A distinct group of north European Aedes vexans as determined by mitochondrial and nuclear markers

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Abstract. The floodwater mosquito Aedes (Aedimorphus) vexans (Meigen, 1830) (Diptera: Culicidae) is common in several areas of Sweden and is predicted to become more abundant in the wake of expected changes in precipitation and temperature caused by climate change. As well as being a nuisance, Ae. vexans can act as a vector of over 30 viruses. In the event of an outbreak of disease caused by a vector-borne virus, knowledge of the distribution, population structure and intermixing of populations from different locations will help direct resources to target locations to prevent spread of the pathogen. The present study analysed individual Ae. vexans from eight locations throughout Sweden. Based on the mitochondrial cytochrome oxidase I (COI) marker, a subset of the analysed mosquitoes cluster apart from the other samples. Similarly, two nuclear loci were sequenced and the same phylogenetic structure observed. These results indicate that this group represents a reproductively isolated population among Ae. vexans. Comparisons with COI sequences held in the Barcode of Life Database (BoLD) for Ae. vexans from around the world show that specimens collected in Belgium and Estonia group together with the Swedish group, suggesting that this genotype is present throughout northern Europe. These results suggest there is a cryptic taxonomic unit related to Ae. vexans in northern Europe.

Key words. Culicidae, barcoding, COI, surveillance, vectors.

Introduction

The floodwater mosquitoes Aedes (Aedimorphus) vexans and Ochlerotatus sticticus (=Aedes sticticus) (Diptera: Culicidae) have the potential to become a nuisance when present in large numbers. In Sweden they occur in several areas and are predicted to spread and become more common over large areas of the country as a result of changes in precipitation and temperature effected by global warming (Schäfer & Lundström, 2009). As well as representing a nuisance, Ae. vexans also has the potential to act as a vector of more than 30 viruses (Horsfall & Novak, 1990), many of which cause important diseases such as West Nile fever, St Louis encephalitis and eastern equine encephalitis (Goddard et al., 2002; Cupp et al., 2004). In the event of an outbreak of a vector-borne disease, previous knowledge of the distribution and population dynamics of the vector can help to focus disease prevention strategies on the right locations (Schaffner et al., 2014). In addition to knowledge of the distributions of vectors such as Ae. vexans, it may be important to have information about the intermixing of mosquito populations from different locations. To estimate the level of intermixing, studies have looked at the genetic differences between mosquitoes from different locations to find out how genetically isolated each population is (Szalanski et al., 2006). Szalanski et al. (2006) examined the genetic diversity of Ae. vexans from Kansas, U.S.A., by sequencing the mitochondrial NADH dehydrogenase subunit 5 (ND5) and found high genetic variance within the studied populations, although no geographical structure to the distribution of genetic variants was apparent. A recent study of European Ae. vexans,
which included mosquitoes from Sweden, Hungary and Serbia (Francuski et al., 2016), used allozyme analysis and wing morphometrics to study intra- and interpopulation variations. This study found a large amount of genetic variation within populations and a low proportion of interpopulation variance.

Some characteristics of *Ae. vexans*, such as its propensity for migrating long distances and its robust eggs, increase its potential to spread over large areas. Females have been shown to migrate up to 48 km (Gjullin et al., 1950; Morhig, 1969; Briegel et al., 2001). The dispersion of *Ae. vexans* also benefits from its eggs’ ability to survive in soil for several years (Maurice & Harwood, 1969). These features are likely to increase the diversity of a local population because they make it less likely that such a population will be subjected to a tight bottleneck that might resolve genetic variants. This conclusion is supported by the genetic stability of *Ae. vexans* in New Orleans, Louisiana, U.S.A., in the face of intense mosquito control efforts after Hurricane Katrina (Solorzano et al., 2010). The present study assessed the genetic variation in Swedish *Ae. vexans* to estimate connections between local populations.

### Materials and methods

Host-seeking female mosquitoes were collected as part of a larger project that aimed to map mosquito populations throughout Sweden. Mosquitoes were collected by members of the public and owners of Mosquito Magnet™ traps (Woodstream Corp., Lititz, PA, U.S.A.), who were invited to collect mosquitoes over any 24-h period during the summer and to send their collections to the National Veterinary Institute of Sweden [Statens Veterinärmedicinska Anstalt (SVA)] for analysis. Mosquito Magnet™ traps were positioned at the locations listed in Table 1. *Aedes vexans* specimens were identified by Eric Blomgren at the National Veterinary Institute using morphological keys (Becker et al., 2010). To allow future morphological verification, only three legs were used for DNA extraction. Specimens from eight locations were analysed (Table 1). In addition, DNA was extracted and cytochrome oxidase subunit I (COI) sequenced from six *Ae. vexans* collected in Estonia to provide a source of comparison with mosquitoes from a distinct region.

