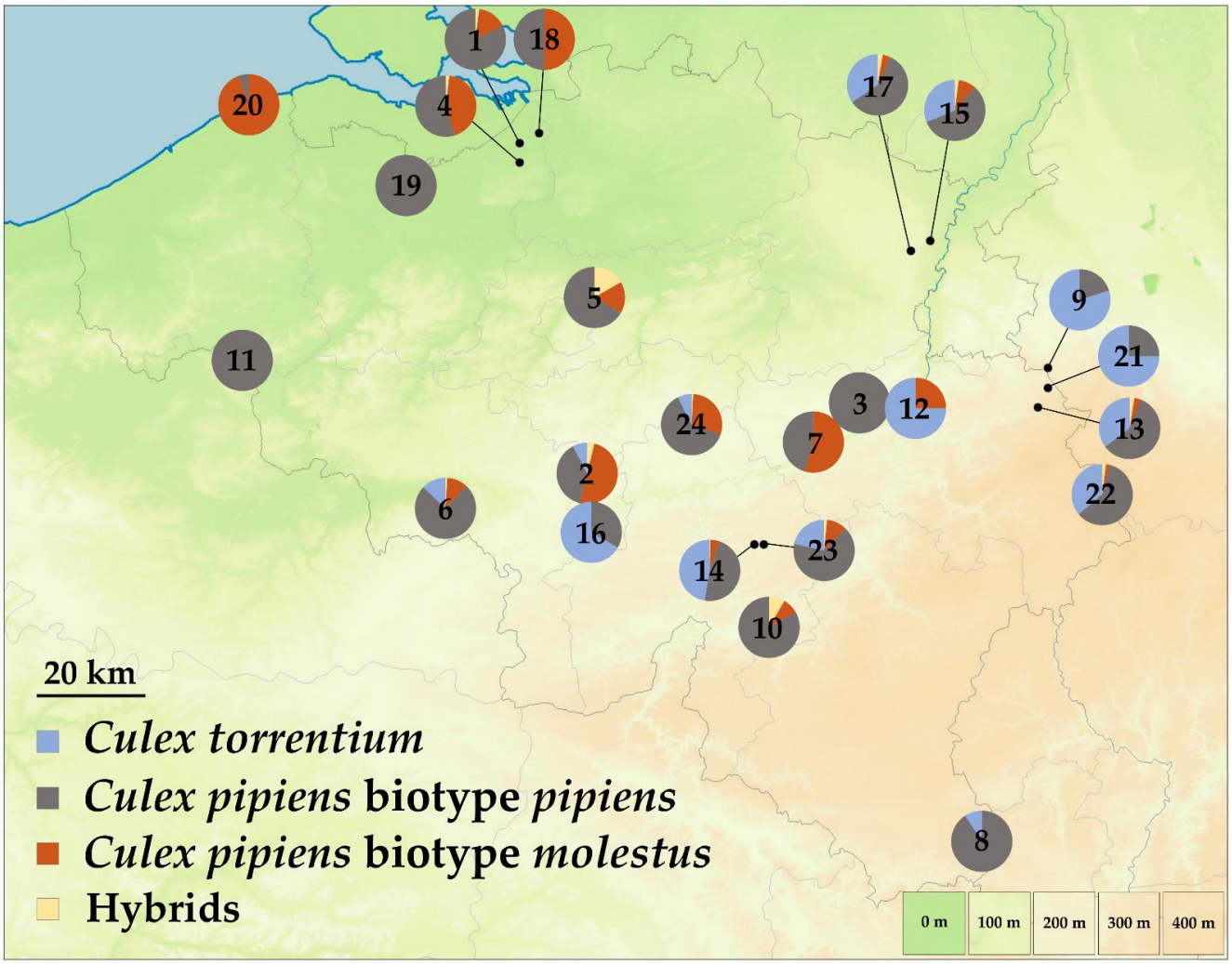

Figure 1. Distribution of *Culex* specimens identified using DNA-based techniques and collected during the MEMO survey 2017–2019 [11]. Hybrids = *Cx. pipiens* biotype *pipiens* X *Cx. pipiens* biotype *molestus*. The numbers indicated on the pie charts are the collection site numbers used in Table S3.

