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ARTHROPODS AND MEDICAL ENTOMOLOGY - ORIGINAL PAPER

Population genetics of the invasive Asian bush mosquito *Aedes japonicus* (Diptera, Culicidae) in Germany—a re-evaluation in a time period of separate populations merging

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Abstract

The Asian bush mosquito *Aedes japonicus*, endemic to East Asia, is one of the most expansive mosquito species in the world and has as yet established in 15 countries of Europe. Within Germany, the species has been spreading tremendously during the last years, and its four once geographically isolated populations were on the verge of merging in 2017. To reveal relationships and carry-over ways between the various populations, and thus, migration and displacement routes, the genetic make-up of *Ae. japonicus* from ten different locations throughout its German distribution area was investigated. For this purpose, a part of the mitochondrial DNA (*nad*4 gene) of collected specimens was sequenced and seven loci of short tandem repeats (microsatellites) were genotyped. When related to similar genetic studies carried out between 2012 and 2015, the results suggest that admixtures had since occurred, but no complete genetic mixture of populations had taken place. At the time of sampling for the present study, the western collection sites were still uniform in their genetic make-up; however, a carry-over of individuals from the southeastern to the northern and southwestern German populations was determined. Further introductions from abroad are possible. In summary, the genetic diversity of *Ae. japonicus* in Germany had grown considerably, thus increasing ecological variability and adaptability of the species. At this point (10 years after the first detection), it is not possible anymore to draw conclusions on the origins of the populations.

Keywords *Aedes japonicus* · Population genetics · Microsatellites · *nad*4 haplotypes

Introduction

The Asian bush mosquito *Aedes japonicus* (Diptera, Culicidae) was first described by Theobald in Tokyo, Japan, in 1901 (Theobald 1901). Its native distribution area is in East Asia (Japan, Korea, China and southeastern Russia) (Tanaka et al. 1979), where temperate climates prevail. The species started to emerge in geographic regions outside Asia in 1993, when it was intercepted in New Zealand (Laird et al. 1994). In 1998, first established populations were detected in New York, New Jersey and Connecticut, USA (Peyton et al. 1999, Andreadis et al. 2001). The first evidence of *Ae. japonicus* in Europe was from France in 2000 (Schaffner et al. 2003). At present, *Ae. japonicus* has colonised at least 34 US states and parts of Canada as well as 15 countries in Europe (Kampen and Werner 2014; Fielden et al. 2015; Jackson et al. 2016; Riles et al. 2017; Ministère de la Santé du Grand-Duché de Luxembourg 2018, Kavran et al. 2018, 2019; Eritja et al. 2019; Koban et al. 2019).

Thus, *Ae. japonicus* is one of the most expansive mosquito species in the world. Its adaption to temperate climates has facilitated establishment in northern America and Central Europe, but populations have also been found in warmer areas, such as Hawaii, Florida and northern Spain (Larish and Savage 2005, Riles et al. 2017, Eritja et al. 2019). The spread of this mosquito species is basically mediated by human activities. A major intercontinental and continental distribution channel is the trade with used tyres and ornamental plants but ground traffic seems to add to this within the USA and Europe (Medlock et al. 2012).

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Aedes japonicus is a potential vector of several viruses. Under laboratory conditions, it is able to transmit West Nile virus, Japanese encephalitis virus, eastern equine encephalitis virus, La Crosse virus, Rift Valley fever virus, chikungunya virus and dengue virus (Takashima and Rosen 1989, Turell et al. 2001, 2013, Sardelis et al. 2002a, 2002b, Schaffner et al. 2011). In the field, specimens of *Ae. japonicus* have been found infected with West Nile virus, Japanese encephalitis virus, Cache Valley virus and La Crosse virus (Chagin and Kondratiev 1943, Novello et al. 2000, Harris et al. 2015; Yang et al. 2018). The species feeds on both mammals and birds (Miyagi 1972, Molaei et al. 2009) and may therefore serve as a bridge vector.

