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Tracking Asian tiger mosquito introductions in the Netherlands using Nextstrain

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RESEARCH ARTICLE

Abstract

The Asian tiger mosquito *Aedes albopictus* is an undesirable invasive mosquito species that causes considerable nuisance through its biting behaviour, and has been proven to transmit more than 22 different viruses under laboratory conditions. Human-aided transportation, the capacity of winter diapause, and possibly global warming have contributed to the global invasion of *Ae. albopictus*. The species was found for the first time in the Netherlands in 2005, and since 2010 has been found introduced at many locations throughout the country. Elucidating the origin of these introduced mosquitoes could help the authorities on the planning and evaluation of the risk-based surveillance of *Aedes* invasive mosquitoes. This study aims to determine the genomic diversity of *Ae. albopictus* that is represented within and between collection sites with a database consisting of *Ae. albopictus* specimens from past introductions in the Netherlands, specimens from populations from other regions in the world, and data from specimens present in databases. In this study, complete mitochondrial genomes were sequenced, a recommended marker for phylogeography analysis of *Ae. albopictus.* Metadata is presented in a Nextstrain build containing 254 *Ae. albopictus* genomes up to October 2020. Overall, the phylogeny results of the Nextstrain build reveals a low mitogenomic diversity within *Ae. albopictus*. Genomic diversity of *Ae. albopictus* specimens found in the Netherlands fall within one main cluster which is hypothesised to represent the globally invasive strain of the species. Other organisations are stimulated to share data or materials for inclusion and improvement of the Nextstrain build, which can be accessed at https://nextstrain.nrcnvwa.nl/Aedes/20210728.

Keywords: *Aedes albopictus*, invasive mosquitoes, mitochondrial DNA, haplogroups, Nextstrain

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1. Introduction

The Asian tiger mosquito *Aedes albopictus* (Diptera: Culicidae) is an invasive mosquito species (IMS), that causes considerable nuisance through its biting behaviour (Kamgang *et al.*, 2012), and has been proven to transmit more than 22 different viruses under laboratory conditions (Gratz, 2004). In the field, it is considered a competent vector of chikungunya and dengue viruses (Delatte *et al.*, 2008). Furthermore, several pathogens were isolated from specimens collected in the field such as West Nile virus, Eastern equine encephalitis virus (Mitchell *et al.*, 1992) and La Crosse virus (Gerhardt *et al.*, 2001) in North America, and the heartworms *Dirofilaria immitis* and *Dirofilaria repens,* Usutu virus in Italy (Cancrini *et al.*, 2007; Puggioli *et al.*, 2017), and Zika in Southeast Asia (Johari *et al.*, 2019).

Aedes albopictus has a hypothesised native range that includes Japan, China, northern India, and parts of Southeast Asia (Hawley, 1988) and it has spread to several islands in the Pacific and the Indian Oceans during the 17th and 18th centuries (Paupy *et al.*, 2009). Due to global trade and human-aided transportation the species has expanded its distribution range to all continents of the world except Antarctica (Benedict *et al.*, 2007). *Aedes albopictus* established populations were discovered for the first time in Albania in 1979 (Adhami and Reiter, 1998), in the USA and Brazil in North and South America, respectively, in 1986 (Forattini, 1986; Sprenger and Wuithiranyagool, 1986), in Fiji in Oceania in 1990 (Laille *et al.*, 1990) and in Nigeria in Africa in 1992 (Savage *et al.*, 1992). In Europe, it is well known that the presence of *Ae. albopictus* in France, Switzerland and recently parts of Germany is related to the spread through the roads and highways from Italy where the species started the European colonisation. The population of *Ae. albopictus* in Italy, whose first presence was reported in the North in 1990 (Sabatini *et al.*, 1990), is suggested to have a double origin: related to the international trade of used tyres from the eastern coast of the USA, and from the spread through Albania and Greece from an Eastern Asian source (Dalla Pozza *et al.*, 1994; Dritsou *et al.*, 2015).

The first detection of *Ae. albopictus* in the Netherlands was at Lucky bamboo greenhouses in 2005 (Scholte *et al.*, 2007). Since 2010, *Ae. albopictus* has been detected each year during routine exotic mosquito species surveillance within the premises of tyre import companies and Lucky bamboo greenhouses (Ibañez-Justicia *et al.*, 2020), and sporadically in other Points of Entry (PoE) such as airports or flower auctions (Ibañez-Justicia, 2020). Surprisingly, in the early summer of 2016 *Ae. albopictus* was discovered in a residential urban area in the municipality of Veenendaal (NL), following a citizen notification to the Netherlands Food and Consumer Safety Authority (NVWA). Further investigation by the Centre for Monitoring of Vectors (CMV) of the NVWA found the species breeding throughout several blocks in the surrounding neighbourhood. Mosquito surveillance revealed the presence of *Ae. albopictus* larvae and pupae in human-made breeding sites (e.g. rain barrels, containers) and adults were found in mosquito traps. In the same summer, the NVWA also received a similar notification related to the presence of *Ae. albopictus*, in this case in an industrial area on the outskirts of the municipality of Weert (NL). Door-to-door mosquito control actions allowed the eradication of the species in both locations. However, from 2016 onwards *Ae. albopictus* specimens have been notified by citizens every year in residential areas in different municipalities. The source of these introductions is uncertain since a link to the main introduction pathways for the species, international trade in used tyres or Lucky bamboo plants (Ibañez-Justicia *et al.*, 2019) is lacking. We hypothesised that the possible sources of the *Ae. albopictus* detections could include accidental introductions by private persons via ground traffic from other regions of Europe where the species is established. Clarification on the origin of these mosquitoes and therefore the pathway of introduction would support the risk-based surveillance of *Aedes* invasive mosquitoes in the Netherlands.

