

COI sequencing for invasive mosquito surveillance in Germany reveals genetically divergent specimens near *Aedes geniculatus* (Diptera: Culicidae)

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Abstract: During routine surveillance for invasive mosquitoes in southern Germany, using BG-Sentinel traps that were run continuously for one to two weeks, specimens were often damaged which prevented morphological species identification. We used DNA sequencing of the COI gene for molecular identification of damaged specimens, and observed two individuals from 2013 with aberrant COI-sequences. These two specimens were collected on separate occasions, and represent COI haplotypes that differ from *Aedes (Finlaya) geniculatus* by 2.0 - 2.4%, and from *Aedes (Finlaya) echinus* by 4.4 - 5.0%. Further analysis revealed a similarity to an *Aedes (Finlaya)* specimen from Macedonia, with these three specimens forming a genetic cluster. Our findings are discussed in the context of the three *Aedes (Finlaya)* species presently reported in Europe, and the potential introduction into Germany by road traffic. *Journal of the European Mosquito Control Association* 32: 22-26, 2014

Keywords: *Aedes (Finlaya) geniculatus*, *Aedes (Finlaya) echinus*, *Aedes (Finlaya) gilcolladoi*, Germany, COI barcoding, invasive species

Introduction

In recent years growing attention has been paid to mosquitoes in Germany and other European countries due to the emergence of invasive species (Medlock *et al.*, 2012), such as the Asian tiger mosquito *Aedes albopictus* (= *Stegomyia albopicta* sensu Reinert & Harbach, 2005), the emergence of exotic mosquito-borne diseases in southern Europe, such as chikungunya and dengue (Rezza *et al.*, 2007; Gould *et al.*, 2010), and first reports of mosquito-borne viruses in German mosquitoes, namely Batai, Sindbis and Usutu (Jöst *et al.*, 2010, 2011a, 2011b).

In order to estimate the risks of introduction of invasive or exotic mosquitoes into Germany, a surveillance programme was initiated in 2012, focussing on the South-North transit motorways in Baden-Württemberg and Bavaria, where increasing numbers of *Ae. albopictus* were indeed captured in 2012 and 2013 (Werner *et al.*, 2012; Becker *et al.*, 2013), while *Aedes japonicus*, which was first recorded in Germany in 2008 (Schaffner *et al.*, 2009), can be regarded as established by now in several parts of Germany (Kampen *et al.*, 2013).

Routine mosquito trapping and surveillance are rather time-consuming and costly when only a limited number of people are available to check the traps in the various locations by car. In order to minimise these efforts and expenses, adult mosquito traps are usually run over weekly or bi-weekly intervals. This has the disadvantage that the specimens caught during the first few days remain dead in the trap for up to two weeks. During that period, all specimens die and remain in the air flow of the sucking device and mixed with by-catch e.g. tipulids, wasps, all of which leads to morphological damage, making their identification a great challenge.

Using both CO₂ and odour-baited traps as well as ovitraps, we detected several Culicine specimens, which were in such bad condition that only a DNA-based identification could

clarify their classification. We choose the cytochrome c oxidase subunit 1 (COI) gene sequencing approach, because there exists a considerable amount of reference data in DNA databases such as Genbank (www.ncbi.nlm.nih.gov/genbank/) or BOLD (www.boldsystems.org). A 648-base pair region of the COI gene has been widely accepted as the standard barcode in animal DNA taxonomy (Ratnasingham & Hebert, 2007). It was shown to be highly conserved intra-specifically (rarely more than 2% variation) and hence allows inter-specific discrimination (Hebert *et al.*, 2003). These authors found in the order Diptera a mean inter-specific divergence of 9.3 % ± 3.5 %, and in 76 % of all analysed species the divergence ranged between eight and 16 %. In an attempt to barcode the main Chinese mosquito species, Wang *et al.* (2012) found that the divergence in over 98% of intra-generic species ranged from 2.3% to 21.8%. Ashfaq *et al.* (2014) applied the technique in order to identify the mosquito species of Pakistan, to reveal their genetic diversity and geographic distribution. Engdahl *et al.* (2013) conducted a COI barcoding analysis on Swedish mosquitoes, including 15 *Aedes* and *Aedes* species, respectively, and found some inconsistencies when compared to the morphological classification. Indeed, the COI barcoding can have certain limitations. In about seven percent of dipteran species the COI divergence is below four percent (Hebert *et al.*, 2003), making a discrimination between inter- and intra-specific variation difficult. On the other hand, COI data may be unsuitable to resolve very closely related species and sibling species, i.e. members of species complexes. Here, other methods, e.g. the analysis of the internal transcribed spacer region of the nuclear DNA could be advantageous, for example for the *Anopheles maculipennis* complex (Weitzel *et al.*, 2012).