In 2008, *Ae. japonicus* larvae were detected in the German federal state of Baden-Württemberg close to the German-Swiss border (Schaffner et al. 2009). A subsequent monitoring programme carried out in 2009 identified a large colonised area in southern Baden-Württemberg (Becker et al. 2011). A study conducted in 2010 found *Ae. japonicus* close to Stuttgart, 80 km north of the previously delimited distribution area (Schneider 2011), which was soon absorbed in a huge population covering large parts of southwestern Germany (Huber et al. 2014). In western Germany, an additional population was identified in 2012 (Kampen et al. 2012), and still another one in a more northern German region in 2013 (Werner and Kampen 2013). In 2015, *Ae. japonicus* was demonstrated in German Upper Bavaria and adjacent Austria (Zielke et al. 2016).

The wider the distribution and the higher the population densities of a mosquito vector, the higher the probability of an introduced pathogen to be transmitted and spread. In the case of an invasive vector species, such as *Ae. japonicus*, it is therefore important to know through which modes and ports it enters a country and spreads within the country (Fonseca et al. 2001). Genetic analyses that unveil degrees of relationships between populations may be useful to elucidate these aspects (Medlock et al. 2012).

Comparative genetic analyses of *Ae. japonicus* populations are scarce. Previous studies have targeted the mitochondrial *nad*4 (NADH dehydrogenase subunit 4) gene and nuclear microsatellites, both of which have relatively high mutation rates and appear to be unique for populations. Mitochondrial DNA has a high mutation rate due to free oxygen radicals and less repair function than nuclear DNA (Richter et al. 1988). It is maternally inherited, evolves very quickly and is characterized by intraspecific polymorphisms (Krzywinski et al. 2006). Sequence differences are caused by mutation and not by recombination. Protein-coding genes of the mtDNA have a mutation rate five to ten times higher than those of the nuclear genome (Brown et al. 1979). Similarly, microsatellites are widely used for genetic analyses because they have a higher mutation rate than other DNA regions (Brinkmann et al. 1998). Insertions or deletions result in a change of the repetitive motif, which modifies the length of the fragment. Through *nad*4 sequence analysis, which works at nucleotide level, changes in population genetics become visible at an early stage, making the method suitable for revealing variations within a population. By contrast, microsatellite analysis is based on differences in DNA fragment length and works at a higher level, taking more time for genetic changes to become visible.

Following the work of Zielke et al. (2014, 2015, 2016), we analysed the *nad*4 gene sequences for nucleotide polymorphisms and the length of informative microsatellite loci of *Ae. japonicus* collections in order to determine differences in the genetic background of the German populations. The main purpose of the present study was to get an overview of the relatedness of these, as they existed in 2017, and to unveil changes developed since performance of similar investigations several years ago (Zielke et al. 2014, 2015, 2016). At that time, two microsatellite signatures and nine different *nad*4 haplotypes were identified in the German populations. Major findings included a close relationship of the West and North German populations, which were genetically isolated from the South German populations. The Southeast German/Austrian population seemed not closely related to the other German populations.

Since the work by Zielke et al. (2014, 2015, 2016), the German *Ae. japonicus* populations have spread considerably (Kampen et al. 2016, 2017, Koban et al. 2019), and previously separated populations were on the verge of merging in 2017. It should be found out whether a genetic mixture of the populations had taken place. Intermixing of separate populations as well as admixture of populations by new introductions increases the genetic variation which in turn leads to a higher adaptability and an associated stronger expansion drive.

Material and methods

Mosquito material

While monitoring the spread of *Ae. japonicus* in Germany, larvae were collected in August and September 2017 from water containers at numerous localities. For the present study, larvae from ten different sites, located in expected merging regions of hitherto separate populations and in new distribution areas, were analysed (Fig. 1). Some of these had been preserved as such in 80% ethanol while others had been reared in the laboratory until adult emergence and stored dry. Adults and larvae were identified morphologically (Schaffner 2003, Becker et al. 2010). To exclude investigating siblings, just one specimen per breeding container was used, but up to 20 specimens per location.