The pairwise F_{ST} values, i.e., a measure of the average genetic differentiation, between *Cx. pipiens* biotype *pipiens* and *Cx. pipiens* biotype *molestus*, were significantly different from zero, which was also the case between the latter and the hybrids (Table 1). The hybrids and *Cx. pipiens* biotype *pipiens* showed the smallest average pairwise nucleotide differences and the lowest average nucleotide diversities (Table 2), despite *Cx. pipiens* biotype *pipiens* being the most widespread taxon in this survey (Figure 1; Table S4). *Culex torrentium* showed higher average pairwise nucleotide differences and nucleotide diversities than *Cx. pipiens* biotype *pipiens* (Table 2).

The most common COI haplotypes in *Cx. pipiens* s.s. were H1 (698 out of 1248 sequences, including 19 hybrids, 91 *Cx. pipiens* biotype *molestus*, and 588 *Cx. pipiens* biotype *pipiens*) and H2 (84 out of 1248 sequences, including 82 *Cx. pipiens* biotype *molestus* and 2 *Cx. pipiens* biotype *pipiens*) (Figure 3). The most common COI haplotype in *Cx. torrentium* was H3 (125 out of 401 sequences) (Figure 3). The haplotype of 509 sequences could not be identified because of ambiguous sites or short fragment lengths ($N_{Cx.\ torrentium}$ = 122; $N_{Cx.\ pipiens}$ biotype *pipiens* = 330; $N_{Cx.\ pipiens}$ biotype *molestus* = 49; $N_{hybrids}$ = 8).

