GENETIC IDENTIFICATION AND PHYLOGENETIC RELATIONSHIP OF CULEX PIPIENS BIOFORMS AND CULEX TORRENTIUM IN SOUTHERN SWEDEN USING MITOCHONDRION AND NUCLEAR DNA MARKERS

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Genetic Identification and Phylogenetic Relationship of *Culex pipiens* Bioforms and *Culext torrentium* In Southern Sweden using Mitochondrion and Nuclear DNA Markers

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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This thesis has been submitted for examination with our approval as University supervisors.

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DEDICATION

This thesis is dedicated to my late grandmother Martha Maero Ndombi.

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LIST OF ABBREVIATIONS AND ACRONYMS

ACE	Acetylcholinesterase
BOLD	Barcode of Life Database
CO1	Cytochrome Oxidase 1
Cx	*Culex*
DNA	Deoxyribonucleic acid
HRM	High Resolution Melting
ITS	Internal Transcribed Spacer
JKUAT	Jomo Kenyatta University of Agriculture and Technology
MEGA	Molecular Evolutionary Genetics Analysis
MSA	Multiple Sequence Alignment
NGS	Next Generation Sequencing
PCR	Polymerase Chain Reaction
SINV	Sindbis virus
SNP	Single Nucleotide Polymorphism
SSRs	Simple Sequence Repeats

STRs	Short Tandem Repeats
SVA	Swedish National Veterinary Institute
USUV	Usutu Virus
WNV	West Nile Virus

ABSTRACT

Culex (Cx.) pipiens bioforms and *Cx. torrentium* are vectors of pathogens known to transmit diseases such as West Nile virus in human populations. Traditional classification has remained a challenge in their identification despite observed ecophysiological differences. Being morphologically indistinguishable, molecular methods have proven reliable for their accurate identification. However, not all molecular methods have been explored to determine the most reliable marker for the identification of the bioforms and *Cx. torrentium*, notwithstanding the evidence of their circulation in Sweden. In this study, mosquito samples were captured in diverse regions (urban, periurban and rural areas) of Southern Sweden in order to genetically identify and determine the geographical distribution of *Cx. pipiens* bioforms and *Cx. torrentium*. This was achieved using mitochondrion and nuclear molecular markers including: the mitochondrial cytochrome C oxidase subunit I (CO1) barcode gene region, the CQ11 microsatellite gene and the CPIJ001674 gene. A total of one hundred and eleven (111) mosquitoes were dissected individually and their respective legs removed, homogenized, and subjected to conventional PCR and sequencing. Amplified Fragment Length Polymorphism (AFLP) was used to distinguish *Cx. pipiens* bioforms and *Cx. torrentium* based on the CQ11 microsatellite gene. Furthermore, a similar approach targeting the CQ11 microsatellite gene was used in identifying hybrids in one southern site. The COI barcode gene was able to distinguish *Cx. pipiens* and *Cx. torrentium* to species level while the CPIJ001674 gene was not a reliable marker for the distinction of the two bioforms and *Cx. torrentium* in Sweden. Phylogenetic results also revealed *Cx. torrentium* and *Cx. pipiens* bioforms clustered separately using the COI barcode and CQ11 genes, but not with the CPIJ001674 gene. The findings further confirmed that the CQ11 microsatellite was a valid diagnostic marker for the distinction of *Cx. pipiens* bioforms and *Cx. torrentium*. These findings may provide new insights into the ecology of the *Cx.* mosquito species since the established molecular markers will serve as proxies for their observed ecophysiological traits. Consequently, aid in virus surveillance against arboviral diseases such as West Nile fever virus, whose key vectors are *Cx.* mosquitoes.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

The *Culex* (*Cx.*) genera comprises of diverse species of mosquitoes including; *Cx. quinquefasciatus*, *Cx. torrentium* and *Cx. pipiens*. These species are known vectors of virus pathogens causing emerging diseases in the world such as West Nile virus (WNV), Sindbis virus (SINV), Japanese encephalitis virus (JEV), St. Louis encephalitis virus, Usutu virus (USUV), Western equine encephalitis virus and Eastern equine encephalitis virus (Van den Hurk *et al.,* 2009, Vogels *et al.,* 2016, Jöst *et al.,* 2011). *Cx. pipiens* commonly known as the northern house mosquito consists of two behaviourally distinct bioforms, *Cx. pipiens* form *pipiens* and *Cx. pipiens* form *molestus* which are capable of forming hybrids (Vogels *et al.,* 2017). *Cx. pipiens* form *pipiens* mosquito is heterodynamic and undergoes a reproductive diapause during winter, preferably feeds on avian blood, mates in open areas, is found above ground habitats in rural areas, and requires a blood meal for its oviposition. The *Cx. pipiens* form *molestus* remains active during winter, mostly feeds on human blood, is found underground habitats in urban areas, mates in confined spaces and does not require a blood meal for oviposition of its first batch of eggs (Beji *et al.,* 2017). They can live in sympatry in surface habitats which promotes hybridization between the species (Vogels *et al.,* 2015). The hybrids are of great epidemiological significance, as they possess an opportunistic behaviour of feeding on both reservoir birds and humans that serve as incidental hosts therefore, facilitating the transmission of diseases (Farajollahi *et al.,* 2011).

Cx. pipiens form *molestus* are a nuisance to human population due to their frequent bites and also for their role together with their hybrids in transmitting arboviral diseases from birds which serve as reservoirs to susceptible hosts (Hesson *et al.,* 2016). They are primary vectors of WNV, SINV, USUV and JEV in Europe (Ravanini *et al.,* 2012). For instance, the number of cases of those infected with JEV annually range between 30,000-50,000 globally and 30%

of the cases who present with symptoms die while 30% who survive present with neurologic clinical forms (Ravanini *et al.,* 2012). In addition, the initial outbreak of WNV reported in US specifically in New York resulted in four fatalities linked with inflammation in the brainstem and meningoencephalitis (Sampson and Armbrustmacher, 2001).

Cx. torrentium is adaptable to cold environment and thrives in high-altitude (Hesson *et al.,* 2014). It oviposits preferably in clean natural habitats (Lühken *et al.,* 2015). *Cx. torrentium* is similar to *Cx. pipiens* form *pipiens* with reference to its morphology, in that, the males can be differentiated by the hypopygium (Becker *et al.,* 2003). However, their females are difficult to distinguish since the pre-alar scales easily fall off rendering the use of the morphometric wing venation pattern not useful as an identification tool (Börstler *et al.,* 2014). In addition, *Cx. torrentium* has a similar breeding ecology to *Cx. pipiens* form *pipiens*, due to their existence in the same geographical area (Weitzel *et al.,* 2011). Studies on *Cx. torrentium* vector capacity for arthropod-borne viruses have gained interest due to its wide distribution and abundance in several parts of the world including Europe (Hesson *et al.,* 2014, Balenghien *et al.,* 2008). Previously, *Cx. torrentium* was presumed to be primarily ornithophilic, therefore not considered as an important vector for zoonotic arthropod-borne viruses (Hesson *et al.,* 2016). However, recent data has proven that *Cx. torrentium* is a vector of both medical and veterinary importance as it has been shown to be a vector of zoonotic arthropod-borne viruses. *Cx. torrentium* feeds on diverse hosts including birds and humans hence considered as a bridge vector for transmission of viruses for instance SINV and WNV (Balenghien *et al.,* 2008, Hesson *et al.,* 2014). *Cx. torrentium* an important epidemiological enzootic vector for SINV in Scandinavia among birds (Hesson *et al.,* 2015). Also, experimental infection studies have demonstrated that *Cx. torrentium* is a competent vector for SINV in Northern Sweden (Lwande *et al.,* 2019). *Cx. torrentium* is also proficient in the transmission of WNV in Central and Northern Europe (Jansen *et al.,* 2019).

Cx. pipiens and *Cx. torrentium* are known to have a wide distribution throughout Sweden (Lundström *et al.,* 2013). Mosquito surveillance data in most parts of Sweden, indicates *Cx. pipiens* distribution in 14 provinces whereas *Cx. torrentium* in 20 provinces, with potential of

further extension to bordering countries (Lundström *et al.,* 2013). Both *Cx.* mosquito species are deemed important in the transmission of zoonotic arthropod-borne viruses most important SINV which has been frequently isolated from both species in nature (Hesson *et al.,* 2015). In Sweden, SINV has been reported to have the highest field infection rate from *Cx. pipiens* and *torrentium* (Hesson *et al.,* 2019).

According to (Hesson *et al.,* 2015), the SINV infection rate in *Cx. torrentium* was (36 infections/1,000) whereas that of *Cx. pipiens* mosquitoes was (8 infections/1000). Recent vector competence studies performed in northern Sweden verify the ability of *Cx. torrentium* and *Cx. pipiens* to transmit SINV (Hesson *et al.,* 2015), and have been linked to SINV outbreaks (Bergqvist *et al.,* 2015).

Morphological approaches have remained a challenge in identifying female *Cx. pipiens* and *Cx. torrentium.* There is lack of appropriate morphological criteria that can distinguish between the *Cx. pipiens* and *Cx. torrentium.* with the male genitalia being the only feature used for identification of the male mosquitoes (Fedorova and Shaikevich, 2007). Consequently, studies on the ecology of arboviruses transmitted by females of *Cx. pipiens* and *Cx. torrentium* have remained challenging. Molecular techniques using genetic markers are needed for their accurate identification and use as proxy for the observed phenotypes (Reusken *et al.,* 2010, Weitzel *et al.,* 2011).

Differentiating between the two *Cx. pipiens* bioforms: *pipiens* and *molestus* and *Cx. torrentium* is significant in taxonomy as well as mapping of their geographical distribution, which is useful in surveillance and control studies that will in turn reduce the level of transmission of diseases to susceptible hosts. This study identified *Cx. pipiens* bioforms: *pipiens* and *molestus* and *Cx. torrentium* in Sweden using DNA barcoding approach utilizing species specific primers that targeted microsatellite loci CQ11, which was used as a marker for differentiating *Cx. pipiens* bioforms, their hybrids and *Cx. torrentium*. The mitochondrial cytochrome C oxidase subunit I (CO1) barcode gene was targeted to differentiate between *Cx. torrentium* and *Cx. pipiens* bioforms: *pipiens* and *molestus* while the nuclear marker

CPIJ001674 gene was evaluated to determine whether it was a valuable marker for discriminating between *Cx. pipiens* bioforms and *Cx. torrentium*.

1.2 Statement of the Problem

The *Cx. pipiens* bioforms and *Cx. torrentium* differ in physiology and behaviour which have an impact in the transmission of disease. They are responsible for the zoonotic spread of arboviruses such as Usutu virus, SINV and WNV from resident and wild birds to susceptible humans and equines. Whilst the former serves as amplifying hosts of the viruses, the latter are dead-end hosts of which the severity of the disease falls upon. Nevertheless, morphological differentiation has proven not to be efficient in identifying and differentiating the *Cx. pipiens* bioforms and *Cx. torrentium*. Consequently, efforts put on surveillance of the arboviruses transmitted by the *Cx. pipiens* bioforms and *Cx. torrentium* have been a challenge thereby resulting in increased transmission levels. Whilst the classification and tracing of the evolutionary descent of *Cx. pipiens* bioforms and *Cx. torrentium* has remained debatable, their preventive and control strategies have been futile. This study used molecular methods to genetically identify *Cx. pipiens* bioforms and *Cx. torrentium*.

1.3 Justification

Knowledge on the physiological and behavioural characteristics of *Cx. pipiens* bioforms and *Cx. torrentium* is vital in understanding the epidemiology of diseases they transmit which will help in targeted surveillance and control efforts. Various genetic approaches have been suggested in differentiating the *Cx. pipiens* bioforms and *Cx. torrentium*. So far, the best way to distinguish the *Cx. pipiens* bioforms has been the CQ11 microsatellite sequence, which differentiates between the two bioforms. This project used CQ11 microsatellite marker, which has two distinct sequences that have previously been used in differentiating *Cx. pipiens* form *pipiens* and *Cx. pipiens* form *molestus* for example in Italy and Morocco but not in Sweden. It was also used to classify *Cx. torrentium* species. The mitochondrial CO1 barcode gene region was also used since it allowed the discrimination of closely related species. It was used in characterizing *Cx. torrentium* from *Cx. pipiens*. Further sequencing of the nuclear gene

CPIJ001674 was performed in order to explore if it was a useful marker for discriminating between *Cx. pipiens* bioforms and *Cx. torrentium*.

The study was conducted in Sweden because *Cx. pipiens* bioforms and *Cx. torrentium* are important disease vectors of public health importance and therefore distinguishing between these two species and bioforms is beneficial in determining the risk areas for potential outbreaks. This will help in developing risk maps and putting necessary measures in place to curb the outbreaks. The techniques developed in this study will be useful in Kenya since entomological surveillance has provided evidence of circulation of WNV among *Cx*. mosquitoes in the North Eastern part of Kenya. Accurate detection of *Cx*. mosquitoes would be useful in knowing their true geographical distribution, the circulation of WNV that they transmit among hosts and accurate detection of human WNV cases that go unnoticed. Identification and mapping of *Cx.* species and their bioforms' will also help in the assessment of their potential risk to the public health.

