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Molecular identification of mosquito species

Evaluation of a rapid DNA extraction method together with DNA barcoding as a tool for identification of species

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Abstract

The current method to determine a mosquito specimen to a certain species is by morphological keys basically following the taxonomy developed by Carl Linnaeus in the 1700. Since Watson and Crick presented their model of the double-helix DNA in 1953, a new era of molecular based taxonomic studies have revolutionized the field. The revolution is not in terms of how the classification of species is done but how the biological diversity is seen. However, morphological, ecological and behavioral characteristics are still important and are used together with the information a gene or whole genome can give. DNA barcoding is one of the promising methods for molecular identification. A small segment of a gene, approximately 400-1000 base pairs (bp), are examined by a Polymerase chain reaction (PCR) and sequencing. Like the barcodes in the grocery store these sequences work like unique ID: s for every species. This thesis shows how a fast DNA extraction method could be combined with DNA barcoding to get a 658-bp segment of the mitochondrial gene cytochrome c oxidase subunit 1 (COI) from different species of the mosquito family Culicidae. A total of 15 thoraxes or wings, from individual specimen of mosquitoes, were examined and 11 different barcode sequences could be retrieved. Six correspond to already published COI sequences and could therefore be determined to the species level, including a sequence from a new species for Sweden, *Aedes (Ochlerotatus) nigrinus*. All mosquitoes were collected during the national inventory of species in summer of 2012 in Sweden, ”Myggjakten”, and have been morphological examined by experts at the National Veterinary Institute (SVA) prior to molecular determination. This thesis also highlights the importance of building a reference library of barcode sequences, so DNA barcoding could become an effective diagnostic tool. Inventory projects like “Myggjakten” may, if repeated, provide excellent material for such a library collection of barcode data.

Sammanfattning

När en stickmyggsart skall artbestämmas är den vanligaste metoden att använda morfologiska nycklar. I princip görs det här efter den taxonomi som Carl von Linne utvecklade på 1700-talet. Men sedan Watson och Crick presenterade sin DNA modell 1953 så har dock en ny era av molykylärt baserade metoder revolutionerat taxonomin. Förändringen består egentligen inte i hur vi klassificerar och använder taxonomin utan mer hur vi ser på den biologiska mångfalden. Morfologiska och ekologiska studier, samt studier av arters beteende, är fortfarande viktiga och komplementerar den molekylära informationen från ett genom eller från en enstaka gen. DNA barcoding är en av de lovande nya molekylära metoderna för artbestämning. Ett litet segment av en gen, på ungefär 400-1000 baspar (bp), undersöks med hjälp av polymeras-kedjereaktion (PCR) och sekvensering. Likt streckkoder i livsmedelsbutiken ger metoden ett unikt ID för varje art. Den här studien visar hur en snabb DNA-extraktionsmetod kan kombineras med DNA barcoding, för att ge en 658-bp lång DNA-sekvens, från den mitokondriella genen cytokrom c oxidas subunit 1 (COI) från olika arter av myggfamiljen Culicidae. I undersökningen ingick 15 mellankroppar eller vingar från individuella stickmyggor och av dessa kunde 11 olika barcode sekvenser utläsas. Sex av dessa stämde överrens med redan publicerade COI-sekvenser och kunde bestämmas till artnivå, varav en av sekvenserna kommer från den nyligen i Sverige funna morfologiskt artbestämda *Aedes (Ochlerotatus) nigrinus*. Stickmyggorna i detta arbete insamlades av privatpersoner på olika ställen i Sverige under sommaren 2012 i det nationella mygginsamlingsprojektet ”Myggjakten”. Dessa artbestämdes morfologiskt av personal på Statens veterinärmedicinska anstalt (SVA) innan de artbestämdes molekylärt. Det här arbetet belyser även vikten av att bygga upp ett referensbibliotek av barcode sekvenser för att DNA-barcoding ska kunna bli ett effektivt diagnostiskt verktyg vid studier av vektorburna zoonoser. Nationella projekt som Myggjakten kan vara mycket användbara för insamling av sådana data.

Contents

Abbreviations **4**

1 Introduction **5**

1.1 Vector borne diseases 5

1.2 Species identification and DNA barcoding 5

1.3 Inventory project “Myggjakten” 7

1.4 Aim of the thesis 7

2 Material and methods **7**

2.1 Specimen collection 7

2.2 DNA extraction 7

2.3 DNA amplification and sequencing 7

2.4 Data analysis 8

3 Results **9**

3.1 DNA amplification 9

3.2 Sequence diversity 10

4 Discussion **11**

5 Appendix **13**

Appendix I 13

Appendix II 14

References **18**

Acknowledgement **21**

Abbreviations

AFLP	Amplified fragment length polymorphism
BOLD	Barcode of Life Database
bp	Base pair
BTI	*Bacillus thuringiensis* ssp. *Israelensis*
COI	Cytochrome c oxidase subunit 1
ITS2	Internal transcribed spacer 2
K2P	Kimura two-parameter
mtDNA	Mitochondrial DNA
NGS	Next-generation sequencing
PCR	Polymerase chain reaction
SNP	Single nucleotide polymorphism
WNF	West-Nile fever
WNV	West-Nile virus

1 Introduction

Mosquitoes are nematocerid flies that belong to the insect order **Diptera** and family Culicidae. There are around 3500 different Culicidae species in the world (Harbach and Howard, 2007), and about 50 species can be found in Sweden. Massive hatching of river floodland mosquitoes, like Aedes (*Ochlerotatus) sticticus* and *Aedes cinereus (Ae. cinereus)*, cause nuisance for humans and animals every year. In some areas (e.g. Nedre Dalälven) the problem is so severe that the authorities chose to take action by spraying the biological insecticide BTI (Lundstrom, 2006). More importantly, these mosquito outbreaks increase the risk for zoonosis as many vector borne diseases (e.g. arbovirus and parasites) are associated with mosquitoes. The mechanisms behind the mosquito outbreaks around Nedre Dalälven are still unclear but it is supposed that a massive accumulation of water, from rain or water plant in the area causes the problem. Efforts towards a more sustainable strategy to the problem, not least from an infection control perspective, have been initiated by the Swedish government (Gerhardt et al., 2013).

1.1 Vector borne diseases

Although the majority of the Swedish Culicidae are potential disease vectors Sweden, has been spared from major mosquito borne diseases. Exceptions include malaria, caused by *Plasmodium* sp., which was common until the 19th century (Hulden and Heliovaara, 2005), and the current "Ockelbosjukan", caused by the Sindbis virus, affecting several people every year. The main vectors are considered to be species from genus *Culex*- and *Culiseta* (Lundstrom et al., 1990), but recent research have shown that *Ae. cinereus* maybe just as important for the virus distribution (Lundstrom et al., 2007; Turell et al., 1990).