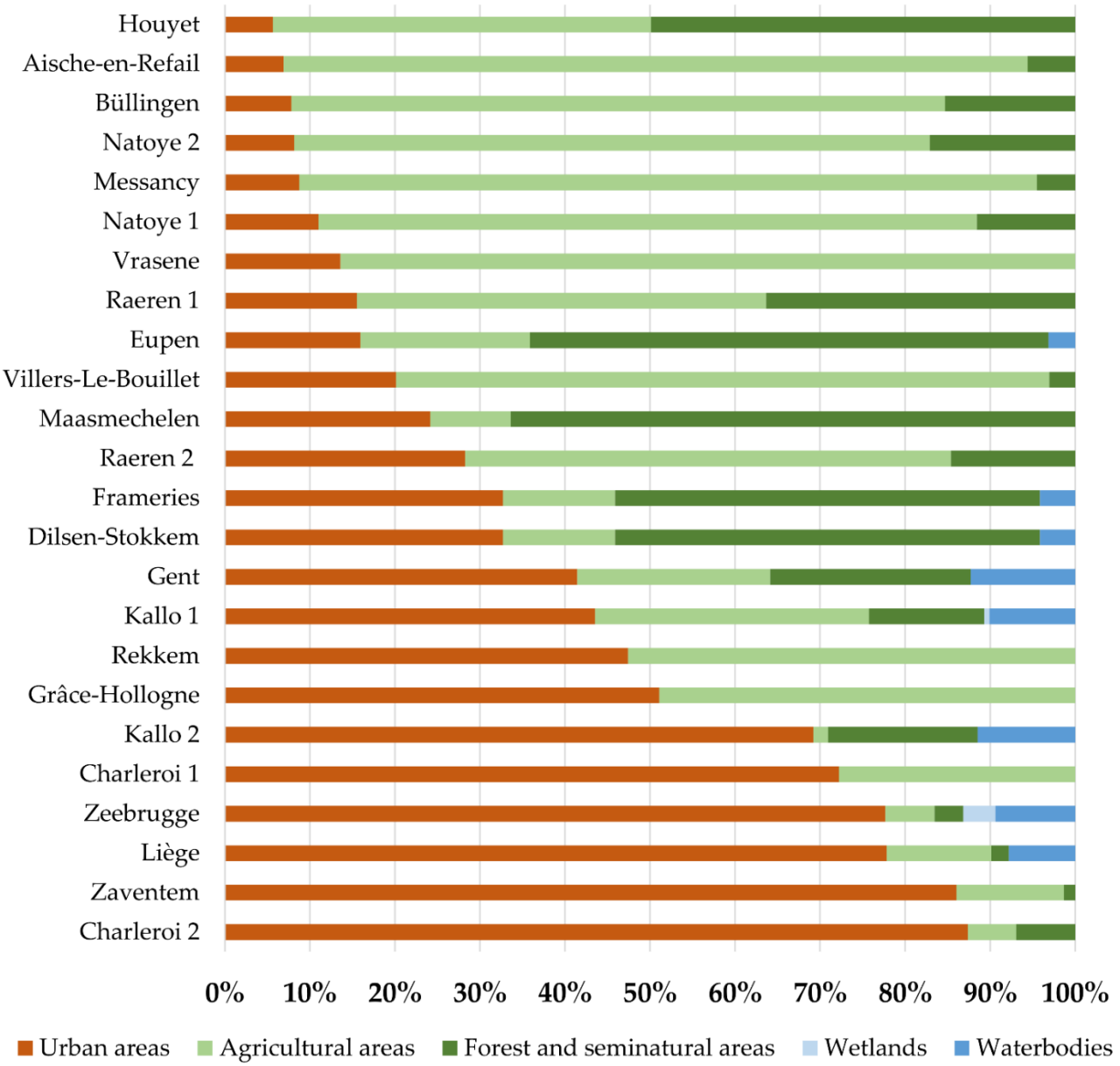

Figure 2. The percentage of main Corine Land-Cover classes in a 2.5 km buffer zone around the sampling locations (group levels based on five classes).

Table 1. Pairwise F_{ST} estimates between biotypes and biotype hybrids of *Culex pipiens* s.s. based on COI sequences, calculated using Arlequin v3.5. Hybrids = *Cx. pipiens* biotype *molestus* X *Cx. pipiens* biotype *pipiens*. Significant values after standard Bonferroni correction marked by an asterisk ($p < 0.0005$).

F_{ST}	Hybrids	Cx. *pipiens* Biotype *molestus*	Cx. *pipiens* Biotype *pipiens*
Hybrids	0	-	-
Cx. pipiens biotype *molestus*	0.2172 *	0	-
Cx. pipiens biotype *pipiens*	0.0164	0.4737 *	0

* = significant values (after Bonferroni correction).

Table 2. COI sequence diversity of *Culex pipiens* s.s. biotypes and *Cx. torrentium*, calculated using Arlequin v3.5. Hybrids = *Cx. pipiens* biotype *molestus* x *Cx. pipiens* biotype *pipiens*, N_{COI} = number of generated COI sequences, N_{Hap} = number of haplotypes, $N_{UnsharedHap}$ = number of haplotypes specific to the taxon, S = number of polymorphic sites, *k* = average pairwise nucleotide differences, *Pi* = nucleotide diversity.

	Hybrids	***Cx. pipiens* Biotype *molestus***	***Cx. pipiens* Biotype *pipiens***	***Cx. torrentium***
N_{COI}	29	227	956	401
N_{Hap}	3	6	24	50
$N_{UnsharedHap}$	1	3	20	50
S	3	8	19	31
k	0.2069 ± 0.2606	0.5458 ± 0.4522	0.0703 ± 0.1418	0.8990 ± 0.6275
Pi	0.0006 ± 0.0008	0.0014 ± 0.0012	0.0002 ± 0.0004	0.0024 ± 0.0018