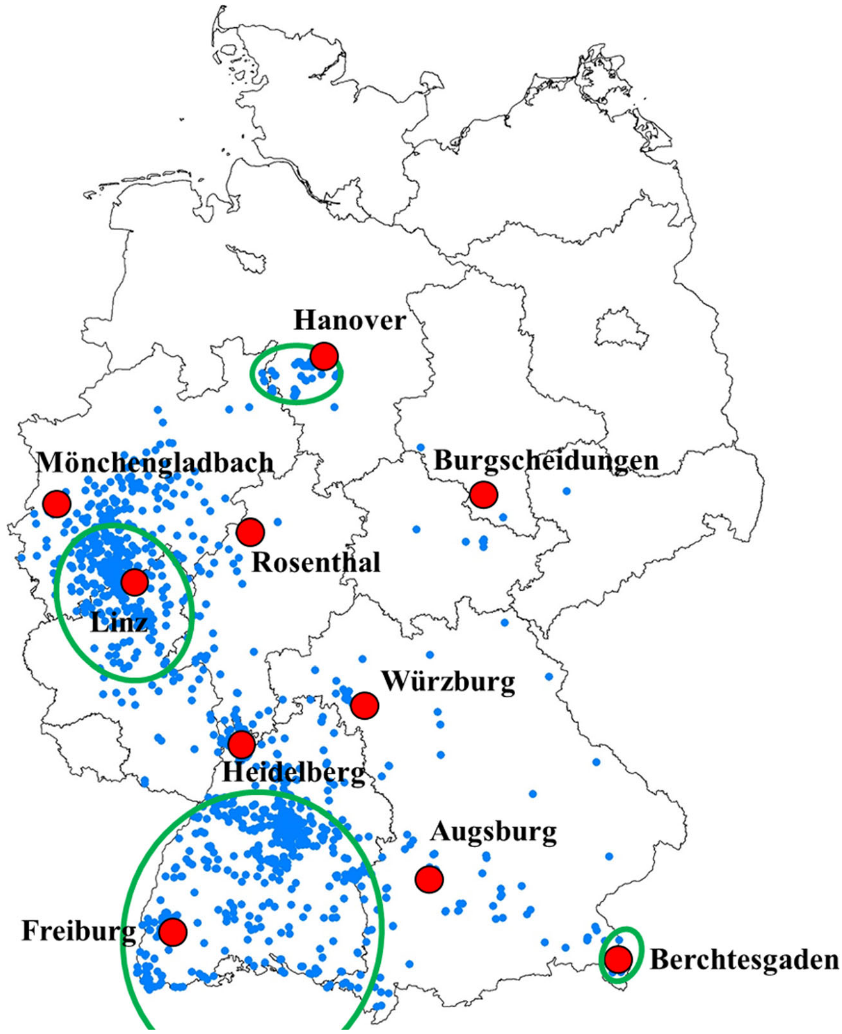


Fig. 1 Geographic distribution of *Ae. japonicus* collection sites analysed in this study (red dots). Blue dots mark *Ae. japonicus* detection sites from 2012 to 2017, and areas encircled in green colour represent approximate population distributions as of 2015

DNA extraction

DNA was extracted by means of the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, using total larvae or adults. DNA was eluted in 80 µl EB buffer and stored at − 20 °C.

*nad*4 gene analysis

For sequence analysis, a 424 bp segment of the mitochondrial *nad*4 gene was amplified. Primers ND4F (5′-CGTAGGAG GAGCAGCTATATT-3′) and ND4R1X (5′-TGATTGCC TAAGGCTCATGT-3′) (Egizi and Fonseca 2015) and a PCR protocol of Fonseca et al. (2010), slightly modified regarding thermoprofile, was used: denaturation 5 min at 96 °C, 35 cycles of 30 s at 94 °C, 30 s at 56 °C and 30 s at 72 °C, and final extension at 72 °C. DNA amplification was performed in a C1000 Touch thermal cycler (Bio-Rad, Munich, Germany). PCR products were run on an agarose gel (1.5%) and visualized by ethidium bromide staining. The bands were excised from the gel, and the DNA was purified by the QIAquick Gel Extraction Kit (Qiagen). Cycle sequencing was conducted bidirectionally with the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's protocol. DNA was cleaned using NucleoSEQ spin columns (Macherey-Nagel, Düren, Germany) and run on a 3130 Genetic Analyzer (Thermo Fisher Scientific, Berlin, Germany). The sequences were edited with Geneious 10.2.3 (Biomatters) and examined for variable sites.

