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Sampling, Distribution, Dispersal

First Detections of *Culiseta longiareolata* (Diptera: Culicidae) in Belgium and the Netherlands

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Abstract

Culiseta (Allotheobaldia) longiareolata (Macquart) (Diptera: Culicidae) is an ornithophilic mosquito species that occurs in the southern Palaearctic Region from the Azores to Central Asia, the Ethiopian Region, India, and Pakistan. Although it has a widespread distribution range, the species was only recently reported in Western and Central Europe. Between 2017 and 2020, larvae, pupae, and adults of *Cs. longiareolata* (*n* = 161) were found at 13 distinct locations in Belgium (*n* = 4) and The Netherlands (*n* = 9). Collected mosquitoes were morphologically identified and the identification was then validated by COI DNA barcoding. These are the first records of the species in the above-mentioned countries. The present results suggest that *Cs. longiareolata* could be increasing its distribution range in temperate regions, indicating a warming climate. As the species might be a potential vector of bird pathogens (e.g., West Nile virus), its spread in Western Europe is noteworthy.

1

Graphical Abstract
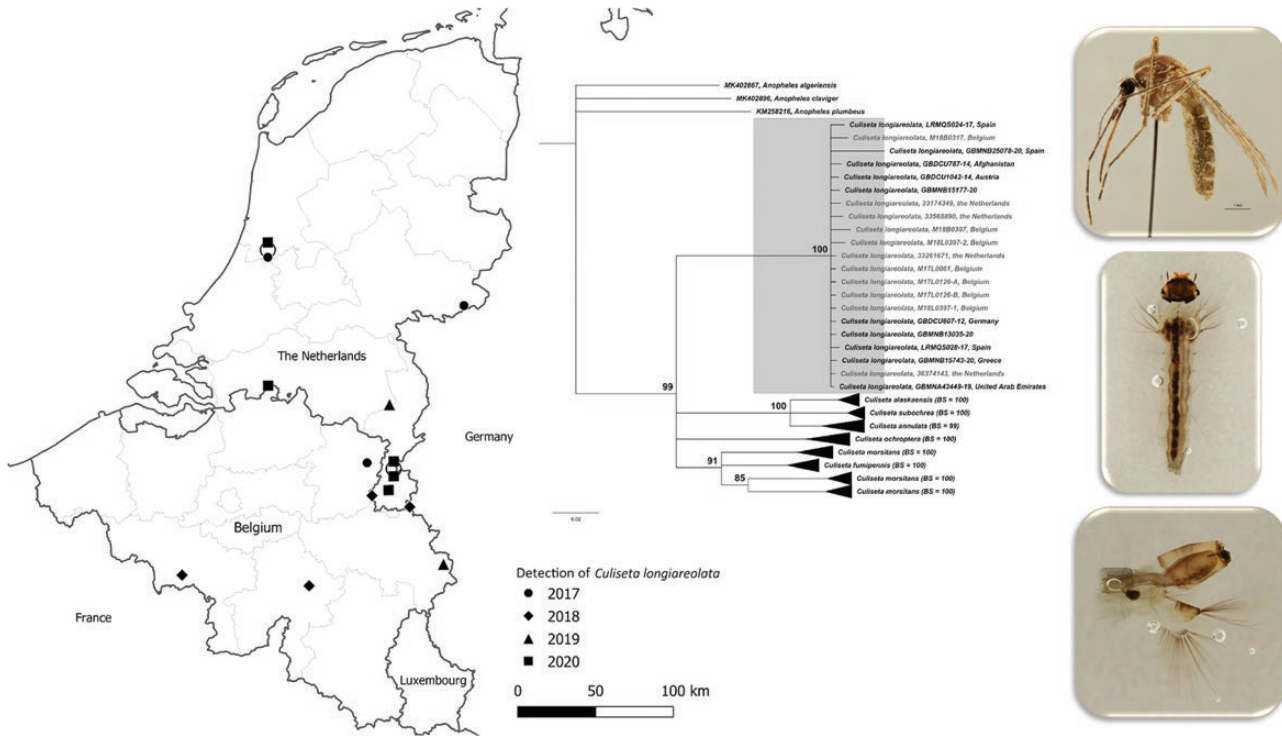
Key words: new species record, DNA barcoding, mosquito surveillance, range expansion, climate change

Over the last 20 yr mosquito-borne diseases (MBD) have been emerging in continental Europe due to a combination of different factors such as globalization, land use change, and global warming (Calzolari 2016, Brugueras et al. 2020). Increasing temperatures, especially warmer winters, promote the range expansion of certain mosquito vectors and the spread of certain pathogens (Brugueras et al. 2020, Osland et al. 2021). Nationwide inventory studies of native mosquito species (NMS) and monitoring the early detection, spread, and density of invasive mosquito species (IMS) are essential for mosquito-borne disease risk assessment and control (European Centre for Disease Prevention and Control (ECDC) 2012, 2014).