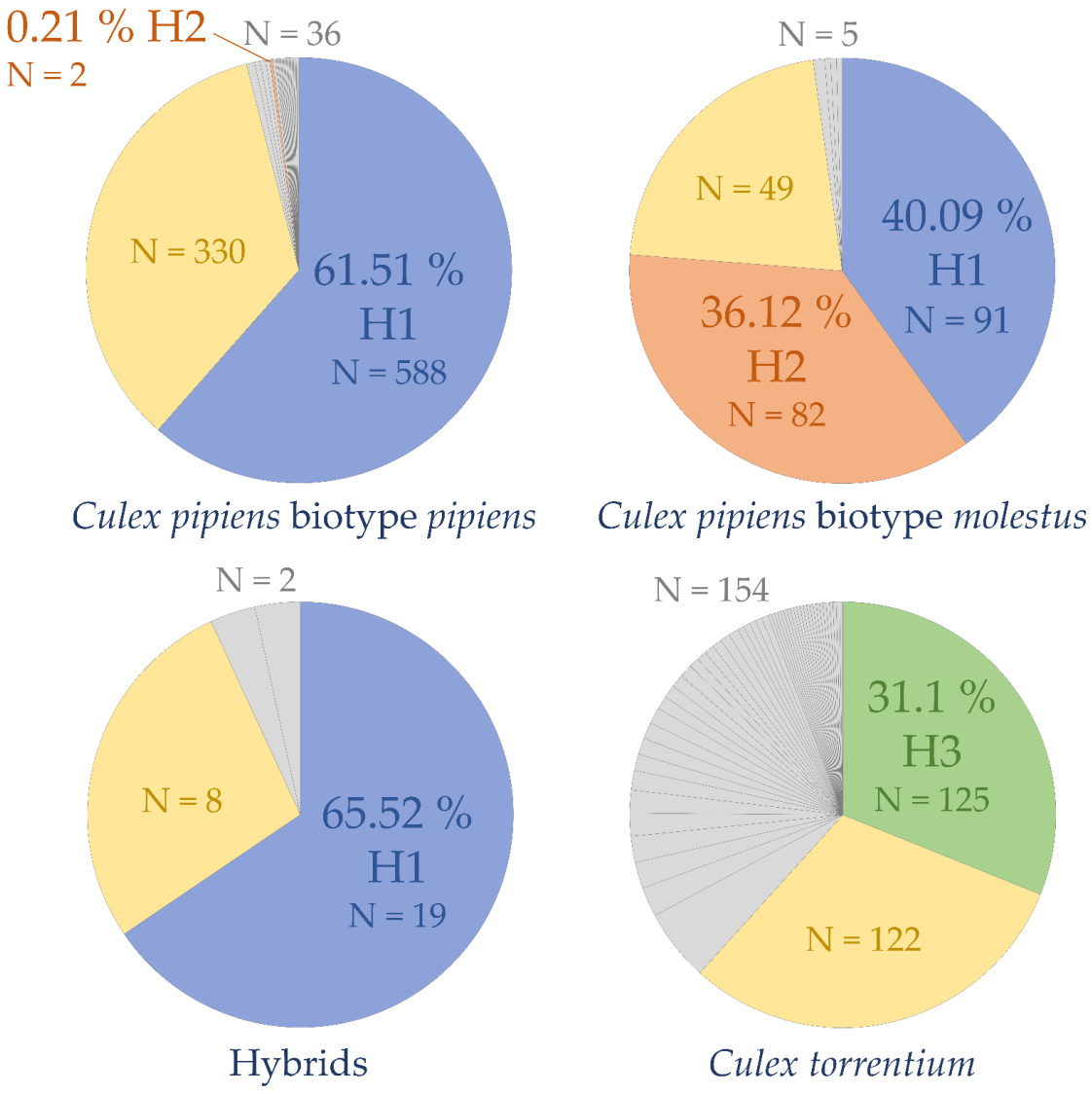

Figure 3. COI haplotype frequencies calculated with Arlequin v.3.5. [46]. Hybrids = *Culex pipiens* biotype *pipiens* X *Cx. pipiens* biotype *molestus*, blue = *Cx. pipiens* s.s. haplotype H1, orange = *Cx. pipiens* s.s. haplotype H2, green = *Cx. torrentium* haplotype H3, yellow = sequences to which no haplotypes could be assigned, grey = haplotypes with a frequency < 6%.

4. Discussion

4.1. Species Occurrence in Belgium

In line with previous studies [8,9,13], *Cx. pipiens* s.s. appears to be more common in Belgium than *Cx. torrentium*. Likewise, *Culex pipiens* biotype *pipiens* is more common and widespread in Belgium than *Cx. pipiens* biotype *molestus*, as the latter comprises only 13% of the specimens (Table S4). However, industrial areas were overrepresented in this survey for the early detection of exotic *Aedes* species [11]; thus, the sampling may have been biased. As such, the *molestus* biotype, with its preference for hypogean habitats [21] and highly eutrophic waters in confined mating spaces [20,21], may be underrepresented, as these environments were not surveyed during the MEMO project [11]. Thus, more targeted surveys are needed to determine the actual prevalence and distribution of *Cx. pipiens* biotype *molestus* in Belgium. Nevertheless, this study confirms the co-occurrence of both biotypes in urban, agricultural, and forest and seminatural habitats [13,47–50] (Figure 2). Despite the sympatric occurrence of both biotypes, only few hybrid specimens were found (1.7%; Tables S3 and S4), i.e., less than in Germany (4.2%) [19], Portugal (8–10%) [47], and Italy (14.4%) [49]. These low hybridisation rates suggest at least partial reproductive/ecological isolation between biotypes [20,47], with some rare haplotypes identified as specific to one biotype (Table 2, see next section). The sympatric co-occurrence of both biotypes and their hybrids with their opportunistic feeding behaviour [47,51] hints at the potential danger of viral transmissions from birds to humans (i.e., to act as bridge for disease vectors). However, the low frequency of hybrids likely limits their potential epidemiological role in WNV outbreaks.