Knowledge about the genetic diversity and the genetic structure of the Asian tiger mosquito can help researchers in identifying the origins and the frequency of introductions (Goubert *et al.*, 2016). Throughout the world, many attempts have been made in order to investigate the genetic structure of native and invasive populations of *Ae. albopictus* using different techniques and molecular markers and providing public available genetic reports (Goubert *et al.*, 2016). One of the most informative and commonly used marker for phylogeography analysis of *Ae. albopictus* is the mitochondrial DNA (mtDNA) (Goubert *et al.*, 2016). Especially, sequencing of a longer *cox1* gene fragment has revealed higher level of variation (Goubert *et al.*, 2016) compared with the shorter fragment of the *cox1* gene and sequences typically used for species identification (Folmer *et al.*, 1994). Previous studies have shown that the variation revealed in short partial mitogenomic gene sequences may be inadequate to identify population level haplogroups (Achilli *et al.*, 2008, 2012; Torroni *et al.*, 2006).

The aim of the current study is to determine the inter-specific and intra-specific mitogenomic diversity of *Ae. albopictus* specimens obtained from locations in the Netherlands to identify potential geographical sources and possible linkages to introduction pathways. Mitogenomic diversity among Dutch *Ae. albopictus* specimens was regarded in the context of the haplotype grouping described by Battaglia *et al.* (2016). Here we present the largest collection of complete *Ae. albopictus* mitogenomes and based on our analysis, a novel and simplified haplogroup naming system is proposed.

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2. Material and methods

Mosquito specimens collection, DNA extraction and Illumina sequencing

To build a genomic DNA world reference panel for *Ae. albopictus*, we first obtained genomic DNA (complete sequence) from published mitogenomes (Battaglia *et al.*, 2016; Ze-Ze *et al.*, 2020; Zhang *et al.*, 2016) from specimens belonging to populations in Europe, America and Asia (n=36). The reference panel included 2 mitogenomes from Albania (1 location), 3 from China (3 locations), 2 from Greece (1 location), 8 from Italy (5 locations), 5 from Philippines (1 location), 2 from Thailand (2 locations), 2 from USA (1 location), and 12 from Portugal (3 locations).

To increase the number of accessions in the reference panel we also added novel mitogenomes (n=24) from specimens collected from permanent populations in Europe and Asia. Fresh specimens were sent to the CMV by mosquito specialists. These additional records included 8 mitogenomes from China (1 location), 2 from Greece (1 location), 9 from Italy (2 locations), and 5 from Spain (2 locations).

Finally, novel mitogenomes were included from specimens (n=195) collected during surveillance in the Netherlands. The novel Dutch mitogenomes included 148 mitogenomes (13 locations) from specimens collected in urban areas, 10 (4 locations) from specimens collected at Lucky bamboo greenhouses, 23 (7 locations) from specimens collected at used tyre companies, 6 (1 location) from specimens collected at an airport, 5 (1 location) from specimens collected at a parking lot, and 3 (1 location) from specimens collected at a flower auction. Location and collection dates of all used samples are available online in the Nextstrain build via: https://nextstrain.nrcnvwa.nl/Aedes/20210728 (last accessed, 4 April 2022).

The sampling included specimens of different life stages: eggs (n=5), larvae (n=50) and adults (n=198). Genomic DNA was extracted from *Ae. albopictus* specimens on an automated KingFisher Flex System (ThermoFisher, MA, USA) using the QuickPick™ SML gDNA Kit (Bio-Nobile, Finland) or manually with the High Pure PCR Template Preparation Kit (Roche, Switzerland) following manufacturer's instructions (S-table 1). Insect tissue was ground in lysis buffer and Proteinase K with a micro-pestle prior to DNA extraction. For whole genome shotgun sequencing, extracted DNA was commercially sequenced by GenomeScan (Leiden, the Netherlands) on an Illumina NovaSeq6000 platform for generation of at least 2 Gb Illumina DNAseq 150PE (paired-end) data per sample.

Reference based assembly of mitogenomic sequences

NovaSeg reads of the *Ae. albopictus* specimens were uploaded to CLC genomics workbench v21.0.3 (Qiagen, Germany) and quality trimmed (quality trim: 0.05; ambiguous limit: 2) prior to read mapping (length fraction = 0.8; similarity fraction = 0.9) to *Ae. albopictus* mitogenome KX383916. Read mappings were visually assessed and consensus sequences with at least 10× average coverage were extracted. Mitogenomic annotations were transferred from the reference sequence and visually assessed in Geneious Prime v20.1.1 (Biomatters, New Zealand). Mitogenomic sequences of the specimens sequenced in this study were submitted to NCBI GenBank under accessions MZ374140 to MZ374358.