The NMS biodiversity has been studied over one 4-yr long project in Belgium and annually in the Netherlands since 2010 (Versteirt et al. 2013, Ibáñez-Justicia et al. 2015b), while surveys focussing on IMS have been implemented yearly since 2006 and 2007, in the Netherlands and Belgium respectively (Scholte et al. 2010, Versteirt et al. 2013, Deblauwe et al. 2014, Ibáñez-Justicia et al. 2020a). On occasion, new or rare species have been detected (Werner et al. 2020). This is illustrated by two newly recorded species during IMS surveys in Belgium, namely *Culex modestus* (Ficalbi) (Diptera: Culicidae) and *Anopheles daciae* (Linton, Nicolescu & Harbach) (Diptera: Culicidae) (De Wolf et al. 2021, Smitz et al. 2021b) and the detection of the IMS *Aedes japonicus japonicus* (Theobald) (Diptera: Culicidae) and *Aedes flavopictus* (Yamada) (Diptera: Culicidae) during NMS and IMS surveys in the Netherlands (Ibáñez-Justicia et al. 2014, 2019). This points to the important contribution of IMS surveillance in the detection of NMS and vice versa and stresses the importance of reliable species identification. Most recent nationwide mosquito surveys have involved both morphology and DNA-based identification techniques (van de Vossenberg et al. 2015, Versteirt et al. 2015, Deblauwe et al. 2020, Ibáñez-Justicia et al. 2020b). Six species of the *Culiseta* genus (Felt) (Diptera: Culicidae) have been reported in Belgium and the Netherlands (Boukraa et al. 2015, Ibáñez-Justicia et al. 2015b, Verdonschot 2019). The present study reports the first findings of *Culiseta (Allotheobaldia) longiareolata* at multiple locations in Belgium and the Netherlands, validated by DNA barcoding.

Material and Methods

Sampling Programs and Collection Methods

In Belgium, mosquitoes were sampled during a three-year nationwide IMS survey (MEMO project, 2017–2020) at 23 locations considered to be possible points of entry for IMS, including premises of used tire and lucky bamboo import companies, airports, and parking lots along highways, and colonized areas of *Ae. j. japonicus* and *Ae. koreicus* (Edwards) (Diptera: Culicidae) (Deblauwe et al. 2020). In the Netherlands, mosquitoes were collected during IMS and NMS surveys. IMS surveys in the Netherlands are conducted at similar types of points of entry as in Belgium, and additionally in urban areas following the detection of an IMS (Ibáñez-Justicia et al. 2020a, b). Allotment gardens in the vicinity of colonized areas of *Ae. koreicus* across the border in Belgium were also surveyed for IMS. Routine NMS surveys in the Netherlands are usually cross-sectional and in randomly generated locations (Ibáñez-Justicia et al. 2015b).

Table 1. Main collection and storage methods used for adults, larvae, and pupae during the invasive and native mosquito species surveys in Belgium and the Netherlands

	Belgium	the Netherlands
Adult trap	Mosquito Magnet Independence trap (Woodstream Corporation, Lititz, PA, USA) with octenol	Mosquito Magnet Liberty Plus trap (Woodstream Corporation, Lititz, PA, USA) with octenol
	BG-Sentinel trap (Biogents, Germany) with BG-Lure and sometimes CO_2	BG-Sentinel trap (Biogents, Germany) with BG-Lure and sometimes CO_2
		BG-Mosquitaire trap (Biogents, Germany) with BG-Lure
	long term storage dry at room temperature; after DNA extraction remaining parts in a –80°C freezer	long term storage dry in a –20°C freezer
Larval sampling	fine mesh aquarium nets	fine mesh aquarium nets
	transported alive to laboratory and killed by a thermal shock	transported in 70% ethanol to the laboratory (dead)
	storage in 80% ethanol at room temperature	storage in 70% ethanol in a –20°C freezer

Larvae, pupae, and adults were collected and preserved as listed in Table 1. Larval sampling was only performed during IMS surveys and mainly in artificial breeding sites.