While *Cx. pipiens* s.s. and *Cx. torrentium* are sympatric in some areas, the latter species was not collected in the north of Belgium (Figure 1). Again, this may be a sampling artefact, since the species was collected all over Belgium during the nationwide MODIRISK mosquito survey (2007–2010) [9,48]. Both species were equally observed in different habitats and have adapted to a life in human neighbourhoods [52], with *Cx. torrentium* and *Cx. pipiens* s.s. larvae often found in small artificial and nutrient-rich bodies of water [7,52]. Thus, both species are widespread in Belgium, but their exact distribution limits in Europe remain to be determined.

4.2. COI Haplotype Composition and Genetic Variability

Belgian *Cx. torrentium* showed a higher COI variability than *Cx. pipiens* s.s., which is in line with [52,53]. However, we found no evidence of further sub-structuring or taxonomic differentiation within *Cx. torrentium*, while previous studies reported some morphological variability within the species [53,54]. The limited intraspecific variation within *Cx. pipiens* s.s. is consistent with [54,55]. Hence, COI haplotype H1 had a prevalence of 61.51% in *Cx. pipiens* biotype *pipiens* and of 40.09% in *Cx. pipiens* biotye *molestus*. Similar prevalences were reported by [52] (H1 = haplotype 1), [3] (H1 = haplotype A/C), and [56] (H1 = haplotype H). As such, haplotype H1 is widespread and most frequent in northern temperate climates (Germany, Japan, North America, and Russia) [3,52,56]. Situations in which populations show limited genetic variation and consist of a highly frequent haplotype, jointly with a few rare haplotypes, can be explained by either a recent population-wide demographic or range expansion, or a recent bottleneck, possibly in combination with a *Wolbachia* infection [52]. Such a *Wolbachia* infection can severely reduce mitochondrial diversity [52,57,58]. This might, in part, explain the limited COI diversity in *Cx. pipiens* s.s., which shows *Wolbachia* infection rates of >90% [52,59–61], whereas COI diversity might have been retained in *Cx. torrentium*, within which *Wolbachia* infections appear to be very rare [59,62].

In Belgium, haplotype H2 was almost exclusively found in *Cx. pipiens* biotype *molestus*. This is somewhat unexpected, as this haplotype is rarely found in temperate climates, but associated with (sub)tropical climates ([3] H2 = haplotype E/E1; [56] H2 = haplotype C). Elsewhere, haplotype H2 is prevalent in *Cx. quinquefasciatus* (42%) and its hybrids with *Cx. pipiens* s.s. (32%) [3]. Hence, COI haplotypes in *Cx. pipiens* s.l. are not species-specific [56].

Currently, the biotypes *pipiens* and *molestus* of *Cx. pipiens* s.s. are regarded as different monophyletic evolutionary units undergoing incipient ecological speciation, so that they may be distinct phylogenetic entities [22,27,47,63–65]. This was supported by the significant F_{ST} estimates found in the present study. The different mating behaviours of both biotypes was considered as an initial factor of a sympatric speciation process [47]. The limited level of hybridisation is not bidirectional, with a mainly male-mediated introgression from *molestus* to the *pipiens* biotype [47], which explains the prevalence of H1 (typical of *pipiens*) and absence of H2 (typical of *molestus*) in hybrids. This asymmetric introgression may reflect a mating strategy wherein stenogamous *molestus* males mate with both *molestus* and *pipiens* females in above-ground habitats, while *pipiens* males mate (via specialised swarming behaviour) in open spaces and, therefore, have a higher disposition to mate with *pipiens* females [47]. An experimental study revealed at least one reproductive isolating mechanism, with females actively avoiding copulation with males of the other biotype, and *pipiens* females being unsuccessful in receiving *molestus* males' sperm [66].