The *Aedes albopictus* Nextstrain build

To identify potential links between genotypes and geographical origin or pathways, a Nextstrain build (Hadfield *et al.*, 2018) was created based on the complete mitogenomic sequences of the 254 *Ae. albopictus* specimens. Augur (github.com/Nextstrain/augur), a bioinformatics toolkit for phylogenetic analysis used in the Nextstrain pipeline, was used to align the mitogenomic sequences with MAFFT (Katoh and Standley, 2013) and to perform a RAxML clustering (Stamatakis, 2014). Repeat regions were masked and excluded from the analysis as copy number variation of small tandem repeats can distort the phylogenetic signal. The initial tree was refined with metadata and a time tree was build using TreeTime (Sagulenko *et al.*, 2018) with optimisation of scalar coalescent time, and using a fixed clock rate of 2.3×10^{-8}/site/year following (Brower, 1994). Internal nodes were assigned to their marginally most likely dates, and confidence intervals for node dates were estimated. Nucleotide and amino acid changes were determined based on the longest annotated *Ae. albopictus* mitogenome present in the dataset, i.e. KX383916: 17,150 bp, specimen ' Rimini isolate 1'. Next, Augur output was exported and visualised in auspice. The *Ae. albopictus* Nextstrain build is deposited on GitHub (https://github.com/NPPO-NL/nextstrain-Aalbo).

We used Nextstrain to create a public interactive webpage in which mitogenomic diversity is visualised in context of haplogroups, detection sites and linkages to introduction pathways. Within Nextstrain, internal node colours indicate the predicted ancestral state of a given trait, and the confidence of that state is conveyed by saturation of the colour of the internal node. The cladogram can be shown in different styles such as rectangular, radial and unrooted. The branch lengths of the tree can be shown based on divergence or in function of time. Based on the information provided in the build, Nextstrain estimates the most likely species spread, which can be animated from the webpage. The genotypes represented in the tree are plotted

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on a map, and users can set different levels of geographical resolution, i.e. continent, country, state and municipality (when this information is available). The use of filters allows simultaneous interrogation of phylogenetic and geographic relationships, with additional relevant metadata. Users can download metadata and tree-files from the webpage, and can create screenshots of their views. The Nextstrain build can be accessed from https://nextstrain.nrcnvwa.nl/Aedes/20210728 (last accessed 4 April 2022).

Clustering analysis

To provide context to the mitogenomic variation among the *Ae. albopictus* specimens, a clustering was performed on the *Ae. albopictus* mitogenome sequences and complete mitogenomes from other *Aedes* species (*Aedes aegypti* EU352212, MK575474; *Aedes alternans* MN389472; *Aedes busckii* MN626443; *Aedes flavopictus* MT501510; *Aedes koreicus* MT093832; *Aedes notoscriptus* KM676218, KM676219; and *Aedes rubrithorax* MN389466). The complete *Culex quinquefasciatus* mitogenome NC_014574 was used to root the *Aedes* spp. phylogenetic tree. Sequences were aligned with MAFFT (repeat region: masked) and clustering was performed with PhyML v3.3.20180621 (Guindon *et al.*, 2010) (masked sites: excluded) using the Generalised Time-Reversible (GTR) model with 100 bootstrap replicates in Geneious Prime v20.1.1.

3. Results

Nextstrain and haplogroup naming system

The *Ae. albopictus* Nextstrain build contains 254 (219 novel and 35 previously published) complete *Ae. albopictus* mitogenomes generated from material sampled between June 2014 and October 2020. Information in the build is presented in three main panels: clustering of genomic diversity, geographical origin of the samples, and diversity relative to *Ae. albopictus* mitogenome KX383916 (Figure 1). The associated metadata, included in the build, allows users to colour external nodes in the tree according to the pathway, haplogroup identity, country and municipality (when available) from which the specimens were obtained.

Phylogenetic analysis shows low *Ae. albopictus* intraspecific mitogenomic diversity relative to other *Aedes* species tested resulting in two major *Ae. albopictus* clusters (Figure 2). The two main clusters were defined as haplogroup 1 (with 249 mitogenomes containing all introduced populations) and haplogroup 2 (with five mitogenomes belonging to five specimens analysed by Battaglia *et al.* (2016) from wild populations in the Philippines). Intraspecific similarity (excluding the mtDNA repeat region) in haplogroup 1 and 2 ranged from 99.7 to 100% and 99.9 to 100% respectively. Haplogroup 1 and 2 interspecific similarity ranged from 99.5 to 99.6% (55 to 71 polymorphic sites). Haplogroup 1

