Identification of Belgian mosquito species (Diptera: Culicidae) by DNA barcoding

V. VERSTEIRT,*‡ Z. T. NAGY,‡ P. ROELANTS,* L. DENIS,* F. C. BREMAN,§ D. DAMIENS,¶ W. DEKONINCK,‡ T. BACKELJAU,** M. COOSEMANS*†† and W. VAN BORTEL‡‡

*Department of Biomedical Science, Vector Biology Group, Medical Entomology Unit, Institute of Tropical Medicine, Nationalestraat 155, Antwerp B-2000, Belgium, †Avia-GIS, Risschotlei 33, Zoersel B-2980, Belgium, ‡Royal Belgian Institute of Natural Sciences, Directorate Taxonomy and Phylogeny, Vautierstraat 29, Brussels B-1000, Belgium, §Royal Museum for Central Africa (RMCA-JEMU), Leuvensesteenweg 13, Tervuren B-3080, Belgium, ¶Ecology and Biodiversity Unit, Université Catholique de Louvain, Place Croix Sud 4/5, Louvain-La-Neuve B-1348, Belgium, **Evolutionary Ecology Group, University of Antwerp, Groenenborgerlaan 171, Antwerp B-2020, Belgium, ††Department of Biomedical Sciences, Faculty of Pharmaceutical, Veterinary and Biomedical Sciences, University of Antwerp, Universiteitsplein 1, Antwerpen (Wilrijk) B-2610, Belgium, ‡‡European Centre for Disease Prevention and Control, Tomtebodavägen 11a, Stockholm 171 83, Sweden

Abstract

Since its introduction in 2003, DNA barcoding has proven to be a promising method for the identification of many taxa, including mosquitoes (Diptera: Culicidae). Many mosquito species are potential vectors of pathogens, and correct identification in all life stages is essential for effective mosquito monitoring and control. To use DNA barcoding for species identification, a reliable and comprehensive reference database of verified DNA sequences is required. Hence, DNA sequence diversity of mosquitoes in Belgium was assessed using a 658 bp fragment of the mitochondrial cytochrome oxidase I (COI) gene, and a reference data set was established. Most species appeared as well-supported clusters. Intraspecific Kimura 2-parameter (K2P) distances averaged 0.7%, and the maximum observed K2P distance was 6.2% for *Aedes koreicus*. A small overlap between intra- and interspecific K2P distances for congeneric sequences was observed. Overall, the identification success using best match and the best close match criteria were high, that is above 98%. No clear genetic division was found between the closely related species *Aedes annulipes* and *Aedes cantans*, which can be confused using morphological identification only. The members of the *Anopheles maculipennis* complex, that is *Anopheles maculipennis* s.s. and *An. messeae*, were weakly supported as monophyletic taxa. This study showed that DNA barcoding offers a reliable framework for mosquito species identification in Belgium except for some closely related species.

Keywords: Belgium, COI, Culicidae, DNA barcoding, invasive species, species identification

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Introduction

In continental Europe, about 100 mosquito species (Diptera: Culicidae) belonging to seven genera are known (Snow 2003; Zgomba & Petric 2008). This number has increased over the last decades as at least six exotic *Aedes* species were intercepted in mainland Europe, viz. *Aedes aegypti*, *Ae. albopictus*, *Ae. atropalpus*, *Ae. japonicus*, *Ae. koreicus* and *Ae. triseriatus* (Medlock et al. 2012; ECDC 2014). The first country-wide inventory of the Culicidae in Belgium in 2007—2008 recorded 23 morphologically identified species (Versteirt et al. 2013), two of which are established alien species, *Ae. japonicus* and *Ae. koreicus*. *Aedes albopictus*, the most notorious invasive alien mosquito species in Europe, has been intercepted twice in Belgium, although without evidence of establishment (Schaffner et al. 2004; Boukraa et al. 2013). Several culicid species in Belgium are potential vectors of pathogens posing a possible threat to human and animal health. Therefore, correct identification in all life stages is essential for effective mosquito monitoring and control.

