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The differentiating power of mitochondrial genes: complete mitogenome sequences of 27 mosquito species present in Europe

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Abstract

The rapid advancement of genomic tools has revolutionised entomological research, offering new insights into insect evolution, population dynamics, and species identification. Much in line with most other insects, mitochondrial DNA has emerged as a key resource in mosquito studies, with the partial *cox1* (cytochrome oxidase c subunit 1, oftentimes referred to as COI) gene commonly used for species identification. However, *cox1*'s limitations in resolving cryptic and sibling species and its challenges in metabarcoding applications underscore the need to explore alternative genetic markers. This study addresses the lack of comprehensive reference mitogenomes for mosquitoes present in Europe, by sequencing and assembling 82 mitochondrial genomes from 27 Northwest European species including 3 invasive *Aedes* species. Two-thirds of the species' mitogenomes were sequenced for the first time. Our results highlight a notable variability of mitochondrial genes, highlighting the potential for development of genetic markers to improve taxonomic resolution. Notably, the *nad6* and *nad2* genes demonstrated more variability compared to *cox1*, exhibiting higher nucleotide diversity, more variable base pairs and greater between-species variability. These genes are flanked by conserved tRNA genes, providing ideal primer sites and enabling efficient amplification for degraded or pooled samples. As such, this study presents a foundation for improved molecular identification techniques, enhancing mosquito monitoring and research across Europe by providing mitogenome sequences of 26% of the 102 European mosquito species. It also highlights the need to sequence the mitogenomes of a much larger fraction of the mosquito species community. By expanding the availability of mitogenomic data, researchers can explore novel genetic markers to improve biomonitoring and address current challenges in species identification.

Keywords

mitochondrial DNA – mosquito genomics – reference genomes – species identification – Culicidae

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1 Introduction

Over the past few decades, there has been a remarkable expansion of the entomologists' genomic toolbox, which has allowed for a deeper understanding of insect evolution, unravel population dynamics, facilitate species identification, and enhance monitoring practices. For all those activities, mitochondrial DNA (mtDNA) genes are ideal markers for demonstrating inter-specific and intra-specific variation, as their inheritance is typically uniparental (mainly maternally), recombination is absent, introns are absent, yet they exhibit a higher nucleotide substitution rate and are available in a high number of copies per cell when compared to nuclear DNA (Ladoukakis and Zouros, 2017).

Like for the vast majority of invertebrate taxa, mitochondrial DNA – specifically the cytochrome oxidase subunit I (*cox1* or COI) barcoding region – has become the predominant genetic marker for mosquito species identification. Currently, *cox1* sequences of about 30% of all mosquito species worldwide have been entered in the major databases (Moraes Zenker *et al.*, 2024). Although the extensive reference dataset is a significant advantage of this gene, *cox1* seems to have several notable disadvantages as well, mostly related to its lack of taxonomic resolution. For example, it appears to lack sufficient discriminatory power to distinguish certain cryptic and sibling species, such as those within many *Culex* (*Culex*) complexes, the *Anopheles maculipennis* complex or between *Culiseta fumipennis* and *Cs. morsitans* (Laurito *et al.*, 2013; Kuhlisch *et al.*, 2019; Smitz *et al.*, 2021).

The rapid expansion of the genetic toolkit, coupled with the increasing affordability of high-throughput DNA sequencing in recent decades, has revolutionised species identification. This advancement has shifted the focus from identifying single specimens to analysing DNA mixtures from pooled specimens or environmental samples (e.g. eDNA from water), a technique known as metabarcoding (Bierman and Lloyd, 2024). Also for this purpose, the *cox1* barcoding region has played a key role, and has also been adopted in mosquito research for species identification in eDNA samples as well as mixed mosquito trap catches (Batovska *et al.*, 2017; Boerlijst *et al.*, 2019; Krol *et al.*, 2019; Gutiérrez-López *et al.*, 2023). To ensure that this method performs well, the target region must be short enough to amplify degraded samples, near-identical within the same species, yet variable between species, and capable of facilitating the equal amplification of the full array of species (Epp *et al.*, 2012). This variable target region should ideally be flanked by conserved genetic sequences, that can be used for annealing of the sequencing primers. *Cox1* poses a number of challenges for this, as well. For example, *cox1* is sometimes lacking conserved areas in the gene, rendering it difficult to detect suitable primer sites, causing (1) unequal amplification of the different species present in bulk samples and (2) hindering the development of group-specific primers (e.g. targeting mosquitoes but no other Diptera) (Deagle *et al.*, 2014).

The issues that occur with *cox1* thereby highlight the need to explore other potential genetic regions. However, the lack of a comprehensive reference database of other mitochondrial genes has led to serious impediment of progression. This is slowly changing as more complete mitochondrial genomes of mosquitoes are increasingly becoming available (Chen *et al.*, 2024; Da Silva *et al.*, 2020; Do Nascimento *et al.*, 2021; Ma *et al.*, 2022). Currently, most available mitogenomes belong to Neotropical species, with a decent representation of the genus *Anopheles*. In contrast, the mitogenomes of European species are poorly resolved. Of the 102 mosquito species present in Europe (Becker *et al.*, 2020), only 14 native and 4 established exotic species have published mitogenomes so far, with only half of the native species sequenced from material collected in Europe (Supplementary Table S1). This is problematic, as many species with large distributions also exhibit substantial genetic variation, resulting in networks of variants within species boundaries that are not yet completely resolved (Aardema *et al.*, 2020; Vargas Espinosa and Aguirre-Obando, 2022).

Because the shortage of reference genomes severely restricts the possibilities to broaden the horizon of molecular identification techniques of mosquitoes, we performed Illumina sequencing to assemble and annotate 82 mitogenomes of 27 Northwest European mosquito species. Two-thirds of the species had their mitogenomes sequenced for the first time. This approach allows for exploring within-species variation, within-family variation and within genus or subgenus variation. We compared the variability of all 13 different protein-coding genes (PCGs), two ribosomal RNA (rRNA) genes, and 22 transfer RNA (tRNA) genes to help researchers select regions of interest for their research. Ultimately, we aim to provide a boost to the genomic research and monitoring of mosquitoes on the European continent by increasing the availability of these mitogenome sequences.

2 Material and methods

Sample collection

For this study, we obtained 92 individuals, initially identified as 29 different Northwest European species and 3 invasive *Aedes* species present in Europe, from the Centre for Monitoring Vectors (CMV) of the Netherlands Institute for Vectors, Invasive plants and Plant Health (NIVIP-NVWA). The majority of mosquitoes were collected between 2010 and 2020 during mosquito monitoring for the Exotic Mosquito Species (EMS) survey (Ibáñez-Justicia *et al.*, 2020) and the National Vector Survey (NVS) (Ibañez-Justicia *et al.*, 2015). Collection methods included the use of various CO_2-baited traps: Mosquito Magnet Liberty Plus trap (Woodstream Corporation, Lititz, PA, USA), BG Sentinel trap (Biogents AG, Regensburg, Germany), and CDC miniature light trap model 512 (John W. Hock Company, Gainesville, FL, USA). Additional mosquito specimens were obtained through incidental larval dipping and rearing, or by hand aspirator. Additional Dutch material was sampled for a project by Prof. dr. P.F.M. Verdonschot in 2019 (Verdonschot, 2020). Non-Dutch specimens of *Aedes aegypti* (German lab strain), *Ae. albopictus* (Spain; Menorca), and *Ae. detritus/coluzzii* (France; Camargue) were opportunistically collected outside standard monitoring efforts. All specimens have been registered in the collection of Naturalis Biodiversity Center, formerly the National Museum of Natural History, Leiden, the Netherlands (RMNH) under collection numbers RMNH.INS.1271298–1389. Storage conditions for all specimens were maintained at -20 °C from collection until genetic processing.