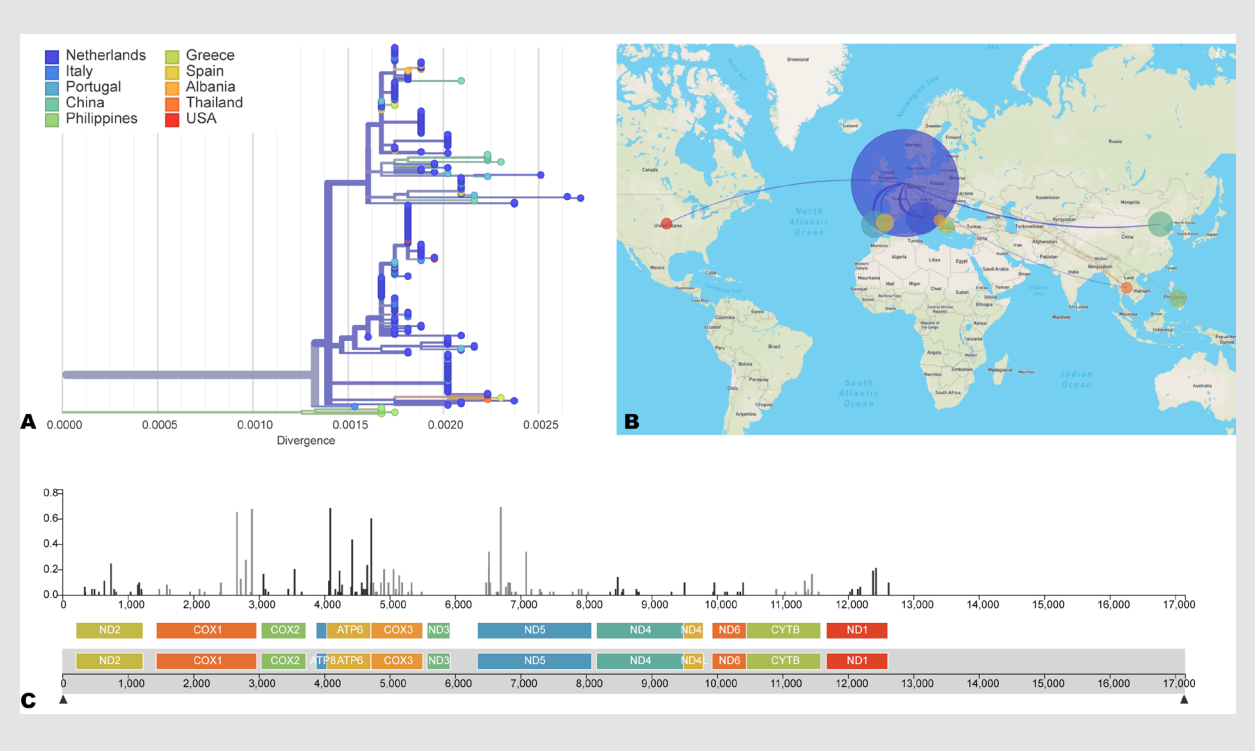

Figure 1. Nextstrain build containing 254 complete *Aedes albopictus* mitogenomes and presented in three main panels: (A) clustering of genomic diversity; (B) geographical origin of the samples; and (C) diversity relative to *Ae. albopictus* mitogenome KX383916.

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was further divided into five subgroups (A – E) based on the main PhyML and RAxML subclusters within haplogroup 1 (Figure 1 and 2). All haplogroups defined by Battaglia *et al.* (2016) were represented in one of the subgroups 1A-E or in haplogroup 2, except Battaglia *et al.* (2016) haplogroup A1a1 specimen Ces2 (KX383923) as this specimen clustered basal to the other haplogroup 1 specimens. Haplogroups 1A (containing Battaglia *et al.* (2016) haplogroups A1a2, A1a2a and A1a2a1) and 1B (containing Battaglia *et al.* (2016) haplogroups A1a1a1, A1a1a1a, A1a1a1a1) with 109 specimens and 103 specimens respectively. Specimens belonging to our haplogroups 1C and 1E were not sampled and sequenced by Battaglia *et al.* (2016).

The *Ae. albopictus* Nextstrain build represents specimens from 10 countries worldwide, i.e. Albania (n=2), China (n=10), Greece (n=4), Italy (n=17), the Netherlands (n=195), Philippines (n=5), Portugal (n=12), Spain (n=5), Thailand (n=2), and the United States of America (n=2). Apart from specimens from the Philippines, all specimens belonged to haplogroup 1. Specimens from European countries other than the Netherlands all possessed mitogenomes belonging

