Human biting *Culex pipiens* bioform *molestus* is spread in several areas in south Sweden.

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Abstract

The mosquito species *Culex pipiens* is a potential vector of several pathogens infecting humans and occurs in two distinct bioforms, *pipiens* and *molestus*. Traditional morphological identification fails to separate the bioforms of *Cx. pipiens* despite their ecophysiological differences since they are morphologically indistinguishable. However, molecular methods can accurately identify the two bioforms. The bioform *molestus* thrives in urban environments and bite all kinds of vertebrates, whereas bioform *pipiens* is more rural and mainly feed on birds.
Mosquito samples submitted in a citizen science project from people experiencing mosquito problems in South Sweden were analyzed to determine the geographical distribution of the bioforms of *Cx. pipiens*. Mosquito specimens were identified to species by DNA barcoding of the cytochrome C oxidase subunit I (COI) gene and the bioforms were determined with the CQ11 microsatellite marker. To establish other differences between the bioforms, the CPIJ001674 gene was sequenced. *Culex pipiens* f *molestus*, was present both within and outside of urban areas in several sites in southern Sweden. In one site, hybrids between the two bioforms were found. *Culex pipiens* f *molestus* has previously been found in urban areas in Sweden, but the detection of the bioform in several rural areas was surprising, indicating that it may be more widely spread than previously thought.

Keywords: Microsatellite, DNA barcoding, *Culex pipiens* f *pipiens*, *Culex pipiens* f *molestus*.

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Introduction

The mosquito species *Culex pipiens* is represented in Europe by two bioforms namely *Cx. pipiens* f *pipiens* Linnaeus (L), 1758 and *Cx pipiens* f *molestus* Forskal (F), 1775 (Diptera: *Culicidae*) (Harbach, Dahl et al. 1985) They are of fundamental importance in epidemiology since they act as bridge vectors that transmit emerging arboviruses such as West Nile fever virus, Japanese encephalitis virus, St. Louis encephalitis virus and eastern equine encephalitis virus from birds to susceptible mammals (Crabtree, Savage et al. 1995, Fonseca, Keyghobadi et al. 2004, Hubálek 2008, Ravanini, Huhtamo et al. 2012).
The two bioforms differ in their behavioural and physiological characteristics, which in turn has a major influence on the transmission of arboviral diseases to humans. *Cx. pipiens* f *pipiens*, also known as the northern house mosquito, oviposits its first batch of eggs after a bloodmeal (anautogenous), located in aboveground habitats (epigeous) in rural areas. It undergoes diapause during winter (heterodynamic), mates in open space (eurygamous) and is known to preferably feed on avian blood (ornithophilic). *Cx pipens* f *molestus* does not require a blood meal for oviposition (autogenous), breeds in underground habitats (hypogeous) in urban areas, although, there is evidence of it occurring in aboveground habitats. It is active during winter (homodynamic), does not require large space to mate (stenogamous) and feeds on human blood (anthropophilic) and other vertebrates (Byrne and Nichols 1999, Vinogradova 2000, Vinogradova 2003). In areas of sympatry, *Cx. pipiens* bioforms can interbreed to form hybrids which have an opportunistic behavior in their feeding habits, as they feed on both humans and birds thus increasing the transmission cycle of arboviral diseases (Rudolf, Czajka et al. 2013, Osório, ZÉ-ZÉ et al. 2014). There is evidence that they serve as bridge-vectors between humans and birds from blood-meal analysis (Fonseca, Keyghobadi et al. 2004, Hamer, Kitron et al. 2008). In northern Europe, they have separate but overlapping habitats and the hybridization rate between *Cx pipiens* f *pipiens* and Cx *pipiens* f *molestus* is lower than in south Europe where habitats overlap more (Vogels, van de Peppel et al. 2015).
Despite the differences in their ecophysiological characteristics, they cannot be distinguished using morphological methods of identification since they lack clear characteristics that separate them (Byrne and Nichols 1999, Weitzel, Braun et al. 2011). Although the two bioforms are considered to belong to the same species, there is need for their accurate identification using molecular methods (Harbach, Harrison et al. 1984).