1.4 Research Questions

1. Which is the most reliable molecular marker for distinguishing between *Cx pipiens* and *Cx. torrentium* species in Southern Sweden?
2. What nuclear markers (CQ11 and CPIJ001674) can reliably discriminate within *Cx. pipiens* bioforms and between the bioforms *and Cx. torrentium* in Southern Sweden?
3. What are the phylogenetic relationships between *Cx. pipiens* bioforms and *Cx. torrentium* using mitochondrion (CO1 barcode) and nuclear markers (CQ11 and CPIJ001674 markers) in Southern Sweden?

1.5 Objectives

1.5.1 General Objective

To genetically identify *Cx. pipiens* bioforms and *Cx. torrentium* using mitochondrion and nuclear DNA markers and determine their phylogenetic relationship in Southern Sweden.

1.5.2 Specific Objectives

1. To distinguish *Cx pipiens species* from *Cx. torrentium* species using CO1 barcode, CQ11 and CPIJ001674 markers in Southern Sweden
2. To ascertain whether nuclear markers (CQ11 microsatellite and CPIJ001674 gene) can reliably discriminate within *Cx. pipiens* bioforms and between the bioforms and *Cx. torrentium* in Southern Sweden
3. To determine the phylogenetic relationships between the *Cx. pipiens* bioforms and *Cx. torrentium* based on the mitochondrion (CO1 barcode) and nuclear markers (CQ11 and CPIJ001674) in Southern Sweden.

CHAPTER TWO

LITERATURE REVIEW

2.1 Economic importance and mosquito control

Mosquito species are known for their medical importance as they vector many pathogens (Christensen *et al.,* 2005, Hanley *et al.,* 2008). This is facilitated by factors such as; vector competence of the mosquitoes in transmitting pathogens to susceptible hosts, variation in their feeding patterns and numerous diversity of hosts that they feed on (Farajollahi *et al.,* 2011). Female anopheline mosquitoes have been extensively studied and confirmed to be vectors of malaria (Stevenson *et al.,* 2012, Bashar and Tuno, 2014). They are linked with marked increase in morbidity and mortality cases in Africa where financial resources needed for planning interventions and control programmes are limited (Wilcox *et al.,* 2019). *Cx. pipiens fatigans, Anopheles gambiae* and *Anopheles funestus* transmit filarial worms to humans and domestic animals including dogs and donkeys in the Kenyan coast (Kinyatta *et al.,* 2014). *Aedes aegypti* and *Aedes albopictus* are known vectors of several viruses including dengue, chikungunya, yellow fever and Zika (Lambrechts *et al.,* 2010, Ryan *et al.,* 2019, Lwande *et al.,* 2020). They are the major cause of increased morbidity and mortality cases that are reported both regionally and globally hence raising the need for effective interventions (Carrington & Auguste, 2013).

Control measures have been put in place with an effort on reducing the transmission of mosquito-borne diseases to the human population (WHO, 2017). Genetic manipulation of the mosquito by knocking out some genes that are upregulated or targeting genes that are downregulated during infection with the virus and using them to develop antivirals that are useful in the treatment of these viral diseases has been employed (Chen *et al.,* 2008, Hammond *et al.,* 2016). This approach is useful although only to a smaller scale. Essential oils extracted from different parts of plants such as; *Foeniculum vulgare Mill, Ferula hermonis Boiss, Citrus sinensis Osbeck, Pinus pinea L, Laurus nobilis L* and *Eucalyptus spp* contain chemical compounds which act as mosquito repellents (Ghosh *et al.,* 2012). This management

tool is preferable to insecticide application, which has harmful toxins that have deleterious effects on humans and the environment at large. Proper management and treatment of mosquito larvae habitats such as; stagnant and moving water bodies, are essential in reducing mosquito population since their survival rate is low when exposed to such conditions. Utilization of treated bed nets is also encouraged since it protects vulnerable populations including: pregnant women and children who are more susceptible to malaria (Traboulsi *et al.,* 2005).

2.2 Mosquitoes in the *Cx. pipiens complex* and *Cx. torrentium*

Mosquitoes in the Cx. *pipiens* complex includes Cx. *quinquefasciatus, Cx. globocoxitus, Cx. pipiens pallens, Cx. australicus* and *Cx. pipiens bioforms* (Farajollahi *et al.,* 2011). *Cx.* mosquitoes are of great epidemiological significance due to the pathogens they transmit to susceptible populations both regionally and globally. For instance, *Cx. quinquefasciatus* is the main vector of lymphatic filariasis in Southeast Asia and China besides transmitting avian malaria among birds in Hawaii (Fonseca *et al.,* 2006, Farajollahi *et al.,* 2011). *Cx. pipiens* is a vector of WNV in North America and in European countries including Italy and Greece (Turell *et al.,* 2002, Sudomo *et al.,* 2010, Chaskopoulou *et al.,* 2016). SINV is largely distributed in diverse continents namely; Africa, Asia, Europe and Australia causing intermittent outbreaks (Adouchief *et al.,* 2016). For instance, in Kenya, five strains of SINV have been isolated from diverse geographical regions including: Kisumu, Budalangi, Naivasha and Boni (Sigei *et al.,* 2018). This is attributable to migratory birds that facilitate the distribution of SINV across the regions.

Cx. pipiens consists of two bioforms namely; *Cx. pipiens* form *pipiens* and *Cx. pipiens* form molestus which are capable of forming hybrids (Di Luca *et al.,* 2016). These bioforms have different ecophysiological characteristics and often occur in sympatry increasing the potential for hybridization (Luande *et al.,* 2020). Despite these differences, it is difficult to distinguish between *Cx. pipiens* bioforms as well as the hybrids using traditional methods since their female lack clear morphological traits and also their hybrids present intermediate features

from both parents, which are challenging to discriminate (Joyce *et al.,* 2018). *Cx. pipiens* form *pipiens* play a major role in transmitting USUV among wild birds (Jöst *et al.,* 2011) whereas *Cx. pipiens* form *molestus* and the hybrids serve as bridge vectors for transmission of WNV from avian hosts to humans (Reusken *et al.,* 2010).

In Sweden, *Cx. torrentium* is the primary vector while *Cx. pipiens* is the secondary vector of SINV causing Ockelbo disease, known to cause debilitating effects to patients presenting with symptoms ranging from mild to chronic illnesses associated with stroke (Ahlm *et al.,* 2014). Furthermore, the female *Cx. torrentium* which is closely related to *Cx. pipiens,* is also morphologically indistinguishable just like in the case of *Cx. pipiens* bioforms and their hybrids (Smith & Fonseca, 2004). These medically important sibling species tend to coexist in Europe and overlap human settlements posing threat to susceptible populations (Weitzel *et al.,* 2011). As a consequence, molecular approach for the accurate identification of *Cx. pipiens* bioforms and *Cx. torrentium* is critical in knowing their true geographical distribution and occurrence, which in turn has a direct influence on the epidemiology of viruses they transmit to humans.

2.3 Traditional approaches of identifying mosquitoes

Classification of diverse mosquito species using dichotomous keys have been employed in mosquito surveillance initiatives (Batovska *et al.,* 2016). Anatomical features illustrated with photomicrographs of both larvae and adult mosquitoes are used in the keys that aid in their morphological identification (Mohamed *et al.,* 2017). Therefore, microscopic examination of the larvae and adult mosquitoes is necessary besides the photographs of their anatomical features (Mohamed *et al.,* 2017). Diverse mosquito species have been identified to species level using morphological traits in Mexico and also along the Kenyan coast (Adeniran *et al.,* 2021, Mwangangi *et al.,* 2013).

Although there are challenges that emanate from this technique such as difficulty in identifying morphologically similar species or damaged specimens, and need for specialized training, it has been utilized together with molecular approach to support species identification (Batovska *et al.,* 2016, Hernandez *et al.,* 2017). Relying on both approaches may be efficacious in ascertaining hidden differences within species populations and in overcoming the limitations of morphological and genetic typing of mosquitoes (Hernandez *et al.,* 2019). This will in turn be essential in surveillance programs of mosquito species, the pathogens they transmit and also in effective disease control initiatives (Hernandez *et al.,* 2019).

2.4 Molecular markers for studying mosquito diversity

Morphological identification has remained a challenge and cannot be relied on for accurate identification of mosquitoes that share similar morphological features (Bickford *et al.,* 2007). Molecular methods have provided a means through which useful molecular markers for the accurate identification of mosquito species have been generated (Bennet *et al.,* 2015, Garros *et al.,* 2004). Through DNA barcoding approach, genetic differences between interspecific and intraspecific mosquito populations has been achieved (Murugan *et al.,* 2016). A number of genetic markers including Mitochondrion markers such as mitochondrial Cytochrome oxidase C subunit I and II (COI and COII), cytochrome oxidase B and ribosomal DNA genes (12S and 16S rDNA) and nuclear DNA markers such as ribosomal internal transcribed spacer (ITS-2), acetylcholinesterase 2 (ACE-2) and alpha amylase have been used in studying genetic lineages within different mosquito species complex (Suguri *et al.,* 2009, Dhananjeyan *et al.,* 2010, Puslednik *et al.,* 2012). In addition, the sequences of nuclear ribosomal DNA genes in arthropods are repeatedly arranged in series; each unit comprises the genes encoding for 18S, 5.8S and 28S ribosomal RNA (Gillespie *et al.,* 2006). The conserved structural units of the genes are separated by internal transcribed spacers (ITS). The ITS-1 and ITS-2 regions are also widely used in taxonomic and phylogenetic analyses of mosquitoes and have been proved to be useful in distinguishing morphologically similar species. For example, a PCR assay targeting the ITS-2 region of the rDNA has been used to identify five species of *Anopheles maculatus* in the north-western part of Thailand (Walton *et al.,* 2007).

Acetylcholinesterase 2 gene (ACE-2) gene has been used to study the genetic ancestry of *Cx. annulirostris* and *Cx. palpalis* species in Australasia (Jansen *et al.,* 2013). The CO1 gene has also been used in equating the differences obtained with those of ACE-2 marker (Hemmerter *et al.,* 2009). These markers have been useful in tracing the evolutionary history of the sibling species which aid in mosquito surveillance and control programmes aiming at managing the spread of arboviral diseases to both human and animal population (Hemmerter *et al.,* 2009). High-resolution melting analysis (HRM) which is an accurate and specific genetic method has also been adopted for the identification of the genetic differences between and within *Cx.* complex mosquito population (Kang & Sim, 2013, Ajamma *et al.,* 201, Kim *et al.,* 2018). HRM assay has been considered faster and reliable in detecting single nucleotide variations in the sequence of ACE-2 gene that is utilized in differentiating species within the *Cx.* complex.

2.4.1 Identification of *Cx. pipiens* Bioforms and *Cx. torrentium* using mitochondrial cytochrome Oxidase Subunit 1 (CO1) Gene

Morphological approaches such as the conformation of the genitalia (*phallasoma*) in males have been the only reliable feature for distinguishing between the males of *Cx. pipiens* bioforms and *Cx. torrentium* (Kent *et al.,* 2007). Whilst *Cx. pipiens* bioforms differ greatly in their behaviour and physiology, their females cannot be morphologically typed since their morphological features are similar or even identical (Shahhosseini *et al.,* 2018). In addition, *Cx. torrentium* presents with the same ecophysiological characteristics as that of *Cx. pipiens* form *pipiens* but their females cannot be differentiated morphologically from the *Cx. pipiens* bioforms (Rudolf *et al.,* 2013, Krüger *et al.,* 2014).

Relying on morphological differentiation of the two-sibling species is laborious, time consuming especially, during microscopic examination of male genitalia and leads to misidentifications of females. As a consequence, precise genetic identification methods are needed for discrimination of these medically important vectors (Versteirt *et al.,* 2012). DNA barcoding using the cytochrome C oxidase subunit 1 (COI) gene has been used to identify some mosquito species (Wang *et al.,* 2012, Chan *et al.,* 2014). It is preferred to other target

genes because it occurs in hundreds of copies per cell, contains no insertions or deletions and its recombination rate is very low (Kumar *et al.,* 2007). Furthermore, the mitochondrial DNA of the offspring is maternally inherited, and its sequence region is conserved across diverse taxa, making it a useful marker for distinguishing the *Cx. pipiens* bioforms and *Cx. torrentium* (Shaikevich *et al.,* 2007, Becker *et al.,* 2012, Simonato *et al.,* 2016). The COI gene sequences of diverse mosquito species has been employed extensively in their classification and taxonomic information referenced from voucher specimens in BOLD database which has 1,510 barcoded Culicidae species from a total of 2,043 described species (Jinbo *et al.,* 2011).