Identification and taxonomic treatment

Individuals were identified by three experts based on their expertise and morphological characteristics outlined in Culicidae identification keys (Becker *et al.*, 2020; Gunay *et al.*, 2020; Schaffner *et al.*, 2001). These identifications were subsequently verified using *cox1* sequences retrieved from the genome assembly, which were compared against GenBank via BLAST (Altschul *et al.*, 1990). All specimens were photographed from at least eight different angles and detailed close-ups using a ZEISS SteREO Discovery.V12 and V.20 (Carl Zeiss AG, Oberkochen, Germany) motorised stacking microscope equipped with a AxioCam MRc 5 (5-megapixel camera). Image acquisition, including automated stacking, was performed using AxioVision software (v.4.8). The software automatically determined the number of stacks and stacking window based on magnification and object size, providing a comprehensive reference for morphological identification. Images are publicly available and can be accessed as a full dataset in Zenodo (https://doi.org/10.5281/zenodo.14672457; Van der Beek *et al.*, 2025).

In instances of uncertainty, either morphologically or genetically, an additional blind verification was performed by three independent experts using high-resolution pictures. *Anopheles maculipennis* s.l. was not identified to the species level morphologically. Instead, species-level identification relied on retrieving the Internal Transcribed Spacer 2 (ITS2) sequence from the raw Illumina sequencing data. Reads were mapped to a consensus of all European species within the complex, using the same method as described for the mapping assemblies below, and comparing the results with the Single Nucleotide Polymorphisms (SNPs) in Kronefeld *et al.* (2014).

Throughout this paper, we followed the species nomenclature and higher taxonomy for mosquitoes as accepted by Wilkerson *et al.* (2021). For the genus *Aedes*, we adopted a composite treatment, with groupings represented at the subgeneric level, as outlined by Wilkerson *et al.* (2015).

DNA extraction and sequencing

Genomic DNA was isolated from the entire body of each individual using a magnetic bead-based approach with the NucleoMag Tissue kit (Macherey-Nagel, Düren, Germany) on a KingFisher Flex system (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturers protocol and application note for the system. DNA was eluted in 150 µl of the kit's Elution Buffer MB6.

Library preparation, including fragmentation, A-tailing, ligation of the sequencing adapters, and PCR, was conducted based on the NEBNext Ultra II FS DNA library construction workflow (NEB, Ipswich, MA, USA), following the procedures outlined in the NEBNext Ultra II FS DNA module and NEBNext Ultra II Ligation module Instruction Manual. The resulting products were approximately 500–700 bp. A concentration of 1.1 nM of DNA was utilised for Illumina sequencing performed on a NovaSeq6000 (Illumina, San Diego, CA, USA) with a paired-end 2x150 bp run, in accordance with the manufacturer's protocols. Library preparation and sequencing were conducted at GenomeScan B.V. (Leiden, the Netherlands).

Genome assembly, mapping, and annotation

Samples with multiple read files for both directions were combined into a single read file for the forward

and reverse sequences of each specimen. We trimmed the low-quality ends of the reads with fastp (v.0.20.1; Chen *et al.*, 2018). Subsequently, the read coverage was normalised to create a more manageable dataset and to mitigate potential sequencing errors. We down-sampled reads in high-depth regions (i.e. areas with many fully overlapping k-mers) to an average depth of 100x and removed reads with coverage below 5x×, using the BBNorm tool of BBMap (v.38.95; Bushnell, 2022).

De Novo mitogenome assembly was performed with the GetOrganelle toolkit (v.1.7.5.3; Jin *et al.*, 2020), using SPAdes (v.3.13.1; Bankevich *et al.*, 2012) as the core assembler. De novo assemblies were seeded based on a local reference database with 139 mitogenomes (139 species; 17 genera) available on the NCBI Reference Sequence Database (RefSeq, accessed: January 2022) (Supplementary Table S2). The seed was only used to collect the initial target-associated reads using Bowtie2. These seed-mapped reads further extended themselves with more overlapped reads without the starter in the following extending rounds, without using the seed as a reference.

Contigs larger than 12,000 bp were selected and queried against the same reference library with 139 mitogenomes using a local nucleotide BLAST (v.2.12.0; Altschul *et al.*, 1990). Contigs with a match in the database were then circularised by removing overlapping sequences at the tails using the Python script: Simple-Circularise (Kitson, 2018). The sequences were then manually inspected using Unipro UGENE (v. 41). Sequences were adjusted to the forward orientation, and all sequences were reset to start with the tRNA for Isoleucine (*trnI*) as the starting point for their linear form. While no formal consensus on the starting point for linear visualisation of mosquito mitogenome sequences is widely cited in the literature, 92% of the 139 reference mitogenomes used begins at the *trnI* locus. Adopting a consistent starting point streamlines subsequent data utilisation.

Sequences were grouped by belonging to the same species, species-group, or subgenus, and those sequences were aligned using the MUSCLE alignment tool in UGENE using the default settings. Next, the assembled sequences were inspected for having large insertions or deletions (> 50 bp) compared to the same or closely related species in our dataset or, if present, among the reference sequences.

Specimens that had those duplications, large insertions and/or deletions in the sequences compared to the sequences from the same and closely related species were re-assembled by mapping the trimmed Illumina reads with minimap2 (v.2.24; Li, 2018, 2021) to a (consensus) sequence of the same species or a consensus sequence of multiple closely related species. The mapped reads were cleaned using SAMtools (v.1.13; Danecek *et al.*, 2021) functions 'fixmate', 'sort', and 'markdup'. The functions 'mpileup' and 'call' from BCFtools (v.1.13) were used to call the variants and extract the consensus sequence.

The mitogenome sequences were annotated using the MitoFinder pipeline (v.1.4.1; Allio *et al.*, 2020) which allows annotating of mitochondrial genes utilising existing genomic assemblies. Specifically, we used the 139 RefSeq mosquito mitogenomes as references for the gene annotation. The mitochondrial tRNA finder (MiTFi) script (v.0.1; Jühling *et al.*, 2012) was employed for the tRNA annotation step within the MitoFinder pipeline.

The full UNIX workflow can be found on the author's GitHub repository: https://github.com/JordyvdB97/mosquito-genomes-pipeline/.

Genome analysis and visualisation

A comparative map of the assembled mitogenomes was built using BLAST Ring Image Generator (BRIG) (v.0.95; Alikhan *et al.*, 2011), with the longest mitogenome (*Ae. punctor*) serving as the reference. The resulting image was post-processed in INKSCAPE (v.1.3.2) to change the colour scheme, titles and markings, as well as to remove the gene similarity gradient, as the focus was on visualising genome structure rather than direct sequence similarity to *Ae. punctor* due to high sequence divergence.

We generated a pairwise distance matrix per gene in GeneiousPrime(v.2023.2.1;https://www.geneious.com/), based on MAFFT alignments (v.7.490; Katoh *et al.*, 2002; Katoh and Standley, 2013) with default settings. The gene level matrices were analysed together with taxonomic annotation with R (v.4.2.1) in RStudio (v.2023.12.1; http://www.rstudio.com/) using additional packages dplyr (v.1.1.4; Wickham *et al.*, 2023) and tidyr (v.1.3.1; Wickham *et al.*, 2024). A sliding window plot of nucleotide diversity (π) was constructed using PopGenome (v.2.7.7; Pfeifer *et al.*, 2014) using a 200 nucleotide window size and 10 nucleotide increments. All plots were visualised using the packages ggplot2 (v.3.5.1; Wickham, 2016), gridExtra (v.2.3; Auguie, 2017) and cowplot (v.1.1.3; Wilke, 2024).

3 Results

The sequenced mosquito samples generated 82 mitogenomes, representing 27 different species from five

genera: *Aedes* (15 spp.), *Anopheles* (3 spp.), *Coquillettidia* (1 sp.), *Culex* (3 spp.), *Culiseta* (5 spp.) (Table 1). Ten mitogenomes were excluded: two did not yield sufficient DNA to produce a complete mitogenome, while the remaining eight were omitted due to the inability to achieve a sufficiently certain morphological identification. The number of mitogenomes generated per species ranged from one to six. Three-quarters of the mitogenomes (62) were *de novo* assembled, while the others were consensus generated from reads mapped against a closely related reference genome. Mitogenomes from 17 species were assembled for the first time: *Aedes annulipes/cantans, Ae. cinereus/geminus, Ae. communis, Ae. detritus/coluzzii, Ae. flavescens, Ae. geniculatus, Ae. leucomelas, Ae. punctor, Ae. rusticus, Ae. sticticus, Anopheles claviger, Coquillettidia richiardii, Culiseta annulata, Cs. longiareolata, Cs. morsitans, Cs. ochroptera,* and *Cs. subochrea* (Table 1, Supplementary Table S1).