Nevertheless, for routine purposes aiming at confirmation of damaged specimens of invasive species, surveillance

programmes can rely successfully on the barcode technique (Werner *et al.*, 2012; Becker *et al.*, 2013). Beside *Ae. albopictus* and *Ae. japonicus*, both of which are also referred to as exotic container-breeding species, there is *Aedes* (Finlaya) *geniculatus* (= *Dahliaena geniculata* sensu Reinert *et al.*, 2006) which is an endemic dendrolimnic species in the surveillance area. While this species has clearly been confirmed by COI sequencing (Krüger *et al.*, 2014), two specimens remained unresolved due to aberrant COI sequences. On the basis of these specimens the present study offers explanations for taxonomic problems with *Ae. geniculatus* and its closest relatives.

Materials and Methods

Sampling protocol

In order to estimate the risk of introduction of invasive mosquitoes into Germany, a routine surveillance at potential routes of entry, such as highly frequented motorways, was carried out since 2012, focussing on south-north routes originating in countries such as Italy, where *Ae. albopictus* is already established. One out of 16 respective sampling locations at service stations in Bavaria was along the motorway 8 south of Munich, at Irschenberg (co-ordinates N47.828° E11.90°, altitude 700m a. s. l.). Two female specimens, collected during July 3rd-18th and September 4th-9th, 2013, respectively, were of particular concern. The collection device was a continuously running BG-Sentinel trap baited with BG-lure and CO₂ from gas cylinders (Biogents, Regensburg, Germany) that was sampled bi-weekly. In total, 27 mosquito specimens of suspected invasive species had to be analysed from Bavaria in 2013.

Mosquito diagnostics

Since their scaling characters (Becker *et al.*, 2010) were usually not sufficiently preserved and hence a clear classification as one of several similarly looking species, e.g. *Ae. albopictus*, *Ae. japonicus* or *Ae. geniculatus* was impossible, a PCR-amplification and direct sequencing of 597 base pairs (bp) of the mitochondrial cytochrome oxidase subunit 1 (COI) gene was conducted (Mousson *et al.*, 2005). The resulting sequence of 543 bp does not cover a full barcode length (648 bp), but has been successfully used for phylogeographic studies with *Ae. albopictus* by Mousson *et al.* (2005). Since our initial goal was also aimed at phylogeographic analyses of *Ae. albopictus*, introduced into Germany, the same protocol was used for all routine DNA identifications. This procedure was applied to the 27 questionable specimens from Bavaria (adults from BGS traps, eggs from ovitraps, not all data shown) as well as assumed *Ae. geniculatus* adults from the federal state of Baden-Württemberg from 2012 and 2013. For reference, two female *Ae. geniculatus*, which had been reared from larvae collected in beech tree holes in Hamburg, Germany in 2012 (data not shown), and one larva of *Ae. echinus* were used. The latter was collected from a black poplar dendrothelm in June 2011 in Enciso (La Rioja, Spain) at 765 meters of altitude (coordinates N42.147389° W2.270139°).

The received COI sequences of the two specimens of concern were first classified using the identification tool of BOLD (Barcoding of Life) (Ratnasingham & Hebert, 2007), which allowed for the best match with BOLD barcode index number AAM5898 (specimen MBIL418-10, field ID BMNH-ARCPLO4YL) from Macedonia. In addition, a BOLD based neighbour-joining taxon ID tree with specimen UBA28

(“unknown specimen”) was also estimated. Subsequently, the sequences of the two specimens of concern (UBA28, UBA75), of MBIL418-10, one *Ae. echinus*, and nine *Ae. geniculatus* were aligned online using MAFFT (www.ebi.ac.uk/Tools/msa/mafft/), together with *Aedes caspius* HM535300 as “nearest neighbour” taxon, and *Aedes japonicus* JQ404435 as “outgroup”. The resulting alignment was used to generate a simple taxon identification (ID) tree, conducted by using MEGA version 5 (Tamura *et al.*, 2011), with the following analysis settings: neighbor-joining methods, bootstrapping with 1000 replications, Kimura 2-parameter substitution model. All sequences generated in this study are available upon request from the corresponding author. The Genbank accession numbers of the sequences of UBA28 and UBA75 are KM522856-KM522857.

Results

The initial step was requesting a barcode identification using BOLD. Figure 1A shows the resulting original taxon ID tree for one of the questionable specimens from Bavaria (“unknown specimen”), which was close to a Macedonian *Ae. geniculatus* (=MBIL418), but more distant from 13 other *Ae. geniculatus*. The following analyses of the COI sequences of 11 specimens from various sites in Germany in addition to *Ae. echinus* from Spain and two outgroup taxa revealed again two clearly divided clusters within the subgenus *Finlaya*, plus a third branch represented by *Ae. echinus* (Fig. 1B).