The cytochrome C oxidase subunit I (COI) gene has been used to accurately identify a number of mosquito species although closely related species could not be identified due to inadequate variation in the gene (Lilja, Nylander et al. 2017). Restriction enzyme digest of the COI marker have been used to differentiate between *Cx. pipiens* bioforms and *Cx. torrentium* (Shaikevich 2007). The COI difference between *Cx. pipiens* bioforms has later been questioned (Danabalan, Ponsonby et al. 2012). A rapid assay has also been able to differentiate hybrids of *Cx. pipiens* and *Cx. quinquefasciatus* by examining numerous polymorphisms in the second intron of ACE-2 region in North America. (Smith and Fonseca 2004).
A collection of eight microsatellite loci (CQ11, CQ26, CxqGT4, CxqGT6b, CxpGT4, CxpGTP, CxpGT12 and CxpGT46) have been used for the identification of the *Cx. pipiens* complex and their hybrids (Fonseca, Keyghobadi et al. 2004). One microsatellite loci, CQ11 has two distinct sequences which makes it possible to separate the *Cx. pipiens* bioforms (Bahnck and Fonseca

2006). Despite CQ11 microsatellite locus being considered a diagnostic marker for the accurate distinction of the two bioforms and their hybrids (Bahnck and Fonseca 2006, Amraoui, Tijane et al. 2012, Osório, Zé-Zé et al. 2014), it may misidentify *Cx. torrentium* (Danabalan, Ponsonby et al. 2012). Thus, where *Cx. torrentium* is present, analysis of COI complements CQ11. Given that the two bioforms can mate and form hybrids and that the CQ11 marker is not shown to be linked to *Cx. pipiens* f *molestus* phenotype. It is possible that *Cx. pipiens* f *pipiens* populations can carry a "*molestus* form" CQ11 genotype. Nevertheless, the CQ11 marker has given robust results when compared to multilocus methods predicting *pipiens* or *molestus* form (Farajollahi, Fonseca et al. 2011, Gomes, Sousa et al. 2013).
The nuclear gene CPIJ001674 was recently identified as having a high degree of variation between *Cx* species (Kim, Lee et al. 2018). This could be a potential new marker to distinguish the two bioforms of *Cx. pipiens*.

Cx. pipiens is found in most parts of Sweden (Lundström, Schäfer et al. 2013). *Cx. pipiens* f *molestus* has been found previously in Gothenburg (Hesson, Schäfer et al. 2016) and has been reported from Stockholm in 1934, reviewed in (Lindström 2017). There are also findings of three *Cx. pipiens* f *molestus* from a farm and two wetlands outside Linköping in central south Sweden and hybrids in Linköping (Vogels, Möhlmann et al. 2016). However, there are no other studies that suggest that *Cx. pipiens* f *molestus* is present outside of urban areas in Sweden.
In this study, we collected *Culex* mosquitoes through citizen science outreach, mainly to people in urban areas experiencing mosquito nuisance. Specimens were also sampled in some areas in the south of Sweden. We determined the specimens as *Cx. pipiens* using COI and distinguished the two bioforms; *Cx. pipiens* f *pipiens* and *Cx. pipiens* f *molestus* using CQ11 microsatellite marker. We further tested whether the CPIJ001674 nuclear DNA marker (Kim, Lee et al. 2018) could distinguish the two bioforms.

Materials and Methods

Ethical Approval
Not required because the study involved mosquitoes.

Mosquito collection
Mosquito samples were captured as part of a citizen science outreach (Fånga Myggan) in diverse regions of Sweden where people experiencing mosquito nuisance problems were encouraged to submit specimens. In addition, mosquitoes were collected in several areas in south of Sweden (table 1). From the collections, only mosquitoes morphologically identified as *Culex pipiens* sl. were included in the study. From the samples sent in by the public some samples were in such state that morphological identification was not possible. These specimens were nevertheless included. In total, 111 mosquito samples were analyzed.

Sample Processing and DNA Preparation
Two to three legs were picked from each mosquito and 30 µL PrepMan ultra reagent solution (Applied Biosystems) was added to each tube. Legs were homogenized with a pellet pestle using a hand-held motorized pestle holder for 30secs. The homogenates were then boiled in a

heat block at 100˚C for 10 mins, chilled on ice for 2 mins and centrifuged for 3 mins at 15000xg. 20 µL of the supernatant from each tube was transferred to a clean tube marked with name and date.