Microsatellite analysis

Microsatellite analysis was performed on seven loci, OJ5, OJ10, OJ70, OJ85, OJ100, OJ187 and OJ338 (Widdel et al. 2005). For their amplification, two multiplex PCRs were carried out (multiplex A: OJ5, OJ10, OJ85, OJ187; multiplex B: OJ70, OJ100, OJ338), using primers described by Widdel et al. (2005), with a modified forward primer for the locus OJ5 (Egizi and Fonseca 2015). PCR conditions were the same as in Fonseca et al. (2010). The amplification was conducted again using a C1000 Touch thermal cycler. The lengths of the microsatellite loci were determined by capillary gel electrophoresis (amedes genetics, Hanover, Germany), and the chromatograms were analysed with Geneious 10.2.3.

Statistical analysis

A phylogenetic tree (HKY model) of the detected *nad*4 haplotypes was built using Geneious 10.2.3. The genetic signature of the microsatellites was determined using Bayesian algorithm in the software Structure 2.3 (Pritchard et al. 2000). To get the most probable number of genetic clusters, i.e. the highest ΔK (Evanno et al. 2005), the software Structure Harvester (Earl and vonHoldt 2012) was applied. The programme GenAIEx was used to perform a principal coordinate analysis (PCoA) on the microsatellite data, based on Nei's genetic distance and pairwise F_{ST} values (Peakall and Smouse 2012).

Results

Within this study, 249 *Ae. japonicus* specimens from 10 different collection sites in Germany were analysed (Table 1).

*nad*4 sequencing

*nad*4 sequences could be determined for 209 specimens (Table 1). Because of mitochondrial heteroplasmy (simultaneous presence of several haplotypes in one organism), 27 specimens could not be assigned to one haplotype. Alignment of the sequences demonstrated 12 variable nucleotide positions (Fig. 2). Each of these was a transition, with two being located at the first position and ten at the third position of the amino acid codon. All substitutions were silent, not leading to a change in amino acid sequence.

The *nad*4 haplotypes determined were H1, H4, H5, H9, H10, H11, H17, H21, H33, H43 and H45 (Fonseca, pers. comm., Fonseca et al. 2001, 2010, Zielke et al. 2015), plus one haplotype which had previously not been described (Fig. 2). The new haplotype was named H46 (GenBank accession no. MK613841), continuing the numbering of haplotypes (Zielke et al. 2015).

The most common *nad*4-haplotype found was H1, detected 69 times at eight collection sites. Haplotype H21 was detected exclusively in Heidelberg, and the haplotypes H11 and H43 were identified only in Burgscheidungen (Fig. 3). The number of haplotypes per site varied from one (Rosenthal) to seven (Burgscheidungen, Freiburg). The collection sites Hanover and Berchtesgaden resemble in their haplotype configuration, with H9 being the dominant haplotype. The collection sites Heidelberg, Würzburg and Augsburg also look similar, with large numbers of specimens with haplotypes H45 and H46. In conclusion, the western and northern collection sites were more homogeneous while the western and southern sites showed a higher diversity.

Figure 4 depicts the genetic relatedness between the various haplotypes found. In most cases, only one base differs in the nucleotide sequences of different haplotypes. H21 has the biggest direct genetic distance (i.e. without intermediate haplotypes) from H1 (three bases). H9 has the same number of differences compared to H1, but intermediate haplotypes exist (H33, H4).

Microsatellite analysis

The microsatellite analysis produced analysable data in 234 samples (Table 1). The cluster analysis suggests the existence of two genotype groups (Fig. 5). According to this, the individuals from Mönchengladbach, Linz and Rosenthal belonged almost exclusively to the same genetic cluster, named here 'genotype 1'. The individuals from the collection sites Burgscheidungen, Würzburg, Heidelberg and Augsburg show a high probability of belonging to a second genetic signature, 'genotype 2'. Specimens from the collection site Freiburg mainly display genotype 2, but with significant admixture of genotype 1. The individuals of the locations Hanover and Berchtesgaden have a high probability of belonging to genotype 1 (ca. 75%), but also display a clear admixture by genotype 2 (ca. 25%).