Morphological and Molecular Identification

All adults and larvae were identified using common morphological Culicidae keys (Schaffner et al. 2001, Becker et al. 2010, Gunay et al. 2018). The identification of the collected pupae and validation of a subset of specimens (first detected and randomly selected larval and adult specimens collected in 2017 and 2018) was performed using COI (Cytochrome *c* oxidase subunit I) DNA barcoding (Hebert et al. 2003), following the methodology as described in Smitz et al. (2021b). Additionally, a fragment of the COII (Cytochrome *c* oxidase subunit II) and mitochondrial *nad4* (Nicotinamide adenine dinucleotide dehydrogenase subunit 4) gene was amplified and sequenced following the methodology in Supp File S1 (online only).

Data Analysis

For the six *Culiseta* species reported in Belgium and The Netherlands, all available COI DNA sequences from BOLD were downloaded and aligned using ClustalW as implemented in the software Geneious Prime (Biomatters Ltd.), together with the *Cs. longiareolata* COI consensus sequences generated in this study and three outgroups. The alignment was trimmed to retain the 658 bp COI Folmer region and checked for stop codons. Duplicate sequences per species, as well as sequences of less than 300 bp, were discarded. Possible COI gene variants (i.e., different forms of a specific region of the mitochondrial genome) were identified. Based on Kimura 2-parameter (K2P) distances (Kimura 1980), a rooted neighbor-joining tree (NJ) was constructed using MEGA X (Kumar et al. 2018), with branch support assessed by 1,000 bootstrap replicates (Felsenstein 1985). Clustering of the sequences generated was compared to other species. Generated consensus sequences were searched against the Identification System of BOLD, with Species Level Barcode Records option (www.boldsystems.org). Finally, average interspecific and maximum observed K2P distances between conspecific COI sequences were calculated with the package Spider v3.6.2 (Brown et al. 2012). Mean numbers of pairwise nucleotide differences (*k*) and average gene diversities over nucleotide positions (*H*) were calculated with Arlequin v3.5 (Excoffier and Lischer 2010).

Calculation of the Land Cover Classes

To characterize the habitat where *Cs. longiareolata* was found, 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 location where the species was collected. The latest raster file (CLC 2018) was used and calculation was done in Q-GIS and R v4.03 (R Development Core Team 2018). The levels were grouped into five main Corine land cover classes, i.e., artificial or urban areas, agricultural areas, forest and seminatural areas, wetlands, and water bodies.

Results

Sampling and CLC Results

In total, 161 *Cs. longiareolata* specimens were found at 13 distinct locations during monitoring activities in Belgium and the Netherlands between 2017 and 2020 (Fig. 1 and Table 2). However, never at the same location during two consecutive monitoring years (Table 2). Eight locations were in cities or villages, while five were point of entry locations (used tire import companies, airport, flower auction, parking lot along highway). Ten locations were situated in predominantly agricultural areas, while three were in predominantly urban areas (Fig. 2). Males (*n* = 7) were collected with both the Mosquito Magnet Liberty Plus trap and BG-Sentinel trap. Females (*n* = 4) were collected with the BG-mosquitaire and BG-Sentinel trap. Adults were collected in April (2020), July (2018), August (2017, 2020), September (2018), October (2018, 2019), and November (2020). Larvae (*n* = 133) and pupae (*n* = 17) were found in a variety of artificial breeding sites: rain water barrels, road drains, used tires and plastic and metal buckets. They were collected in August (2018), September (2017, 2019, 2020), and October (2020).