DNA amplicon sequencing:

COI gene

A Bio-Rad PCR machine (Applied Biosystems, Thermo Fisher Scientific) was used to amplify the samples. Two primer pairs targeting the mitochondrial COI gene (658bp) were used. The universal primers LCO1490 (5´-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5´- TAAACTTCAGGGTGACCAAAAAATCA-3') (DNA primers for amplification of the 5' part of COI from diverse metazoan invertebrates). Primers amplifying a longer fragment of the COI gene were also used. Fwd_5_COI (GAAGGAGTTTGATCAGGAATAGT) and Rev_3_COI (TCCAATGCACTAATCTGCCATATTA). PCR amplification reactions, targeting COI, using 2.5 µL of 10x PCR buffer, 1 µL of 25-µM $MgCl_2$, 0.5 µL of 10-µM dNTPs, 1.5 µL each of forward primer and reverse primer, 0.15 µL AmpliTaq DNA polymerase (5 U/µL) (Thermo Fisher Scientific), 1 µL of DNA template and 16.85 µL of water to total volume of 25 µL were set up. PCR amplifications were performed, with an initial denaturation at 95°C for 3 mins followed by 39 cycles of denaturization at 95°C for 30 secs, annealing 55°C for 1 min and extension at 72°C for 1 min. Final extension was then performed at 72°C for 5 mins and held at 4°C indefinitely. PCR products and a negative control were then visualized on a 1% agarose gel with a 100-bp molecular weight ladder (Invitrogen, Thermo Fisher Scientific, MA, USA).

CQ11 Microsatellite Gene

PCR amplification reactions targeting the flanking region of CQ11 microsatellite with primers pipCQ11R (5'-CATGTTGAGCTTCGGTGAA-3'), molCQ11R (5'-CCCTCCAGTAAGGTATCAAC-3') and CQ11F2 (5'-GATCCTAGCAAGCGAGAAC-3') were carried out using 2 µL of 10x PCR buffer, 1 µL of 50-µM $MgCl_2$, 0.2 µL of 10-µM dNTPs, 10-µM primers (0.2 µL of pipCQ11R ,0.3 µL molCQ11R and0.3 µL of CQ11F2), 0.15 µL AmpliTaq DNA polymerase (5 U/µL), 1 µL of DNA template and 14.65 µL water to a total volume of 20 µL. PCR was performed with an initial denaturation at 94°C for 3 mins followed by 40 cycles of denaturization at 94°C for 30 secs, annealing 54°C for 30secs and extension at 72°C for 40secs. Final extension was performed at 72°C for 5 mins and held at 4°C indefinitely. PCR products and a negative control were visualized on a 2% agarose gel with a 100-bp molecular weight ladder (Bahnck and Fonseca 2006).

CPIJ001674 Gene

CPIJ001674 fragment was targeted for amplification by PCR using primers CPIJ001674_fwd (TGTACGTGGAGCACAAGAGC) and CPIJ001674_rev (TCCGAGTAGACCGAGACCAG) in a final volume of 25 µL using 2.5 µL of 10x PCR buffer, 2 µL of 25-µM $MgCl_2$, 0.5 µL of 10-µM dNTPs, 10-µM primers (1 µL each of CPIJ001674 forward and reverse primers), 0.15 µL AmpliTaq DNA polymerase (5 U/µL), 1 µL of DNA template and 16.85 µL of water. The thermocycler reaction comprised of an initial denaturation at 94°C for 2mins and 15 secs followed by 40 cycles of denaturisation at 95°C for 30 secs,

annealing 55°C for 30secs and extension at 72°C for 30secs. Final extension was performed at 72°C for 5 mins and held at 4°C indefinitely. Results were visualized on a 2% agarose gel with a 100-bp molecular weight ladder.

Purification of PCR products

Amplified PCR products were treated using ExoSap, Exonuclease I (thermo fisher scientific) Fast Alkaline phosphatase (Fermentas life sciences) in order to remove unwanted primers and degrade unincorporated nucleotides. Purified products were then sent for Sanger sequencing by Macrogen, South Korea, Inc and sequenced in both directions.

Data Analysis

DNA sequences were edited using Bioedit software, analyzed in MEGA version 6.06 after aligning them with ClustalW (Tamura, Stecher et al. 2013). COI sequences that were obtained were identified in Barcode of Life Data (BOLD) System Database which is an integrated bioinformatic platform (Ratnasingham and Hebert 2007). Genetic signatures targeted for the COI fragment described in Shaikevich (Shaikevich 2007) were assayed to differentiate between bioforms *pipiens* and *molestus*. CPIJ001674 gene sequences were aligned and compared (Kim, Lee et al. 2018) while CQ11 microsatellite sequences were aligned and compared with publicly available sequences.