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d14060486/s1, Figure S1: Neighbour-Joining tree based on the COI sequences available on BOLD/GenBank for *Culex* species present in Belgium [12,45] including the sequences generated in this study, and identified based on the ACE2 fragment-size analysis. Bootstrap values are indicated at the branches. Sequences were collapsed in species clusters. N = total number of unique sequences included, following Geneious® v10.0.4; Figure S2: Example of ACE2 fragment-size analysis on a 2.5% agarose gel. PCR multiplex including the primers B1246s (Forward), ACEpip (Reverse), ACEtorr (Reverse), and ACEquin (Reverse). Cycling conditions are provided in Table S2. P = *Cx. pipiens* s.s. (610 bp); T = *Cx. torrentium* (416 bp); Figure S3: Example of CQ11 fragment-size analysis on a 2.5% agarose gel. PCR multiplex including the primers CQ11F2 (Forward), molCQ11R (Reverse) and pipCQ11R (Reverse). Cycling conditions are provided in Table S2. PP = *Culex pipiens* biotype *pipiens* (200 bp); PM = *Culex pipiens* biotype *molestus* (250 bp); H = *Cx. pipiens* biotype *pipiens* X *Cx. pipiens* biotype *molestus* (200 bp and 250 bp); Table S1: Detailed list of DNA-based identified specimens, including their life stage at collection and the trapping method; Table S2: Summary of PCR cycling conditions for the amplification of the COI, ACE2, and CQ11 loci; Table S3: Map codes, municipalities, and coordinates of sampling localities with taxon occurrence; Table S4: Overview of the COI sequencing success per year. N = number of specimens. Reference [67] are cited in the Supplementary Materials.

Author Contributions: Conceptualization: N.S., I.D., K.D.W., W.D., R.M. and W.V.B.; Methodology: N.S., K.D.W., I.D., A.V. (Adwine Vanslembrouck), J.D.W., A.S., I.V. and W.V.B.; Formal Analysis and Software: N.S., A.V. (Ann Vanderheyden), K.M. and S.G.; Data Curation: W.D., N.S. and I.D.; Writing—Original Draft Preparation: A.V. (Ann Vanderheyden) and N.S.; Writing—Review and Editing: I.D., K.D.W., W.D., K.M., S.G., A.V. (Adwine Vanslembrouck), J.D.W., A.S., I.V., M.D.M., T.B., R.M. and W.V.B.; Visualization: A.V. (Ann Vanderheyden) and N.S.; Supervision: I.D., W.V.B., R.M., T.B. and M.D.M.; Project Administration and Funding Acquisition: I.D., W.V.B., R.M., T.B. and M.D.M. All authors have read and agreed to the published version of the manuscript.

Funding: This work is part of the MEMO project (tender: CES-2016-02), funded by the Flemish, Walloon and Brussels regional governments and the Federal Public Service (FPS) Public Health, Food Chain Safety and Environment in the context of the National Environment and Health Action Plan (NEHAP) (Belgium). The Barcoding Facility for Organisms and Tissues of Policy Concern (BopCo-http://bopco.myspecies.info/, accessed 15 May 2022) is financed by the Belgian Science Policy Office (Belspo). The Outbreak Research Team of the Institute of Tropical Medicine is funded by the Department of Economy, Science and Innovation of the Flemish government.

Institutional Review Board Statement: Not applicable.

Data Availability Statement: The data presented in this study are openly available in GenBank at https://www.ncbi.nlm.nih.gov/genbank/, accessed 15 May 2022, reference number: OM747883-OM749568.

Acknowledgments: We would like to thank all cooperating companies for giving access to their private property during mosquito sampling surveys. We would like to thank the laboratory and technical staff at the Institute of Tropical Medicine Antwerp (ITM), at the Royal Belgian Institute of Natural Sciences (RBINS), and at the Royal Museum for Central Africa (RMCA).

Conflicts of Interest: The authors declare no conflict of interest.

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