DNA was extracted from legs from 99 mosquitoes homogenized in 30 μL PrepMan Ultra (Applied Biosystems, Inc., Foster City, CA, U.S.A.). In brief, the homogenized solution was incubated at 100 °C for 10 min and centrifuged at 15,000g for 3 min, after which 20 μL of the supernatant was deposited in a new tube and used as a template for polymerase chain reaction (PCR) assays. Because of problems with DNA
was determined according to the number of base substitutions per site by averaging over all sequence pairs between locations. Analyses were conducted using the Tamura 3-parameter model (Tamura, 1992), which accounts for C-content bias as has been seen in mitochondrial DNA, was used for the COI sequence. The rate variation among sites was modelled with a discrete gamma distribution (shape parameter: 0.19). For ITS2 sequences, the model assumes equal base frequencies and equal mutation rates. Evolutionary divergence of the COI sequence within and between locations was determined according to the number of base substitutions per site by averaging over all sequence pairs between locations. Analyses were conducted using the Tamura–Cantor model (Jukes & Cantor, 1969). The rate variation among sites was modelled with a discrete gamma distribution (shape parameter: 1.22).

Phylogenetic analysis was performed using the maximum composite likelihood model for COI sequences. The Tamura-3 model (Tamura, 1992) with a discrete gamma distribution was used to model evolutionary rate differences among sites in five categories (+G, parameter: 0.19). For ITS2 sequences, the model assumes equal base frequencies and equal mutation rates. Evolutionary divergence of the COI sequence within and between locations was determined according to the number of base substitutions per site by averaging over all sequence pairs between locations. Analyses were conducted using the Jukes–Cantor model (Jukes & Cantor, 1969). The rate variation among sites was modelled with a gamma distribution (shape parameter: 0.19). Evolutionary divergence of the ITS2 sequence within and between locations was determined according to the number of base substitutions per site by averaging over all sequence pairs between locations. Analyses were conducted using the Jukes–Cantor model (Jukes & Cantor, 1969). The rate variation among sites was modelled with a discrete gamma distribution (shape parameter: 1.22).

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The proportion of the collected mosquitoes identified as *Ae. vexans* varied from 78.0% in Kävlinge to 23.0% in Ottenby, 70.0% in Gotland, 4.3% in Vara, 10.7% in Tibro, 21.0% in Vindeln, 8th 0.247 0.221 0.309 0.187 0.355 0.149 0.147
Gotland 3rd 0.394 0.366 0.036 0.051 0.033 0.034 0.031
Vara 4th 0.255 0.195 0.335 0.038 0.010 0.008 0.021
Tibro 5th 0.446 0.387 0.469 0.310 0.037 0.037 0.041
Sala 6th 0.212 0.159 0.300 0.068 0.286 0.005 0.017
Söderhamn 7th 0.209 0.150 0.300 0.051 0.282 0.023 0.018
Vindeln 8th 0.247 0.221 0.309 0.187 0.355 0.149 0.147

The proportion of the collected mosquitoes identified as *Ae. vexans* varied from 78.0% in Kävlinge to 23.0% in Ottenby, 70.0% in Gotland, 4.3% in Vara, 10.7% in Tibro, 21.0% in Vindeln. However, each of these locations was sampled for only 24 h and hence conditions in some locations may have been more favourable for *Ae. vexans* than in others at the time the samples were collected. For example, the same location in Gotland was sampled 1 year earlier and no *Ae. vexans* was collected at that time. Three loci were sequenced from Swedish *Ae. vexans*: COI in the mitochondrial DNA; ITS2 in the rRNA locus, and the homologue of *S. aegypti* gene AAEL000094 (referred to as AVh-AAEL000094) from the nuclear DNA. These sequences were compared in order to estimate the genetic distance between individuals in Sweden, a country with diverse habitats from south to north. Morphologically identified *Ae. vexans* from eight locations throughout Sweden (Kävlinge, Ottenby, Gotland, Vara, Tibro, Sala, Söderhamn and Vindeln) (Fig. 1) were compared to assess genetic differences that may correlate to geographical distance. DNA was extracted and the barcoding region of COI, as well as the ITS2 of the rRNA region, was sequenced. COI sequences were identified as *Ae. vexans* in BoLD. Both COI and ITS2 sequences were available for 68 specimens, for 49 of which AVh-AAEL000094 was sequenced to further analyse differences between the mosquitoes.