Results

Taxonomy of submitted specimens

In this study, we asked members of the public experiencing problems with mosquitos in urban areas to submit specimens. In addition to this, mosquitoes were collected in rural control areas in southern Sweden. All in all 111 specimens were analyzed. Of the specimens, 58 were classified as *Cx. pipiens* f *molestus*, 3 were hybrids, 32 were *Cx. pipiens* f *pipiens*, 12 were *Cx. torrentium*, 2 were not *Culex* mosquitoes and 4 failed to be classified. Most of the *Culex pipiens* f *molestus* specimens were collected in Gothenburg and the bioform was more spread throughout the city than previously reported and was also present in neighbouring suburban locations. The molestus bioform was also present in the northern Malmoe suburb Burlov and in rural locations such as Sollebrunn and Horby. Hybrids were found in Simrishamn (Fig 1).

Species identification using the COI gene

Specimens were classified to species level by comparing the COI sequence to published sequences in the Barcode of life database (BOLD.org). No specimens had a *Cx. pipiens* f *molestus* variant on the polymorphic site, as suggested by Shaikevich (2007) for Russian *Cx. pipiens* mosquitoes, suggesting that this marker was a poor predictor of human biting *Cx. pipiens* f *molestus* in Sweden. Interestingly, one specimen of *Cx. torrentium* shared the same variation as that of *Cx. pipiens* f *pipiens* on the same nucleotide indicating a higher intraspecies variation in COI in *Cx. torrentium* than *Cx. pipiens* as observed in three cases in the UK specimens(Danabalan, Ponsonby et al. 2012). Nevertheless, there were 12 specimens classified as *Cx.torrentium*. (Table 2).

Bioform classification using the CQ11 microsatellite marker

CQ11 sequences from the mosquitoes were compared with publicly available sequences in GenBank and there was a clear distinction between the two *Cx. pipiens* bioforms. Of all the mosquitoes sequenced (n=111), thirty specimens (27%) were classified as *Cx. pipiens* f *pipiens* while fifty-nine specimens (53%) were classified as *Cx. pipiens* f *molestus*. three sequences (3%) were from *Cx. torrentium* and fifteen specimens failed to produce sequence (14%) (Table 1).

Potential bioform classification using the CPIJ001674 gene

The CPIJ001674 gene was sequenced from 64 mosquito specimens to evaluate this marker as a potential new marker for the *molestus* bioform. However, there was no distinction between *Cx. pipiens* f *pipiens* and *Cx. pipiens* f *molestus* despite several specimens sharing the same polymorphic sites. A phylogenetic tree of the sequences had low bootstrap values and showed no stable clusters (Fig 2). Whilst the CPIJ001674 gene was published as a gene that showed polymorphisms among the *Culex* complex species mosquitoes, there was no clear distinction in our hands, even between *Cx. pipiens* and *Cx. torrentium* mosquitoes.

Discussion

Human biting *Cx. pipiens* f *molestus*, is a known vector for several pathogens, and can be an efficient bridge vector for flaviviruses such as West Nile fever virus and Usutu virus due to its wide range of hosts. As such, it is of interest to distinguish *Cx. pipiens* f *molestus* from the more strictly ornitophilic*Cx. pipiens* f *pipiens* which is an important amplification vector but does not spread the virus to mammals.

In southern Europe, the division between the two bioforms is less pronounced where they share many ecological niches. In northern Europe, *Cx. pipiens* bioforms have been thought to be more isolated from each other and hybrids are less common (Osório, Zé-Zé et al. 2014, Vogels, van de Peppel et al. 2015, Vogels, Möhlmann et al. 2016). Compared to the few previous reports of *Culex pipiens* f *molestus* in Sweden we have found specimens in several rural areas where we would not have expected it to be present. Our study only analyzed specimens sent in by people experiencing mosquito nuisance in the south of Sweden, which leads to a biased sample. Most of the *Cx pipiens* f *molestus* specimens were collected in Gothenburg where the bioform has been recently described (Hesson, Schäfer et al. 2016). This makes it hard to evaluate the true distribution of the molestus bioform. However, it is clear that it is more common than prevously thought. This indicates that the bioform may be present in even more places if it is investigated more closely.