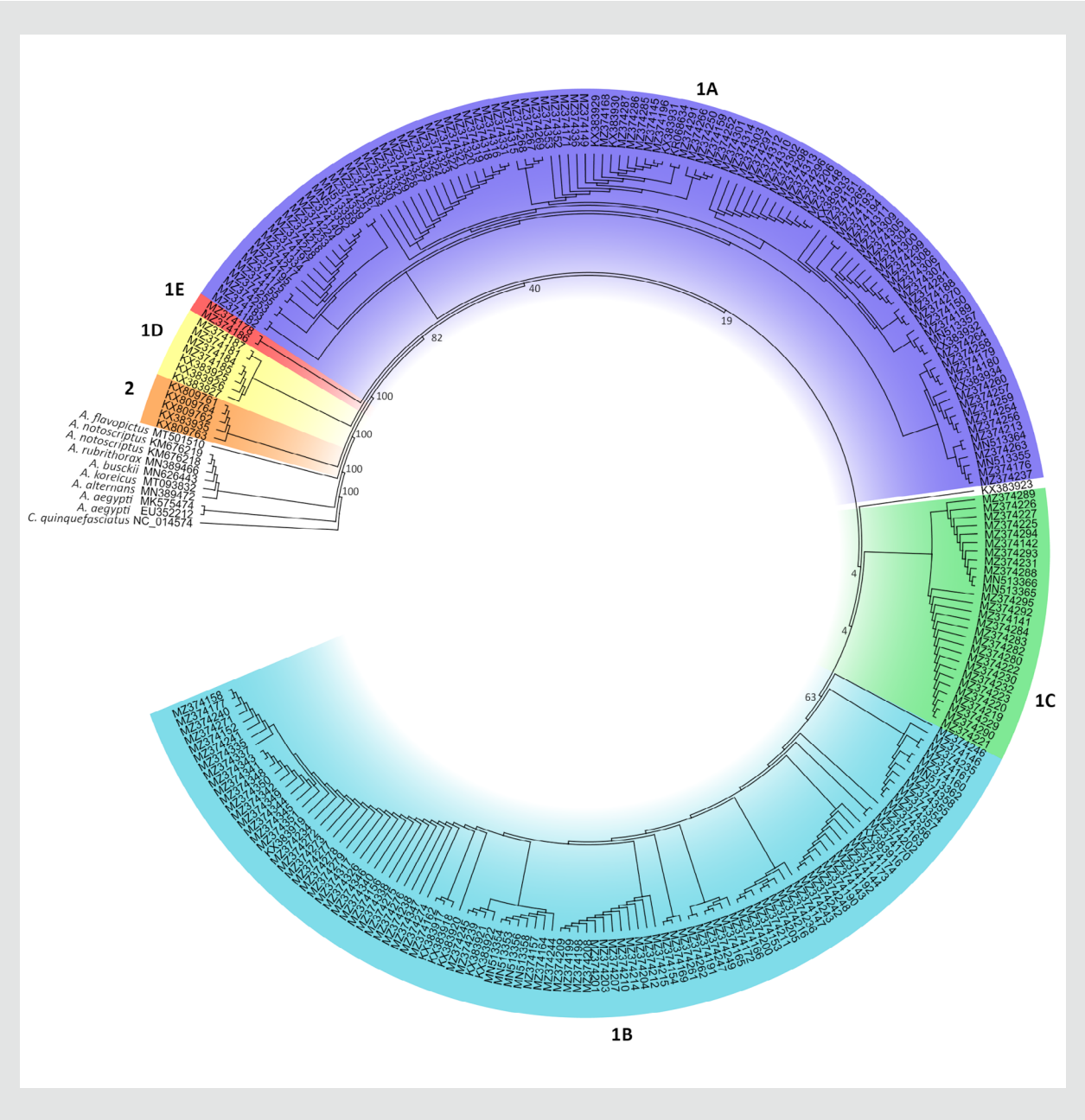

Figure 2. PhyML phylogeny of the 254 complete *Aedes albopictus* sequences and complete mitogenomes from other *Aedes* species and *Culex quinquefasciatus*. Haplogroups and subgroups obtained are indicated with colours.

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to haplogroups 1A, 1B and 1C, except for Greece which also had two haplogroup 1D specimens. The representation of different haplotype subgroups on country-level is presented in Supplementary Figure S1.

Specimens introduced into the Netherlands

Between June 2014 and October 2020, 195 *Ae. albopictus* specimens were sampled and sequenced from 26 different localities in the Netherlands. Here haplogroup 1A was found to be most prominent with 79 specimens, followed by 1B (n=85), 1C (n=25), 1D (n=4) and 1E (n=2). The majority of specimens were obtained from foci in urban areas (n=148), followed by specimens associated with used tyre import (n=23), Lucky bamboo importers (n=10), airports (n=6), highways (n=5) and flower auctions (n=3).

Specimens from foci in urban areas within municipality haplogroup variation

When considering foci in urban areas where 18 or more specimens were sampled, sequenced and included in the Nextstrain build, seven municipalities can be further analysed to determine the within-municipality diversity (Figure 3, Supplementary Figure S2). Only in a single municipality (Uithoorn) all specimens belonged to a single haplogroup (1A). Specimens representing haplogroups 1A and 1B were found in Sittard, Valkenburg, Veenendaal and Weert, and the municipality Aalten represented mitogenomes from haplogroups 1A and 1C. In the municipality Alblasserdam haplogroups 1A, 1B and 1C were found. In Hoogeveen, sampling was limited to three specimens but both haplogroups 1A and 1C were found to be present.

Haplogroup – pathway association

When considering different introduction pathways, specimens introduced via used tyre trade represented the biggest group with 31 specimens from seven different municipalities (Figure 4, Supplementary Figure S3). Mitochondrial haplogroups represented in these samples were restricted to 1A and 1B. Similar to the foci in urban areas, locations where multiple specimens were sampled and sequenced (e.g. Assen, Emmeloord and Lelystad) showed presence of both haplogroups 1A and 1B at a single used tyre site. Specimens collected at Lucky bamboo importers (Figure 4, Supplementary Figure S4) represented the second biggest group with 10 specimens from four different municipalities (Amstelveen, Bleiswijk, Leimuiden and 's-Gravenzande). These specimens represented haplogroups 1A, 1D and 1E. The latter haplogroup (1E) was only found at Lucky bamboo import sites, but both at the locations Leimuiden and 's-Gravenzande. Similarly, haplogroup 1D specimens from the Netherlands were only associated with Luck bamboo import. Specimens from Greece and Thailand were found to also possess an haplogroup 1D mitogenome. Specimens from airport surveys (n=6) represented both