While inadequate variation in the COI gene sometimes makes it difficult to identify closely related mosquito species (Lilja *et al.,* 2017), Russian mosquito specimens have been identified using restriction enzymes that digest varying restriction sites of the gene. The *Hae*III restriction enzyme has been used to differentiate the two *Cx. pipiens* bioforms *pipiens* and *molestus* and the *Bcll* digestion to distinguish *Cx. torrentium* from *Cx. pipiens* (Shaikevich *et al.,* 2007). This was performed after primers that are complementary to the targeted sequence were designed and used to amplify the selected sequences.

Therefore, genetic tools using the COI gene have been employed in differentiating members of the *Cx. pipiens* complex (Shaikevich *et al.,* 2016). Utilization of the COI gene in mosquito species identification in diverse regions is owned to its conserved sequence across regions (Kumar *et al.,* 2007). Several species have been identified concurrently with the use of high throughput technology (Lilja *et al.,* 2017) and also through DNA-barcoding and Single Nucleotide Polymorphism (SNP) analysis (Engdahl *et al.,* 2014).

2.4.2 CQ11 Gene Marker for the Identification of *Cx. pipiens* Bioforms and *Cx. torrentium*

Microsatellites also referred to as Simple Sequence Repeats (SSRs) or Short Tandem Repeats (STRs), consist of short nucleotides of 2-6 base pairs with tandem repeats that have high rates of mutations, hence making them highly polymorphic (Wuyun *et al.,* 2019, Huang *et al.,*

2008). Studies have been done that target the GT repeats in the loci of *Cx. pipiens* and *Cx. quinquefaciatus* species (Edillo *et al.,* 2007). Due to high polymorphism between the loci, seven to nineteen alleles have been found for every locus indicating that the microsatellites serve as good markers for the differentiation of *Cx. pipiens* species complex (Huang *et al.,* 2011). Primers complementary to the microsatellite sequences have been designed and used to amplify variable regions between the species (Keyghobadi *et al.,* 2004).

So far, the use of microsatellites has been shown to be useful in identifying hybrids between *Cx. pipiens* form *pipiens* and *Cx. pipiens* form *molestus* in Germany (Rudolf *et al.,* 2013) and Morocco (Amraoui *et al.,* 2012). In Germany, mosquito surveillance using multiplex PCR targeting the acetylcholinesterase 2 (ace2) gene locus was able to differentiate between two closely related and morphologically indistinguishable mosquitoes belonging to the *Culex* genera: *Cx. torrentium and Cx. pipiens* whereas the bioforms and hybrids of *Cx. pipiens* were discriminated utilizing the CQ11 locus (Rudolf *et al.,* 2013). Both studies utilized the CQ11 microsatellite locus to distinguish *Cx. pipiens* bioforms and their hybrids. However, the German study further distinguished *Cx. torrentium* and *Cx. pipiens* using the ACE-2 gene locus (Rudolf *et al.,* 2013). The sizes of the amplicons varied between the two bioforms and their hybrids; with a 250-bp fragment identifying the *molestus* bioform, a 200-bp fragment characterizing the *pipiens* bioform and their hybrids possessing both fragment sizes (Amraoui *et al.,* 2012).

Microsatellite analysis has also been conducted in mosquito studies focused on *Culex pipiens* targeting different loci such as CQ11, CxpGT9, CxpGT20, CxpGT40, CxpGT51, and CxpGT53 (Fonseca *et al.,* 2009). Bayesian clustering analysis revealed *Cx. pipiens* form *pipiens* populations from the US constitute more than 40% hybrids with *Cx. pipiens* form *molestus* from the European ancestry (Farajollahi *et al.,* 2011). However, *Culex pipiens* form *molestus* population in the US was not identical to either southern Europe or northern Africa *Cx. pipiens* form *molestus* (Huang *et al.,* 2008). Moreover, several alleles were not shared between US *Cx. pipiens* form *molestus* and the other two populations from southern Europe and northern Africa. Despite CQ11 microsatellite locus being considered a diagnostic marker

for the accurate distinction of the two *Cx* bioforms and their hybrids, it was reported to misidentify *Cx. torrentium* in southern England and Wales in the UK (Danabalan *et al.,* 2012). Two genetic markers CQ11 and COI were used to identify wild caught *Cx.* sampled above ground habitats from selected counties across southern England and Wales. COI sequences showed *Cx. torrentium* was misidentified as *Cx. pipiens* in more than half of the cases and *Cx. pipiens* sampled were deemed *Cx. pipiens* form *pipiens*. It was therefore deduced that, in sympatry *Cx. torrentium* and *Cx. pipiens* could not be distinguished using CQ11 (Danabalan *et al.,* 2012).

In Sweden, discrimination between the *Cx. pipiens* bioforms and their hybrids has remained a challenge due to lack of a diagnostic molecular marker in spite of their circulation. It has been shown that geographical distance decreases the transfer of alleles between and within species population thereby altering their evolution (Bouchemousse *et al.,* 2016). This makes it difficult for the same molecular marker to be used in species identification in geographically distinct regions.

2.4.3 Nuclear Marker CPIJ001674 used in the Identification of *Cx. pipiens* Bioforms and *Cx. torrentium*

A number of nuclear markers have been used to study diverse mosquito populations' genetic structure and differentiation including: ITS-2, ACE-2, Alpha amylase and CQ11 among others (Suguri *et al.,* 2009, Dhananjeyan *et al.,* 2010, Puslednik *et al.,* 2012).

SNPs are rapid and efficient in the differentiation of mosquito species and their hybrids (Neafsey *et al.,* 2010). In the case of *Cx. pipiens* bioforms and their hybrids, SNP markers CxpG2T and CxpA2d have been shown to be reliable and cost-effective for the detection of *Cx. pipiens* complex mosquitoes and their hybrids due to the low cost incurred by the additional restriction digestion step (Huang *et al.,* 2011). Also, the intergenic spacer region has been applied in the development of useful SNP markers together with other microsatellite markers since it can detect genetic variation in morphologically similar incipient species, and

aid in the understanding of the evolution of *Cx. pipiens* complex mosquitoes (Huang *et al.,* 2008).

The nuclear gene CPIJ001674 which is a SNP marker, was recently identified as having a high degree of variation between *Cx* species (Kim *et al.,* 2018). SNP markers were designed from two genes namely; CPIJ005487 and CPIJ001674 with an aim of discriminating *Cx* species including *Cx. pipiens* and *Cx. torrentium*. Sequencing results indicated A/T transition in gene CPIJ005487 and A/G transition in CPIJ001674 between the *Cx. pipiens* form *pipiens* and *Cx. pipiens* form *molestus* (Kim *et al.,* 2018). In addition, the SNP markers on the gene CIPJ002074 showed F1 hybrid distinction (Kim *et al.,* 2018). Nuclear genes carrying SNPs can serve as markers for grouping individuals into population groups since the DNA has more than one allele in the studied population (Schmidt *et al.,* 2017). Therefore, they are useful for studying the evolution of mosquito species (Wondji *et al.,* 2007).

2.5 Phylogenetic Relationship between *Cx. pipiens* Bioforms and *Cx. torrentium*

To infer the phylogenetic relationship between *Cx. pipiens* bioforms: *pipiens* and *molestus*, and *Cx. torrentium*, Barcoding sequence data is important in the molecular taxonomy that enables the definition of the genetic structure among and within groups of these closely related *Cx.* mosquito populations (Talaga *et al.,* 2017, Chan-Chable *et al.,* 2019). The COI gene is the most commonly used barcode for classification and evaluation of the genetic diversity among closely related species for example: *Cx. pipiens* and *Cx. torrentium* (Hernandez *et al.,* 2019, Engdahl *et al.,* 2014). Further the barcode is used to compare the phylogenetic patterns within the *Cx. pipiens* bioforms: *pipiens* and *molestus* (Yurchenko *et al.,* 2020). In order to perform phylogenetic analyses a number of tools have been used as models of sequence evolution including MEGA6 based on Akaike information criterion (AIC), the Tamura-3-parameter Model as a suitable model for the analysis (Tamura *et al.,* 2013). Further, the model has been applied in the calculation of genetic distances within and between both species namely: *Cx. pipiens* and *Cx. torrentium.*

Diverse allele frequencies at numerous enzyme gene loci have been found to have significant genetic distances and genetic variances between *Cx. pipiens* bioform *pipiens* and *Cx. pipiens* bioform *molestus* populations (Becker *et al.,* 2012). The loci are as a result of inheritance which is solely independent from each *Cx. pipiens* bioform: *pipiens* and *molestu*s with regards to the Mendelian rules and the Hardy-Weinberg-equilibrium. Potential hybridization of *Cx. pipiens* bioform: *pipiens* and *molestu*s as detected by Fonseca *et al* (2004) in North America and Gomes *et al* (2009) in Southern Europe, is alleged to have no role in allelic divergence and does not have a role in the gene flow of the bioforms in Europe (Gomes *et al.,* 2013). To date, the taxonomy of *Cx. pipiens* bioform: *pipiens* and *molestu*s and *Cx. torrentium* is presumed to fall under the monophyletic evolutionary lineage including *Cx. quinquefasciatus*, which are the main members of the *Cx. pipiens* complex. These species are discriminated by alleles frequencies. It is believed that the reproductive exchange between the *Cx. pipiens* bioforms *pipiens* and *molestus* does not have an influence on the independence of the respective gene pools (Becker *et al.,* 2012).

The ITS nuclear gene sequences of diverse members of the *Cx.* species of mosquitoes have been used to study their evolutionary development. Sibling relationships were determined through monophyletic groups that formed between *Cx. salinarius* and *Cx. erythrothorax*. In addition, *Cx. pipiens* and *Cx. torrentium* also formed a monophyletic group (Ruiz-Arrondo *et al.,* 2020). *Cx. torrentium* has been shown to form a sister clade with the mosquitoes belonging to the *Cx. pipiens* complex implying it as a distinct but closely related species based on phylogenetic analysis of the ITS gene (Hernandez *et al.,* 2019).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Sites

Mosquitoes were sampled in thirteen regions in South of Sweden namely, Göteborg (57°41'40.5"N 11°55'11.6"E), Stockholm (59°20'05.5"N 18°04'31.8"E), Uppsala (59°51'17.8"N 17°38'29.4"E), Malmö (55°35'29.8"N 13°00'29.2"E), Burlöv (55°38'11.7"N 13°03'45.9"E), Lund (55°41'30.7"N 13°27'00.8"E), Simrishamn (55°33'23.9"N 14°21'10.6"E), Hörby (55° 50' 22.596" N 13°43'26.0148"E), Lindome (57°34'48.5"N 12°05'41.6"E), Laholm (56°27'38.7"N 12°55'30.5"E), Mölndal (57°36'30.0"N 12°03'50.0"E), Vellinge (55°23'00.2"N 12°49'03.1"E) and Sollebrunn (58°06'44.7"N 12°33'44.1"E) (Figure 3.1). Mosquito magnet® Patriot traps (Woodstream Corp) were used in the sampling process since it was reliable and efficient in trapping adult mosquitoes. Citizens were also motivated to capture mosquito samples through a citizen science project. The Mosquito magnet® Patriot trap utilized propane gas to create CO_2 that attracted and pulled mosquitoes into the traps, where they were retained in a catch bag. Mosquitoes collected per site were thirty-five (35) in Simrishamn, fifty six (56) in Göteborg, five (5) in Burlöv, four (4) in Hörby, two (2) each in Vellinge and Sollebrunn, and one (1) each in Lindome, Mölndal, Lund, Malmö, Stockholm, Laholm and Uppsala. Morphological typing of the collected mosquitoes was then performed by experts at the National Veterinary Institute (SVA) and only *Cx. pipiens* and *Cx. torrentium* adults were included in the study. Nevertheless, five (5) *Cx. torrentium* mosquitoes could not be classified genetically despite being included in the study.