If the climate changes to rainier and warmer in northern Europe, it´s possible that vectors like *Anopheles* could start to carry *Plasmodium* species again (Jaenson et al., 1986). The risk for introduction of new arboviruses in Sweden increases as well. WNF is a zoonotic disease, caused by WNV which is a single stranded RNA virus, of genus *Flavivirus*. The main hosts are birds and the virus can spread to new areas when infected birds migrate. Female mosquitoes pick up the virus and infect other birds, reptiles and mammal species including humans. Humans infected with WNV are usually asymptomatic but around 20 % get WNF and 1 % get West-Nile neuroinvasive disease with encephalitis (Davis et al., 2006). No human vaccine is currently available. The virus is widely distributed and established in Africa, Oceania, North America, South and Central Asia and southern Europe (Dauphin et al., 2004; Zeller and Schuffenecker, 2004) and causes epidemics every year. Mosquito species from the genus *Culex* together with *Coquillettidia richiardii* (*Cq. richiardii*) are considered to be the main vector for WNV (Hayes et al., 2005). *Cq. richiardii* and the vector complex of *Culex pipiens* (*Cx. pipiens*) *are* potential carriers of WNV in Sweden. Tularemia is caused by the bacterium *Francisella tularensis holarctica* in Sweden and causes epidemic every year (Ryden et al., 2012). The mechanism behind the spreading of the bacteria is currently unknown, but research suggested that larvae from *Ae. sticticus* are exposed to the bacterium through protozoa (Lundstrom et al., 2011; Ryden et al., 2012). Another risk with a warmer climate is that mosquito species could be introduced to new areas. The "Tiger mosquito", *Aedes albopictus,* a tropic and subtropic species that is a vector for around 20 different arboviruses that can cause diseases such as WNF, Rift Valley fever and Dengue fever, has been found in southern Germany (Scholte and Schaffner, 2007).

1.2 Species identification and DNA barcoding

The method to identify, label and classify organisms is largely built around morphological characteristics. It was developed by Carl Linnaeus in the 18th century and his taxonomic system is to a large extent still used. Today taxonomists also consider physiology, behavior and population biology

in the classification of new species. Since the discovery of DNA and recognition of its role in inheritance, genetic variation plays a major role to distinguish the diversity of life. Morphological identification of species is obviously limited since it does not consider phenotypic plasticity, genetic variation of individuals or morphological complexity (e.g. cryptic taxa or keys only developed for certain gender or life stage) (Hebert et al., 2003a). DNA based identification could fill these gaps and most importantly add new biological diversity to the already known. Furthermore, molecular based methods are generally used for species identification of viruses, bacteria and protozoa, in taxonomic studies (Adl et al., 2007; Edwards and Rohwer, 2005; Pace, 1997). In a more general way, whole genome sequencing and other sequencing based methods are used to identify diversity. Another approach are screening for biological markers such as microsatellites, AFLP or SNP (Arif et al., 2010). This approaches is however not suitable for phyla like Arthropoda because of the extensive biological diversity. It would be expensive and time consuming. A more suitable way to discriminate species is to analyze small gene segments, so called DNA barcoding (Hebert et al., 2003a; Stoeckle, 2003; Stoeckle et al., 2003). What would be the most appropriate gene segment for this kind of analysis has been debated, because the rates of molecular evolution differ depending on which part of a genome is being investigated. However, in animals, the mitochondrial genome seems to be better suited for barcoding than the nuclear genome because it has no introns and rarely undergoes recombination. Mitochondrial genes' maternal inheritance is also beneficial for barcoding (Saccone et al., 1999). Previous work on barcoding has focused on mitochondrial ribosomal (12S, 16S) DNA genes. However, insertions and deletions (indels) are common in these genes, thus complicating the sequence alignments (Doyle and Gaut, 2000). In plants and fungi the ITS2 region of nuclear ribosomal DNA, seems to be a better choice for barcoding (Chen et al., 2010).

There are 13 mitochondrial genes encoding protein in animals and the choice which one to use is not obvious. Some previous barcoding studies have been done on the cytochrome b oxidase gene, but another gene that should be considered is COI (Hebert et al., 2003b). It has areas with relatively conserved sequences and therefore universal primers developed for this gene are very robust (Folmer et al., 1994). In comparison to other mitochondrial genes, the phylogenetic signal from COI seems to have greater output. Base substitution in third-positions nucleotide occurs more frequently which makes its rate for evolution in the gene fairly high (Knowlton and Weigt, 1998; Stoeckle, 2003). Although intra specific variations are less than 2 % in the COI gene (Simon et al., 1994), it´s enough to separate species phylogeographic and still discriminate closely related species (Palenko et al., 2004). This is because divergence in inter specific variation is more than 5-fold higher in average (Stoeckle et al., 2003).

DNA barcoding is today done routinely using ordinary PCR and Sanger sequencing. However a new generation of sequencing techniques, referred as NGS, has been developed in the past decade. The technique combines DNA capturing, PCR and sequence reaction, and hence could be suitable for DNA barcoding.

To support the barcoding movement and make sequence data more accessible to other DNA barcoders, researchers, and to the public the international Consortium for the Barcode of Life (CBOL) has been founded. This is an effort to make DNA barcoding a global standard for biological identification and around 200 associated organizations from 50 countries work together to promote barcoding. One of the consortium members is the Mosquito Barcode Initiative (MBI) and their goal is to have DNA barcodes from at least 5 specimens of 80 % of all Culicidae species. BOLD is the supporting portal. It provides the users with analytical tools, computational resources and a structured repository for data. Data from barcoding is linked to taxonomy, geographical sites and images. The aim is to have a reference database with validated sequence records for all species, from all taxa (Ratnasingham and Hebert, 2007).

1.3 Inventory project "Myggjakten"

The knowledge of the geographic distribution of the mosquito fauna in Sweden is poor. The last major survey was 1977 which was based on mosquito collections from museums. In the summer of 2012, the National Veterinary Institute (SVA) started a national project, "Myggjakten" (SVA, 2013), to determine the geographical frequency and collect as many species as possible. A broader knowledge of the mosquito species and prevalence is of major importance for an effective control of vector borne diseases. Individuals from all around Sweden kindly agreed to collect mosquitoes and sent them in for counting and morphological characterization. For most of the specimens a morphological determination is relatively uncomplicated. However, the morphological determination could be difficult if the specimens are in bad condition or species exists in complexes, where the adults are inseparable based on mosquito morphological characteristics. In these cases DNA barcoding with universal mtDNA primers could be a valuable tool. In Sweden only one, not published, barcoding study of Swedish mosquitoes, has been done before.

1.4 Aim of the thesis

The aim of this thesis has been to test the concept of DNA barcoding for species identification of Swedish mosquitoes. It has been based on a rapid DNA extraction method together with different body parts of mosquitoes and a modified PCR thermal protocol from a barcoding studies of biting midges (*Culicoides*)(Ander et al., 2012).