With the known limitations of using one molecular assay, this study encompassed three molecular markers, which aimed at distinguishing *Cx. pipiens* bioforms (CQ11 microsatellite gene, COI gene and CPIJ001674 gene). The COI gene was able to identify *Cx. pipiens* and *Cx. torrentium* specimens. However, it was not able to separate the two *Cx. pipiens* bioforms from each other since *Cx. pipiens* mosquitoes shared a single nucleotide variant in the COI gene that previously identified Russian *Cx. pipiens* form *pipiens* (Shaikevich 2007). This was similar to what was observed in the UK and US (Kothera, Godsey et al. 2010, Danabalan, Ponsonby et al. 2012) hence it is useful in the identification of *Cx. pipiens* to species level. The separation of bioforms in our study relied on the CQ11 microsatellite gene that has previously been

established as a good marker which correlates well with the ecophysiological characteristics of the bioforms (Smith and Fonseca 2004, Di Luca, Toma et al. 2016). The nuclear DNA marker (CPIJ001674) was not reliable for the distinction of the *Cx. pipiens* biotypes in our study, despite that the gene region was previously suggested to have a nucleotide variation that could be used for differentiating between the bioforms (Kim, Lee et al. 2018).

Conclusions

Cx. pipiens f *molestus* is present in several urban and rural locations in Sweden. Further studies of *Cx. pipiens* in Sweden could further clarify the spatial distribution and how this might affect future transmission of mosquito borne viruses such as West-Nile virus and Usutu virus that are spreading in Europe.

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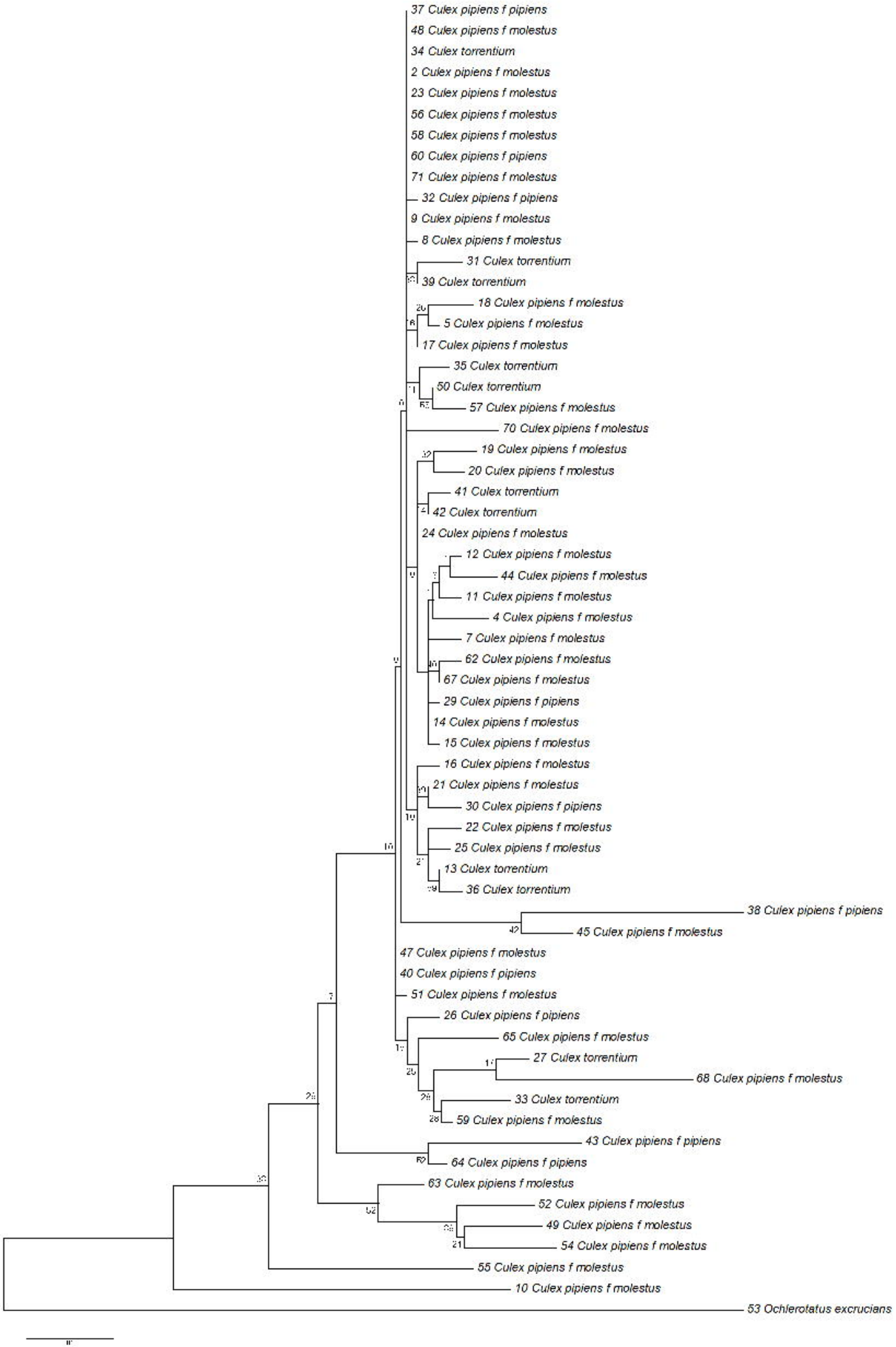
Table 1:
Sample locations included in the study.