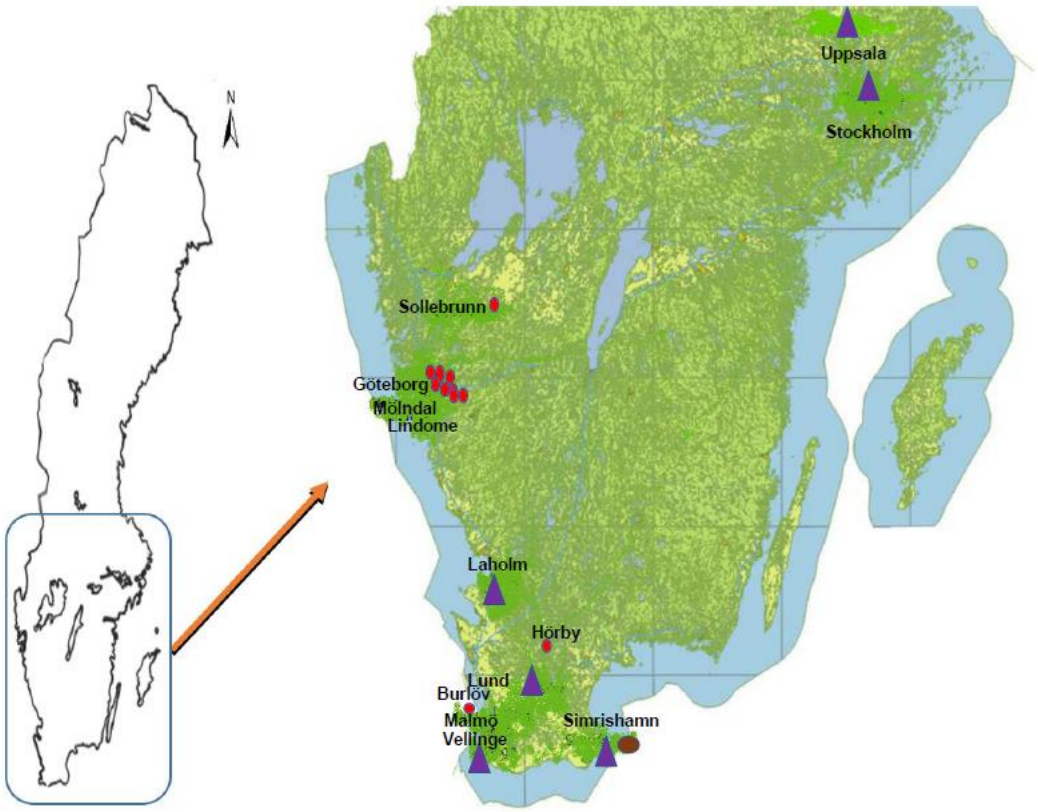

Figure 3.1: Map of South Sweden showing the sampling sites classified according to presence of *Cx. pipiens* form *molestus* (red dot), *Cx. pipiens* form *pipiens* (purple triangle) and hybrids (brown dot).

3.2 Sample Processing and DNA Isolation

Three legs were picked from each individual mosquito, placed in a 1.5mL well-labelled eppendorf tubes and 30 µL PrepMan ultra reagent solution added to each respective tube (Thermo Fisher Scientific). Legs were homogenized using a hand-held motorized pestle for 30 seconds. The homogenates were then incubated in a heat block at 100˚C for 10 minutes, chilled on ice for 2 minutes and centrifuged for 3 minutes at 13000rpm. The supernatant (20 µL) which contained genomic DNA was transferred to each clean tube marked with name and date.

3.3 Amplification of Mitochondrion and Nuclear DNA Markers

3.3.1 Mitochondrial Cytochrome C Oxidase Subunit 1 Gene Marker

The extracted genomic DNA was PCR amplified using COI primer pair targeting mitochondrial COI barcode gene region (Table 3.1) in a Bio-Rad PCR machine (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). The PCR master mix comprised of 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 universal primers LCO 1490 forward and HCO 2198 reverse (Table 3.1), 0.15 µL AmpliTaq DNA polymerase (5 U/µL), 1 µL of DNA template and 16.85 µL of water to total volume of 25 µL. The cycling conditions were as follows; initial denaturation at 95°C for 3 minutes followed by 39 cycles of denaturation at 95°C for 30 seconds, annealing 55°C for 1 minute and extension at 72°C for 1 minute. Final extension was performed at 72°C for 5 minutes and held at 4°C indefinitely. PCR products, positive and negative controls were then visualized on a 1.2% agarose in 1x TAE with GelRed (Biotium Inc. Hayward, CA, US) with a FastRuler Middle Range DNA Ladder (ThermoFisher Scientific, Vilnius, Lithuania).

Table 3.1: Primers used for amplification of the mitochondrion and nuclear DNA markers

Primer name	Sequence (5´-3´)	Product size	Target gene	Reference
LCO1490	GGTCAACAAATCATAAAGATATTGG	658-bp	COI barcode	(Lilja *et al.*, 2017)
HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	658-bp	COI barcode	(Lilja *et al.*, 2017)
PipCQ11R	CATGTTGAGCTTCGGTGAA	200-bp	CQ11	(Bahnck and Fonseca, 2006)
molCQ11R	CCCTCCAGTAAGGTATCAAC	250-bp	CQ11	(Bahnck and Fonseca, 2006)
CQ11F2	GATCCTAGCAAGCGAGAAC	250-bp	CQ11	(Bahnck and Fonseca, 2006)
CPIJ001674_fwd	TGTACGTGGAGCACAAGAGC	178-bp	CPIJ001674	(Kim *et al.*, 2018)
CPIJ001674_rev	TCCGAGTAGACCGAGACCAG	178-bp	CPIJ001674	(Kim *et al.*, 2018)

3.3.2 CQ11 Microsatellite Gene

PCR amplification reactions were done targeting the flanking region of CQ11 microsatellite using pipCQ11R, molCQ11R and CQ11F2 primers (Table 3.1). The master mix comprised of 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 and 0.3 µL of CQ11F2), 0.15 µL AmpliTaq DNA polymerase (5 U/µL), 1 µL of DNA template and 14.65 µL water to make a total volume of 20 µL. PCR was performed with an initial denaturation at 94°C for 3 minutes followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing 54°C for 30 seconds and extension at 72°C for 40 seconds. Final extension was performed at 72°C for 5 minutes and held indefinitely at 4°C. PCR products, positive and negative controls were then visualized on a 1.2% agarose in 1x TAE with GelRed (Biotium Inc. Hayward, CA, US) with a GeneRuler Middle Range DNA Ladder (ThermoFisher Scientific, Vilnius, Lithuania) (Bahnck and Fonseca, 2006).

3.3.3 Nuclear Marker CPIJ001674

CPIJ001674 fragment was targeted for amplification by PCR using CPIJ001674 forward and reverse primers (Table 3.1) in a final volume of 25 µL that consisted of 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 2 minutes and 15 seconds followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing 55°C for 30 seconds and extension at 72°C for 30 seconds. Final extension was performed at 72°C for 5 minutes and held at 4°C indefinitely. PCR products, positive and negative controls were then visualized on a 1.2% agarose in 1x TAE with GelRed (Biotium Inc. Hayward, CA, US) with a GeneRuler Middle Range DNA Ladder (ThermoFisher Scientific, Vilnius, Lithuania).

3.4 Purification of PCR Products

PCR products that amplified were purified using ExoSap-IT reagent (Thermo Fisher scientific, Carlsbad, CA, USA). The reaction volume for each product was incubated at 37°C for 15 minutes following mixing of 2 µL of ExoSap-IT reagent with 5 µL of post-PCR product. These were performed aiming at degrading primers and unincorporated nucleotides. The ExoSap-IT reagent was inactivated by incubating at 80°C for 15 minutes. Amplified purified product (5 µL) was then added to 5 µL of the relevant forward or reverse primer (5 pmol). Purified products were sent to Macrogen, South Korea, Inc for Sanger sequencing in both forward and reverse directions with the respective primers.

3.5 Bioinformatics Analysis

DNA sequences were edited using BioEdit software. Consensus sequences were generated from both forward and reverse DNA sequences of each sequenced mosquito specimen after conducting a pairwise alignment. Primer sequences in both forward and reverse direction were screened and removed from the alignment. Multiple sequence alignment was performed using MAFFT version 7 using Smith-Waterman algorithm for the sequences generated for COI, CQ11 and CPIJ001674 amplicons and visualized in Jalview (Jalview, 2018). Identification of single nucleotide polymorphisms (SNPs) was performed on CQ11 sequences to identify *Cx. pipiens* bioforms (*pipiens* and *molestus*) and *Cx. torrentium*. Genetic signatures targeted for the COI fragment as described in Shaikevich (Shaikevich, 2007) were assayed to differentiate between *Cx. pipiens* bioforms (*pipiens* and *molestus*) and *Cx. torrentium*.

3.5.1 Phylogenetic Analysis of COI Gene Sequences Obtained from the *Cx.* Mosquitoes Analyzed in the Study

Phylogenetic relationship of the COI sequences obtained from the PCR products of the mosquito samples screened in the study, were estimated by Bayesian Evolutionary Analysis by Sampling Trees (BEAST version 2.6.4). Hasegawa-Kishino-Yano (HKY) nucleotide substitution model was used with a Relaxed Clock Log Normal mode (Bouckaert et al., 2019).

Gamma Site Model was applied and Markov chain Monte Carlo (MCMC) chain length of 10,000,000 with a Pre Burnin of 10,000 while logging in parameters every 1000 step.

3.5.2 Phylogenetic Analysis of CPIJ001674 Gene Sequences Obtained from the *Cx.* Mosquitoes Analyzed in the Study

BEAST2 inferred the phylogeny of CPIJ001674 gene using the HKY nucleotide substitution model that employed Relaxed Clock Log Normal mode (Bouckaert et al., 2019). Markov chain Monte Carlo (MCMC) chain length of 10,000,000 and Gamma Site Model was applied with a Pre Burnin of 10,000 while logging in parameters every 1000 step.

3.5.3 Phylogenetic Analysis of CQ11 Microsatellite Sequences Obtained from the *Cx.* Mosquitoes Analyzed in the Study

The CQ11 phylogenetic tree was estimated using BEAST2 applying the HKY nucleotide substitution model with Relaxed Clock Log Normal mode (Bouckaert et al., 2019). Gamma Site Model was applied with a Pre Burnin of 10,000. The MCMC chain length of 10,000,000 steps was executed, logging in tree parameters every 1000.

3.6 Ethical Approval

This study involved mosquitoes and therefore ethical approval was not required.

CHAPTER FOUR

RESULTS

4.1 Distinguishing *Cx. pipiens* from *Cx. torrentium* species using the mitochondrial CO1 barcode gene

A 658-bp fragment of mitochondrial cytochrome C oxidase subunit 1 gene was used for identification of *Cx. pipiens* to differentiate them from *Cx. torrentium* (Figure 4.1 a-d). However, the COI gene could not distinguish the two *Cx. pipiens* bioforms; *pipiens* and *molestus.*

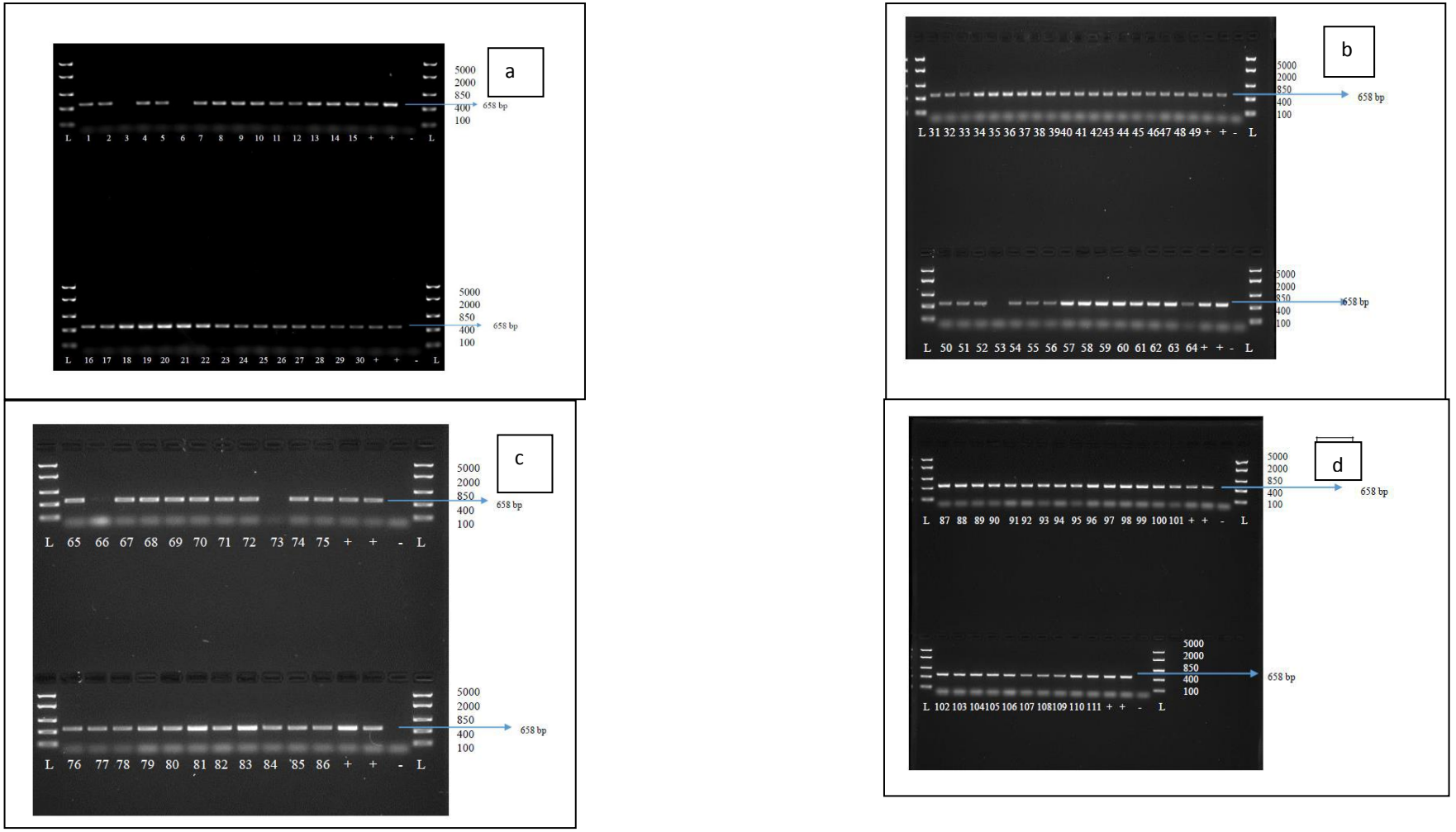

Figure 4.1: a-d: Gel electrophoresis of PCR products targeting a 658bp COI gene- L- Fast ruler Middle -range ladder, (1-111) represents mosquito samples screened, (+) is the positive control for *Cx torrentium* and *Cx. pipiens* and (–) is the negative control. Numbers 1 to 111 represent individual mosquitoes sampled in all the thirteen sites – no mosquito identified, P= *pipiens*, M=*molestus and* T=*torrentium*