Mosquito species identification is mostly achieved using morphological characters, which may be difficult...
to interpret without specialized taxonomic expertise and which are regularly lost during collection and storage (Cook et al. 2005; Patsoula et al. 2006). Moreover, morphological variability often complicates identification (Verna & Munstermann 2011; Versteirt et al. 2012b). Hence, there is a need of a straightforward, reliable and easy-to-use identification tool. DNA barcoding might be such a tool (Hebert et al. 2003). It employs a short standard fragment of about 650 bp of the mitochondrial cytochrome c oxidase I (COI) 5’ region (Folmer et al. 1994) to discriminate species of a wide range of taxa (Jinbo et al. 2011). Recently, this method has been applied in numerous taxonomic studies on different dipteran taxa (e.g. Stahls et al. 2009; Pramual et al. 2011) and as such it has been used to identify mosquito species (Gonzalez et al. 2010; Ruiz et al. 2010; Laboudi et al. 2011; Ruiz-Lopez et al. 2012). Regional studies were carried out in Canada (Cywinska et al. 2006), India (Kumar et al. 2007), Pakistan (Ashfaq et al. 2014), the Persian Gulf region (Azari-Hamidian et al. 2010) and China (Wang et al. 2012), showing the usefulness of DNA barcoding for Culicidae. In Europe, a number of studies used a molecular identification approach to address specific questions regarding the identification of, for example the Anopheles maculipennis complex or Culex pipiens. Another study used DNA barcoding to identify Culicidae from Sweden (Engdahl et al. 2014). Nevertheless, the number of barcode sequences of European mosquito specimens in the Barcoding of Life Database (BOLD) is still limited. Only 7% of the Culicidae records in BOLD with proper information on country and species name are coming from Europe (BOLD 2014). Hence, this study aimed to establish a DNA barcode library for the Belgian mosquito fauna and to evaluate its utility in species identification.

Material and methods

Sampling and a priori identification

Mosquitoes were sampled during a 2-year survey in Belgium (MODIRISK project) in 2007 and 2008 (Versteirt et al. 2013). Specimens were collected using CO2-baited Mosquito Magnet Liberty Plus traps (Woodstream Corporation, Lititz, PA, USA). Mosquitoes were collected from 910 randomly selected sites in three key habitat types (urban, rural and natural) (Versteirt et al. 2013). For this study, these sites were subsampled to include different geographical locations and habitats in order to maximize the geographical coverage and include possible genetic variation. A minimum of five (whenever possible) and a maximum of 20 specimens per species were included. For Culex pipiens, more specimens were included as it was collected in 698 sites during the MODIRISK project and was therefore the most common species found (Versteirt et al. 2013). All specimens were kept dry or frozen at −20 °C in individual vials. Specimens were identified morphologically using the digital identification key of Schaffner et al. (2001) and the printed keys of Schaffner (1993) and Becker et al. (2010). All specimens were double checked by Francis Schaffner without prior knowledge of the initial identification. Only specimens for which the morphological identification was confirmed were included in the study. Members of the Anopheles maculipennis complex were identified by the PCR-RFLP assay of the nuclear ribosomal ITS2 region (Nicolaescu et al. 2004).

Recently, a number of taxonomic revisions have been published (Reinert 2000; Reinert et al. 2004, 2006, 2008, 2009) which are not commonly applied (Savage 2005). Table 1 provides an overview of the species names used in this study and the names according to these recent revisions. Species names were verified using the Mosquito Taxonomy Inventory (Harbach 2014)

DNA sequencing

One to four legs were removed from mosquito specimens and were subjected to DNA extraction based on the method of Collins et al. (1987) or the protocol of the Phire Animal Tissue Direct PCR Kit (Thermo Scientific). In the latter protocol, a small piece of mosquito tissue was briefly incubated (5 min) in the provided incubation buffer and 1 μL of this buffer was subsequently added to the PCR. The standard barcoding fragment of the mitochondrial cytochrome oxidase I (COI) gene was amplified using the PCR protocol of Versteirt et al. (2012a). All PCR products were checked on a 2% agarose gel and visualized after ethidium bromide staining on an Image master VDS (Amersham, Uppsala, Sweden). Positive PCR products were purified on NucleoFast PCR plates (Macherey-Nagel) using vacuum filtering. Cleaned PCR products were sequenced in both directions on an ABI 3130xl capillary DNA sequencer using the BigDye Terminator v1.1 chemistry (both Life Technologies).