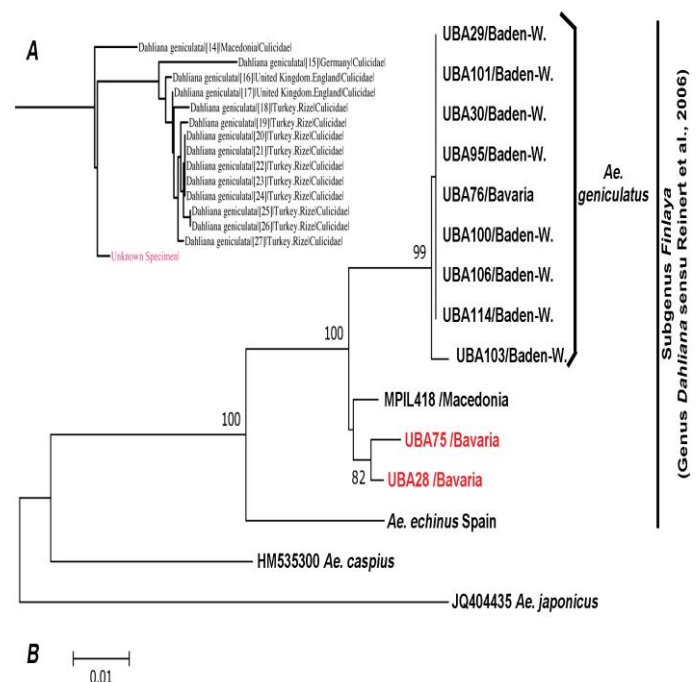


Figure 1. Neighbour-joining taxon identification trees derived from partial COI sequences. A) Original BOLD tree requested online for classification of an “unknown” Bavarian specimen. B) Taxon ID tree for all specimens from the present study, including previously published sequences MPIL418 (BOLD), JQ404435, and HM535300 (NCBI Genbank). Numbers on branches are bootstrap values >80%, i.e. statistical node support after 1000 replications (100 = 100% support). Scale: Kimura 2-parameter distance.

Since the main cluster showed up to 100% similarity with nine out of 13 *Ae. geniculatus* sequences in BOLD, the remaining nine specimens were also assumed to belong to *Ae. geniculatus*, with all nine showing a maximum intra-specific variation of 0.36% (nucleotide difference within 543 base pairs). For comparison: the 13 *Ae. geniculatus* sequences in the BOLD database (excluding MBIL418 from Macedonia) exhibit a maximum variation of 1.7%. The second cluster included only the two questionable specimens from Irschenberg in Bavaria and BOLD specimen MBIL418, which varied from the former by only 1.1% (within 543 base pairs), suggesting conspecificity of all three. On the other hand, this cluster differed between 2.0–2.4% from the *Ae. geniculatus* clade (2.1–3.8% in BOLD), and 4.4–5.0% from *Ae. echinus*. The latter showed 5.3–5.7% difference to *Ae. geniculatus*.

Discussion and conclusion

The unequivocal identification of mosquito specimens is one of the backbones of mosquito and vector surveillance programmes (Schaffner *et al.*, 2013). As the invasive species *Ae. albopictus* had already been detected in Bavaria (Becker *et al.*, 2013), and the two questionable specimens were caught in the context of the same ongoing surveillance programme for invasive mosquitoes in southern Germany, their unambiguous identification was mandatory. The result, however, remains puzzling. The closest COI barcode match is a specimen from Macedonia designated as *Ae. geniculatus* (Fig. 1A), which clearly deviates from the other BOLD entries of *Ae. geniculatus* from Turkey, UK and Germany. The closest relatives of *Ae. geniculatus* in Europe are *Ae. echinus*, and possibly *Ae. gilcolladoi*, neither of which have been observed in Germany (Becker *et al.*, 2011), but are considered to be Mediterranean species.

For neither *Ae. echinus* nor *Ae. gilcolladoi*, public reference DNA sequence data were available, and the present study provides the first COI sequence for *Ae. echinus*. Regarding *Ae. gilcolladoi*, Becker *et al.* (2010) stated: “The position regarding the validity of this species is unsatisfactory. Type specimens were not deposited, and material is not available for examination.”