Fifty out of the one hundred and eleven *Cx. pipiens* mosquitoes screened (50/111; 45%) were present in Göteborg as compared to Malmö (1/111: 0%) which had only one *Cx. torrentium* (Table 4.1). Simrishamn and Göteborg were the only regions that had both twenty six and fifty out of the one hundred and eleven *Cx. pipiens* (pipiens 26/111; 23% and 50/111; 45%) and nine and two *Cx. torrentium* (9/111; 8%, 2/111; 2%) specimens respectively, whereas *Cx. pipiens* specimens were identified in Hörby, Uppsala, Sollebrunn, Burlöv, Laholm, Lindome, Lund, Mölndal, Stockholm and Vellinge regions.

Table 4.1: Specimens from thirteen localities in Sweden

	COI Gene Specimens		CQ11 Gene Specimens			CPIJ001674 Gene Specimens		
Locality	**P or M**	**T**	**P**	**T**	**M**	**P**	**M**	**T**
Göteborg	1, 2, 4, 5, 7, 8, 9, 10, 11, 12, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24,25, 44, 45, 46, 47, 48, 49, 51, 52, 54, 59, 60, 61, 62, 63, 64, 65, 68, 69, 70, 71, 72, 78, 79, 85, 86, 87, 91, 92	13, 50	60, 64	50	1, 2, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 37, 44, 45, 46, 47, 48, 49, 51, 52, 54, 59, 61, 62, 63, 65, 68, 69, 70, 71, 72, 78, 79, 85, 86. 87, 91, 92	-	-	-
Hörby	55, 56, 57, 58	-	-	-	55, 56, 57, 58	-	-	-
Uppsala	26	-	26	-	-	-	-	-
Simrishamn	28, 29, 30, 32, 37, 38, 40, 43, 90, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109	27, 31, 33, 34, 35, 36, 39, 41, 42	28, 29, 30, 32, 38, 40, 42, 43, 90, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109	31, 33, 34, 36, 35, 39, 41	-	-	-	-
Sollebrunn	67	-	-	-	67	-	-	-
Burlöv	76, 77, 80, 82, 83	-	-	-	76, 77, 80, 82, 83	-	-	-
Laholm	84	-	84	-	-	-	-	-
Lindome	74	-	-	-	74	-	-	-
Lund	81	-	81	-	-	-	-	-
Malmö	-	89	-	89	-	-	-	-
Mölndal	75	-	-	-	75	-	-	-

Stockholm	88	-	88	-	-	-	-	-
Vellinge	110,111	-	110, 111	-	-	-	-	-

4.2 Distinguishing *Cx. pipiens* bioforms and *Cx. torrentium* using the nuclear (CQ11 and CPIJ001674) markers

The CQ11 microsatellite gene was used to characterize *Cx. pipiens* form *pipiens* and *Cx. pipiens* form *molestus* by PCR. *Cx. pipiens* form *pipiens* had a 200-bp amplicon size whereas the *Cx. pipiens* form *molestus* resulted in a product size of 250-bp (Figure 4.2 a-e). *Cx. torrentium* had multiple bands sizes with the highest having a 850-bp fragment. Göteborg had both bioforms; fifty *Cx. pipiens* form *molestus* and two *Cx. pipiens* form *pipiens* (50/111; 45%), (2/111; 2%) respectively as compared to Uppsala (1/111; 0%), Simrishamn (26/111; 23%), Laholm (1/111; 0%), Lund (1/111; 0%), Stockholm (1/111; 0%) and Vellinge (2/111; 2%) regions which had only *Cx. pipiens* form *pipiens* (Table 4.2). One (1/111; 0%) *Cx. torrentium* was identified in Malmö as compared to Hörby, Burlöv, Uppsala, Laholm, Sollebrunn, Lund, Lindome, Mölndal, Stockholm and Vellinge which had no *Cx. torrentium* specimens. Moreover, two hybrids (2/111; 2%) were identified in Simrishamn (Table 4.2). Although the CPIJ001674 gene amplified by gel electrophoresis, it could neither identify the bioforms (*Cx. pipiens* form *molestus* and two *Cx. pipiens* form *pipiens*) nor *Cx. torrentium* (Table 4.2).

Figure 4.2 a-e indicates a gel electopheresis of *Cx. pipiens* bioform *pipiens* and *molestus*. The two bioforms were able to be distinguished by different amplicon sizes. The *Cx. pipiens* bioform *molestus* had an amplicon size of 250 bp whilst 200 bp denoted the *Cx. pipiens* bioform *pipiens*.

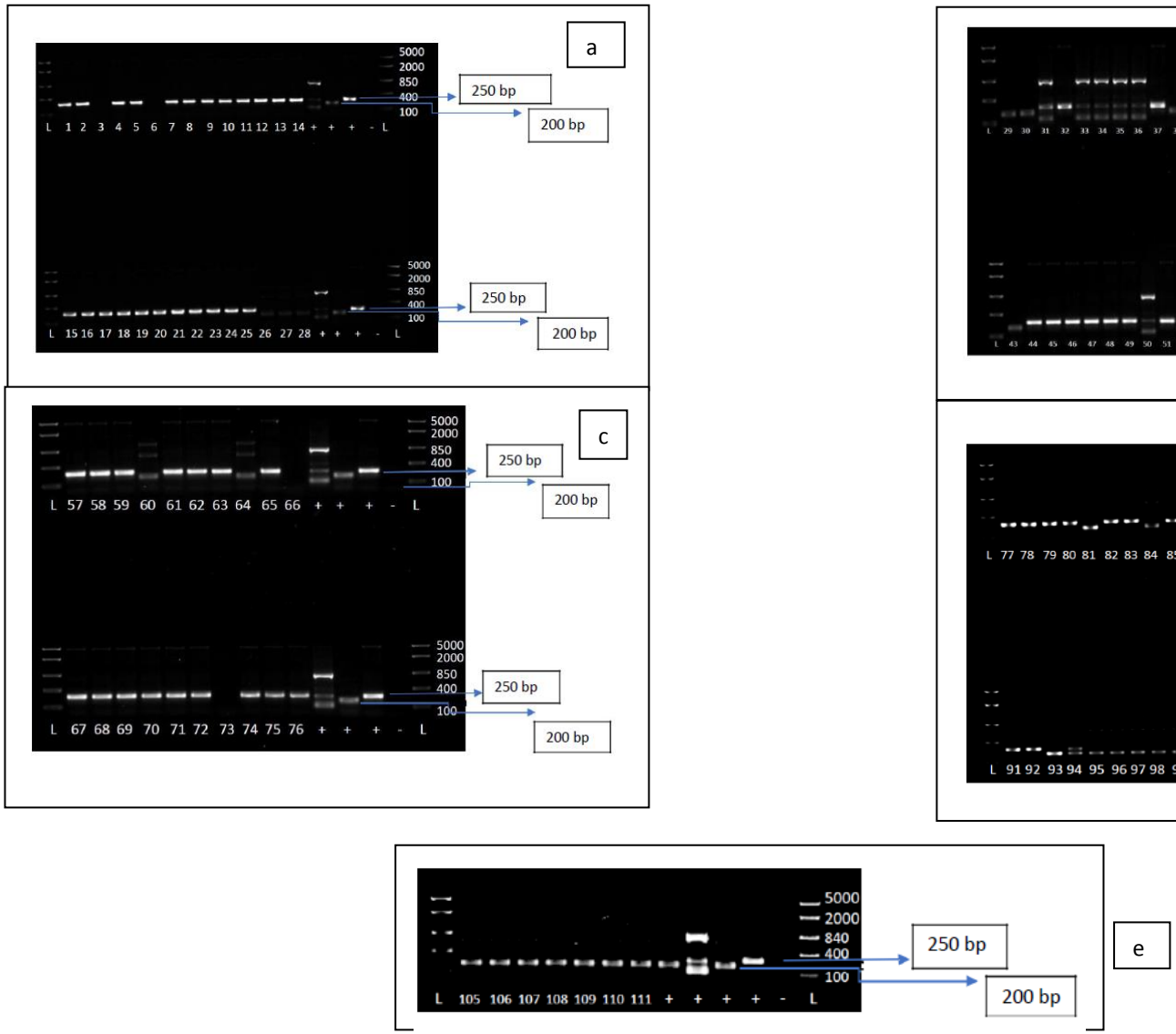

Figure 4.2: a-e: Gel electrophoresis of PCR products targeting 200bp of *Cx. pipiens* form *pipiens* and 250bp of *Cx. pipiens* form *molestus* CQ11 gene - L- Fast ruler Middle -range ladder, (1-111) represents mosquito samples screened, (+) is the positive control for *Cx torrentium Cx. pipiens* form *pipiens,* and *Cx. pipiens* form *molestus* respectively and (–) is the negative control. Double bands represent hybrids.

Whilst one hundred and eleven mosquitoes were amplified by CPIJ001674 gene, the generated sequences did not match with either the *Cx. pipiens* bioforms; *pipiens* and *molestus* or *Cx. torrentium* after blasting against NCBI sequences (Table 4.2). It was not a valuable marker for identification or differentiation of the *Cx. pipiens* bioforms; *pipiens* and *molestus* and *Cx. torrentium* in our study.

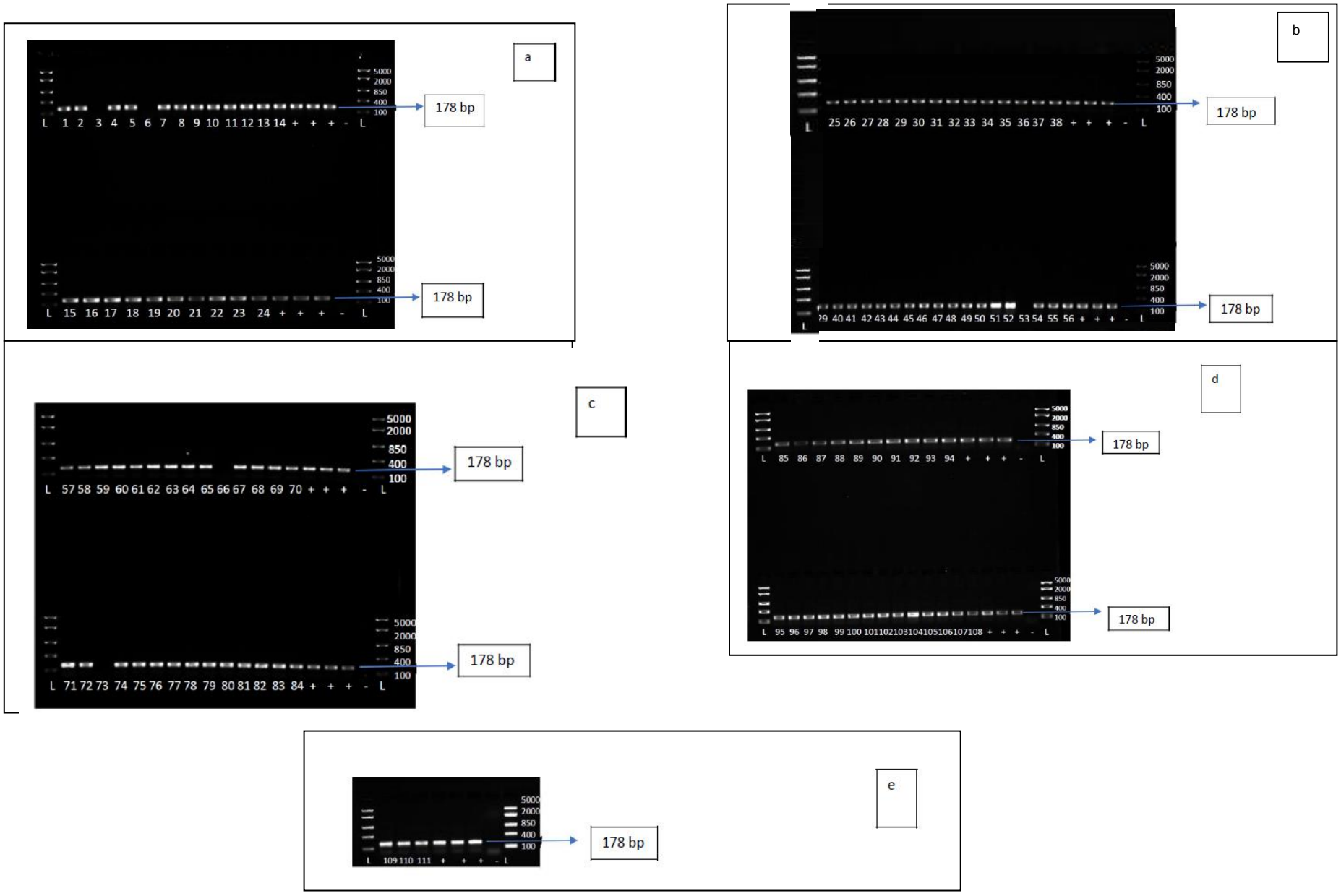

Figure 4.3: a-e. Gel electrophoresis of PCR products targeting 178bp of CPIJ001674 gene - L-Fast ruler Middle -range ladder, (1-111) represents mosquito samples screened, (+) is the positive control for *Cx torrentium Cx. pipiens* form *pipiens,* and *Cx. pipiens* form *molestus* respectively and (–) is the negative control.