Data analysis

DNA sequences were checked, edited and assembled using CodonCode Aligner v3.7.1 (CodonCode, USA), and subsequently aligned with ClustalW (Thompson et al. 1994). Based on Kimura 2-parameter (K2P) distances (Kimura 1980), a neighbour-joining (NJ) tree was reconstructed in MEGA 6 (Tamura et al. 2013), with branch support assessed by 1000 bootstrap replicates. Haplotype networks were constructed using SplitsTree v4 (Huson & Bryant 2006). The proportion of correctly identified specimens was estimated with SpeciesIdentifi er v1.5 (Meier et al. 2006) using the criteria ‘best match’
(BM), 'best close match' (BCM) and `all species barcodes` (ASB). According to BM, each sequence that has to be identified (i.e. a `query`) is assigned the species name of its best-matching DNA sequence in the database (i.e. the entire sequenced data set), regardless of how (dis)similar the query and reference sequences are. Under this criterion false-positive assignments are almost inevitable because all query barcodes will be linked to reference barcodes, even if they are very divergent, a problem inherent to the use of the BLAST algorithm (Kelly et al. 2007). The BCM was used to avoid most of the false-positives produced by the BM criterion. With BCM, the best-matching reference barcode is identified, but the query is only assigned the taxon name of that barcode if the query is within the threshold distance below which 95% of all intraspecific pairwise distances are found. This threshold is estimated `ad hoc` for the given data set (Meier et al. 2006). The ASB criterion assembles a list of all barcode sequences similar to the query sequence using the same threshold as BCM. Identification is considered: (i) correct if the query sequence is associated with all conspecific barcode sequences; (ii) ambiguous if at least one allospecific sequence is more similar to the query than the least similar conspecific sequence; and (iii) erroneous if the query is associated to barcode sequences that do not belong to the correct species.

The ABGD method was used to identify Molecular Operational Taxonomic Units (MOTU's) (Puillandre et al. 2012). This method infers the divergence at which a barcode gap occurs and sorts the sequences in putative species based on this. All pairwise K2P distances calculated with MEGA 6 were uploaded at http://wwwabi.snv.jussieu.fr/public/abgd/ and ABGD was run using the default settings.

**Results**

A 658 bp fragment of the mitochondrial COI gene was analyzed in a total of 260 specimens of 23 morphologically identified culicid species from Belgium. Within the An. maculipennis s.l. species complex, two species, An. maculipennis s.s. and An. messeae, were recognized based on the ITS2 PCR-RFLP identification method (File S1, Supporting information).
The mean intraspecific K2P distances was 0.7%, with a maximum observed value of 6.2% for *Ae. koreicus* (Table 2). The divergence between congeneric species averaged 11.0% (range of the pairwise K2P distances: 0.8–17.7%) and an overlap between the intra- and interspecific K2P divergences of congeneric sequences was observed (Fig. 1). The distance below which 95% of all intraspecific pairwise distances were found was 2.6% and was applied as threshold by SpeciesIdentifier. The overall identification success based on BM and BCM was higher than 98% (no erroneous identifications). The ASB criterion yielded 20% ambiguous identifications (Table 3). Such ambiguous ABS identifications were observed in *Ae. annulipes* (all specimens), in *An. maculipennis* s.s. (7 of the 11 specimens) and in *Cx. pipiens* (33 of the 34 specimens) (File S2, Supporting information). Both *Aedes koreicus* specimens did not match and one non-matching specimen was observed in each *Ae. punctor* and *Cx. territans*.