Genome organisation

Almost all assemblies were circular (containing overlapping ends), only three *de novo* assemblies of *Anopheles plumbeus* were non overlapping and therefore referred to as 'partial sequences'. The genome size ranged between 15,347 bp to 17,186 bp, with an average genome size of 15,916 bp (Table 1). All assembled and annotated genomes covered all the 13 different protein-coding genes (*atp6*, *atp8*, ATP synthase subunits 6 and 8 genes; *cytb*, cytochrome oxidase b gene; *cox1–cox3*, cytochrome oxidase c subunit 1–3 genes; *nad1–nad6*, *nad4L*, NADH dehydrogenase subunits 1–6 and 4 L), two ribosomal RNA genes (the large and small ribosomal RNA subunit gene: *rrnS* and *rrnL*), and 22 transfer RNA genes (each of them hereafter referred to as *trn* + the single-letter IUPAC-IUB code for their respective amino acids) genes. All assembled and annotated genomes included the 13 protein-coding genes (PCGs): the ATP synthase subunits 6 and 8 (*atp6* and *atp8*); cytochrome b oxidase (*cytb*); the cytochrome c oxidase subunits 1–3 (*cox1–cox3*); and the NADH dehydrogenase subunits 1–6 and 4L (*nad1-nad6*, *nad4L*). They also covered two ribosomal RNA genes (the large and small subunit genes: *rrnS* and *rrnL*) and 22 transfer RNA genes (tRNAs). The order and orientation of the genes were exactly the same for all species (Figure 1). Only the control region of the genome, positioned after the *rrnS* gene, and a noncoding region between the tRNA's for cysteine (*trnC*) and tyrosine (*trnY*), contribute to the varying genome sizes among the mosquito species sequenced. The noncoding region being exceptionally long in *Ae. punctor* (907 bp; Figure 1).

Discriminative power different coding genes

We assessed the genetic similarity within and between species for each of the PCGs and rRNAs (Figure 2). Eleven mitochondrial genes (PCGs: *atp6*, *cox1*, *cox2*, *cox3*, *cytb*, *nad1*, *nad2*, *nad4*, *nad5*, *nad6*; and the rRNA gene: *rrnS*) showed no overlap in the extent of the intra- and interspecific genetic similarity. For two PCGs, *cox1* and *nad2*, there was a significant gap of at least 1.5% between the lowest intraspecific similarity and the highest interspecific similarity (Supplementary Table S3). For the remaining nine genes, this gap was smaller.

In general, low intraspecific variation was observed, but the level of intraspecific variation differed considerably across genes. The largest range observed in *nad4* (97.5–100% similarity) and the smallest in *rrnL* (99.7–100% similarity). The greatest intraspecific variation was found in *Culex modestus* specimens for *nad4*, but also other genes of these species show relatively low similarity (Supplementary Table S4). At the species level, five species showed a relatively low level of intraspecific similarity (<99% for the *cox1* barcoding region), which include *Ae. aegypti*, with a similarity of 96.2–97.87%, *Cx. modestus*, with a similarity of 98.1–99.6%, *Ae. detritus/coluzzii*, with a similarity of 98.2%, *Ae. annulipes/cantans*, with a range of 98.3–99.9%, and *An. messeae*, with a range of 98.8–99.9% (Supplementary Table S4).

At higher taxonomic levels, we find that the genetic similarity varies significantly between genes. The lowest average similarity between different species of the same subgenus was found in *nad6* (90.0%) and *nad2* (91.3%), while the highest occurred in the two rRNAs (*rrnL*: 97.7%, *rrnS*: 97.5%). Some genes fail to distinguish certain species. For example, the *atp8* gene (162 bp) of *Ae. leucomelas* was 100% identical with all three sequences of *Ae. caspius*. However, PCGs did vary between these species, with similarities ranging from 91.5% in *cox1* to 97.3% in *nad4L*.

All tRNAs (Supplementary Figure S1) appear highly conserved, exhibiting high similarities between closely related species (within the same subgenus). In some cases, different species displayed completely identical sequences across all tRNAs. Even at higher taxonomic levels, the similarities remained substantial, with the most dissimilar sequences (across species of different subfamilies) for the tRNAs of Leucine (*trnL1*), Lysine (*trnK*), Methionine (*trnM*), Serine (*trnS1*), Tryptophan (*trnW*), and Valine (*trnV*) remaining above 90% similar.

The variability across mitochondrial genomes was quantified using nucleotide diversity (π), which measures the mean pairwise genetic difference among all possible pairs of individuals in the sample (Figure 3;

TABLE 1 Overview of generated genomes and generation methods.[1]

Species	Reference number	GenBank acquisition number	Assembly method	Reference sequence for mapping	Genome length (bp)
Aedes (*Aedes*) *cinereus/geminus*	RMNH.INS.1271319	PV094688	mapped against reference	consensus *de novo* assemblies *Ae. cinereus/geminus*	16,104
Aedes (*Aedes*) *cinereus/geminus*	RMNH.INS.1271320	PV094689	*de novo*		16,103
Aedes (*Aedes*) *cinereus/geminus*	RMNH.INS.1271321	PV094690	*de novo*		16,100
Aedes (*Aedes*) *cinereus/geminus*	RMNH.INS.1271386	PV094747	*de novo*		16,101
Aedes (*Aedimorphus*) *vexans* (Meigen, 1830)	RMNH.INS.1271304	PV094673	*de novo*		15,912
Aedes (*Aedimorphus*) *vexans* (Meigen, 1830)	RMNH.INS.1271305	PV094674	*de novo*		15,913
Aedes (*Aedimorphus*) *vexans* (Meigen, 1830)	RMNH.INS.1271306	PV094675	mapped against reference	consensus *de novo* assemblies *Ae. vexans*	15,909
Aedes (*Dahliana*) *geniculatus* (Olivier, 1791)	RMNH.INS.1271340	PV094709	*de novo*		15,790
Aedes (*Dahliana*) *geniculatus* (Olivier, 1791)	RMNH.INS.1271341	PV094710	*de novo*		15,790
Aedes (*Dahliana*) *geniculatus* (Olivier, 1791)	RMNH.INS.1271342	PV094711	*de novo*		15,791
Aedes (*Hulecoeteomyia*) *japonicus* (Theobald, 1901)	RMNH.INS.1271379	PV094741	*de novo*		15,779
Aedes (*Hulecoeteomyia*) *japonicus* (Theobald, 1901)	RMNH.INS.1271380	PV094742	mapped against reference	RMNH.INS.1271379	15,777
Aedes (*Hulecoeteomyia*) *japonicus* (Theobald, 1901)	RMNH.INS.1271381	PV094743	mapped against reference	RMNH.INS.1271379	15,792
Aedes (*Ochlerotatus*) *annulipes/cantans*	RMNH.INS.1271325	PV094694	*de novo*		15,855
Aedes (*Ochlerotatus*) *annulipes/cantans*	RMNH.INS.1271326	PV094695	mapped against reference	consensus *de novo* assemblies *Ae. annulipes*/*cantans* & *Ae. flavescens*	15,854
Aedes (*Ochlerotatus*) *annulipes/cantans*	RMNH.INS.1271327	PV094696	*de novo*		15,841
Aedes (*Ochlerotatus*) *annulipes/cantans*	RMNH.INS.1271328	PV094697	*de novo*		15,853
Aedes (*Ochlerotatus*) *annulipes/cantans*	RMNH.INS.1271329	PV094698	*de novo*		15,846

[1]Reference numbers correspond to internal institution identifiers assigned to specimens at Naturalis Biodiversity Center. While original specimens were destructively sampled and are no longer available in the collection, their DNA extracts are stored in the DNA reference collection. These numbers link the specimens' DNA extracts, associated images, metadata, and genetic sequences. *Anopheles plumbeus* (*) is referred to as a partial genome, where no circularisation was detected, but did recover all coding regions in its entirety and the genome size is comparable with other members from the genus.