When combining the phylogenetic tree of the *nad*4 haplotypes with bars showing the probability of the haplotypes to belong to the two microsatellite clusters, the haplotypes H1 and H5 are likely to correspond to genotype 1 (Fig. 6). The remaining haplotypes rather correspond to genotype 2. Haplotype H4 shows a balanced probability for both genotypes but is based on the analysis of two mosquito specimens only.

Table 1 Overview of *Ae. japonicus* samples analysed. N_S is the number of specimens analysed for the *nad*4 region and N_M the number of individuals processed by microsatellite analysis. The order of the collection sites (top to bottom) corresponds to the geographical location of the populations (north to west, to east, to south)

Federal state	Location	Number	N_S	N_M
Lower Saxony	Hanover	27	21	27
North Rhine-Westphalia	Mönchengladbach	21	19	17
Rhineland-Palatinate	Linz	30	25	24
Hesse	Rosenthal	26	24	26
Saxony-Anhalt	Burgscheidungen	23	20	23
Bavaria	Würzburg	30	22	30
Baden-Württemberg	Heidelberg	32	32	29
Bavaria	Augsburg	20	14	20
Baden-Württemberg	Freiburg	20	19	20
Bavaria	Berchtesgaden	20	13	18
Total		249	209	234

Fig. 2 Variable positions of the 424 bp *nad*4 gene region

Haplotypes	001	055	058	073	076	091	135	136	163	300	376	403
H1	A	C	T	A	C	T	A	T	T	A	C	C
H4	.	.	.	.	.	C	.	.	.	.	.	T
H5	.	.	.	.	T	.	.	.	.	.	.	.
H9	.	.	.	.	.	C	.	.	.	.	T	T
H10	.	.	.	.	T	.	.	.	.	G	.	.
H11	.	T	.	.	T	.	.	.	.	.	.	.
H17	.	.	C	.	.	.	.	.	.	.	.	.
H21	G	.	.	G	.	.	.	C	.	.	.	.
H33	.	.	.	.	.	.	.	.	.	.	.	T
H43	.	.	.	.	.	.	G	.	.	.	.	.
H45	.	.	.	.	.	C	.	.	C	.	.	.
H46	G	.	.	.	.	.	.	.	.	.	.	T

H43 was detected only in one single individual for which no result could be obtained in the microsatellite analysis.

Based on pairwise F_{ST} values and Nei's genetic distance of the microsatellite data, the results of the PCoA indicate a clear separation of the western collection sites (Mönchengladbach, Rosenthal, Linz) from the other collection sites (Fig. 7). The specimens from Hanover and Berchtesgaden appear to be closely related. Although having a high probability of belonging to microsatellite genotype 1, the PCoA rather associates them with sites where genotype 2 prevails.

Discussion

Aedes japonicus specimens collected in 2017 at various sites throughout the German distribution area of this species were genetically analysed regarding *nad*4 haplotypes and microsatellite signatures. The results generated by the two analytical approaches are more or less conform and complement one another: with one exception, every *nad*4 haplotype can be assigned to one of the two microsatellite genotypes found. Only for *nad*4 haplotype H4, which was represented by only two specimens for which microsatellite data could be obtained, no unambiguous assignment was possible.

According to the presented microsatellite analyses, the German *Ae. japonicus* populations clearly fall into two genetic clusters (genotypes). The most admixed populations are Hanover and Berchtesgaden. The microsatellite signature from Hanover does not resemble the signatures of the closest collection sites in this study (Rosenthal, Burgscheidungen) but instead is most similar to the southeastern collection site