<table>
<thead>
<tr>
<th>Species</th>
<th>Number of specimens</th>
<th>Average K2P distance (%)</th>
<th>Maximum observed K2P difference between conspecific specimens (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aedes annulipes</em></td>
<td>12</td>
<td>0.92</td>
<td>2.06</td>
</tr>
<tr>
<td><em>Aedes cantans</em></td>
<td>12</td>
<td>0.28</td>
<td>0.63</td>
</tr>
<tr>
<td><em>Aedes caspius</em></td>
<td>14</td>
<td>1.32</td>
<td>3.03</td>
</tr>
<tr>
<td><em>Aedes cineus</em></td>
<td>13</td>
<td>0.61</td>
<td>1.42</td>
</tr>
<tr>
<td><em>Aedes communis</em></td>
<td>13</td>
<td>0.10</td>
<td>0.31</td>
</tr>
<tr>
<td><em>Aedes detritus</em></td>
<td>9</td>
<td>0.24</td>
<td>0.94</td>
</tr>
<tr>
<td><em>Aedes geniculatus</em></td>
<td>15</td>
<td>0.25</td>
<td>0.63</td>
</tr>
<tr>
<td><em>Aedes japonicus</em></td>
<td>4</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td><em>Aedes koreicus</em></td>
<td>2</td>
<td>6.22</td>
<td>6.22</td>
</tr>
<tr>
<td><em>Aedes punctor</em></td>
<td>19</td>
<td>1.21</td>
<td>4.03</td>
</tr>
<tr>
<td><em>Aedes rusticus</em></td>
<td>10</td>
<td>1.33</td>
<td>0.31</td>
</tr>
<tr>
<td><em>Aedes sticticus</em></td>
<td>8</td>
<td>1.25</td>
<td>2.88</td>
</tr>
<tr>
<td><em>Aedes vexans</em></td>
<td>16</td>
<td>0.88</td>
<td>1.58</td>
</tr>
<tr>
<td><em>Anopheles claviger</em></td>
<td>8</td>
<td>1.64</td>
<td>2.72</td>
</tr>
<tr>
<td><em>Anopheles maculipennis</em></td>
<td>11</td>
<td>1.00</td>
<td>1.90</td>
</tr>
<tr>
<td><em>Anopheles messeae</em></td>
<td>6</td>
<td>0.67</td>
<td>3.19</td>
</tr>
<tr>
<td><em>Anopheles plumbeus</em></td>
<td>5</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td><em>Coquillettidia richiardii</em></td>
<td>15</td>
<td>0.10</td>
<td>0.63</td>
</tr>
<tr>
<td><em>Culex pipiens</em></td>
<td>34</td>
<td>0.67</td>
<td>3.19</td>
</tr>
<tr>
<td><em>Culex territans</em></td>
<td>4</td>
<td>2.52</td>
<td>3.99</td>
</tr>
<tr>
<td><em>Culex torrentium</em></td>
<td>5</td>
<td>0.25</td>
<td>0.47</td>
</tr>
<tr>
<td><em>Culiseta annulata</em></td>
<td>16</td>
<td>0.04</td>
<td>0.31</td>
</tr>
<tr>
<td><em>Culiseta fumipennis</em></td>
<td>3</td>
<td>0.52</td>
<td>0.63</td>
</tr>
<tr>
<td><em>Culiseta morsitans</em></td>
<td>6</td>
<td>0.31</td>
<td>0.63</td>
</tr>
</tbody>
</table>

The ABGD method identified 21 MOTU’s at the prior maximal distances of 0.0359. The following species were classified within the same MOTU: *Anopheles maculipennis* s.s. and *An. messeae*, *Cx. pipiens* and *Cx. torrentium*, *Ae. cantans* and *Ae. annulipes*, and *Ae. punctor* and *Ae. communis*. The two *Ae. koreicus* specimens were assigned to different MOTUs.

Most species were characterized by a distinctive set of COI sequences that formed well-supported clusters in the NJ-tree (bootstrap values of 99%) (Fig. 2 and File S2, Supporting information). The *An. maculipennis* s.l. species complex formed a well-supported clade in the NJ-tree. The two sibling species, *An. maculipennis* s.s. and *An. messeae*, identified within this complex were supported as monophyletic taxa by bootstrap values of 83% and 75%, respectively (Fig. 2). The haplotype network clearly differentiated these two groups (File S3, Supporting information). Further, within *An. maculipennis* s.s., two well-supported subgroups were recognized with a maximum observed intraspecific K2P distance of 2.7% (File S2, Supporting information). *Aedes annulipes* and *Ae. cantans* are two closely related species which can be confused using morphological identification. The samples of these two species were split in three subgroups, that is two groups within *Ae. annulipes* and one group for *Ae. cantans* (Fig. 2, File S2 and S3, Supporting information). Mean intraspecific sequence divergence was 0.92% in *Ae. annulipes* and 0.28% in *Ae. cantans*. The interspecific divergence between specimens of both species ranged from 0.8% to a maximum of 1.6%. The entire group and one of the subgroups were well supported in the NJ analysis (99% bootstrap support) (Fig. 2, File S2, Supporting information). *Culex torrentium* formed a well-supported group in the NJ-tree with bootstrap value of 99% (Fig. 2). The intraspecific K2P distance in *Cx. pipiens* had a maximum value of 3.2% and well-supported subgroups with bootstrap values above 90% could be identified (Files S2 and S3, Supporting information). Further, within *Ae. punctor* and *Cx. territans* well-supported groups were observed in each taxon (File S2, Supporting information), with maximum observed intraspecific K2P distances between conspecific specimens of 4.03% and 3.99%, respectively. In the NJ-tree, *Ae. j. japonicus* clustered with *Ae. koreicus* and both taxa formed a well-supported group.