When drawing conclusions on the taxonomic identity of the new cluster there are the following possible interpretations:

i) The two specimens from Bavaria and MBIL418 from Macedonia represent a hitherto unknown cryptic sibling species of *Ae. geniculatus*, which then itself constitutes a species complex.

ii) The two specimens from Bavaria and MBIL418 from Macedonia represent true *Ae. echinus*, whereas our “*Ae. echinus*” from Spain could be *Ae. gilcolladoi*. This possibility is supported by several circumstances: the type locality of *Ae. echinus* is Greek Macedonia, hence near the collection site of MBIL418. In addition, Eritja *et al.* (2000) stated: “Statistical and biometric comparisons [of *Ae. gilcolladoi*] with European specimens of *Ae. echinus* led the authors [i.e. Sánchez-Covisa *et al.*, 1985a] to conclude that the latter did not occur in Spain, where *Ae. gilcolladoi* replaced *Ae. echinus*.” Edwards (1920), in the description of *Ae. echinus*, also included specimens from North Africa, but stated: “The Moroccan and Algerian specimens ... are somewhat doubtfully conspecific with the type.” This may suggest the presence of *Ae. gilcolladoi* in North Africa, hence in the western Mediterranean.

iii) The two specimens from Bavaria and MBIL418 from Macedonia represent *Ae. gilcolladoi*, which would hence not be restricted to Spain only, although Sánchez-Covisa *et al.* (1985a,b) proposed such restriction. This is certainly the least likely possibility.

Eventually, the issue of the presence or absence of *Ae. echinus* and *Ae. gilcolladoi* in Spain remains controversial. Some authors claim solely *Ae. gilcolladoi* to be present (Sánchez-Covisa *et al.*, 1985a,b; Schaffner *et al.*, 2001), others list both species for Spain (Melero-Alcibar *et al.*, 2009; Bueno-Marí *et al.*, 2012), or emphasize the uncertainties (Eritja *et al.*, 2000). Further careful studies throughout Europe are mandatory in order to elucidate the systematics of these Aedine taxa. Figure 2 summarizes the published distribution records for all three species including the type localities. In this context it should be emphasized that the two Bavarian specimens have most probably been imported from the Balkan, e.g. Macedonia, by road traffic, as a similar route of introduction has also been suggested for the German *Ae. albopictus* findings, originating in Italy, where this species is common (Becker *et al.*, 2013).

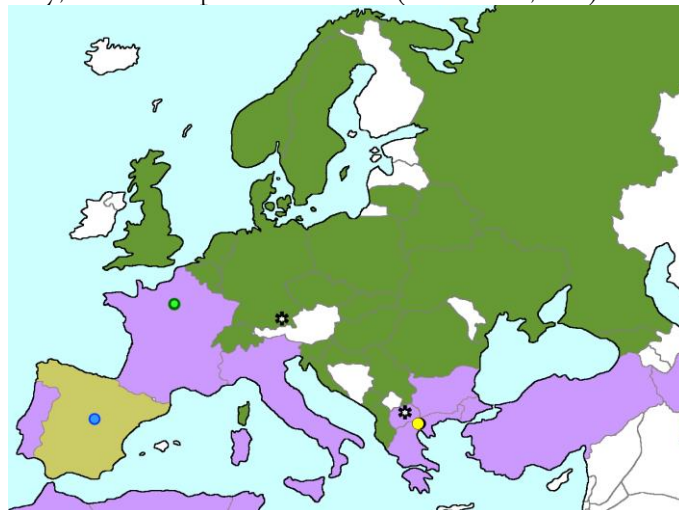


Figure 2. Country record map of three *Aedes* species. Green: *Ae. geniculatus*; violet: *Ae. geniculatus* and *Ae. echinus*; olive: *Ae. geniculatus*, *Ae. gilcolladoi* and *Ae. echinus*; White: negative or unknown. Asterisks mark the new findings in Bavaria and their closest BOLD match in Macedonia. Other dots: the type localities, green= *Ae. geniculatus*, blue= *Ae. gilcolladoi*, yellow= *Ae. echinus*.

Several studies have concluded that for dipteran culicid COI gene DNA sequences, the critical genetic distance of >2% applies to most species delimitations (Wang *et al.*, 2012; Ashfaq *et al.*, 2014). Hebert *et al.* (2003) reported a mean inter-specific divergence of 9.3% in more than 1400 dipteran species pairs, with more than 92% of species exceeding four percent distance. Similarly, Wang *et al.* (2012) found in over 98% of 122 Chinese mosquito species that the inter-specific divergence exceeded 2.3%. Applying these values to our data, the two specimens from Bavaria and the BOLD record from Macedonia, which differ by 2%–2.4% from *Ae. geniculatus*, just fall into the threshold zone of intra- and inter-specific delimitation, which prohibits final conclusions on their taxonomic status. This uncertainty is further aggravated by the lack of reference data for *Ae. gilcolladoi*. Eventually, the present study was based on only 543 base pairs, which represents only 84% of the full COI barcode and may thus result in slight over-estimations of the

distances. If that was the case and the two questionable specimens would differ by less than 2% from *Ae. geniculatus*, the first explanation given above would indeed be the most likely one, i.e. that *Ae. geniculatus* could represent a species complex, or at least a highly variable species. Future analyses of other genomic regions, and careful searching for more specimens in a European framework may resolve this question.

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