2 Materials and methods

2.1 Specimen collection

The mosquitoes chosen for molecular processing were collected during "Myggjakten" in 2012 in Sweden using Mosquito Magnets (Woodstream Corp., USA). The collected mosquitoes were exclusively adult females. The specimens chosen were first identified using standard taxonomic keys (Becker, 2010) and then checked in Genbank (Benson et al., 2013) and Bold Systems v3 (Ratnasingham and Hebert, 2007) for mtDNA sequences matching the COI barcode region of choice. A full list of the specimen and its geographical site where it was collected is shown in *Appendix I*.

2.2 DNA extraction

For the first experiments a wing, or (the innermost) part of a wing, from individual insects was used. In the second part, the thorax from individuals was used. The part chosen was transferred to a plastic tube, to which 30 µL of PrepMan Ultra (Life Technologies, Inc., Carlsbad, CA, U.S.A.) was added. The wing or thorax was then homogenized with a motorized polypropylene pestle (Sigma-Aldrich Corp., St Louis, MO, U.S.A.) for 30 s. The homogenate was briefly centrifugated before it was lysed at 100°C for 10 min. Samples were kept on ice for 2 min and remaining tissue debris was removed by centrifugation at 13 200 x g for 3 min, after which 20 µL of the supernatant was transferred to a fresh tube (Ander et al., 2012).

2.3 DNA amplification and sequencing

An approximately 710-bp fragment including primers of the barcoding region of COI was amplified using the universal primers LCO1490 (5´-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5´-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al., 1994). The PCR volume was 15 µL consisting of 1x PCR buffer, 2.5 mM $MgCl_2$, 200 µM each dNTP, 0.5 µM of each primers, 0.5 units of AmpliTaq DNA polymerase (Life Technologies, Inc.), 1 µL of DNA template and Super-Q water for the

remainder(Ander et al., 2012). The PCR thermal protocol was a single cycle of 94°C for 2 min and 15 s, followed by 30 cycles at 94°C for 30 s, 57°C for 25 s, 72°C for 45 s, and a final elongation step 72°C for 5 min. The optimal temperature for annealing was determined with thermal gradient PCRs and the gradients consisted of 8 temperatures between 45 and 65°C or between 52 and 62°C. The PCR thermal protocol was 94°C for 2 min and 15 s, followed by 30 cycles at 94°C for 30 s, 45/65°C or 52/62°C for 25 s, 72°C for 45 s, and a final elongation step 72°C for 5 min.

The PCR products were visualized on a 1.5 % agarose gel (BioNordika, Denmark) stained with GelRed (Biotium, USA). If a clean 710-bp band was seen, 10 µL of the product were cleaned from unused primers and nucleotides by JetQuick PCR Purification Spin Kit (Genomed, Germany), according to the manufacturer's protocol.

Sequence reactions were carried out in both directions using the same primers. The sequence reaction volume was 20 µL and consisted of 2 µL Big Dye Terminator v3.1 (Life Technologies, Inc.), 3 µL sequencing buffer, 1.8 µL primer (2 µM), 10 µL clean PCR product and Super-Q water for the remainder. The thermal protocol was a single cycle at 96°C for 1 min, 25 cycles at 96°C for 10 s, 50°C for 5 s and 60°C for 4 min. Sequence products were cleaned up using ethanol precipitation. 2 µL sodium acetate (3 M, pH 5.2) and 50 µL 99 % ethanol were added to the products. A short vortex was performed before incubation at room temperature for 20 min followed by centrifugation for 20 min at 13 200 x g (4°C). The ethanol was removed without interfering with the pellet. 250 µL of 75 % ethanol was added and the pellet was displaced by tapping on the tube followed by centrifugation for 20 min at 13 200 x g (4°C). All ethanol was then removed and the pellet was dried in the dark for 20-30 min at room temperature before storage at -20°C. Before the sequence analysis 13 µL formamide was added to the tube with the pellet. The clean resolubilized products were then injected into a Genetic Analyzer 3100 (Life Technologies, Inc.) under standard conditions.

2.4 Data analysis

Sequences from forward and reverse primers, from individual mosquitoes, were aligned and assembled to a consensus (contig) sequence using CLC Main Workbench 6 (CLC bio, Denmark) and then trimmed to 658-bp barcode-sequences using BioEdit (Hall, 1999). The Barcode sequences were compared and aligned, using ClustalΩ (McWilliam et al., 2013) with standard parameters, with sequences from Genbank and BOLD Systems v3. A neighbor-joining phylogenetic tree based of K2P genetic distance (Kimura, 1980) was created, with MEGA version 5 (Tamura et al., 2011). This was to visualize and demonstrate the divergence in sequence obtained and to compare them to sequences held in Genbank and BOLD Systems v3. Primarily sequences from Scandinavian specimens were chosen and after that sequences of specimens from United Kingdom or Germany. Additionally, a number of sequences from Canada and United States, available in Genbank or BOLD systems v3, were included.

3 Results

3.1 DNA amplification

The result of the first gradient PCR (45/65°C) indicated that the optimal temperature is around 55°C (not shown). To find the optimal temperature a second gradient PCR was performed with temperatures ranging from 52 to 62°C as shown in Figure 1. In ratio of strong fragment, and appearance of unspecific fragment, 57°C were chosen for annealing temperature for the rest of the PCR experiments.

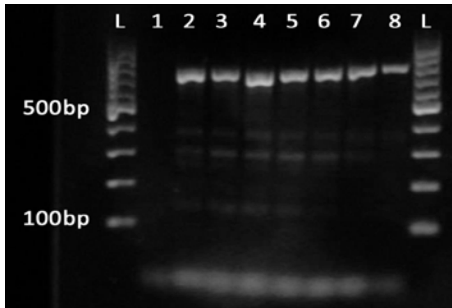

Figure 1. 1.5 % agarose gel of PCR thermal gradient of annealing temperature (52/62°C) from DNA of innermost part of wing from *Cq.richiardii* (A). Ladder (L) (O´GenRuler 100bp DNA Ladder (Thermo Fisher Scientific, USA)), lane 1: PCR negative control, lane 2: 52°C, lane 3: 52.9°C, lane 4: 54.3 lane 5: 56°C lane 6: 58.3°C lane 7: 60.1°C and lane 8: 61.3°C. 62°C was not available.

Amplification gave approximately 710-bp fragments from all amplified DNA templates shown in figures 2 and 3, aside from lane 5 (Figure 3). The lack of amplification in this case is probably because of too small amounts of DNA or the presence of PCR inhibitors. Lane 4 (figure 2) has a weak band of approximately 710-bp, but also a stronger band, around 500-bp. This may be due to an unspecific annealing of primers, caused by presence of contaminating (non vertebrates or non invertebrates) DNA in the sample.