Sequences from each location were compared to estimate genetic distances between local populations. For this analysis, 14 specimens from Ottenby, 33 from Kävlinge, five from each of Sala and Vindeln, three from each of Tibro and Söderhamn and four from each of Vara and Gotland were used. The population with the highest intrapopulation genetic distance according to the COI marker comes from Ottenby, followed by those from Tibro and Kävlinge. Interpopulation genetic distances for the COI marker between the samples from Vindeln, Gotland and Kävlinge were small. Genetic distances between the populations from Söderhamn, Sala, Tibro and Vara were also small. The population from Ottenby has intermediate interpopulation genetic distances from all the other populations (Tables 2 and 3). The same analysis was performed for the ITS2 locus, but, because of PCR failures, Kävlinge was represented by 32 specimens and Gotland was represented by three specimens. Based on the ITS2 marker, the genetic distance was larger within the Gotland population, but the overall result showed small distances between the groups from Söderhamn, Sala, Tibro and Vara, similarly to the COI result (Tables 4 and 5). No discernable correlation was discovered when interpopulation genetic differences were plotted against the geographical distances between the catch locations (Fig. 2).

The phylogenetic analysis of the COI sequences showed that the specimens group into two distinct clusters: Group 1 and Group 2. All sequenced specimens from six of the locations group together, but in specimens from Kävlinge and Ottenby, both sequence variants are represented. A phylogenetic tree of the COI region constructed with all 68 specimens confirms the division into two groups (Fig. 3A). The Swedish specimens were compared with six specimens collected in Estonia, as well as with COI sequences from *Ae. vexans* from both Europe and North America available in BoLD. This comparison shows that the Group 1 specimens form a clade together with two sequences from Belgian *Ae. vexans* and four from Estonian specimens. All *Ae. vexans* sequences group into four distinct groups: Group 1; *Aedes vexans nipponii*; North American specimens, and European specimens. The Group 1 specimens are divergent from both other European *Ae. vexans* and North American specimens and are more closely associated with, but distinct from, *Ae. v. nipponii* specimens (Fig. 3C). Additional comparisons of ITS2 sequences from the Swedish specimens and four available ITS2 sequences from other countries showed a similar clustering of these sequences (Fig. 3B). The bootstrap values for the Group 1 cluster in the ITS2 sequences are low, indicating that the phylogenetic signal for this node is low. For 49 of these specimens the nuclear gene AAEL000094 was also sequenced, showing an insertion of 6 bp compared with Group 2 in those specimens that cluster in Group 1 for COI and ITS2. Thus, three markers, one in the mitochondrial DNA and two nuclear loci, group these
specimens together as distinct from other Swedish *Ae. vexans* (Fig. 4).

Results from the sequencing of one mitochondrial locus and two nuclear loci show the same specimens form a clade, which suggests that these specimens are reproductively isolated from other *Ae. vexans* and represent a separate taxonomic unit.

**Discussion**

When COI sequences from tested mosquitoes were used to generate a phylogenetic tree, a group of mosquitoes that clustered apart from other specimens was revealed. At six locations, only one group was represented, but in Kävlinge and Ottenby both groups were found. Further specimens from these collections were included and the resulting sequences were compared with publicly available *Ae. vexans* sequences and specimens from Estonia sequenced within this study. Some of the Swedish specimens, four of six Estonian specimens and two Belgian specimens were found to group together.