DNA-Based Species Validation

COI fragments were sequenced in 11 specimens (GenBank accession numbers: MT862911-MT862921). The obtained similarity percentages between these sequences and those extracted from the online reference repository BOLD ranged from 99.85 to 100%. Additionally, the *Cs. longiareolata* COI sequences formed a well-supported monophyletic cluster (100 BS) on the NJ-tree (Fig. 3), which included ten unique *Cs. longiareolata* COI variants downloaded from BOLD. Within our COI dataset, four new gene variants were identified (Supp Table S1 [online only]). Average COI K2P distances between *Culiseta* species varied from 3.5 to 11.8%, and the largest intraspecific K2P distance was 10.0%, within *Cs. morsitans* (Theobald) (Diptera: Culicidae) (Table 3 and Supp Fig. S1 [online only]). *Culiseta longiareolata*, in particular, displayed the largest average interspecific (11.8%) and one of the lowest intraspecific (0.4%) K2P genetic distances.
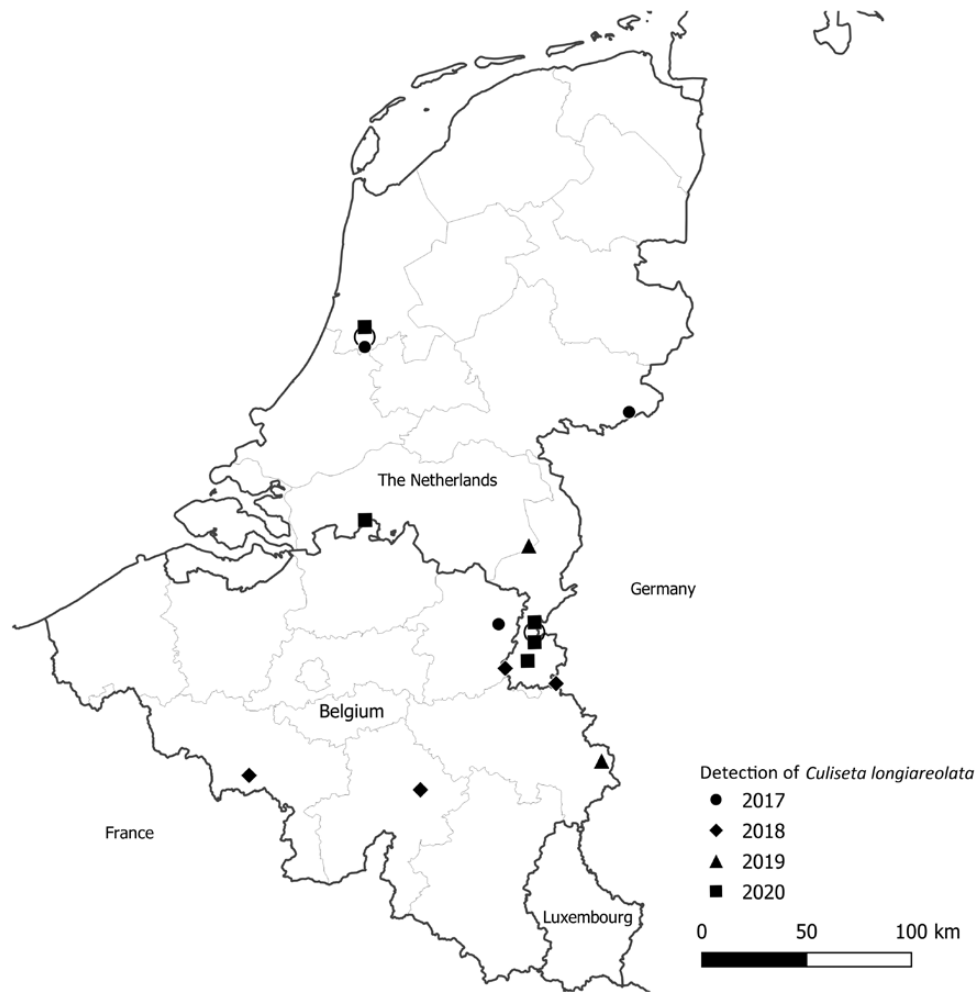
Fig. 1. Map of Belgium and the Netherlands indicating the locations where *Culiseta longiareolata* was collected per year (locations overlapping on the map are connected by a circle (point displacement links)).