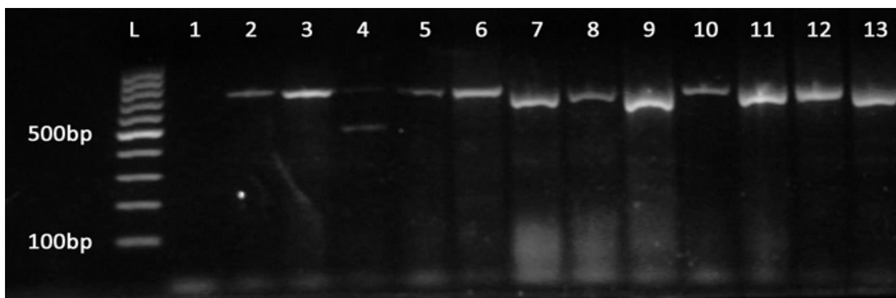

Figure 2. 1.5 % agarose gel of PCR amplicons from DNA of thorax of individual mosquito specimen derived from primer pair LCO1490 and HCO2198. Ladder (L) (O´GenRuler 100bp DNA Ladder (Thermo Fisher Scientific)), lane 1: PCR negative control, lane 2: *Cq. richiardii* (A, positive control), lane 3-5: *Ae. cinereus/geminus* (B to D), lane 6-8: *Ae. (Oc.) punctor/punctodes* (E to G), lane 9-11: *An. maculpenis sl.* (J to L), lane 12: *Cx. sp*. (P) and lane 13: *Cx. pipiens/torrentium*(M to O).

Figure 4 shows purified PCR amplicons before sequence reactions. All purified amplicons were sequenced, including the product from lane 16.

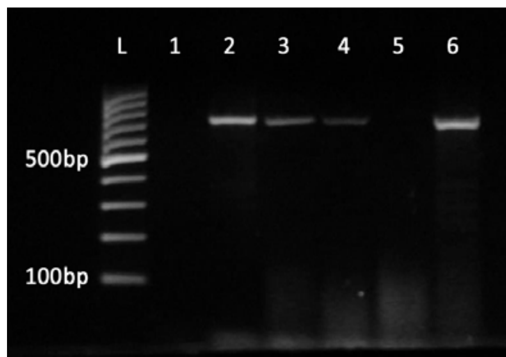

Figure 3. 1.5 % agarose gel of PCR amplicons from DNA of thorax of individual mosquito specimen derived from primer pair LCO1490 and HCO2198. Ladder (L) (O´GenRuler 100bp DNA Ladder (Thermo Fisher Scientific)), lane 1: PCR negative control, lane 2: *Cq. richiardii* (A, positive control), lane 3-4: *Cx. pipiens/torrentium* (N and O) and lane 5-6: *Ae. (Oc.) nigrinus* (H and I).

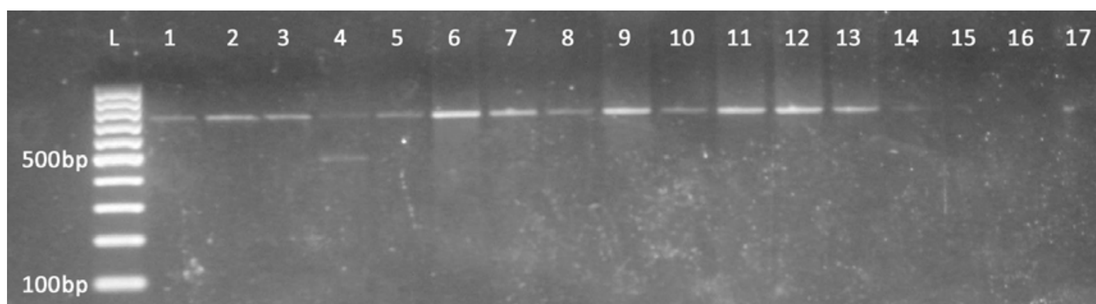

Figure 4. 1.5 % agarose gel of purified PCR amplicons. Ladder (L) (O´GenRuler 100bp DNA Ladder (Thermo Fisher Scientific)), lane 1 and 2: *Cq. richiardii* (A), lane 3-5: *Ae. cinereus/geminus* (B to D), lane 6-8: *Ae. (Oc.) punctor/punctodes* (E to G), lane 9-11: *An. maculpenis sl.* (J to L), lane 12: *Cx.* sp. (P), lane 13-15: *Cx. pipiens/torrentium* (M to O) and lane 16-17: *Ae. (Oc.) nigrinus* (H and I)

3.2 Sequence diversity

Sequences were obtained from both primers from almost all of the samples, except specimen (C) and (H), *see Appendix II*. Genbank or BOLD v3 yields that species morphologically determined to *Cx. pipiens/torrentium* (M to O) or *Cx.* sp. (P) are in fact *Cx. pipiens* and (A) is *Cq. richiardii*. It could also be found that two of the specimen (J and L) are *Anopheles messeae (An. messeae)* and (K) is *Anopheles beklemishevi (An. beklemishevi)*. Barcodes from specimen (B) and (C) were determined as a species from genus *Aedes (Aedes)* Meigen probably *Ae. cinereus* or possibly *Aedes geminus.* Specimen (E) to (G) could be determined as species from genus *Aedes,* subgenus *Ochlerotatus* Lynch Arribalzaga. Probably *Aedes (Ochlerotatus) punctor* or *Aedes (Ochlerotatus) punctodes* but it could also be the closely related species *Aedes (Ochlerotatus) hexodontus.* Specimen (I) is probably the new morphological determined species for Sweden, *Aedes (Ochlerotatus) nigrinus* (*Ae. (Oc.) nigrinus)*.