Table 2:
Classification of each sample according to the COI and Cq11 markers, divided by sample location.

Figure 1:
Map of South Sweden with sample locations classified according to presence of *Cx. pipiens* f *molestus* (red dot), *Cx. Pipiens* f *pipiens* (Black triangle) or hybrids (purple dot).

Figure 2:
Phylogenetic tree of CPIJ001674 gene sequences. Sequences were aligned and phylogenetic analysis was done in MEGA6. Maximum Likelihood was used to infer the evolutionary history of the species based on the Tamura-Nei model. The bootstrap values were inferred from 500 replicates.


37 Culex pipiens f pipiens
48 Culex pipiens f molestus
34 Culex torrentium
2 Culex pipiens f molestus
23 Culex pipiens f molestus
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58 Culex pipiens f molestus
60 Culex pipiens f pipiens
71 Culex pipiens f molestus
32 Culex pipiens f pipiens
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51 Culex pipiens f molestus
26 Culex pipiens f pipiens
65 Culex pipiens f molestus
27 Culex torrentium
68 Culex pipiens f molestus
33 Culex torrentium
59 Culex pipiens f molestus
43 Culex pipiens f pipiens
64 Culex pipiens f pipiens
63 Culex pipiens f molestus
52 Culex pipiens f molestus
49 Culex pipiens f molestus
54 Culex pipiens f molestus
55 Culex pipiens f molestus
10 Culex pipiens f molestus
53 Ochlerotatus excrucians

Location:	Latitude:	Longitude:
Simrishamn	55.556637	14.352959
Hörby	57.6718589	11.926202500000045
Sollebrunn	57.58013320000001	12.094897100000025
Lindome	57.677168	11.926871
Mölndal	55.636584	13.062753
Burlöv	55.556637	14.352959
Lund	55.691855	13.450239
Malmö	55.5916	13.008100000000013
Vellinge	55.556637	14.352959
Göteborg	55.691855	13.450239
Stockholm	59.334877	18.075499
Laholm	56.460751	12.92515
Uppsala	59.8549456	17.6414992

	COI Gene Specimens				CQ11 Gene Specimens			
Locality	**no sequence**	**other species**	**pipiens**	**torrentium**	**no sequence**	**torrentium**	**pipiens**	**molestus**
Burlöv	80		76, 77, 82, 83					76, 77, 80, 82, 83
Göteborg	3, 6, 18, 48, 49, 51, 68-73	53, 61	1, 2, 4, 5, 7-12, 14-17, 19-25, 44-47, 52, 54, 59, 60, 62-65, 78, 79, 85-87, 91, 92	13, 50	3, 6, 13, 53, 61, 73	50	60, 64	1, 2, 4, 5, 7-12, 14-25, 44-49, 51, 52, 54, 59, 62, 63, 65, 68-72, 78, 79, 85-87, 91, 92
Hörby			55-58					55-58
Laholm			84				84	
Lindome			74					74
Lund			81				81	
Malmö				89	89			
Mölndal			75					75
Simrishamn	90		*28-30, 32, 37, 38, 40, 43, 93-109*	*27, 31, 33-36, 39, 41, 42*	27, 29, 31-34, 36, 37, 41, 99, 100	35, 39	28, 30, 38, 40, 42, 43, 90, 93, 94-98, 101-109	
Sollebrunn	66		67		66			67
Stockholm			88				88	
Uppsala			26				26	
Vellinge			110, 111				110, 111	