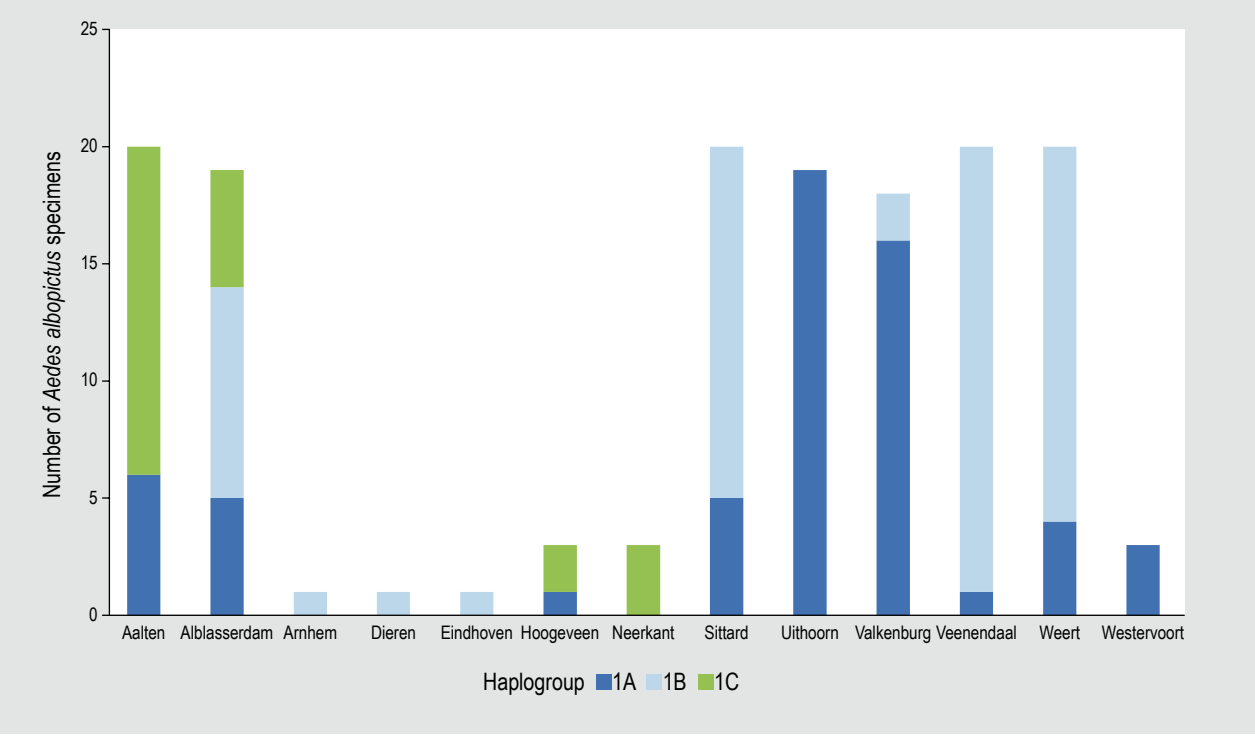

Figure 3. Mitochondrial haplogroups represented in the *Aedes albopictus* specimens found from foci in urban areas at municipality level.

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haplogroups 1A and 1C, specimens from highway surveys (n=5) represented haplogroup 1B, and specimens obtained at flower auctions (n=3) represented haplogroups 1A and 1B.

4. Discussion and conclusion

Aedes albopictus is a mosquito species considered to be native to Southeast Asia (Hawley, 1988). This assumption is based on the fact that this mosquito is present everywhere in the forested areas of this region and that many species close to the 'albopictus' subgroup, member of the Scutellaris group, are present in Southeast Asia. However, the notoriety of *Ae. albopictus*, compared to relatives, comes from the fact that it has moved out of its area of origin, becoming cosmopolitan in 50 years, adapting perfectly to urbanisation, temperate climates, and international transport, as well as being involved in several dengue and chikungunya epidemics (Fontenille and Powell, 2020).

The recent and very successful spread of *Ae. albopictus* has been depicted in the results shown in this study. Our results show that the genetic variability on the analysed specimens of *Ae. albopictus* is very low, even if the specimens were collected from populations from remote locations in different continents. Endosymbionts such as *Wolbachia* sp. could introduce a selective sweep resulting in low mitogenomic variation (Jiggins, 2003). In *Ae. albopictus* infections with *Wolbachia* are known to occur and their prevalence was shown to differ on a regional level (Hu *et al.*, 2020). Almost identical mtDNA sequences were obtained in different locations, and single locations represented specimens with mtDNA sequences from multiple haplogroups. Results show that mtDNA did not significantly diverge at these locations. However, based on mtDNA we can consider that *Ae. albopictus* can be divided in two major haplogroups. A haplogroup 1 characterised by very low diversity, but very successful for invasion of the world, and a haplogroup 2 defined by five mitogenomes from the Philippines determined by Battaglia *et al.* (2016).

Based on mitogenomic variation within haplogroup 1, this cluster was further subdivided in subgroups A, B, C, D and E. Subgroups were chosen based on clusters present in both the PhyML and RAxML trees. However, the subgroups mainly serve a dissemination purpose to communicate diversity within haplogroup 1, but do not have a strong phylogenic basis as is reflected by the bootstrap values from some of the internal nodes. Battaglia *et al.* (2016) suggested that haplogroup 2 specimens might be limited to the Philippines, Indonesia, Papua New Guinea, and Northern Australia. They also suggested that haplogroup A2 appears to have played a role in the spread of *Ae. albopictus* from South-East Asia restricted to the context of Oceania. When compared with haplogroup 1 mainly present in adventive populations, haplogroup 2 is present nearby the species geographical origin and shows genetic diversity. Our results provide molecular support for the recent progressive