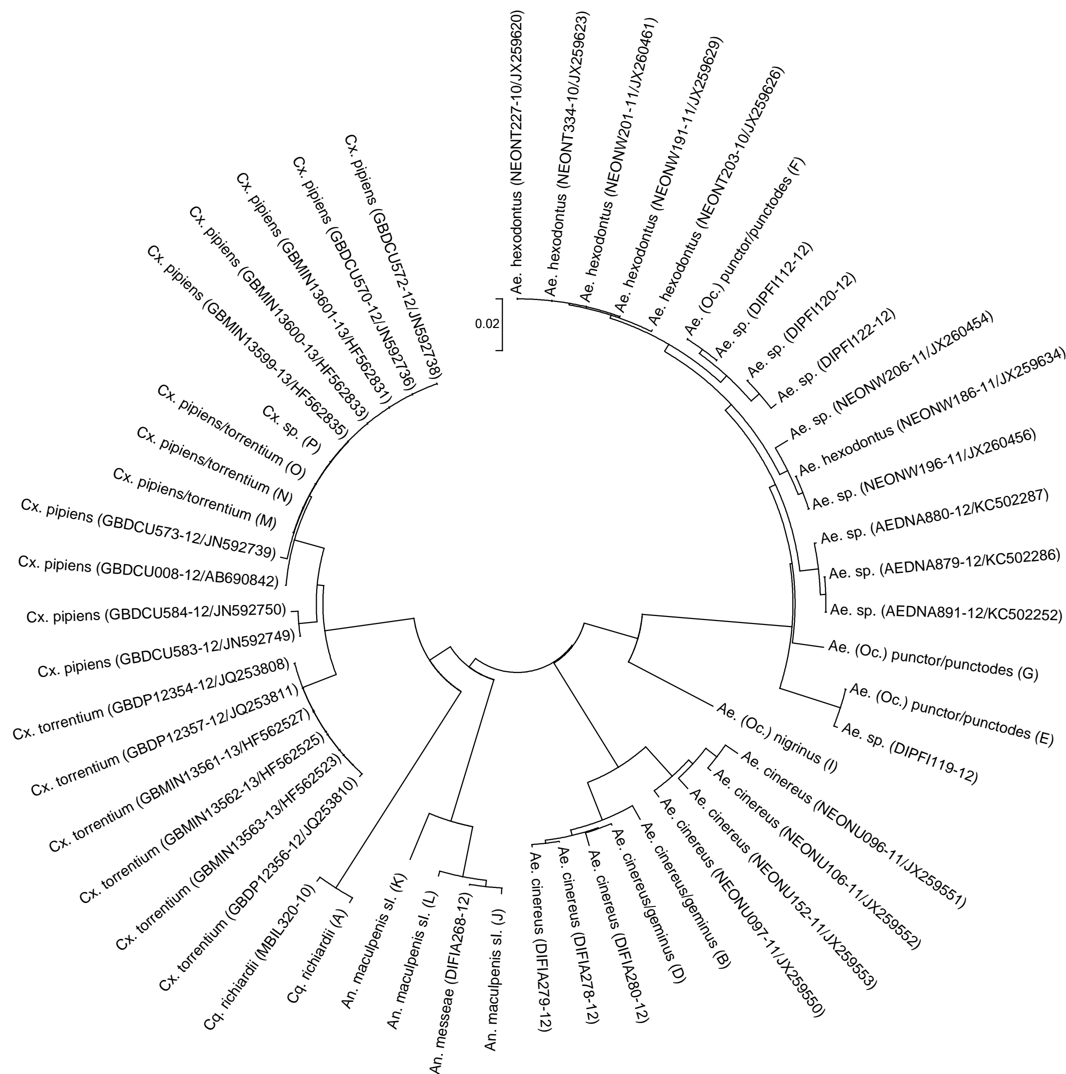

Figure 5. Neighbor-joining tree based on K2P genetic distances of sequences from the mitochondrial COI gene of family Culicidae. Sequences from Genbank and BOLD Systems v3 are displayed with both Genbank accession number and BOLD record number (- / -) or just BOLD record number (-). Sequences from current work are displayed by morphological name and (A) to (P).

Figure 5 show the phylogenetic tree of K2P genetic distances of the mitochondrial COI gene and demonstrate the sequence diversity obtained.

4 Discussion

The rapid DNA extraction method for DNA barcoding may be used as a complimentary tool for characterization and discrimination of species however some issues need to be carefully considered. Firstly, its lack of use of RNase and Proteinase and no phase separation or separation by magnetic beads, makes that the DNA is contaminated. Especially, the DNA contains lots of inhibitors for PCR, which could prevent a correct annealing of primers or correct polymerase activity. Some parts of the mosquito do not give enough good quality DNA. Especially wings and legs are difficult parts of the mosquito to extract DNA of sufficient quality from. Use of extracted mtDNA from a whole mosquito specimen does not give satisfying PCR results. The abdomen with extremities is full of inhibitors for PCR and the shell has lots of chitin. In previous DNA barcoding studies, abdomen or a whole mosquito was used as start material and the DNA extraction was performed with kits that are

commercially available (Cywinska et al., 2006; Ruiz et al., 2010). However, the result in this thesis shows that qualitative barcode sequences from mtDNA can be extracted from thoraxes of mosquitoes and the anticipated problems with inhibitors could thus be overcome.

The concept DNA barcoding also has molecular limitations. The examination is based on one gene or a segment of a gene, which means that the molecular information available is limited. If a gene with a relatively rapid evolution is chosen it might be sufficient for species determination. In a deeper evolutionary understanding for taxonomy, characterization and discrimination of species, ordinary DNA taxonomy, transcriptome studies, behavior and morphological studies are preferred. When new species are identified or new research reveals a change in current Linnaean taxonomy it should always apply a holistic perspective. DNA barcoding shall be seen as a complement to that perspective and has great potential to be a good diagnostic tool, either used by itself or together with morphological examination. This topic has greatly concerned some researchers who were afraid that DNA barcoding would be an accepted method for more extensive taxonomy work (Will et al., 2005). Some of them even called DNA barcoders “anti-taxonomist” (Ebach and Holdrege, 2005). Despite the problems and limitations DNA barcoding obviously have advantages. The new sequencing technologies introduced in the last decade like NGS or third generation sequencing provides a good platform. The ability to pool many samples and mixed-sequence samples makes it a very cost effective and time-efficient method. The method should be suitable for species determination, through DNA barcoding and especially for huge and unknown materials. A big concern would however be the large amount of data generated from NGS. It´s exceeding the development of computer capacity according to Moore´s law (which state that the capacity of the computer hardware would increase exponentially and be doubling every two years) (Moore, 1965) and data are produced in an accelerating manner. The capacity to store and process data should at least be doubling each year, to meet the requirements of NGS (Kahn, 2011).

This thesis also tangent another big issue, partly demonstrated by the distance tree (figure 5). Although, the clustering of obtained barcoding sequences is definitive in comparison to each other and show the sequence diversity, it could not demonstrate a close relationship down to species level. This is because most of the mosquito species lack reference sequences from their nuclear or mitochondrial genome. This problem is shared with genus from other families. To make DNA barcoding an effective diagnostic tool, reference sequences from so called voucher specimens are required. Since the same sort of species could be found in different parts of the world it would be desirable to get sequences from voucher specimens from all around the world. The geographical differences in the DNA sequence of a gene from same species are very small, but still significant. Closely related species could also be a problem to separate with mitochondrial genes like COI. Even if COI: s evolutionary rate is fairly rapid it does not seem to be fast enough. Nuclear genes, for example ITS2, may therefore be used as a complement to COI for barcoding, because of its rapid evolving rate (Yao et al., 2010).

“Myggjakten” gives a unique opportunity for Swedish entomologist to build a national reference library of barcode sequences from Swedish mosquitoes. This would help the taxonomist but also the epizootiologist as well. The ultimate goal would be to use DNA barcoding as the main vector identification method when screening for vector borne diseases is done in the future.

6 Appendix

Appendix I. List of specimen (morphological determined) and its site of collection.