Public Barcode Index Numbers (BINs) (http://www.boldsystems.org/index.php/databases) were found for 19 of the 24 species. For some species more than one BIN was available (File S4, Supporting information). *Aedes cantans* and *Ae. annulipes* shared the same BIN cluster and two representatives of the cluster were included in the NJ-tree (File S4, Supporting information). The BIN clusters of *Culex territans* were from populations from Canada and the USA and were clearly distinct from the
Belgian conspecifics (K2P distance ≥ 4.8%). The Culiseta morsitans BIN did not cluster with the conspecifics from Belgium. This cluster comprises specimens from the Americas and Sweden. Using the BOLD species identifier, the Belgian specimens were identified as Culiseta morsitans morsitans and as such they matched with specimens from Germany and the U.K. (these specimens are not publically available and could not be included in the NJ-tree).

Discussion

The current study assessed the use of DNA barcoding for Belgian mosquitoes (Diptera: Culicidae). Overall, the identification success using BM and BCM was high, that is above 98%. These two criteria performed better than the ASB criterion, a pattern also observed by Virgilio et al. (2010). An overlap between the intra- and interspecific K2P divergence of congeneric sequences was observed resulting in ambiguous identifications in some closely related species. The An. maculipennis s.l. species complex consists of several species. Three of them might occur in Belgium, that is An. maculipennis s.s., An. messeae and An. atroparvus (Schaffner et al. 2001). Anopheles messeae was recently subdivided into An. messeae and An. dacie (Djadid et al. 2007), although morphological and genetic similarities between these two taxa are high and there is yet no irrefutable evidence of their specific status (Versteirt 2012). This study identified only An. messeae based on PCR-RFLP. Moreover, there is no evidence for the current presence of An. atroparvus, a species that was formerly known to occur in Belgium (Versteirt et al. 2013). The current study showed that An. maculipennis s.s. and An. messeae are distinct but weakly supported groups in the NJ-tree (<85% bootstrap). No false assignments of these two species were made based on the DNA barcoding, although a high percentage of ambiguous identifications according to ASB criterion were observed. Furthermore, reference specimens of An. messeae and An. maculipennis s.s. available in BOLD clustered with their conspecifics from Belgium. Anopheles maculipennis s.s. consisted of two subgroups and a K2P divergence of up to 2.7% was observed within this species. This is above the threshold of 2.6% calculated by SpeciesIdentifier.

Culex torrentium was clearly separated from the closely related Cx. pipiens in the NJ-tree. Within Cx. pipiens two ‘forms’ discriminated by a number of life history

Table 3 Identification success using the Kimura two-parameter distances with three different criteria: Best Match, Best Close Match and All Species Barcodes based on the threshold of 2.6 calculated by SpeciesIdentifier*

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Correct identification (%)</th>
<th>Ambiguous identification (%)</th>
<th>Incorrect identification (%)</th>
<th>No match (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Best Match</td>
<td>100.00</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Best Close Match</td>
<td>98.46</td>
<td>0.0</td>
<td>0.0</td>
<td>1.53</td>
</tr>
<tr>
<td>All Species Barcodes</td>
<td>78.46</td>
<td>20.1</td>
<td>0.0</td>
<td>1.53</td>
</tr>
</tbody>
</table>

*The a priori threshold of 3% yielded the same results.
traits are recognized (Becker et al. 2010). Although the subdivision into these two forms is widely accepted, their taxonomic status is unresolved and it is unclear if the divergence observed in this study can be linked to these forms.