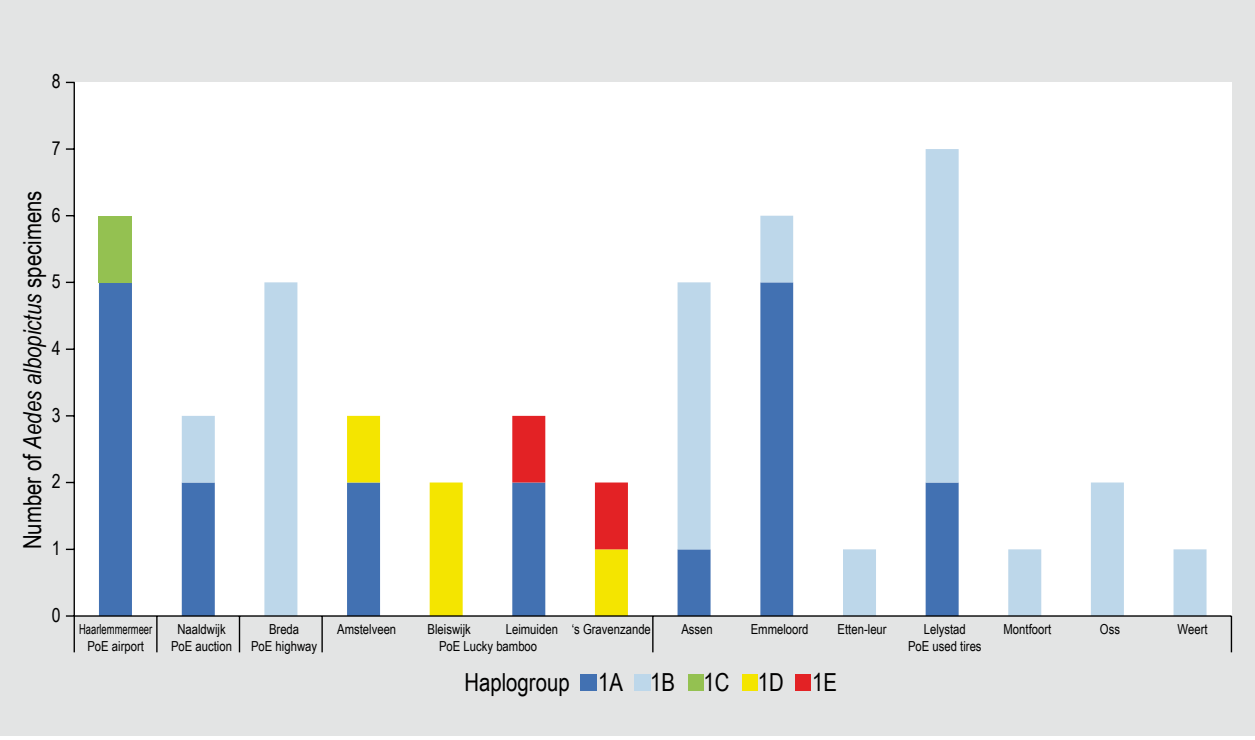

Figure 4. Mitochondrial haplogroups represented in the *Aedes albopictus* specimens found in different Points of Entry (PoE) in the Netherlands at municipality level.

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invasion which represents a subset of the genetic diversity present at the geographical origin of the species.

To the best of our knowledge, this study is one of the first large trials that has implemented Illumina sequencing for an *Ae. albopictus* population-level study. Instead of sequencing only a small part of the genome, Illumina DNAseq data allows the assembly of the (near) complete *Ae. albopictus* genome. The complete mitogenome offers additional resolution to determine potential linkages between genotypes and potential geographical sources. To allow a good comparison of assembled sequence data, only complete *Ae. albopictus* mitogenomes from public databases were included in this study, as recommended by Goubert *et al.* (2016). We used Nextstrain to present mitogenomic diversity in the context of geographical distribution and epidemiological traits such as pathway. Nextstrain is has been mainly used for creating interactive views for viruses causing disease in humans (Gardy and Loman, 2018) and plants (Kangabam *et al.*, 2021), but also for complete bacterial genomes (https://nextstrain.org/tb/global, last accessed 4 April 2022). In essence, any genomic region from any species can be used provided it is suitable for the specific research question addressed and taking into account the substitution rate of the genomic region investigated to produce meaningful time trees. We applied the 'standard' molecular clock for insect mitogenomes estimated at 2.3% per million years (Brower, 1994). It should be noted that since the mutation rate of insect mitochondrial genomes is not constant (Papadopoulou *et al.*, 2010), a single universal molecular clock rate is not feasible and the estimated time of divergence of the different clusters in the time tree is indicative.

Sample size of the study included specimens for the reference panel and the specimens found introduced in the Netherlands. These included the life stages egg, larva and adult. In this study we chose to mostly use samples of adults, as adults are more likely to represent the sampling site diversity than the other stages from the same breeding site. Immature stages could more likely belong to the same progeny. For example, in our study five eggs from the same substrate of an ovitrap were used from one location in a parking lot (Breda) which all had the same mtDNA sequence belonging to haplogroup 1B. This result could indicate that the specimens could belong to the same progeny from the same ovipositing *Ae. albopictus* female. Similarly, emerging adults from eggs collected using ovitraps were analysed from the location Los Banos (Philippines) by Battaglia *et al.* (2016). In this case, three of the five mitogenomes belonging to haplogroup 2 were found to have identical mtDNA sequences.