	Genus	Subgenus	Species (morphological)	City	Counties
A	*Coquillettidia*	*Coquillettidia*	*richiardii*	Tierp	Uppsala
B	*Aedes*	*Aedes*	*cinereus/geminus*	Värmdö	Stockholm
C	*Aedes*	*Aedes*	*cinereus/geminus*	Hagfors	Värmland
D	*Aedes*	*Aedes*	*cinereus/geminus*	Jokkmokk	Norrbotten
E	*Aedes*	*Ochlerotatus*	*punctor/punctodes*	Jokkmokk	Norrbotten
F	*Aedes*	*Ochlerotatus*	*punctor/punctodes*	Skellefteå	Västerbotten
G	*Aedes*	*Ochlerotatus*	*punctor/punctodes*	Vimmerby	Kalmar
H, I	*Aedes*	*Ochlerotatus*	*nigrinus*		Halland
J	*Anopheles*	*Anopheles*	*maculpenis sl.*	Umeå	Västerbotten
K	*Anopheles*	*Anopheles*	*maculpenis sl.*	Jokkmokk	Norrbotten
L	*Anopheles*	*Anopheles*	*maculpenis sl.*	Vaxholm	Stockholm
M	*Culex*	*Culex*	*pipiens/torrentium*	Mörbylånga	Kalmar
N	*Culex*	*Culex*	*pipiens/torrentium*	Varberg	Halland
O	*Culex*	*Culex*	*pipiens/torrentium*	Hedemora	Dalarna
P	*Culex*	*Culex*	sp.	Uppsala	Uppsala

Appendix II. 658-bp Barcode sequences of mitochondrial cytochrome c oxidase subunit 1 (COI) gene from specimen of family Culicidae.

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                                          10        20        30        40        50
                                  ....|....|....|....|....|....|....|....|....|....|
Cq. richiardii (A)                TACATTATATTTTATTTTTGGAGCTTGATCTGGAATAGTTGGGACTTCTT
Ae. cinereus/geminus (B)          A...C..................T......C........A..A..A....
Ae. cinereus/geminus (D)          A...C..................T......C........A..A..A....
Ae. (Oc.) punctor/punctodes (E    A.................C...T..................A..A..A.
Ae. (Oc.) punctor/punctodes (F    G.................C...T..................A..A..A.
Ae. (Oc.) punctor/punctodes (G    G.................C...T..................A..A..A.
Ae. (Oc.) nigrinus (I)            A........C..C........T.T......A........A..A..A..A.
An. maculpenis sI. (J)            A..T..............C.........G.A........A..A.....A.
An. maculpenis sI. (K)            A..T...........C..C.........G.A........A..A.......
An. maculpenis sI. (L)            A..T..............C.........G.A........A..A.....A.
Cx. pipiens/torrentium (M)        A....................G......G.............A.......
Cx. pipiens/torrentium (N)        A....................G......G.............A.......
Cx. pipiens/torrentium (O)        A....................G......G.............A.......
Cx. sp. (P)                       A....................G......G.............A.......

                                          60        70        80        90       100
                                  ....|....|....|....|....|....|....|....|....|....|
Cq. richiardii (A)                TAAGTATTCTTATTCGAGCAGAATTAAGTCAACCTGGGATTTTTATTGGA
Ae. cinereus/geminus (B)          .......AT.A.....T..............T..AA.T..A.........
Ae. cinereus/geminus (D)          .......AT.A.....T..............T..AA.T..A.........
Ae. (Oc.) punctor/punctodes (E    ....A...T.A.....T..T........C..C..A..A..A........T
Ae. (Oc.) punctor/punctodes (F    ....A...T.A.....T..T........A.....A..A..A........T
Ae. (Oc.) punctor/punctodes (G    ....A...T.A.....T..T........A.....A..A..A........T
Ae. (Oc.) nigrinus (I)            ....A...T.A.....T..T..............A..T..A.........
An. maculpenis sI. (J)            ........T.A.....T..T......G.A..C.....AGC..........
An. maculpenis sI. (K)            ........T.A.....T..T......G.A..C..C..AGC.........T
An. maculpenis sI. (L)            ........T.A.....T..T......G.A..C.....AGC..........
Cx. pipiens/torrentium (M)        .....T.A..A.......................A..TG.A.........
Cx. pipiens/torrentium (N)        .....T.A..A.......................A..TG.A.........
Cx. pipiens/torrentium (O)        .....T.A..A.......................A..TG.A.........
Cx. sp. (P)                       .....T.A..A.......................A..TG.A.........

                                         110       120       130       140       150
                                  ....|....|....|....|....|....|....|....|....|....|
Cq. richiardii (A)                AATGACCAAATTTATAATGTTATTGTAACAGCTCATGCTTTTATTATAAT
Ae. cinereus/geminus (B)          ....................A.................A...........
Ae. cinereus/geminus (D)          ....................A.................A...........
Ae. (Oc.) punctor/punctodes (E    ....................A.....T.....A.....C...........
Ae. (Oc.) punctor/punctodes (F    ....................A.....T.....A.....C...........
Ae. (Oc.) punctor/punctodes (G    ....................A.....T.....G.....C...........
Ae. (Oc.) nigrinus (I)            ....................A.....T.......................
An. maculpenis sI. (J)            G....T..........................C.................
An. maculpenis sI. (K)            G....T............................................
An. maculpenis sI. (L)            G....T..........................C.................
Cx. pipiens/torrentium (M)        .....T.......................T....................
Cx. pipiens/torrentium (N)        .....T.......................T....................
Cx. pipiens/torrentium (O)        .....T.......................T....................
Cx. sp. (P)                       .....T.......................T....................

                                         160       170       180       190       200
                                  ....|....|....|....|....|....|....|....|....|....|
Cq. richiardii (A)                TTTTTTTATAGTTATACCTATTATAATTGGAGGATTTGGTAATTGATTAG
Ae. cinereus/geminus (B)          ...C..........................G...............C.T.
Ae. cinereus/geminus (D)          ...C..........................................C.T.
Ae. (Oc.) punctor/punctodes (E    ...C........A.....C..............G.....A..........
Ae. (Oc.) punctor/punctodes (F    ...C........A..........................A..........
Ae. (Oc.) punctor/punctodes (G    ...C........A..........................A..........
Ae. (Oc.) nigrinus (I)            ...C........A..........................A..........
An. maculpenis sI. (J)            ...C...................................A..C.......
An. maculpenis sI. (K)            ...C...................................A..........
An. maculpenis sI. (L)            ...C...................................A..C.......
Cx. pipiens/torrentium (M)        ............A.....A..C.................A..........
Cx. pipiens/torrentium (N)        ............A.....A..C.................A..........
Cx. pipiens/torrentium (O)        ............A.....A..C.................A..........
Cx. sp. (P)                       ............A.....A..C.................A..........