TABLE 1 Overview of generated genomes and generation methods. (*cont.*)

Species	Reference number	GenBank acquisition number	Assembly method	Reference sequence for mapping	Genome length (bp)
Aedes (*Ochlerotatus*) *annulipes/cantans*	RMNH.INS.1271330	PV094699	*de novo*		15,845
Aedes (*Ochlerotatus*) *caspius* (Pallas, 1771)	RMNH.INS.1271346	PV094714	*de novo*		15,888
Aedes (*Ochlerotatus*) *caspius* (Pallas, 1771)	RMNH.INS.1271347	PV094715	*de novo*		15,883
Aedes (*Ochlerotatus*) *caspius* (Pallas, 1771)	RMNH.INS.1271348	PV094716	*de novo*		15,887
Aedes (*Ochlerotatus*) *caspius* (Pallas, 1771)	RMNH.INS.1271369	PV094733	*de novo*		15,884
Aedes (*Ochlerotatus*) *communis* (De Geer, 1776)	RMNH.INS.1271334	PV094703	*de novo*		16,722
Aedes (*Ochlerotatus*) *communis* (De Geer, 1776)	RMNH.INS.1271335	PV094704	mapped against reference	RMNH.INS.1271334	16,723
Aedes (*Ochlerotatus*) *communis* (De Geer, 1776)	RMNH.INS.1271336	PV094705	mapped against reference	RMNH.INS.1271334	16,722
Aedes (*Ochlerotatus*) *detritus/coluzzii*	RMNH.INS.1271367	PV094731	*de novo*		15,981
Aedes (*Ochlerotatus*) *detritus/coluzzii*	RMNH.INS.1271368	PV094732	*de novo*		15,988
Aedes (*Ochlerotatus*) *flavescens* (Müller, 1764)	RMNH.INS.1271370	PV094734	mapped against reference	consensus *de novo* assemblies *Ae. annulipes*/*cantans* & *Ae. flavescens*	15,846
Aedes (*Ochlerotatus*) *flavescens* (Müller, 1764)	RMNH.INS.1271371	PV094735	mapped against reference	consensus *de novo* assemblies *Ae. annulipes*/*cantans* & *Ae. flavescens*	15,849
Aedes (*Ochlerotatus*) *flavescens* (Müller, 1764)	RMNH.INS.1271372	PV094736	*de novo*		15,847
Aedes (*Ochlerotatus*) *leucomelas* (Meigen, 1804)	RMNH.INS.1271375	PV094737	*de novo*		15,347
AedessOchlerotatus) *punctor* (Kirby in Richardson, 1837)	RMNH.INS.1271337	PV094706	*de novo*		16,279
Aedes (*Ochlerotatus*) *punctor* (Kirby in Richardson, 1837)	RMNH.INS.1271339	PV094708	*de novo*		16,280
Aedes (*Ochlerotatus*) *sticticus* (Meigen, 1838)	RMNH.INS.1271361	PV094728	*de novo*		16,061
Aedes (*Ochlerotatus*) *sticticus* (Meigen, 1838)	RMNH.INS.1271362	PV094729	*de novo*		16,060
Aedes (*Ochlerotatus*) *sticticus* (Meigen, 1838)	RMNH.INS.1271363	PV094730	*de novo*		16,059

TABLE 1 Overview of generated genomes and generation methods. (*cont.*)

Species	Reference number	GenBank acquisition number	Assembly method	Reference sequence for mapping	Genome length (bp)
Aedes (*Rusticoidus*) *rusticus* (Rossi, 1790)	RMNH.INS.1271331	PV094700	*de novo*		16,046
Aedes (*Rusticoidus*) *rusticus* (Rossi, 1790)	RMNH.INS.1271332	PV094701	*de novo*		16,044
Aedes (*Rusticoidus*) *rusticus* (Rossi, 1790)	RMNH.INS.1271333	PV094702	mapped against reference	consensus *de novo* assemblies *Ae. rusticus*	16,047
Aedes (*Rusticoidus*) *rusticus* (Rossi, 1790)	RMNH.INS.1271338	PV094707	*de novo*		16,045
Aedes (*Stegomyia*) *aegypti* (Linnaeus, 1762)	RMNH.INS.1271382	PV094744	mapped against reference	RMNH.INS.1271383	16,256
Aedes (*Stegomyia*) *aegypti* (Linnaeus, 1762)	RMNH.INS.1271383	PV094745	*de novo*		16,256
Aedes (*Stegomyia*) *aegypti* (Linnaeus, 1762)	RMNH.INS.1271384	PV094746	mapped against reference	RMNH.INS.1271383	16,249
Aedes (*Stegomyia*) *albopictus* (Skuse, 1895)	RMNH.INS.1271376	PV094738	*de novo*		16,099
Aedes (*Stegomyia*) *albopictus* (Skuse, 1895)	RMNH.INS.1271377	PV094739	mapped against reference	RMNH.INS.1271376	16,099
Aedes (*Stegomyia*) *albopictus* (Skuse, 1895)	RMNH.INS.1271378	PV094740	mapped against reference	RMNH.INS.1271376	16,057
Anopheles (*Anopheles*) *claviger* (Meigen, 1804)	RMNH.INS.1271310	PV094679	*de novo*		15,469
Anopheles (*Anopheles*) *claviger* (Meigen, 1804)	RMNH.INS.1271311	PV094680	*de novo*		15,470
Anopheles (*Anopheles*) *claviger* (Meigen, 1804)	RMNH.INS.1271312	PV094681	*de novo*		15,471
Anopheles (*Anopheles*) *messeae* Falleroni, 1926	RMNH.INS.1271298	PV094667	*de novo*		15,452
Anopheles (*Anopheles*) *messeae* Falleroni, 1926	RMNH.INS.1271299	PV094668	*de novo*		15,451
Anopheles (*Anopheles*) *messeae* Falleroni, 1926	RMNH.INS.1271300	PV094669	*de novo*		15,457
Anopheles (*Anopheles*) *plumbeus* Stephens, 1828*	RMNH.INS.1271349	PV094717	*de novo*		15,473
Anopheles (*Anopheles*) *plumbeus* Stephens, 1828*	RMNH.INS.1271350	PV094718	*de novo*		15,474
Anopheles (*Anopheles*) *plumbeus* Stephens, 1828*	RMNH.INS.1271351	PV094719	*de novo*		15,472
Coquillettidia (*Coquillettidia*) *richiardii* (Ficalbi, 1889)	RMNH.INS.1271301	PV094670	mapped against reference	RMNH.INS.1271303	15,629

TABLE 1 Overview of generated genomes and generation methods. (*cont.*)