The *Ae. albopictus* introduced in Lucky bamboo greenhouses included the invasive haplotype 1A, but also 1D and 1E. The haplogroup 1E was only found at Lucky bamboo import sites in the Netherlands. The specimens captured in Lucky bamboo greenhouses most likely arrived as eggs from tropical areas where the plants are cultivated, such as Zhanjiang city or Taishan city in the Guangdong province of China. For this reason, the specimens introduced with Lucky bamboo are expected to belong to the tropical strain of the species, having distinct kinetics of embryogenesis, that differs from the temperate strain (Lacour *et al.*, 2014). Tropical strains are also unable to perform diapause, contrary to temperate and subtropical strains which perform photo-induced diapause (Pumpuni, 1989).

In our study, the specimens from Virginia (eastern coast of the USA) belong to the haplogroup 1B, and Albanian and Greek specimens mainly belong to haplogroup 1A. Interestingly, haplogroup 1D is also representing Greek specimens, being a haplogroup that is also found in specimens that are supposed to arrive directly from Eastern Asia with the Lucky bamboo import. In specimens detected in Dutch urban areas, we often find specimens of the same location clustering in different haplogroups. For example in the location Alblasserdam three different haplogroups, 1A (n=5), 1B (n=9) and 1C (n=5) were found. The presence of multiple haplogroups in small detection sites in the Netherlands could be explained by a single introduction of several individuals from different subgroups (e.g. container with eggs oviposited by different females), or repeated introduction events that started building the population as suggested by Ze-Ze *et al.* (2020). For the small outbreaks at urban foci in the Netherlands, the latter explanation is regarded far less likely than the first. For used tyre import however, the latter explanation might be more likely.

In the municipality Uithoorn, nineteen specimens were sampled, 16 of which were larval specimens. All sequenced specimens belonged to haplogroup 1A. Furthermore, 10 specimens had an identical mtDNA sequence, and the remaining 9 specimens also had an identical (but different) mtDNA sequence. The specimens with identical mtDNAs could belong to the same offspring.

We set out to determine the presence of links between mtDNA haplotypes and pathway or origin. Our results show however that no clear links exist. Without such clear links, determining the origin of new outbreaks or detections, or the potential of overwintering of mosquito populations are not feasible. The mitogenome is frequently used as population level marker given its presumed clonal (maternal) inheritance, neutrality of accumulated mutations, and constant mutation rate across different species. There could be limitations to the use of the mitogenome as recent opinions have questioned this central dogma (Galtier *et al.*, 2009). For instance, mitogenomic intramolecular recombination was shown for the nematode species *Meloidogyne javanica* which was facilitated by the

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presence of tandem repeats (Lunt and Hyman, 1997). Also for the fruit fly genus *Bacterocera* (Tephritidae) strong evidence of recombination was provided (Tsaousis *et al.*, 2005). Mitogenomic recombination could hamper the usability of the mitogenome to determine population-level relationships. Other authors have explored the use of microsatellites to determine linkages between Small Simple Repeats (SSRs) on the nuclear genome and geographical origin (Manni *et al.*, 2015; Porretta *et al.*, 2006). The potential for the use of SSRs on our current sample set could be further explored. Alternatively, the potential of the nuclear genome could be explored using SRAs generated in this study allowing direct comparison to our current analyses.

Our analyses reveal that all worldwide introduced mosquito populations analysed in our study belong to a single major haplogroup which could be further divided into five subgroups. At Dutch introduction locations where intensive sampling and sequencing was performed, multiple subgroups were found to be present. This indicates that introduced populations represent high intra-specific mitogenomic diversity suggesting the introduction of multiple mosquito specimens at any life stage. As this was observed at multiple sites of introduction, it is likely that the same applies to other introduction sites worldwide potentially restricting the use of mitogenomic diversity for source attribution for these introduced populations.

Sharing data for inclusion in this interactive online tool enables the vector entomology field to better understand and communicate the diversity and spread of this important invasive mosquito species. The reliability of a track and trace tool depends on sampling and metadata completeness. The Nextstrain build currently holds mitogenomes from various locations in Europe, Asia and North-America. However, there is a strong bias towards genotypes obtained from the Dutch locations. Sampling bias and lack of data can (in addition to the limited genomic variation and ease of spread) hamper the determination of reliable transmission links. The predictive power of the tool will improve with the addition of additional genomes. Therefore, we encourage other organisations to share data or biological materials together with relevant metadata in order to improve the build. Entomologists can contribute to the further build by providing preferably *Ae. albopictus* specimens from the origin area in Asia, or providing Illumina sequence datasets or assembled mitogenomes together with relevant metadata to the National Reference Centre (NRC), which is part of the Netherlands Food and Consumer Safety Authority (NVWA).

Supplementary material

Supplementary material can be found online at https://doi.org/10.52004/JEMCA2021.0006.

Figure S1. Country-municipality level representation of *Aedes albopictus* haplotype subgroups. Countries other than the Netherlands are included in this plot.

Figure S2. Nextstrain built of mitochondrial haplogroups represented in the *Aedes albopictus* specimens found from foci in urban areas in the Netherlands.

Figure S3. Nextstrain built of mitochondrial haplogroups represented in the *Aedes albopictus* specimens found in points of entry used tires in the Netherlands.

Figure S4. Nextstrain built of mitochondrial haplogroups represented in the *Aedes albopictus* specimens found in points of entry Lucky bamboo in the Netherlands.

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Conflict of interest

The authors declare no conflict of interest.

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