Discussion

This work provides the first solid evidence on the occurrence of *Cs. longiareolata* in both Belgium and the Netherlands and stresses the importance of IMS and NMS surveillance programs in the detection of new species, which might be potential vectors of pathogens and/or indicators of climate or environmental changes. This cosmopolitan species is widely occurring in the Mediterranean area (Becker et al. 2010, Becker and Hoffmann 2011). In the last decade, it has been increasingly reported from Western and Central European countries with records in Germany, Austria, Slovenia, Switzerland, northern France, and Luxembourg (Schaffner et al. 2009, Becker and Hoffmann 2011, Kampen et al. 2013, Seidel et al. 2013, Zittra et al. 2014, Schaffner and Ries 2019, Werner et al. 2020). In the past, *Cs. longiareolata* has been rarely observed so far north as in the current study, except for some limited findings in northern France and southern England (Moussiegt 1986, Cranston et al. 1987). In Germany *Cs. longiareolata* has been associated with used tires, a common larval habitat of the species (Schaffner 2003, Roiz et al. 2007, Becker and Hoffmann 2011). In this study, larvae and adults of *Cs. longiareolata* were also collected at used tire import companies in Belgium indicating used tire trade as a possible pathway for introduction. On one occasion, an adult of this species was detected in the baggage handling basement at the Schiphol airport (Amsterdam, The Netherlands) and could imply aircraft transport as another

introduction pathway. Two adult mosquitoes were collected in traps placed at a flower auction and a parking lot in the Netherlands and could point to an introduction through the import of fresh flowers or vehicle transport, respectively. In this study *Cs. longiareolata* has also been found at locations without immediate introduction history or risk, such as farms, cemeteries, private or allotment gardens, and industrial parks. Some authors state that the presence of this species could have been underestimated in the past due to the lack of nationwide inventory studies, its low population densities or behavioral traits (e.g., ornithophilic, late seasonal peak), and the use of adult traps specifically designed to attract anthropophilic IMS (Kampen et al. 2013, Seidel et al. 2013, Schaffner and Ries 2019). However, before 2017, IMS and NMS surveys were conducted in both countries using similar adult traps each year and *Cs. longiareolata* was not detected (Scholte et al. 2010; Versteirt et al. 2013; Deblauwe et al. 2014, 2015; Ibáñez-Justicia et al. 2015a, b, 2020a). In Belgium, the used tire import company premises at Natoye were sampled in 2008, 2012, 2017, and 2018, and the company at Frameries since 2016, using the same monitoring techniques (Versteirt et al. 2009; Deblauwe et al. 2014, 2020; Smitz et al. 2021a). *Cs. longiareolata* was not detected in either location before 2018. In the Netherlands, the airport of Schiphol has been sampled since 2013 (Ibáñez-Justicia et al. 2017), but *Cs. longiareolata* was not found until 2017. Despite the fact that these locations have been monitored for several years

Table 2. Overview of the *Culiseta longiareolata* collections in Belgium and the Netherlands with information on the collection period, target, location, method, and specimens

Country	Year	Collection date	Target of surveillance*	Location name	Coordinates	Years monitored	No. specimens and life stage	Collection method	Description location/breeding site
The Netherlands	2017	01–15 Aug. 2017	IMS	Schiphol	52.30739, 4.76593	2013–2020	1 adult female	BG-Mosquitaire trap (without CO_2)	Placed indoors in the basement of the baggage handling area of Schiphol airport
		5 Sep. 2017	IMS	Aalten	51.92081, 6.59306	2016–2019	2 pupae	larval sampling	Road drain in street
	2018	24–30 Jul. 2018	NMS	Vaals	50.77165, 6.00965	2018	1 adult male	Mosquito Magnet Liberty Plus trap	Near private garden
		23 Aug. 2018	IMS	Maastricht	50.84533, 5.66925	2016, 2018	2 larvae, 4 pupae	larval sampling	Rain barrel in allotment garden
	2019	25 Sep.–3 Oct. 2019	IMS	Neerkant	51.36812, 5.86117	2019–2020	1 adult male	BG-Sentinel trap (without CO_2)	In private garden
	2020	14–28 Apr. 2020	IMS	Aalsmeer	52.26175, 4.77672	2017–2020	1 adult male	BG-Sentinel trap (without CO_2)	Inside flower auction, near dock service
		29 Jul.–12 Aug. 2020 and 12–26 Aug. 2020	IMS	Hazeldonk	51.49932, 4.74321	2010, 2011, 2020	3 adult male, 1 adult female	BG-Sentinel trap (without CO_2)	Parking lot along highway
		30 Sep. 2020 and 6 Oct. 2020	IMS	Sittard	50.99545, 5.88394 and 50.99501, 5.88384	2020	97 larvae, 11 pupae	larval sampling	Metal container in allotment garden and plastic bucket in the cemetery next to the allotment garden
		27 Oct.–3 Nov. 2020	IMS	Valkenburg	50.87358, 5.82337	2019–2020	1 adult female	BG-Sentinel trap (without CO_2)	Outside garage in an industrial park near cemetery
Belgium	2017	5 Sep. 2017	IMS	Maaseik	51.03582, 5.63497	2017	4 larvae	larval sampling	Plastic buckets next to an old barn
	2018	30 Aug. 2018	IMS	Natoye	50.33591, 5.07158	2008, 2012, 2017, 2018	3 larvae	larval sampling	Used tires at used tire import company
		13–27 Sep. 2018	IMS	Natoye	50.33591, 5.07158	2008, 2012, 2017, 2018	1 adult female	BG-Sentinel trap (with CO_2)	Used tire import company
		3–17 Oct. 2018	IMS	Frameries	50.41259, 3.92343	2016–2019	1 adult male	BG-Sentinel trap (with CO_2)	Used tire import company
	2019	5 Sep. 2019	IMS	Rocherath	50.43259, 6.29435	2017–2019	27 larvae	Larval sampling	Road drain next to cemetery