Species	Reference number	GenBank acquisition number	Assembly method	Reference sequence for mapping	Genome length (bp)
Coquillettidia (*Coquillettidia*) *richiardii* (Ficalbi, 1889)	RMNH.INS.1271302	PV094671	mapped against reference	RMNH.INS.1271303	15,698
Coquillettidia (*Coquillettidia*) *richiardii* (Ficalbi, 1889)	RMNH.INS.1271303	PV094672	*de novo*		15,708
Culex (*Barraudius*) *modestus* Ficalbi, 1890	RMNH.INS.1271313	PV094682	*de novo*		15,575
Culex (*Barraudius*) *modestus* Ficalbi, 1890	RMNH.INS.1271314	PV094683	*de novo*		15,580
Culex (*Barraudius*) *modestus* Ficalbi, 1890	RMNH.INS.1271315	PV094684	*de novo*		15,578
Culex (*Culex*) *pipiens* Linnaeus, 1758	RMNH.INS.1271355	PV094722	*de novo*		15,586
Culex (*Culex*) *pipiens* Linnaeus, 1758	RMNH.INS.1271356	PV094723	*de novo*		15,587
Culex (*Culex*) *pipiens* Linnaeus, 1758	RMNH.INS.1271357	PV094724	*de novo*		15,585
Culex (*Neoculex*) *territans* Walker, 1856	RMNH.INS.1271316	PV094685	*de novo*		16,091
Culex (*Neoculex*) *territans* Walker, 1856	RMNH.INS.1271317	PV094686	*de novo*		16,086
Culex (*Neoculex*) *territans* Walker, 1856	RMNH.INS.1271318	PV094687	*de novo*		16,088
Culiseta (*Allotheobaldia*) *longiareolata* (Macquart, 1838)	RMNH.INS.1271322	PV094691	*de novo*		15,729
Culiseta (*Allotheobaldia*) *longiareolata* (Macquart, 1838)	RMNH.INS.1271323	PV094692	*de novo*		15,729
Culiseta (*Allotheobaldia*) *longiareolata* (Macquart, 1838)	RMNH.INS.1271324	PV094693	mapped against reference	consensus *de novo* assemblies *Cs. longiareolata*	15,729
Culiseta (*Culicella*) *morsitans* (Theobald, 1901)	RMNH.INS.1271358	PV094725	mapped against reference	RMNH.INS.1271360	15,858
Culiseta (*Culicella*) *morsitans* (Theobald, 1901)	RMNH.INS.1271359	PV094726	mapped against reference	RMNH.INS.1271360	15,857
Culiseta (*Culicella*) *morsitans* (Theobald, 1901)	RMNH.INS.1271360	PV094727	*de novo*		15,857
Culiseta (*Culicella*) *ochroptera* Peus, 1935	RMNH.INS.1271389	PV094748	*de novo*		16,176
Culiseta (*Culiseta*) *annulata* (Schrank, 1776)	RMNH.INS.1271307	PV094676	mapped against reference	consensus *de novo* assemblies *Cs. annulata* & *Cs. subochrea*	15,790

TABLE 1 Overview of generated genomes and generation methods. (*cont.*)

Species	Reference number	GenBank acquisition number	Assembly method	Reference sequence for mapping	Genome length (bp)
Culiseta (*Culiseta*) *annulata* (Schrank, 1776)	RMNH.INS.1271308	PV094677	*de novo*		15,783
Culiseta (*Culiseta*) *annulata* (Schrank, 1776)	RMNH.INS.1271309	PV094678	mapped against reference	consensus *de novo* assemblies *Cs. annulata* & *Cs. subochrea*	15,776
Culiseta (*Culiseta*) *annulata* (Schrank, 1776)	RMNH.INS.1271345	PV094713	*de novo*		15,785
Culiseta (*Culiseta*) *annulata* (Schrank, 1776)	RMNH.INS.1271352	PV094720	*de novo*		15,785
Culiseta (*Culiseta*) *annulata* (Schrank, 1776)	RMNH.INS.1271354	PV094721	*de novo*		15,784
Culiseta (*Culiseta*) *subochrea* (Edwards in Wesenberg, 1921)	RMNH.INS.1271344	PV094712	*de novo*		15,791

Table 2). The highest π values were observed in the PCGs (π = 0.090–0.157), while much lower diversity was recorded in rRNAs (π = 0.045 and 0.056) and tRNAs (π = 0.015–0.088). Among the PCGs, both *nad6* and *nad2* exhibited greater variability than the *cox1* barcoding region, with both a higher nucleotide diversity (respectively π = 0.157 and π = 0.134, compared to π = 0.126 for *cox1* barcoding region) and a higher percentage of variable base pairs (respectively 54.7% and 51.5% compared to 37.1%). All PCG, except *nad1*, have a higher percentage of variable base pair sites than *cox1*.

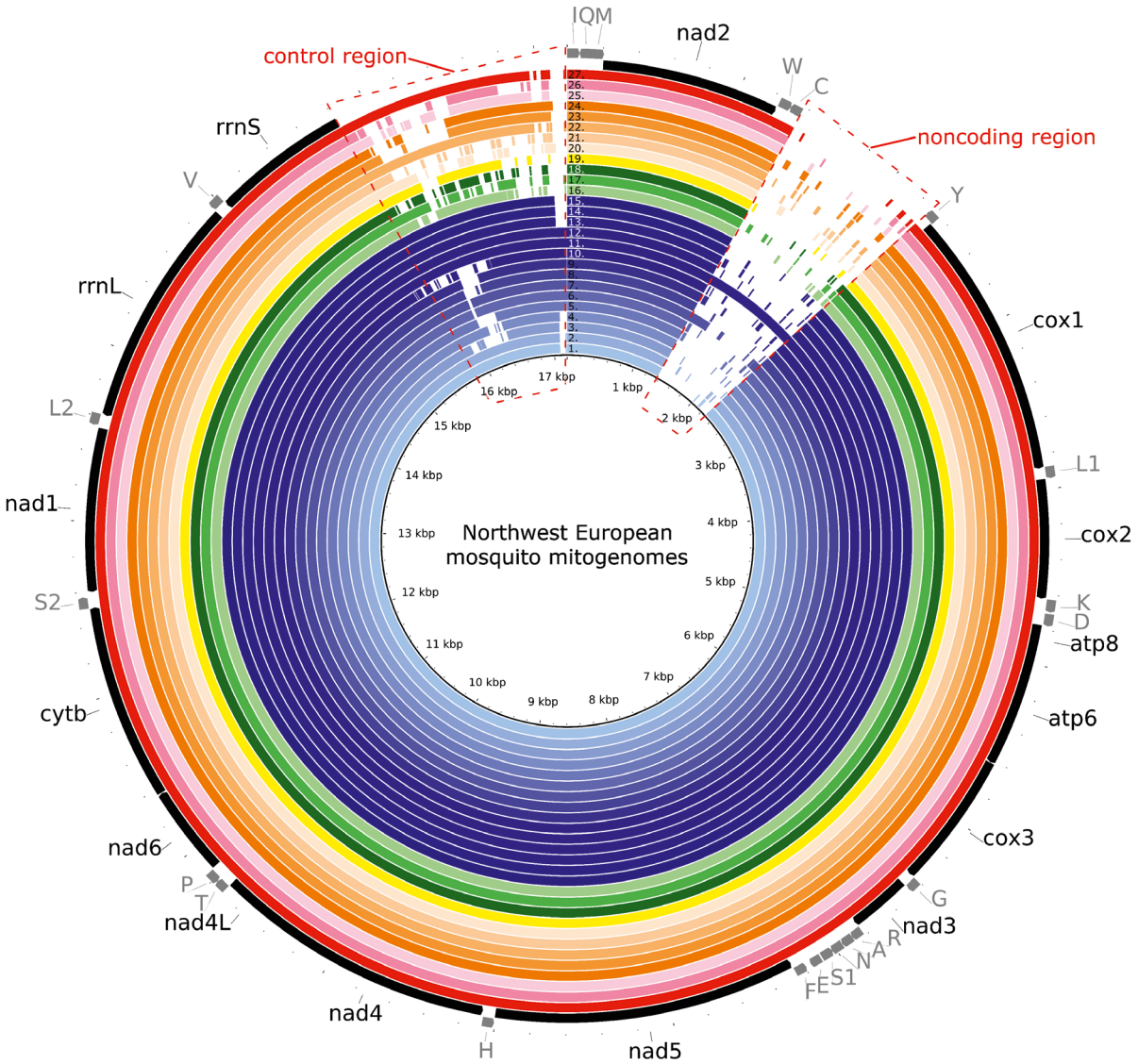
In particular, the genera *Anopheles* and *Culiseta* showed peaks in nucleotide diversity for *nad6*, whereas in the genera *Culex* and *Aedes*, the variability in *nad6* was more comparable to other genes. Overall, the variability patterns of genes were consistent across genera (Figure 3). However, some regional differences were noted. For instance, in *nad3*, *Culex* exhibited a dip in π that was lower than its neighbouring genes, while other genera displayed π that was similar to those of their neighbouring regions.