4.3 Multiple Sequence Alignment of COI, CQ11 and CPIJ001674 genetic markers

Multiple sequence alignment (MSA) of COI, CQ11 and CPIJ001674 genes revealed presence of high conserved nucleotides in Cx. *torrentium* and *Cx. Pipiens* (Figures 4.4, 4.5 and 4.6). Multiple Sequence Alignment for the COI gene showed that *Cx pipiens* and *Cx. torrentium* shared the same nucleotides at similar positions designating their close evolutionary relationship. However, there were some notable A-G mutations in the *Cx. pipiens* sequence at position 342, G-A mutation at position 409 and A-C mutation at position 431 in the *Cx. torrentium* sequence (Figure 4.4). The MSA for the CQ11 gene revealed that *Cx. torrentium* had SNPs that were similar to either *Cx. pipiens* form *molestus* (A-A) or *Cx. pipiens* form *pipiens* (A-T) at position 131 (Figure 4.5). At position 157, both *Cx. pipiens* bioforms (pipiens and molestus) shared the same G-G polymorphism (Figure 4.5) The MSA for CPIJ001674 gene showed no distinction between *Cx. pipiens* and *Cx. torrentium* (Figure 4.6)

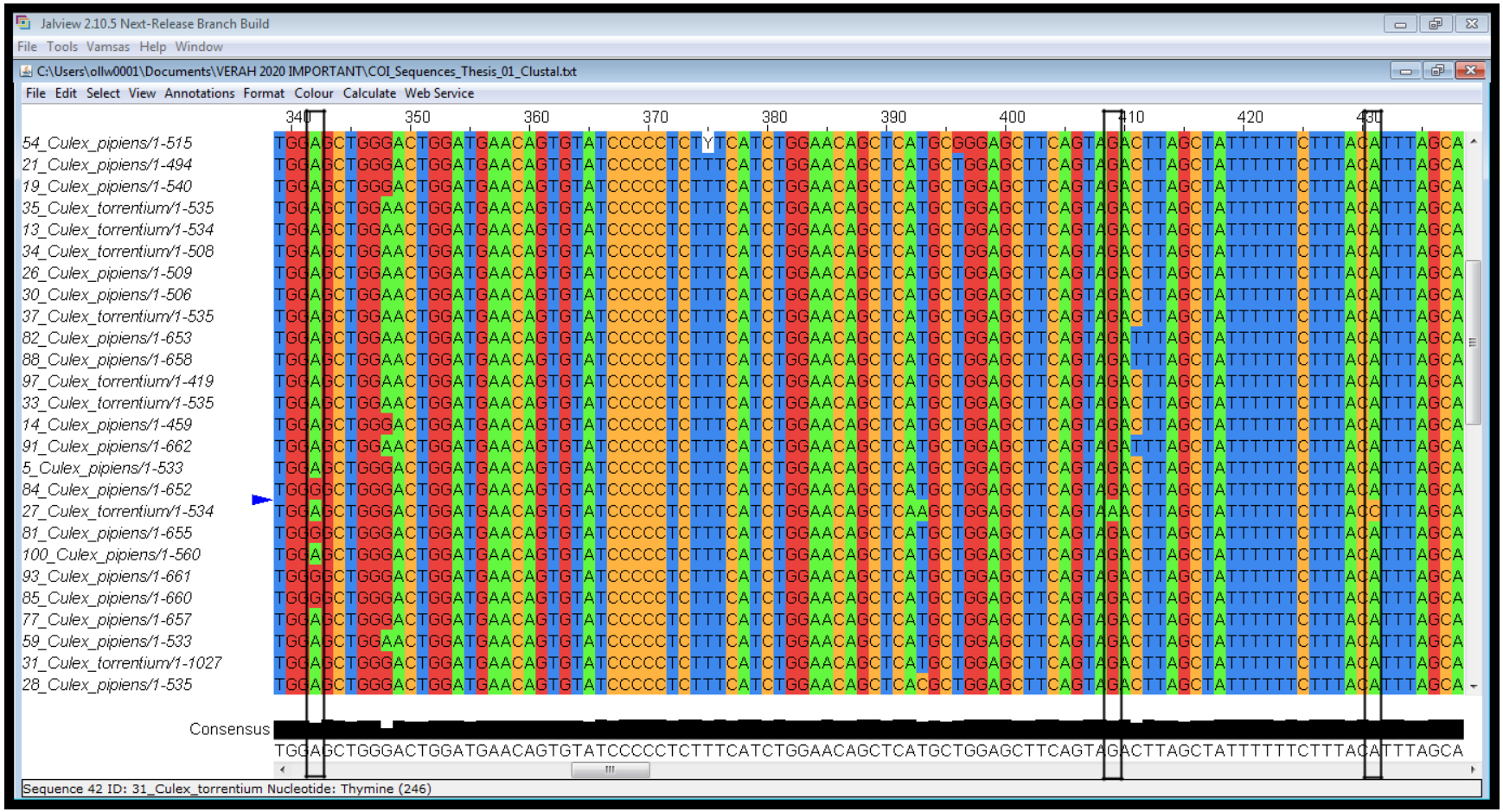

Figure 4.4: Multiple Sequence Alignment of the *Cx.* mosquito sequences generated from the COI gene

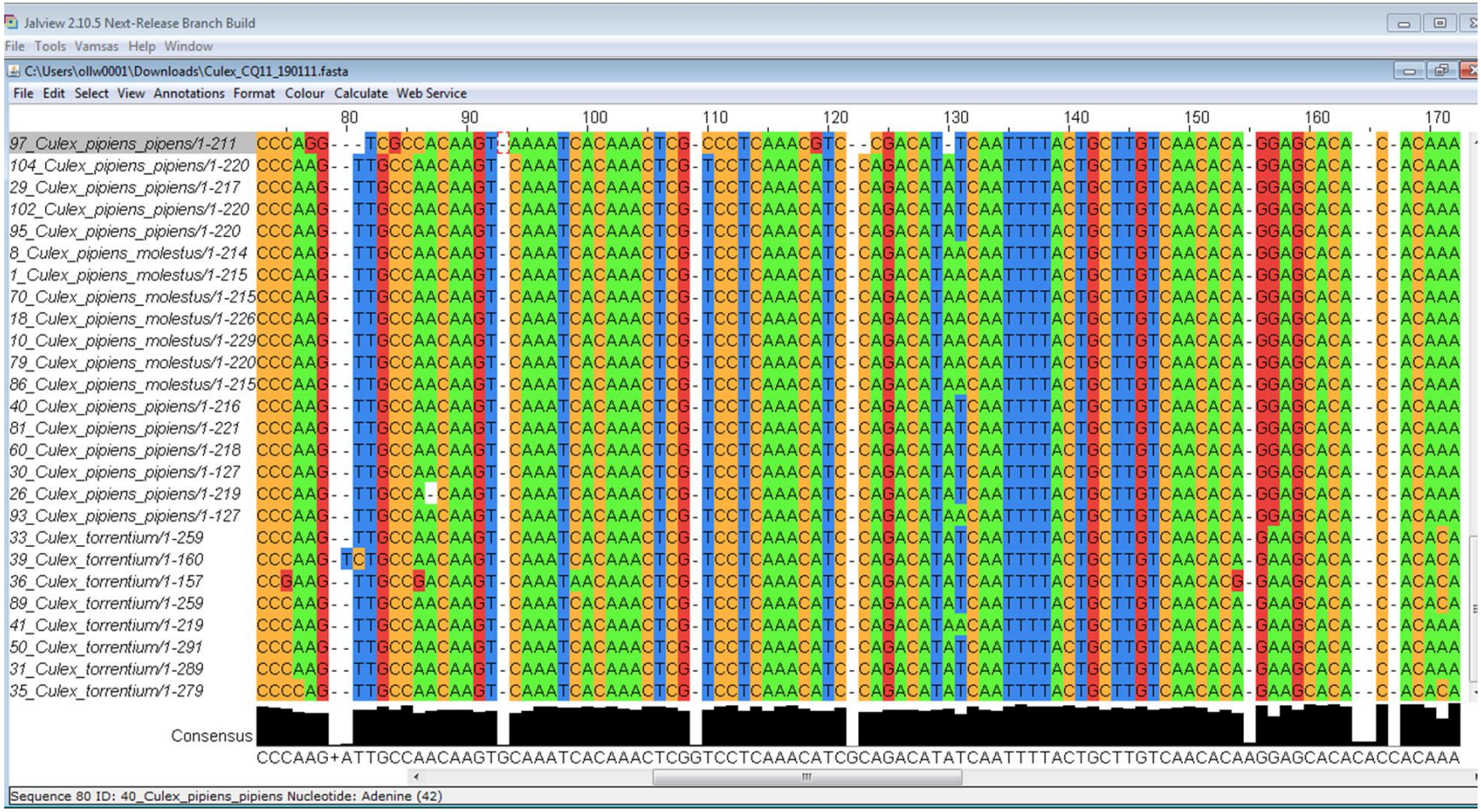

Figure 4.5: Multiple Sequence Alignment of the *Cx.* mosquito sequences generated from the CQ11 gene

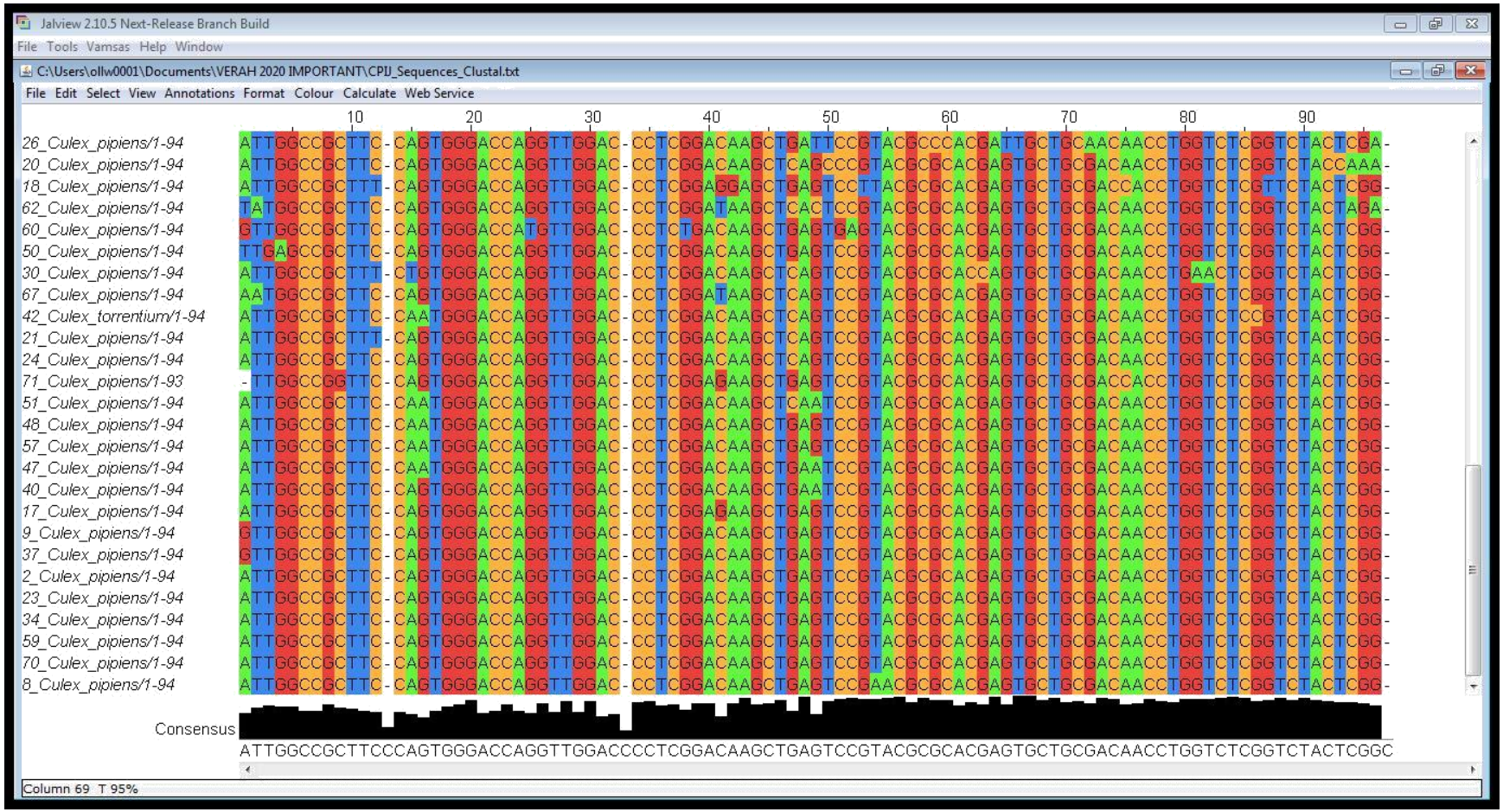

Figure 4.6: Multiple Sequence Alignment of the *Cx.* mosquito sequences generated from the CPIJ001674 gene