The differences in COI sequence are interesting, but divergent mitochondrial lines on their own do not necessarily indicate separate taxa because mitochondrial genes are inherited maternally and no recombination occurs. Interestingly, the present analysis of two separate nuclear genes shows a similar pattern for all 68 specimens in which both COI and ITS2 were analysed and in
Fig. 3. (A) Phylogenetic analysis of COI sequences from specimens from Sweden. Publicly available Stegomyia albopicta (=Aedes albopictus) sequences are used as outgroup data. Nodal values represent bootstrap values for each node. (B) Phylogenetic analysis of ITS2 sequences from specimens from Sweden and publicly available sequences from the U.S.A., China, Iran and Spain. Publicly available S. albopicta sequences are used as outgroup data. Nodal values represent bootstrap values for each node. (C) Phylogenetic analysis of Swedish, Estonian and publicly available sequences from around the world. Publicly available S. albopicta sequences are used as outgroup data. Nodal values represent bootstrap values for each node.
Fig. 4. Sequence comparison for the AVEL000094 locus. A 6-bp insertion distinguishes specimens in the outgroup from other Swedish *Aedes vexans*.

the 49 specimens in which AVEL000094 was also analysed. For ITS2, the phylogenetic signal distinguishing the groups was low. This can be attributed to the fact that only a few mutations differ between the groups. However, these changes are consistent and distinguish the groups from one another. The clustering of the two groups is clear for the COI region and is supported by similar results from the two nuclear genes. As mitochondrial and nuclear genes are inherited through different mechanisms, the fact that the three alleles occur together in all specimens suggests that the two variants have not mixed. Because these specimens were collected in areas in which both groups co-localize, the present finding that the same specimens group together for all three loci suggests they represent a group of mosquitoes that might be reproductively separated. Of the 16 publicly available *Ae. vexans* sequences from Belgium present in BoLD (Versteirt et al., 2015), two fall within the Group1 COI sequence variant described herein, demonstrating that this variant is present elsewhere in Europe. Other sequences from Russia (Khrabrova et al., 2013) and Hungary (Zittra et al., 2015) group together with the other *Ae. vexans* in Sweden in Group 2. The present authors conclude that the Group 1 specimens may represent a taxonomic unit with a northwest European distribution. However, they group close to, but are still distinct from, the publicly available *Ae. v. nipponii* specimens (Fig. 3C). *Aedes v. nipponii* has been found as far west as Ukraine (Sheremet, 1975) and has been considered an invasive species in North America (Cywinska et al., 2006).

If the two groups identified in the present study are considered as different taxonomic units, the current observations, although performed with a different method, agree with those of a study conducted in Swedish, Hungarian and Serbian *Ae. vexans* (Francuski et al., 2016). Francuski et al. (2016) used allozyme loci to identify large intrapopulation variation and low levels of interpopulation variation, indicating a substantial exchange between populations. The study did not include sequencing of the COI barcoding region and hence no direct comparison with the present populations can be made. The studied Swedish populations were collected in Deje, which is not represented in the present study, and in Gysinge, which is relatively close to Sala (approximately 45 km), at which *Ae. vexans* of the Group1 variant were collected in the present study. Although Francuski et al. (2016) concluded that the data represented populations with continuous exchange, the fact that the Swedish populations were most divergent, especially the population in Gysinge, suggests this may be a population similar to those sequenced in the present study.

The specimens in the current study were taken from samples collected during a national survey of mosquitoes which, because of its collection methods, biases the collection towards host-seeking adult females. These collections are not suitable for detailed morphological study, which would potentially identify morphological differences between the two genetic groupings. No males or immature stages contributed to this study. It is possible there are potential morphological differences between
Group 1 and Group 2 in some life stage or in either males or females. For these differences to be determined, sufficient larvae belonging to both groups would have to be collected, reared to adults, and then critically examined. This was not possible within the limits of the current study.

Taken together, the present data do not indicate any differences between local populations of previously described Ae. vexans in different parts of Sweden, which represents a conclusion similar to those of earlier studies. However, the current findings indicate a clear difference between two groups of morphologically determined Ae. vexans that seem to be reproductively separated from one another, even when collected from the same location.

The taxonomic rank of this population is unclear. Speculation on the taxonomic nature of this new group requires the collection of a larger sample and study of the morphological characters of all life stages in both groups.

Acknowledgements

This work was financed by the Swedish Board of Agriculture and Formas project 2014-1556, MOBOZO (TL). The work carried out by TL and AL was conducted within the framework of EurNegVec COST Action TD1303. The authors thank Eric Blomgren, Statens Veterinärmedicinska Anstalt, for the morphological identification of the mosquitoes used in this study.

The authors report no conflicts of interest.

Funding information – online only.

This study was supported by the Swedish Board of Agriculture and Formas project 2014-1556, MOBOZO (TL).

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Accepted 7 December 2017
First published online 16 January 2018