4 Discussion and conclusion

In this study, we sequenced and assembled 82 mitochondrial genomes of 27 mosquito species occurring or introduced in Northwestern Europe. The material was primarily collected in the Netherlands, supplemented by *Ae. albopictus* from Menorca (Spain), *Ae. detritus/coluzzii* from the Camargue (France), and a laboratory population of *Ae. aegypti*. These newly sequenced genomes will enable researchers to explore novel genetic markers beyond the widely used *cox1* barcoding region, providing new opportunities for addressing species identification and monitoring challenges. Our results show large differences in genetic variation between the various mitochondrial genes, not only at the species level, but also at higher taxonomic levels. This raises a number of novel ideas and opportunities regarding further use of these mitogenomes for dedicated species analysis. In particular, the *nad2* and *nad6* gene appear to be promising regions to improve species identification of mosquito species complexes.

Coverage of genetic variation and quality control

The species used in this study represent a large portion of the indigenous mosquito species found in Northwestern Europe, but also include widespread *Aedes* invasive species across Europe, such as *Ae. albopictus* and *Ae. japonicus* (ECDC and FSA, 2024), as well as *Aedes* species that are less common but occasionally introduced in the Netherlands, such as *Ae. aegypti* (Ibáñez-Justicia *et al.*, 2020). The material used in this study was primarily obtained from adult mosquito traps, which largely capture female specimens. While effective for broad sampling, specimens collected in traps are often damaged, potentially complicating morphological identification.



1. *Ae. (Adm.) vexans*
2. *Ae. (Aed.) cinereus*
3. *Ae. (Dah.) geniculatus*
4. *Ae. (Hul.) japonicus*
5. *Ae. (Och.) annulipes/cantans*
6. *Ae. (Och.) caspius*
7. *Ae. (Och.) communis*
8. *Ae. (Och.) detritus/coluzzii*
9. *Ae. (Och.) flavescens*
10. *Ae. (Och.) leucomelas*
11. *Ae. (Och.) punctor*
12. *Ae. (Och.) sticticus*
13. *Ae. (Rus.) rusticus*
14. *Ae. (Stg.) aegypti*
15. *Ae. (Stg.) albopictus*
16. *An. (Ano.) claviger*
17. *An. (Ano.) messeae*
18. *An. (Ano.) plumbeus**
19. *Cq. (Coq.) richiardii*
20. *Cs. (All.) longiareolata*
21. *Cs. (Cuc.) morsitans*
22. *Cs. (Cuc.) ochroptera*
23. *Cs. (Cus.) annulata*
24. *Cs. (Cus.) subochrea*
25. *Cx. (Bar.) modestus*
26. *Cx. (Cux.) pipiens*
27. *Cx. (Ncx.) territans*

protein-coding and rRNA genes

tRNA genes

FIGURE 1 Comparative map of the assembled mitogenomes in relation to the longest genome (i.e. *Aedes punctor*). Showing the highly conserved arrangement of the protein-coding genes, ribosomal RNA genes, and transfer RNA genes. tRNA genes are represented by the single-letter IUPAC-IUB code for their respective amino acids. Only the control region and a non-coding region between *trnC* en *trnY* seems to be variable in length. *Anopheles plumbeus* (*) was derived from a partial sequence, where no circularisation was detected, but did recover all coding regions in its entirety.

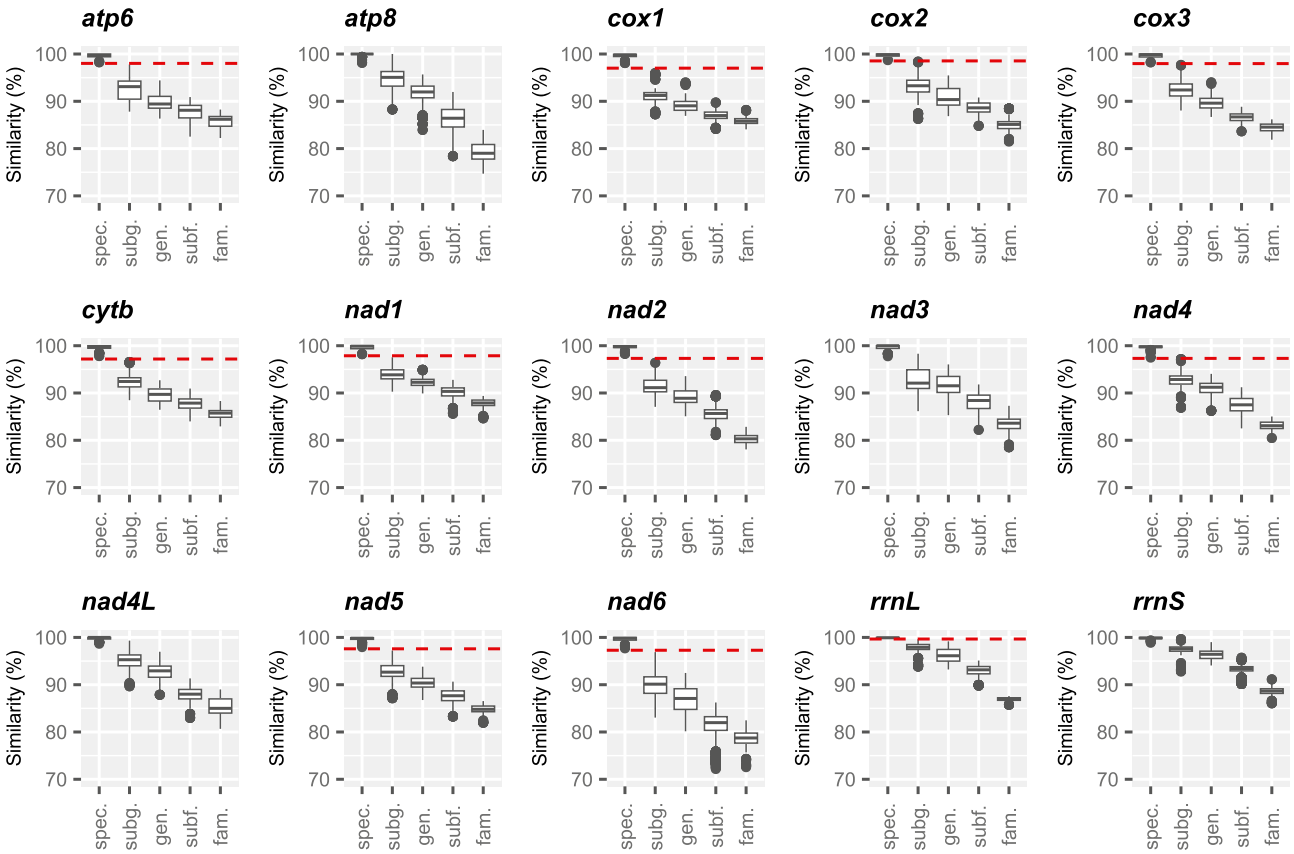


FIGURE 2 Box plot illustrating pairwise genetic similarity across different taxonomic levels for all mitochondrial protein-coding genes and ribosomal RNAs. Red dashed line indicates no overlap between variance of genetic similarity at the species level and higher taxonomic levels.

Although adult females are commonly used for identification, they may not always be the most straightforward sex or life stage for accurate differentiation. For example, morphologically distinguishing between *Ae. cinereus* and *Ae. geminus* can only be achieved by examining male genitalia (Schaffner *et al.*, 2001). Similarly, the differentiation of *Ae cantans* and *Ae. annulipes, Cx. pipiens* and *Cx. torrentium*, as well as *Cs. morsitans* and *Cs. fumipennis*, is much easier and more reliable when based on larvae or male genitalia (Becker *et al.*, 2020). To address these challenges, we implemented a rigorous identification process that included initial assessments by three experts in morphological mosquito identification, followed by genetic confirmation using *cox1*. In cases of uncertainty, additional blind verification was conducted by three independent experts. Despite this, incorporating larval or male specimens, which can sometimes provide clearer morphological traits for identification, might benefit the certainty of identifications even more.

A common deficit in mosquito mitogenome studies, and in mosquito research in general, is the lack of vouchered specimens. The absence of non-destructive sampling or storage of remaining specimens in public collections, or if destructive sampling is necessary, the lack of detailed photographic reference of the species, can limit the ability to verify the identification of previously published mitogenomes.