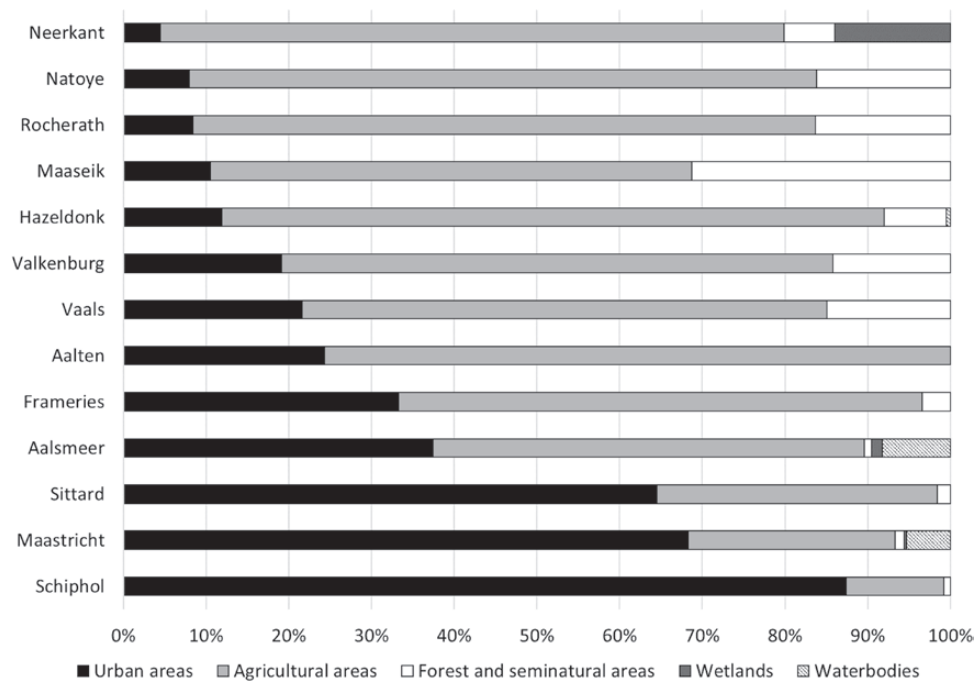*IMS=Invasive Mosquito Species; NMS=Native Mosquito Species

Fig. 2. The percentage of main Corine Land Cover classes in a 2.5 km buffer zone around the locations with *Culiseta longiareolata* collections (group levels based on five classes).

using similar collection methods, including both adult and larval sampling techniques, *Cs. longiareolata* was found only recently at these locations, probably indicating relatively recent colonization. However, we were not able to confirm the presence of the species in consecutive years at those locations where sampling was done in the years of the first detection. Similarly, repeated findings over the years in Germany only recently demonstrated the species to occur at the same places in subsequent years (Kampen et al. 2017, Werner et al. 2020).