4.4 Single Nucleotide Polymorphisms (SNPs) in the CQ11 gene

When the PCR products of the mosquito specimens were sequenced, 2 single-nucleotide polymorphisms (SNPs) were found in the CQ11 gene which were used as signatures to¨ identify and discriminate between *Cx. pipiens* forms *pipiens* and *molestus* and *Cx. torrentium*. Interestingly, at position 131, *Cx. torrentium* had similar SNP with that of *Cx. pipiens* form *pipiens*. There was similarity in SNPs was observed in both *Cx. pipiens* form *pipiens* and *Cx. pipiens* form *molestus* at position 157. There were differences in SNPs at position 131 between *Cx. pipiens* bioform; *pipiens* and *molestus* (Table 4.2).

Table 4.2: Genetic signatures and Single-Nucleotide Polymorphism in *Cx. torrentium, Cx. pipiens* form *pipiens* and *Cx. pipiens* form *molestus*

SNP position	*Cx. torrentium*	*Cx. pipiens* f *pipiens*	*Cx. pipiens* f *molestus*
131	CATA**T**C	CATA**T**C	CATA**A**C
157	ACA-G**A**A	ACA-G**G**A	ACA-G**G**A

Underlined bold letters indicate the nucleotide found in the CQ11 gene of the *Cx. torrentpipiens* bioforms *pipiens* and *molestus*. When the mosquitoes were sequenced 2 SNPs were found in the CQ11 gene and used in this study to distinguish *Cx. torrentium* from *Cx. pipiens* bioforms (Table 4.2).

4.5 Phylogenetic analyses of *Cx.* bioforms *pipiens* and *molestus* and *Cx. torrentium* using the CO1, CQ11 and CPIJ001674

Overall, the CPIJ001674 gene phylogenetic tree showed random clustering of *Cx. torrentium* across all clades despite amplification of the specimens. There was no order in the clustering of the *Cx.* species mosquitoes as a result of the gene not being able to distinguish between *Cx. pipiens* form *pipiens* and *Cx. pipiens* form *molestus*, despite several specimens sharing the same polymorphic sites (Figure 4.6). COI gene specimens of *Cx. pipiens* grouped closely together regardless of the collection site (Figure 4.8). In addition, *Cx. torrentium* also clustered together with *Cx. pipiens* species. CQ11 sequences obtained from the PCR products from the mosquito samples analysed in the study indicated a clear distinction between the two *Cx. pipiens* bioforms and *Cx. torrentium* forming separate clades (Figure 4.9). Although the COI gene identified one specimen as *Cx. torrentium* in Simrishamn, the CQ11 microsatellite identified the same specimen as *Cx. pipiens* form *pipiens*. The tree corresponding to CQ11 gene showed well-supported clades and distinct clusters of *Cx. pipiens* form *pipiens* and *Cx. pipiens* form *molestus*. One clade had both *Cx. pipiens* bioforms (*pipiens* and *molestus*) because of putative interbreeding events that result in recombination. *Cx. torrentium* also clustered together with *Cx. pipiens* form *molestus* suggesting a possibility of hybridization events. Nodes linking sequences of individuals of the same *Cx.* species had good bootstrap support or high bootstrap values (91% -100%) whereas some linking sequences of different *Cx.* species had lower bootstrap values (59% -100%).

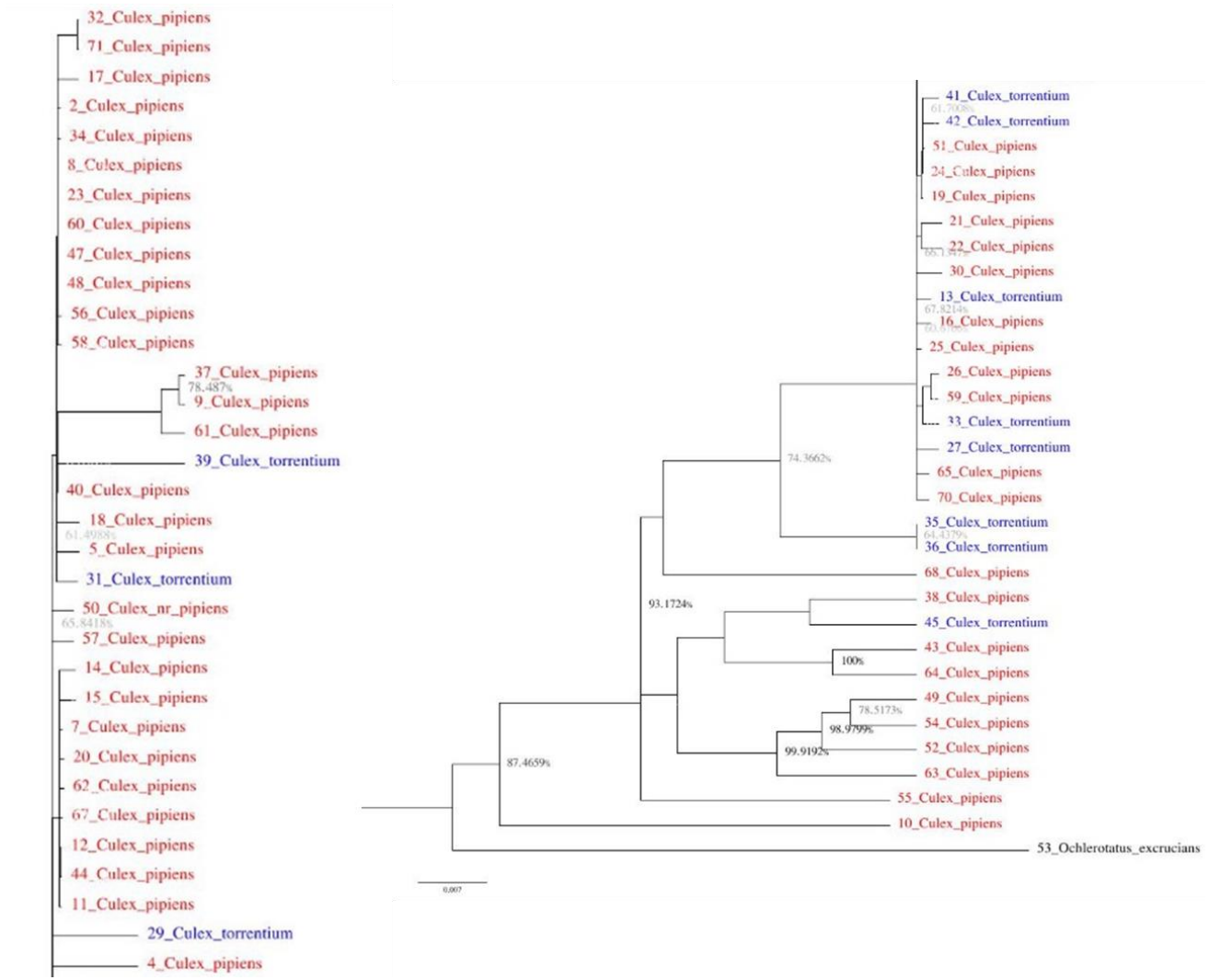

Figure 4.7: Phylogenetic analysis of CPIJ001674 gene. Red colour represents *Cx. pipiens* and blue colour indicates *Cx. torrentium*

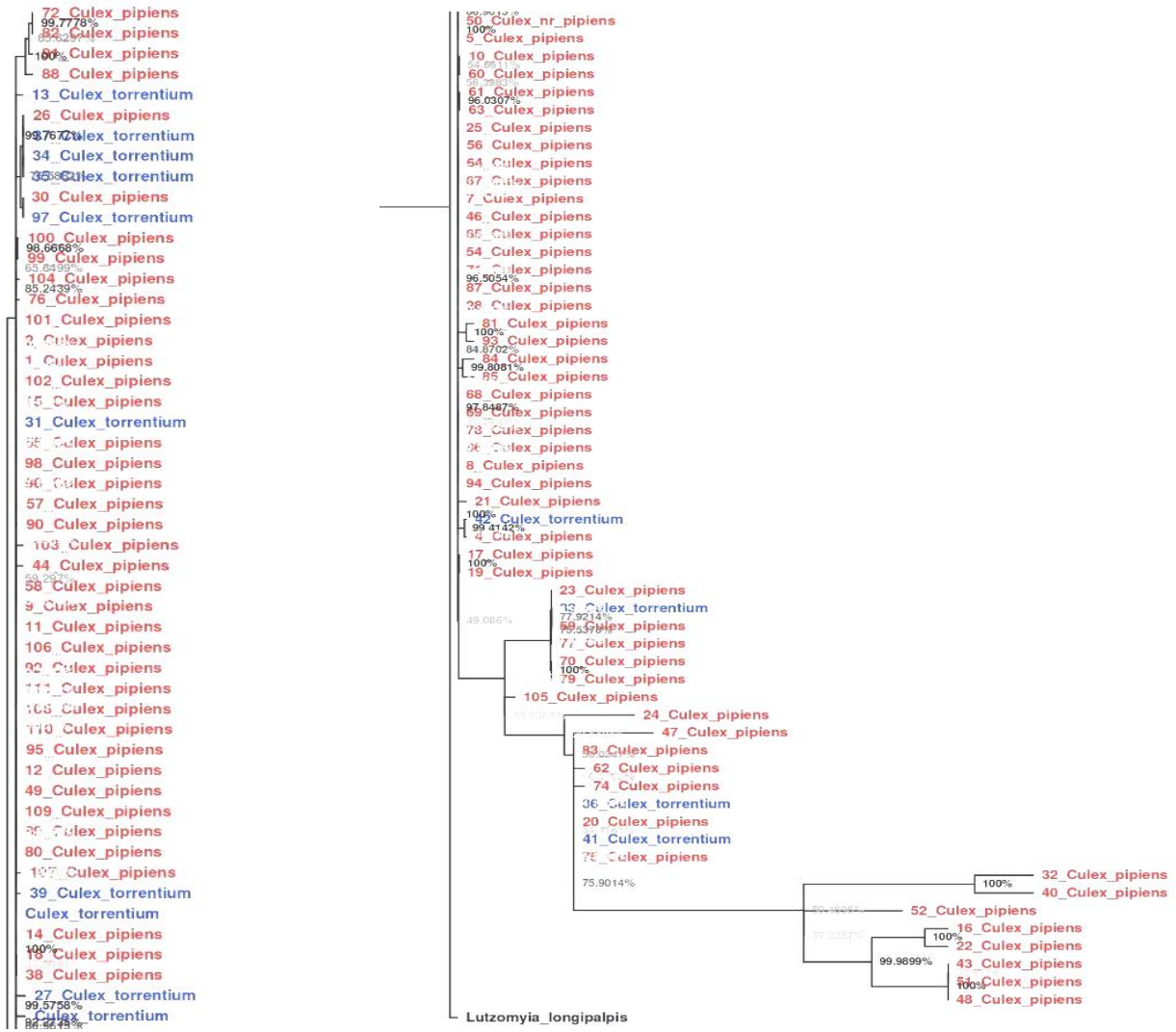

Figure 4.8: Phylogenetic analysis of COI gene. Red colour indicates *Cx. pipiens* while blue colour indicates *Cx. torrentium*