Additionally, some mitogenomic studies rely on material derived from long-established laboratory colonies of mosquitoes (e.g. Luo *et al.*, 2016; Peng *et al.*, 2016), which may have undergone numerous generations in captivity. This can lead to unusual genetic variability, as seen in our high within-species variation in many different genes of our laboratory reared specimens of *Ae. aegypti* mitogenomes. The population history and potential selective pressures may have introduced unusual genetic variability, that lacks in natural populations, increasing mismatches between the reference library and field-collected specimens.

Other mitochondrial genes potentially suitable for species identification

Our results suggest that mitochondrial genes other than *cox1* may also be suitable for species identification of mosquitoes. In other insect groups, such as beetles and butterflies, it has been previously reported that certain genes in the mitochondrial genome evolve more rapidly and offer greater taxonomic resolution (Ma *et al.*, 2019;

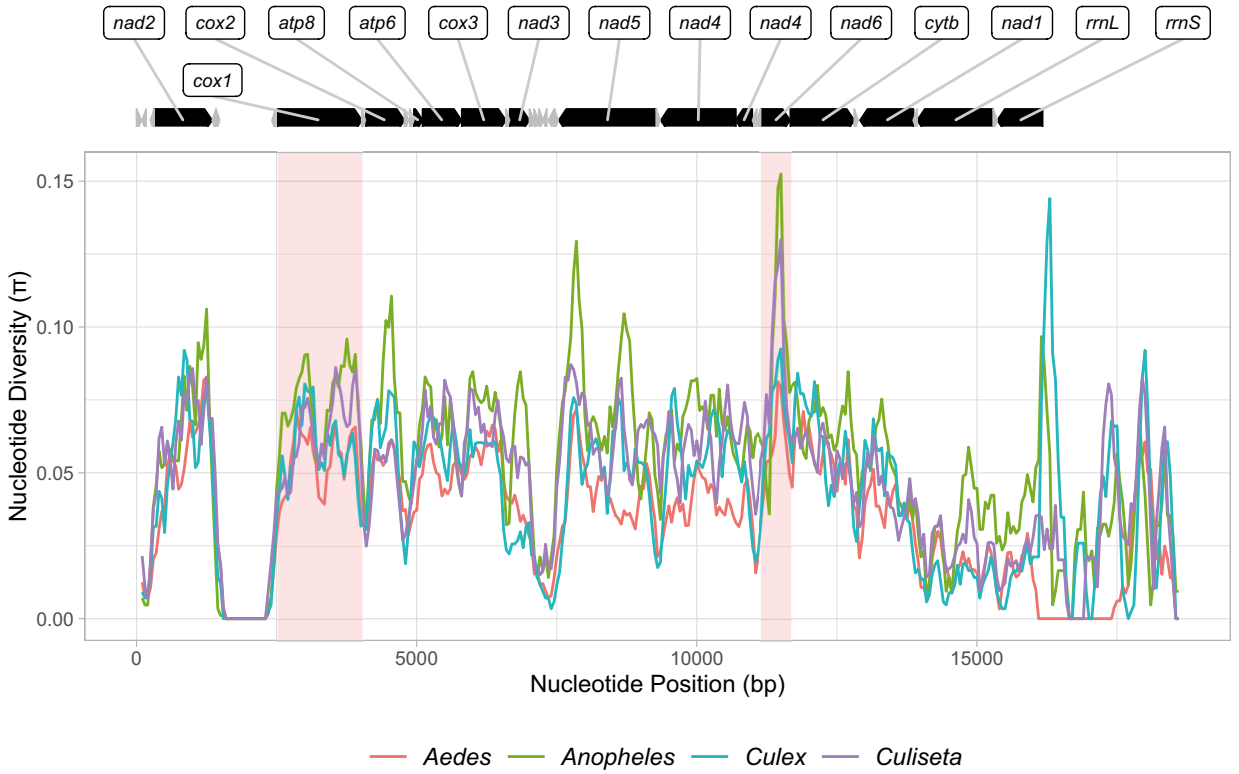


FIGURE 3 Sliding window plots of nucleotide diversity (π) among the species of the different genera, showing potential loci with most genetic variation. Highlighted in red is the most widely used gene for genetic identification (*cox1*) and the gene showing the highest nucleotide diversity (*nad6*). Window sizes are 200 nucleotides with 10 nucleotide increments.

Li *et al.*, 2024). While *cox1* is a widely used marker due to its high variability and the clear gap it provides between intraspecific and interspecific variation at higher taxonomic levels, our analysis highlights the potential of *nad2* and *nad6* as superior alternatives for (northwestern) European mosquito identification. A previous study demonstrated that the gene *nad2* exhibits greater genetic divergence than *cox1* in a pairwise comparison of nine mosquito species from four different genera, making it useful for designing species-specific probes for mosquitoes (Wang *et al.*, 2017). Our study shows, that *nad6* exhibits greater nucleotide diversity in *Anopheles* and *Culiseta* compared to *cox1*. For the genus *Anopheles*, *nad6* has helped in other studies resolving phylogenetics in difficult species groups of *Anopheles* in combination with other genes such as: with *cox1* in Leucosphyrus Group of *Anopheles* (*Cellia*) (Sallum *et al.*, 2007; Takano *et al.*, 2010); or with a nuclear marker in three species groups of *Anopheles* (*Nyssorhynchus*) (Bourke *et al.*, 2010). Our study shows that *nad2* demonstrates a comparable gap between within- and between-species similarity to *cox1*, with a similarly high nucleotide diversity. Notably, *nad2* exhibited greater dissimilarity among closely related species within the same subgenus, resulting in a higher taxonomic resolution compared to *cox1*. *Nad6* emerged as another excellent candidate, showing a slightly smaller within- and between-species gap than *cox1* but surpassing it in dissimilarity among closely related species. Furthermore, *nad6* displayed the highest nucleotide diversity of all the genes analysed. Both *nad6* and *nad2* genes also featured a greater percentage of variable base pairs compared to *cox1*. Both genes *nad2* is on both sides and *nad6* on one side bordered by highly conserved tRNAs which allow for ideal primer sites, with full *nad6*-gene being a great size (about 519 bp) for a barcode sequence as well. Using the conserved tRNAs as primer sites has resulted in high recovery rates (Helleman *et al.*, 2025; Park *et al.*, 2010). Together, these observations underscore the potential utility of using *nad6* and *nad2* as alternative genetic markers for species identification of mosquitoes, particularly when addressing taxonomic challenges or resolving cryptic species complexes.

Resolving species complexes

Our results highlight the challenges posed by species complexes, where intra-species similarity can closely approach inter-species levels. Notable examples in our data include *Ae. detritus/coluzzii, Ae. annulipes/cantans*, and *An. messeae*, all of which exhibited relatively low within-species similarity (<99%) in *cox1* and other mitochondrial genes. These species are all part of recognised

TABLE 2 Variability of the 13 different protein-coding genes, two ribosomal RNA genes, 22 transfer RNA genes, as well as the barcoding region of *cox1*.