Larval sampling has proven to be an effective method for the first detection of *Cs. longiareolata* in an area where the species has not been previously reported (Becker and Hoffmann 2011, Seidel et al. 2013, Zittra et al. 2014, Schaffner and Ries 2019). Although the BG-Sentinel trap did not always manage to collect adults (Seidel et al. 2013, Zittra et al. 2014), it detected the species in Germany (Werner et al. 2012), Belgium, and the Netherlands. In this study, *Cs. longiareolata* was collected for the first time with BG-mosquitaire traps and the Mosquito Magnet trap. In accordance with previous studies, the larvae of *Cs. longiareolata* were found in man-made waterbodies, often associated with a high level of pollution (Schaffner 2003, Roiz et al. 2007, Becker et al. 2010, Zittra et al. 2014, Schaffner and Ries 2019, Toma et al. 2020). These artificial containers are typically included in IMS surveillance programs as they form an ideal larval habitat for IMS from the *Aedes* genus (Meigen) (Diptera: Culicidae). In Belgium and the Netherlands, *Cs. longiareolata* was mainly collected in agricultural areas, followed by urban areas, which is also observed in the Mediterranean region (Schaffner et al. 2010, Möhlmann et al. 2017, González et al. 2020). This mosquito species is associated with vegetable gardens, farms, and domestic hen houses (Möhlmann et al. 2017, Toma et al. 2020). However, *Cs. longiareolata* is also found in fresh water rock pools and other waterbodies in natural area (Schaffner et al. 2010, Möhlmann et al. 2017).

Culiseta longiareolata has been described as a thermophilic and ornithophilic species that rarely bites humans (Becker et al. 2010, Martínez-de la Puente et al. 2020). The species seems not to be involved in the transmission of pathogens to humans nor is considered as a nuisance. Its vector role has been poorly investigated (Martinet et al. 2019). Despite its preference for birds and its suggested vector competence for bird blood parasites (e.g., avian *Plasmodium* (Welch) (Haemosporida: Plasmodiidae)) and West-Nile virus (Hurlbut 1956, Maslov 1967, Van Pletzen and Van Der Linde 1981, Seidel et al. 2013), the species is not causing any veterinary problem in countries where it is well established. It was suggested that *Cs. longiareolata* could act as a potential vector of the Usutu virus (Seidel et al. 2013), but this is not yet proven.

In conclusion, although population densities might still be too low to allow frequent collections, the high number of detections in Belgium and the Netherlands suggests that the species has only recently been established in both countries. The detections of *Cs. longiareolata* might result from a combination of multiple introduction events and a northward expansion of the species distribution range possibly linked to global warming (Becker and Hoffmann 2011, Kampen et al. 2017), which would make *Cs. longiareolata* a possible indicator species for climate change.