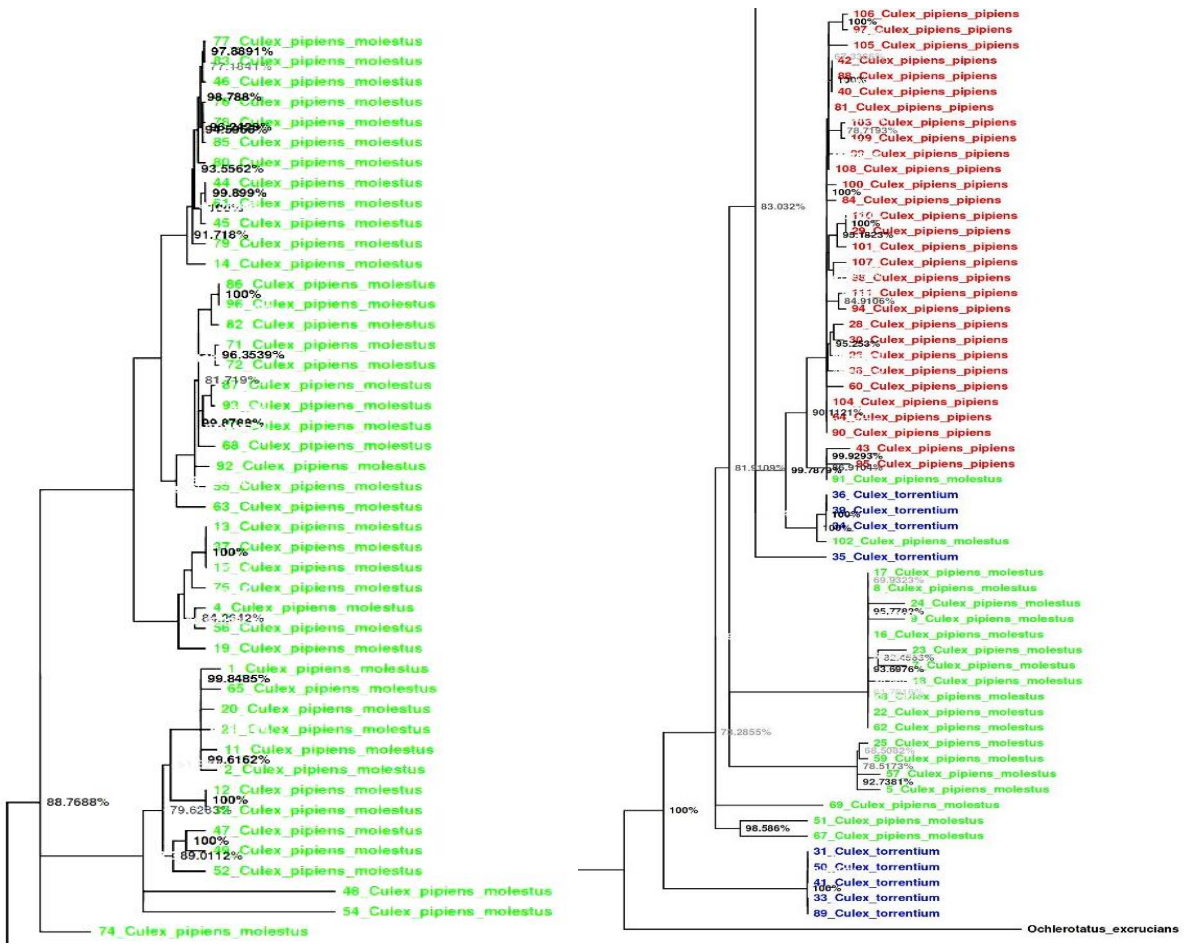

Figure 4.9: Phylogenetic tree of CQ11 gene sequences. Green colour represents *Cx. pipiens* form *molestus*, red indicates *Cx. pipiens* form *pipiens* and blue colour indicates *Cx. torrentium*

CHAPTER FIVE

DISCUSSION

This study used molecular approach in the form of DNA barcoding to discriminate between *Cx.* species mosquitoes from thirteen sites in Southern Sweden. With the known limitations of using one DNA marker, this study encompassed three molecular markers, which aimed at distinguishing between the *Cx. pipiens* bioforms and *Cx. torrentium*. DNA based markers such as mitochondrion (COI) and two nuclear markers (CQ11 and CP1J001674) were targeted in the assay for the purpose of differentiating the *Cx. pipiens* bioforms and *Cx. torrentium*. The mitochondrial COI gene identified *Cx.* mosquitoes to species level, whereas one nuclear microsatellite marker (CQ11) classified the *Cx. pipiens* bioforms, their hybrid and *Cx. torrentium*. However, the nuclear DNA marker (CP1J001674) was deemed not useful for identification of the bioforms and *Cx. torrentium*. The findings implied that despite the mitochondrial COI gene having some SNPs that are widely distributed within its sequence, it is not highly polymorphic like the CQ11 microsatellite. Therefore, it could not be utilized in differentiating the closely related *Cx. pipiens* bioforms. Nevertheless, it served as a good marker for discriminating between *Cx. pipiens* and *Cx. torrentium* species due to its conserved sequence. Moreover, the high variability in the CQ11 microsatellite loci, enabled *Cx. pipiens* bioforms, their hybrid and *Cx. torrentium* to be differentiated. The inability of the CPIJ001674 gene to distinguish *Cx*. mosquitoes from Southern Sweden may be due to high mutations and divergence in its sequence that resulted due to geographical distance. The findings highlighted the COI and the CQ11 genes as effective markers that could be used to characterize the Swedish *Cx.* mosquitoes. Moreover, data on epidemiologically important but morphologically indistinguishable *Cx. pipiens* bioforms (*pipiens* and *molestus*) and *Cx. torrentium,* and their distribution in selected sites in Sweden was also provided.

The detection of *Cx*. mosquitoes species (*Cx. pipiens* and *Cx. torrentium*) by the mitochondrial COI gene in Southern Sweden, revealed the ability of the gene to classify diverse species owing to its conserved sequence region across disparate taxa (Simonato *et al.,* 2016). Whilst

the COI barcode marker lacks distinctive variation in its sequence, which could otherwise be used in distinguishing the *Cx. pipiens* bioforms (Kothera *et al.,* 2010), it has identified the Russian *Cx. pipiens* bioforms using the PCR-RFLP method (Shaikevich *et al.,* 2007). Previous studies have pointed the ability of the CQ11 microsatellite marker to distinguish between the *Cx. pipiens* bioforms and their hybrids thus also provided insights into their ecology (Amraoui *et al.,* 2012, Krida *et al.,* 2015, Reusken *et al.,* 2010). Nevertheless, CQ11 locus has been reported to misidentify *Cx. torrentium* in southern England and Wales in the UK (Danabalan *et al.,* 2012). Efforts have been made to identify the hybrids of the *Cx. pipiens* bioforms (*pipiens* and *molestus*) with the ACE-2 assay besides CQ11 microsatellite marker (Bahnck and Fonseca, 2006) due to its rapid detection of the hybrid specimens.

In southern Europe, the division between the two *Cx. pipiens* 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 et al., 2014, Vogels et al., 2015, Vogels et al., 2016). Compared to the few previous reports of *Cx pipiens* form *molestus* in Sweden (Hesson *et al.,* 2016), this study found specimens in several rural areas in Sweden, namely Sollebrunn and Horby where we would not have expected the *molestus* bioform to be present. Nevertheless, this study only analyzed specimens sent in by people experiencing mosquito nuisance in the south of Sweden, which leads to a biased sample which makes it hard to evaluate the true distribution of the *molestus* bioform. Interestingly, in this study, both bioforms were found co-existing in Göteborg suggesting the possibility of hybrid populations as supported by other studies (Rudolf *et al.,* 2013, Osório *et al.,* 2014). This could also indicate that the bioform may be present in more places if investigated more widely and with a variety of collection methods. In addition one hybrid was also detected in Simrishamn by the CQ11 locus.

Detection of both bioforms and their hybrids in sympatry, especially in Gothenburg which is the second largest city in Sweden, is a key finding considering that these bioforms exhibit unique behavioral, physiological, and reproductive traits (Osório *et al.,* 2014, Vogels *et al.,*

2015). These characteristics are important factors that may influence the outcome of disease transmission to humans (Savage *et al.,* 2007). For instance, *Cx. pipiens* form *pipiens* undergoes diapause and lays eggs only after ingesting a blood meal of mainly avian hosts and is mostly found on above ground habitats where it can mate freely (Vinogradova, 2003). In contrast, *Cx. pipiens* form *molestus*, does not undergo diapause, lays eggs without a blood meal, mates in confined spaces, and bites humans to acquire blood (Huang *et al.,* 2008). Each of these bioform-specific traits could guide the understanding of the population structure and distribution of these mosquito complex in Sweden. This will in the long run, guide the design of appropriate preventive and control measures through generation of infectious disease models and risk maps.

Results obtained from the phylogenetic analysis of the CQ11 gene point to the possible hybridisation events in *Cx. pipiens* bioforms, which may lead to the formation of bridge vectors, which consequently could increase the transmission of viruses by *Cx*, e.g., SINV or WNV to humans. Therefore, the distribution and prevalence of the hybrid population of *Cx. pipiens* bioforms need to be further investigated in Sweden.

Besides, hybridization between these two bioforms could encourage opportunistic feeding behaviour in *Cx. pipiens* forms hence increasing the relative importance of host availability and host defensive mechanisms in the feeding pattern of the mosquito population (Fonseca *et al.,* 2004). Therefore, the distribution and prevalence of the hybrid population of *Cx. pipiens* bioforms need to be further investigated in Sweden. The COI marker identified *Cx. pipiens* and *Cx. torrentium* specimens to species level, it lacked the genotype that was observed in Russian *Cx. pipiens* form *molestus* specimens that was used in differentiating the *Cx. pipiens* bioforms (Shaikevich, 2007). Consequently, it is not a reliable marker for differentiating between the two *Cx. pipiens* bioforms in Sweden. The polymorphism found in the CPIJ001674 gene sequences that was diagnostic between the *Cx. pipiens* bioforms in a recent finding was not useful in our study since it could not clearly differentiate between the *Cx. pipiens* and *Cx. torrentium* species (Kim *et al.,* 2018). Surprisingly, the same gene marker

(CPIJ001674) was able to discriminate between bioforms and hybrids in mosquitoes in the US (Kim et al., 2018).

There is a possibility that different markers are influenced by diverse introgression levels that exist in different genomic regions in the hybrids between the two bioforms, a situation that warrants further research. In addition, mechanisms behind hybridization events among the members of *Cx. pipiens* complex species in Sweden compared to other geographically distant populations, for example, could provide insights into better understanding of introgression patterns in this mosquito complex. The design of bioform specific DNA marker using SNPs that target key mosquito genes could be the best way of unravelling challenges emanating from DNA barcoding of the *Cx.* mosquito complex. Knowledge of the distribution and identification of Cx. *pipiens* bioforms and *Cx. torrentium* could potentially be used in disease surveillance and control efforts to curb the risk of spread of arboviruses to humans.

5.1 Conclusions

1. The COI and CQ11 markers were able to differentiate *Cx. pipiens* from *Cx. torrentium* species in Southern Sweden
2. The CQ11 microsatellite was effective in discriminating within *Cx. pipiens* bioforms and between the bioforms and *Cx. torrentium* in Southern Sweden. However, the CPIJ001674 gene was not a valuable marker for differentiating the bioforms and *Cx. torrentium.*
3. Phylogenetic analysis of the CQ11 gene indicated a clear distinction between the two *Cx. pipiens* bioforms and *Cx. torrentium* forming separate clades

5.2 Recommendations

A high throughput sequencing of the entire *Cx* mosquito genome could unravel novel genetic markers that could be useful in accurate characterization of *Cx pipiens* bioforms, their hybrids and *Cx. torrentium*. This will enable further screening and analysis of *Cx.* mosquitoes in the entire regions of Sweden, in order to accurately discriminate between the *Cx. pipiens* bioforms and *Cx. torrentium*.

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2019-04-04

Tobias Lilja, PhD

Letter of recommendation, Verah Nafula Luande

To whomever it may concern:

I write to you in support of Verah Luande who did her master project at SVA under my supervision during the fall of 2018. During this time, she used DNA based methods for species identification of mosquito species involving PCR, sanger sequencing, sequence alignment and phylogenetic methods.
Verah worked independently on molecular biology techniques such as DNA extraction, PCR and agarose gel electrophoresis. She is also familiar with Sequence alignment and phylogenetic analysis of DNA sequences. Verah worked independently and was focused to finalize her project.
Verah quickly gained an understanding of the field and the relevant methods.

I would be happy to answer any further questions via telephone or email.

Best Regards

Tobias Lilja, PhD

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