Genes[1]	Length (bp)	Variable sites		Nucleotide diversity (π)				
		Number	Percentage	Total	*Aedes*	*Anopheles*	*Culex*	*Culiseta*
nad6	523	286	54.7	0.157	0.110	0.109	0.080	0.105
nad2	1035	533	51.5	0.134	0.095	0.088	0.073	0.086
atp8	162	75	46.3	0.126	0.107	0.096	0.077	0.085
cox1 barcoding region	658	244	37.1	0.126	0.068	0.069	0.053	0.092
cox3	789	315	39.9	0.121	0.091	0.086	0.070	0.084
cox1	1506	555	36.9	0.118	0.096	0.091	0.072	0.085
nad4	1344	601	44.7	0.117	0.095	0.083	0.075	0.079
Cytb	1144	447	39.1	0.116	0.078	0.086	0.071	0.082
atp6	698	302	43.3	0.114	0.084	0.091	0.065	0.086
nad5	1743	759	43.5	0.113	0.088	0.087	0.071	0.084
nad3	354	146	41.2	0.110	0.077	0.091	0.041	0.074
cox2	689	282	40.9	0.109	0.081	0.093	0.071	0.065
nad4	300	132	44.0	0.095	0.062	0.073	0.049	0.075
nad1	957	332	34.7	0.090	0.069	0.067	0.064	0.060
rrnL	1356	375	27.7	0.088	0.030	0.033	0.007	0.051
trnE	89	44	49.4	0.077	0.015	0.047	0.040	0.049
trnS2	68	20	29.4	0.067	0.053	0.092	0.005	0.034
trnL2	69	15	21.7	0.066	0.024	0.078	0.026	0.048
trnV	70	20	28.6	0.056	0.028	0.038	0.016	0.031
trnC	79	33	41.8	0.055	0.028	0.016	0.007	0.054
trnS1	67	15	22.4	0.055	0.029	0.030	0.009	0.062
trnY	69	11	15.9	0.053	0.046	0.015	0.022	0.046
trnA	83	31	37.3	0.051	0.036	0.015	0.008	0.047
rrnS	812	235	28.9	0.048	0.033	0.042	0.087	0.092
trnR	75	29	38.7	0.048	0.016	0.041	0.022	0.018
trnG	68	13	19.1	0.045	0.024	0.040	0.019	0.027
trnT	74	25	33.8	0.044	0.029	0.036	0.022	0.020
trnD	71	14	19.7	0.044	0.035	0.032	0.029	0.018
trnM	68	12	17.6	0.044	0.026	0.038	0.011	0.057
trnI	86	33	38.4	0.043	0.027	0.024	0.000	0.048
trnW	68	19	27.9	0.043	0.019	0.031	0.023	0.052
trnK	70	16	22.9	0.042	0.024	0.007	0.000	0.010
trnQ	112	55	49.1	0.037	0.035	0.003	0.022	0.049
trnP	67	10	14.9	0.029	0.033	0.016	0.008	0.037
trnF	71	13	18.3	0.025	0.012	0.025	0.006	0.011
trnH	69	8	11.6	0.022	0.008	0.000	0.000	0.011
trnL1	67	8	11.9	0.017	0.007	0.019	0.003	0.023
trnN	72	9	12.5	0.015	0.006	0.032	0.016	0.008

[1] tRNA genes are represented by as 'trn' plus the single-letter IUPAC-IUB code for their respective amino acids The table is sorted for the total nucleotide diversity.

species complexes: *Ae. detritus* has a sibling species, *Ae. coluzzii*, in the Camargue, where the specimens were collected (Rioux *et al.*, 1998); *Ae. annulipes/cantans* is part of a group of four morphologically similar species in Northwestern Europe (Kuhlisch *et al.*, 2019); and *An. messeae* (98.8–99.8%) belongs to the *Anopheles maculipennis* complex, which consists of four species in the Netherlands (Ibáñez-Justicia *et al.*, 2022). However, we also observed low similarity in *Cx. modestus*, a species that is not part of a species complex in the region where it was collected. Unfortunately, our data did not include all members of these complexes to allow for an in-depth analysis. However, the availability of mitochondrial genomic data provides a foundation for identifying regions in the genome where members of these complexes vary most. Future studies could leverage these genomic regions by sequencing these markers for all species in the complex, represented by specimens across their entire distribution, using gold-standard specimens – fresh samples of the sex or life stage that is most morphologically identifiable and verified by multiple experts. This approach would allow for testing whether consistent genetic patterns can be identified to differentiate these species reliably.

For some species complexes, a higher resolution might be necessary, which nuclear genetic markers can provide. While mitochondrial DNA generally exhibits a higher mutation rate than nuclear DNA (estimates ranging from 10- to 100-fold greater in humans), attributed to factors such as the lack of protective histones, a high replication rate, and less efficient DNA repair mechanisms in the mitochondrion (Serrano *et al.*, 2024), the nuclear genome possesses a significantly larger proportion of non-coding DNA. These extensive non-coding regions in the nucleus, including introns, telomeres, and various regulatory sequences, often harbour greater genetic variability as they are under weaker selective pressure. Consequently, they can provide a different and sometimes more detailed genetic pattern in phylogenetic relationships (Hanemaaijer *et al.*, 2019; Lee *et al.*, 2019). Examples include the Internal Transcribed Spacer 2 (ITS2) widely employed in *Anopheles* mosquitoes (e.g. Beebe *et al.*, 1999; Fang *et al.*, 2017) and highly polymorphic microsatellites (e.g. Laurito *et al.*, 2017).

Broader impacts and future directions

By assembling and annotating multiple mitogenomes of 27 mosquito species, this study significantly expands the reference database for European mosquitoes. Historically, the selection of genetic markers has often been dictated by the availability of primers and reference sequences, rather than the suitability of specific genes for addressing particular research questions. While *cox1* remains a valuable tool for species identification, our result highlight *nad6* and *nad2* as mitochondrial genes that may offer improved resolution and greater discriminative power. Additionally, highly conserved tRNAs, scattered throughout the genome, could address challenges like unequal amplification caused by variable primer binding sites. As such, this study underscores the potential for developing genetic markers tailored to specific identification needs, using mitochondrial reference genomes as a foundation. Ultimately, we hope that the increased availability of these genomes will encourages researchers to explore a wider array of mitochondrial genes and the future use of the obtained results in improving eDNA metabarcoding tools.

Supplementary material

Supplementary material can be found online at https://doi.org/10.52004/2054930X-20251025

Table S1. Overview of available mitogenome sequences in GenBank for species occurring in Europe, as listed by Becker *et al.* (2020)

Table S2. Details of the 139 mosquito mitogenome sequences (representing 139 species across 17 genera) utilised as seeds for the de novo assemblies in this study.

Table S3. Overview of the average genetic similarity at different taxonomic levels (the smallest taxonomy two sequences have in common), split by the 13 protein-coding genes, 2 ribosomal RNAs and the cox1 barcoding region.

Table S4. Overview of the average genetic similarity within species, split by the 13 protein-coding genes, 2 ribosomal RNAs and the *cox1* barcoding region.

Figure S1. Box plot illustrating pairwise genetic similarity across different taxonomic levels for all transfer RNAs.

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work greatly supported this endeavour. We also express our appreciation to Steffanie Teekema for organising the blind identification of the reference photographs, and to Francis Schaffner, Anders Lindström, and Filiz Gunay for their expert reviews of the reference photographs, which helped clarify the identification of some challenging species. Finally, special recognition goes to Frank Stokvis for his diligent efforts in performing the DNA extractions, and to Eveline Metz for her indispensable assistance in organising the sequencing process.

Authors' contributions

All authors have accepted responsibility for the entire content of this manuscript and approved its submission. Conceptualisation: JvdB, AI-J, AS, BvdV, TW, and MS. Funding acquisition: AI-J, KB, AS, and BvdV. Data collection and identification: JvdB and AI-J. Data analysis and visualisation: JvdB. Result interpretation: JvdB, AI-J, KB, EL-F, AS, BvdV, TW, and MS. Writing original draft: JvdB. All authors have read, contributed to, and approved the final version of the manuscript.

Conflict of interest

Adolfo Ibáñez-Justicia serves as a board member of the European Mosquito Control Association at the time of writing; however, he had no involvement in the review process or decision-making related to this manuscript. The remaining co-authors declare no conflict of interest.

Data availability

The assembled mitogenomes are available on GenBank under accession numbers: PV094667-PV094748. The raw sequencing data is deposited in GenBank Sequence Read Archive (SRA), under BioProject PRJNA1219649. The full analysis pipeline, including R scripts and statistics, is available on GitHub at https://github.com/JordyvdB97/mosquito-genomes-pipeline/. Reference images of the sequenced material are hosted on Zenodo and can be accessed at https://doi.org/10.5281/zenodo.14672457.

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Informed consent

The 'use' of specimens collected in France and Spain was formally communicated to the relevant authorities in both countries, in compliance with the Nagoya Protocol on Access and Benefit-sharing.

Research ethics

The collection of these mosquitoes is not subject to restrictions under national or international laws and does not require special permission. All specimens were collected on state-owned properties or, where applicable, with the consent of the landowner.

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