Supplementary Data

Supplementary data are available at *Journal of Medical Entomology* online.
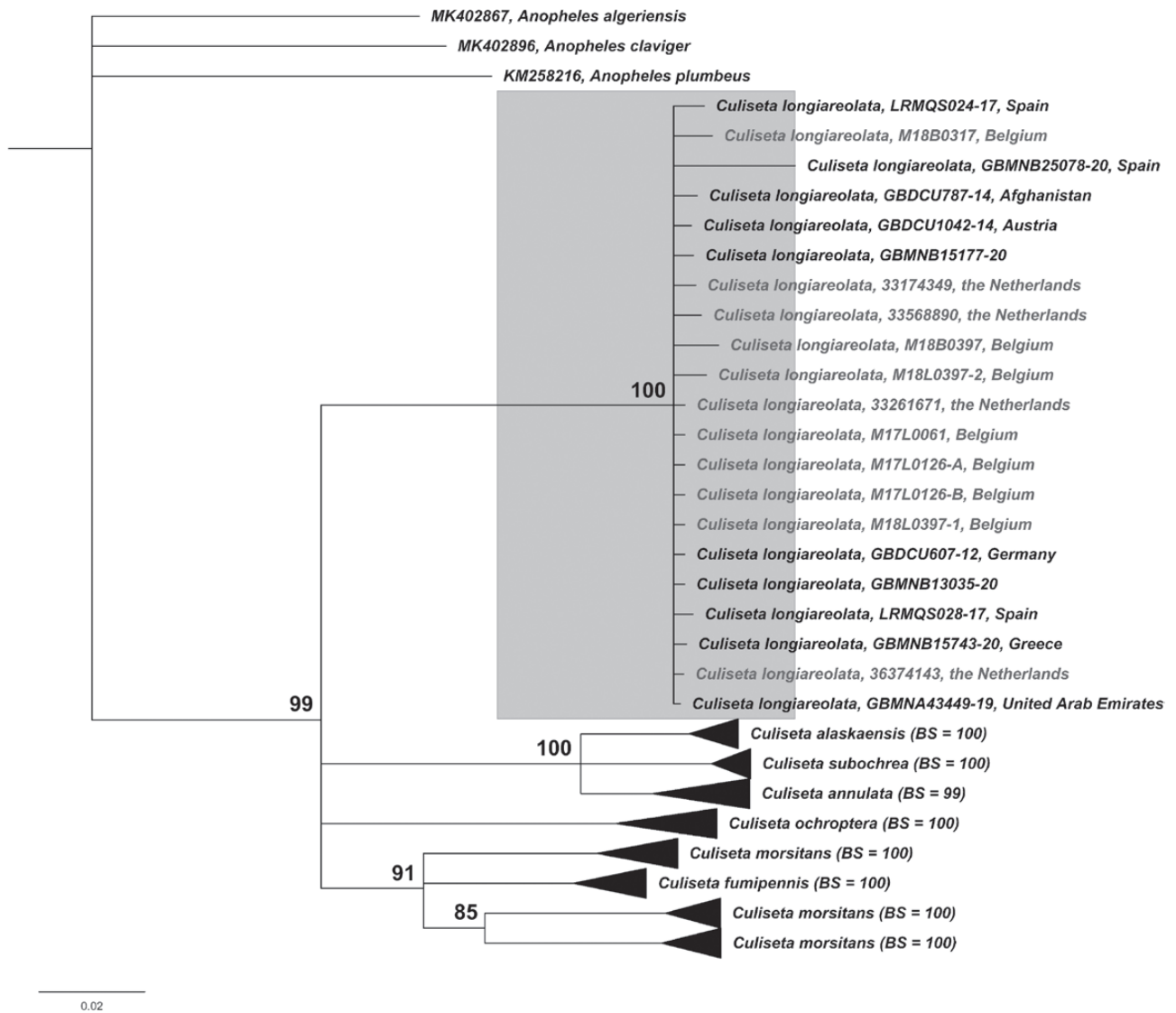
Acknowledgments

Fig. 3. Neighbor-Joining tree including the six *Culiseta* species recorded in Belgium and the Netherlands and the newly found *Cs. longiareolata* species (generated barcodes highlighted in grey). Three *Anopheles* species (Meigen) (Diptera: Culicidae) were included as outgroup (GenBank accession numbers: KM258216, MK402867, MK402896). The tree is based on COI K2P; 658 bp fragment; 164 unique barcodes downloaded from BOLD (2 September 2020). The bootstrap values (BS; 1,000 replicates) are shown in the tip labels for the collapsed sequence clusters. Clusters were collapsed to make the overall tree visually clearer. The minimum bootstrap displayed is 75, other branches are collapsed.

Table 3. Descriptive statistics of the genetic diversity within the genus *Culiseta* (Belgium and the Netherlands), including the overall maximum, observed intraspecific and average interspecific Kimura two-parameter (K2P) distances among COI sequences

	N_{COI} unique*	$k \pm$ SD	$H \pm$ SD	Average interspecific K2P (%)	Max intraspecific K2P (%)
Culiseta alaskaensis (Ludlow)	13	2.667 ± 1.509	0.004 ± 0.003	4.104	0.650
Culiseta annulata (Schrank)	20	1.290 ± 0.840	0.003 ± 0.002	3.515	0.288
Culiseta fumipennis (Stephens)	6	4.867 ± 2.725	0.008 ± 0.005	6.588	1.751
Culiseta longiareolata	21	0.780 ± 0.590	0.002 ± 0.002	11.786	0.396
Culiseta morsitans	92	17.257 ± 7.735	0.032 ± 0.016	7.349	10.037
Culiseta ochroptera (Peus)	8	10.500 ± 5.309	0.017 ± 0.010	9.932	2.522
Culiseta subochrea (Edwards)	15	1.257 ± 0.835	0.002 ± 0.002	3.461	0.576

N_{COI}: number of COI sequences included in the analysis, k: mean number of pairwise nucleotide differences, H: average gene diversity over nucleotide positions, SD: standard deviation. (*) as defined in Geneious Prime.

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