                                         210       220       230       240       250
                                  ....|....|....|....|....|....|....|....|....|....|
Cq. richiardii (A)                TCCCTTTAATATTAGGAGCTCCTGATATAGCCTTTCCTCGAATAAATAAT
Ae. cinereus/geminus (B)          .T.................C..............................
Ae. cinereus/geminus (D)          .T.................C..............................
Ae. (Oc.) punctor/punctodes (E    ...................G..A..C..G..T..C..C............
Ae. (Oc.) punctor/punctodes (F    ...................A..A.....G..T..................
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```
Ae. (Oc.) punctor/punctodes (G     ...................G..A.....G.....................
Ae. (Oc.) nigrinus (I)             .T...........G.....C.................A............
An. maculpenis sI. (J)             .T.................C..A.....G..T..C...............
An. maculpenis sI. (K)             .T....................A........T..C...............
An. maculpenis sI. (L)             .T.................C..A.....G..T..C...............
Cx. pipiens/torrentium (M)         .T........G...........A.....G.....................
Cx. pipiens/torrentium (N)         .T........G...........A.....G.....................
Cx. pipiens/torrentium (O)         .T........G...........A.....G.....................
Cx. sp. (P)                        .T........G...........A.....G.....................

                                           260       270       280       290       300
                                   ....|....|....|....|....|....|....|....|....|....|
Cq. richiardii (A)                 ATAAGATTTTGAATACTTCCCCCTTCATTAACCCTTCTTCTTTCCGGGGG
Ae. cinereus/geminus (B)           .....T.........T.A..T..C...........GT.AT.A..AA.CTC
Ae. cinereus/geminus (D)           .....T.........T.A..T..C...........GT.AT.A..AA.CTC
Ae. (Oc.) punctor/punctodes (E     .....T..C..G..............C.....GT.G..GA.....A.TA.
Ae. (Oc.) punctor/punctodes (F     .....T..C..G..............C.....G..G..G.....TA.TA.
Ae. (Oc.) punctor/punctodes (G     .....T..C..G..............T.....G..G..GA.....A.TA.
Ae. (Oc.) nigrinus (I)             .....T..C..G...T.A..T..C........A..A..A.....TA.TA.
An. maculpenis sI. (J)             .....T.........T.A..T..A..T.....TT.AT.AA....TA.AA.
An. maculpenis sI. (K)             .....T.........T.A........T.....T...T.AA....TA.TA.
An. maculpenis sI. (L)             .....T.........T.A..T..G..T.....TT.AT.AA....TA.AA.
Cx. pipiens/torrentium (M)         .....T...........A..T........G..A..A..A.....AA.TA.
Cx. pipiens/torrentium (N)         .....T...........A..T........G..A..A..A.....AA.TA.
Cx. pipiens/torrentium (O)         .....T...........A..T........G..A..A..A.....AA.TA.
Cx. sp. (P)                        .....T...........A..T........G..A..A..A.....AA.TA.

                                           310       320       330       340       350
                                   ....|....|....|....|....|....|....|....|....|....|
Cq. richiardii (A)                 TATAGTGGAAAGCGGGGCTGGTACTGGATGAACTGTTTATCCCCCACTTT
Ae. cinereus/geminus (B)           A.....A....AT..A........A..G...........C..T..TT.A.
Ae. cinereus/geminus (D)           A.....A....AT...........A..G...........C..T..TT.A.
Ae. (Oc.) punctor/punctodes (E     ...G..T....AT...T.A..G..A..T.....G..G..C.....T....
Ae. (Oc.) punctor/punctodes (F     ...G..T....AT...T.A..G..A..T.....G..G..C..A..T....
Ae. (Oc.) punctor/punctodes (G     ...G..T....AT..CT.A..G..A..T.....G..G..C..A..T....
Ae. (Oc.) nigrinus (I)             ......A....AT...T....G...........A.....C..A..T....
An. maculpenis sI. (J)             ......A....AT..A..C..A..A..G..............T..T..A.
An. maculpenis sI. (K)             ......A....AT..A..A..A..A..............C.....T..A.
An. maculpenis sI. (L)             ......A....AT.....C..A..A..G..............T..T..A.
Cx. pipiens/torrentium (M)         .T....A....AT..A.....G...........A..G........T....
Cx. pipiens/torrentium (N)         .T....A....AT..A.....G...........A..G........T....
Cx. pipiens/torrentium (O)         .T....A....AT..A.....G...........A..G........T....
Cx. sp. (P)                        .T....A....AT..A.....G...........A..G........T....

                                           360       370       380       390       400
                                   ....|....|....|....|....|....|....|....|....|....|
Cq. richiardii (A)                 CCTCTGGCACAGCTCACGCAGGAGCATCTGTAGATCTCTCTATTTTTTCT
Ae. cinereus/geminus (B)           .T.....A..T.....T.......GT.....T..CT.AG.A.....C...
Ae. cinereus/geminus (D)           .T.....A..T.....T.......GT.....T..CT.AG.A.....C...
Ae. (Oc.) punctor/punctodes (E     .T.....A..T.....T........T..A..T...T.AA.A.........
Ae. (Oc.) punctor/punctodes (F     .T.....A..T..C..T........T..A..T...T.AA.A.........
Ae. (Oc.) punctor/punctodes (G     .T..G..A..T..C..T........T..A..T...T.AA.A.........
Ae. (Oc.) nigrinus (I)             .A..A..A..T.....T..C.....T.....T...T.AA.A.........
An. maculpenis sI. (J)             .T.....A.TT...........G..T..A......T.AG..........A
An. maculpenis sI. (K)             .T.....A.TT.....T........T..A......T.AG..........A
An. maculpenis sI. (L)             .T.....A.TT.....T.....G..T..A......T.AG..........A
Cx. pipiens/torrentium (M)         .A.....A........T..T.....T..A.....CT.AG...........
Cx. pipiens/torrentium (N)         .A.....A........T..T.....T..A.....CT.AG...........
Cx. pipiens/torrentium (O)         .A.....A........T..T.....T..A.....CT.AG...........
Cx. sp. (P)                        .A.....A........T..T.....T..A.....CT.AG...........

                                           410       420       430       440       450
                                   ....|....|....|....|....|....|....|....|....|....|
Cq. richiardii (A)                 CTTCATTTAGCGGGAATTTCTTCTATTTTAGGAGCAGTAAATTTTATTAC
Ae. cinereus/geminus (B)           T.A........C...........G...........C..............
Ae. cinereus/geminus (D)           T.A........C..G........G........G..C..............
Ae. (Oc.) punctor/punctodes (E     ...........T..G..C.....A........G.................
Ae. (Oc.) punctor/punctodes (F     ...........T..G..C.....A..........................
Ae. (Oc.) punctor/punctodes (G     ...........T..G........A..........................
Ae. (Oc.) nigrinus (I)             ...........T..TG....A..A..........................
An. maculpenis sI. (J)             T.A........A........C..A...........T..............
An. maculpenis sI. (K)             T.A........A.....C.....A...........T..............
An. maculpenis sI. (L)             T.A........A........C..A...........T..............
Cx. pipiens/torrentium (M)         T.A........A........A..A........T.................
Cx. pipiens/torrentium (N)         T.A........A........A..A........T.................
```

```
Cx. pipiens/torrentium (O)      T.A........A........A..A........T.................
Cx. sp. (P)                     T.A........A........A..A........T.................