Difficulties were encountered with the differentiation between *Ae. annulipes* and *Ae. cantans*, which were clustered in one well-supported group, and all *Ae. annulipes* specimens were classified as ambiguous using the ASB criterion. These two species share the same BIN cluster in BOLD and were identified as one MOTU.

High intraspecific divergence was observed between two specimens of the introduced *Ae. koreicus* (6.2%—no match at the 2.6% threshold and they were identified as two separate MOTU’s). Introduced species are thought to exhibit a reduced genetic diversity (Tsutsui et al. 2000), but the observed divergence in *Ae. koreicus* could reflect multiple introductions from different populations (Fonseca et al. 2010). In the NJ-tree, the species cluster with *Ae. j. japonicus* and both taxa form a well-supported group. The taxonomy of these taxa is currently being revised. Phylogenetic studies in the USA by Cameron et al. (2010) place *Ae. koreicus* and *Ae. j. japonicus* in a single clade and suggest that *Ae. koreicus* belongs to the *Ae. japonicus* species complex.

DNA barcoding is a successful molecular identification tool for insects in general and for mosquitoes in particular (Cywinska et al. 2006; Kumar et al. 2007). The current study shows that it also offers a reliable framework for mosquito species identification despite the identifications of a few closely related species remain ambiguous. The current analysis points to some taxonomic differentiation that needs further investigation such as in *Cx. pietiens* and *An. maculipennis*, but also *Cx. territans* and *Ae. punctor*. Identifying species is an essential step in vector monitoring and control. Our results directly contribute to the further development of a comprehensive and reliable mosquito DNA
identification database. To further improve the DNA Barcode reference library for mosquitoes in Belgium, species with complete series of larval exuvia (L4), pupae and adults should be added to document the link between morphological and molecular identification standards.

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V.V. was responsible for the day-to-day implementation of the study including field sampling, morphological identification and laboratory analysis. She drafted the manuscript. Z.T.N. and F.C.B. performed the sequencing, the sequence alignment and analysis, and contributed to the manuscript. P.R. and L.D. were responsible for the laboratory analysis and reviewed the manuscript. D.D. and W.D. performed the mosquito collections and morphological identification, and contributed to the manuscript. T.B. contributed to the data analysis and interpretation and to the manuscript drafting. M.C. critically reviewed and contributed to the manuscript. W.V.B. was responsible for the design and coordination of the study, reviewed the data analysis and contributed to drafting and reviewing the manuscript. All authors read and approved the manuscript.

Data Accessibility

Information of each individual specimen is provided in supplementary information File S1 including species name, species identification number, coordinates, locality name and habitat where the specimen was collected. Specimens used in this study are stored in the collections of the Institute of Tropical Medicine, Antwerp, Belgium and voucher specimens were deposited at the Royal Belgian Institute of Natural Sciences. Sequence alignment is uploaded as supplementary material. Data were submitted to BOLD and accession nos are following: [CULBE001-14 – CULBE260-14 – and completed in supplementary information File S1].

Supporting Information

Additional Supporting Information may be found in the online version of this article:

File S1 List of specimens with species number, locality and habitat type. The habitat type is based on an aggregated Corine landuse classification as defined in Versteirt (2012).

File S2 Neighbour-joining tree based on the Kimura 2-parameter distances among COI sequences (658 bp fragment) of 24 Culicidae species. Bootstrap values are shown at the branch points. Coloured label before species ID and name: habitat where species was collected based on combined Corine landuse classification: Green: rural; Blue: peri-urban; Red: urban. Circle: specimen correctly identified according to ASB; Triangle: specimen classified as ambiguous according to ASB; square: specimen classified as no-match according to ASB. Collection place between brackets.
File S3 Haplotype network constructed with SplitsTree, *Anopheles maculipennis* s.l.; *Aedes annulipes* and *Aedes cantans*; *Culex pipiens* and *Culex torrentium*

File S4 Neighbour-joining tree based on the Kimura 2-parameter distances among COI sequences of 24 Culicidae species and the BIN clusters identified through http://www.boldsystems.org/index.php/databases. Bootstrap values are shown at the branch points. Sequences marked with black squares: sequence selected from the respective BIN cluster.

File S5 sequence alignment of study specimens.