                                         460       470       480       490       500
                                ....|....|....|....|....|....|....|....|....|....|
Cq. richiardii (A)              TACAGTAATTAATATACGAACTTCAGGAATTACTTTAGATCGTTTACCAT
Ae. cinereus/geminus (B)        A..T..T............T.AG.G........AC.T.....AC.T..T.
Ae. cinereus/geminus (D)        A..T..T............T.AG..........AC.T.....AC.T..T.
Ae. (Oc.) punctor/punctodes (E  ...TA.T............T.......G.....A........A.....T.
Ae. (Oc.) punctor/punctodes (F  ...T..T............T.......G.....A........A.....T.
Ae. (Oc.) punctor/punctodes (G  ...T..T............T.............A........A.....T.
Ae. (Oc.) nigrinus (I)          A..................T.AG.G..T..............A.....T.
An. maculpenis sI. (J)          A..T...............T..C.T.........C.T.....AA....T.
An. maculpenis sI. (K)          A..T...............T..C.T.........C.T.....AA....T.
An. maculpenis sI. (L)          A..T...............T..C.T.........C.T.....AA....T.
Cx. pipiens/torrentium (M)      A..................T..............C.T.....AA....T.
Cx. pipiens/torrentium (N)      A..................T..............C.T.....AA....T.
Cx. pipiens/torrentium (O)      A..................T..............C.T.....AA....T.
Cx. sp. (P)                     A..................T..............C.T.....AA....T.

                                         510       520       530       540       550
                                ....|....|....|....|....|....|....|....|....|....|
Cq. richiardii (A)              TATTTGTTTGATCAGTAGTAATTACAGCAGTTCTATTACTCCTTTCTCTC
Ae. cinereus/geminus (B)        ................T..T.....T..TA..T.......T......T.A
Ae. cinereus/geminus (D)        ................T..T.....T..TA..T.......T......T.A
Ae. (Oc.) punctor/punctodes (E  .......A.....C..T...........TA..T.......AT.A..CT.A
Ae. (Oc.) punctor/punctodes (F  .......A.....C..T...........TA..T.......AT.A..CT.A
Ae. (Oc.) punctor/punctodes (G  .......A.....C..T...........TA..T.......AT.A..CT.A
Ae. (Oc.) nigrinus (I)          .............T..............TA..T.......TT.A...T.A
An. maculpenis sI. (J)          .......A.....T..T..............AT.....T.AT.A..AT.A
An. maculpenis sI. (K)          .......A.....T..T..............AT..C..T.AT.A..AT.A
An. maculpenis sI. (L)          .......A.....T..T...........G..AT.....T.AT.A..AT.A
Cx. pipiens/torrentium (M)      .........................T......T.......T......T.A
Cx. pipiens/torrentium (N)      .........................T......T.......T......T.A
Cx. pipiens/torrentium (O)      .........................T......T.......T......T.A
Cx. sp. (P)                     .........................T......T.......T......T.A

                                         560       570       580       590       600
                                ....|....|....|....|....|....|....|....|....|....|
Cq. richiardii (A)              CCAGTCCTTGCTGGAGCAATTACTATACTTTTAACTGATCGAAATTTAAA
Ae. cinereus/geminus (B)        ..T..TT.A.....G..T.........T.A....................
Ae. cinereus/geminus (D)        ..T..TT.A........T.........T.A....................
Ae. (Oc.) punctor/punctodes (E  .....AT.A........T.........T.A........C...........
Ae. (Oc.) punctor/punctodes (F  .....AT.A..C.....T.........T.A........C...........
Ae. (Oc.) punctor/punctodes (G  .....AT.A........T.........T.A........C...........
Ae. (Oc.) nigrinus (I)          ..T..AT.A........T.........T.A.....A..C.....C.....
An. maculpenis sI. (J)          .....TT.A........T.....A...T.A....................
An. maculpenis sI. (K)          .....TT.A........T.....A...T.A...............C....
An. maculpenis sI. (L)          .....TT.A........T.....A...T.A....................
Cx. pipiens/torrentium (M)      ..T..TT.A.....T..T........GT.A.....A..............
Cx. pipiens/torrentium (N)      ..T..TT.A.....T..T........GT.A.....A..............
Cx. pipiens/torrentium (O)      ..T..TT.A.....T..T........GT.A.....A..............
Cx. sp. (P)                     ..T..TT.A.....T..T........GT.A.....A..............

                                         610       620       630       640       650
                                ....|....|....|....|....|....|....|....|....|....|
Cq. richiardii (A)              CACTTCTTTCTTTGACCCAACAGGAGGAGGAGACCCTATCTTATACCAAC
Ae. cinereus/geminus (B)        T..A..A.............TT...........T..A..T..........
Ae. cinereus/geminus (D)        T..A..A.............TT...........T..A..T.....T....
Ae. (Oc.) punctor/punctodes (E  T..A..A..T........T.TT.................T..........
Ae. (Oc.) punctor/punctodes (F  T..A..A...........T.TT.....G...........T..........
Ae. (Oc.) punctor/punctodes (G  T..G..A...........T.TT..G..............T.....T....
Ae. (Oc.) nigrinus (I)          T.....C.............TT...........T..A..T.....T....
An. maculpenis sI. (J)          T.....A..T..C..T...G................A..T..G..T....
An. maculpenis sI. (K)          T..C..A..T..C......G....G...........A..T.....T....
An. maculpenis sI. (L)          T.....A..T..C..T...G................A..T.....T....
Cx. pipiens/torrentium (M)      T.....A........T....TT...........T..A..T.....T....
Cx. pipiens/torrentium (N)      T.....A........T....TT...........T..A..T.....T....
Cx. pipiens/torrentium (O)      T.....A........T....TT...........T..A..T.....T....
Cx. sp. (P)                     T.....A........T....TT...........T..A..T.....T....

                                ....|...
Cq. richiardii (A)              ATTTATTT
Ae. cinereus/geminus (B)        ........
```

```
Ae. cinereus/geminus (D)        ........
Ae. (Oc.) punctor/punctodes (E  ........
Ae. (Oc.) punctor/punctodes (F  ........
Ae. (Oc.) punctor/punctodes (G  ........
Ae. (Oc.) nigrinus (I)          ........
An. maculpenis sI. (J)          ........
An. maculpenis sI. (K)          .C......
An. maculpenis sI. (L)          ........
Cx. pipiens/torrentium (M)      ........
Cx. pipiens/torrentium (N)      ........
Cx. pipiens/torrentium (O)      ........
Cx. sp. (P)                     ........
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