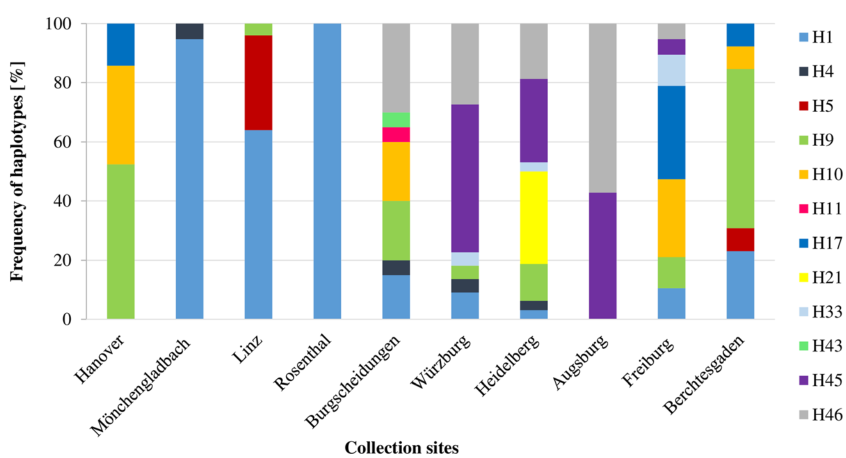


Fig. 3 Frequency of *nad*4 haplotypes detected

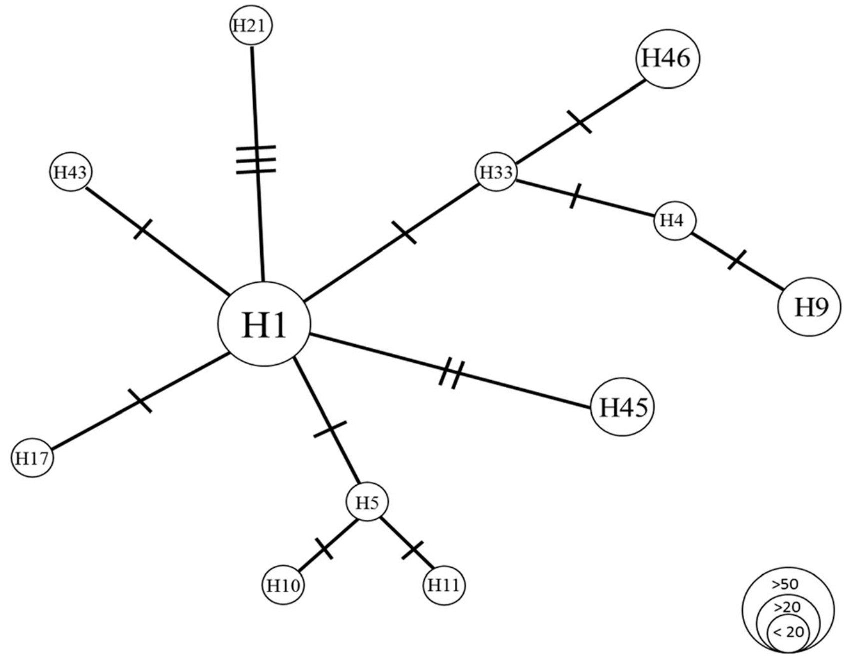


Fig. 4 Spanning tree of *nad*4 haplotypes detected. Every cross line represents one transition. The size of the circles represents the number of detected specimens with the respective haplotypes

Berchtesgaden. Thus, these results are in contrast to a previous study supposing the northern German population around Hanover to be an offshoot of the western one (Zielke et al. 2015) and the southeastern population to have a different origin and to be closely related to the Austrian/Slovenian population (Zielke et al. 2016). It therefore appears that individuals from the southeastern population (Berchtesgaden) were displaced to North Germany and admixed in the Hanover population. This hypothesis is supported by the results of the *nad*4 haplotype analysis. In Hanover, the haplotypes H1 and H5, and in Berchtesgaden, the haplotypes H1, H9 and H10 had been detected by Zielke et al. (2016), with H9 and H10 being present nowhere else in Germany. In the present study, the Hanover population was represented by the haplotypes H9, H10 and H17. The same haplotypes were detected in Berchtesgaden. Thus, the haplotypes previously identified in Hanover were possibly replaced by the new introduced haplotypes, although H1 and H5 may still be present but in a so small number that they escaped sampling.

The results of the PCoA show the genetic alienation of the North and West German populations even more clearly. According to this evaluation, which is a measure of genetic differentiation within the complete dataset and calculates the genetic distance between the subsets (here: locations of collection), the specimens of the northern location (Hanover) are genetically closer to the specimens collected in Central and South Germany (Augsburg, Berchtesgaden) than to those from West Germany (Rosenthal, Linz, Mönchengladbach).

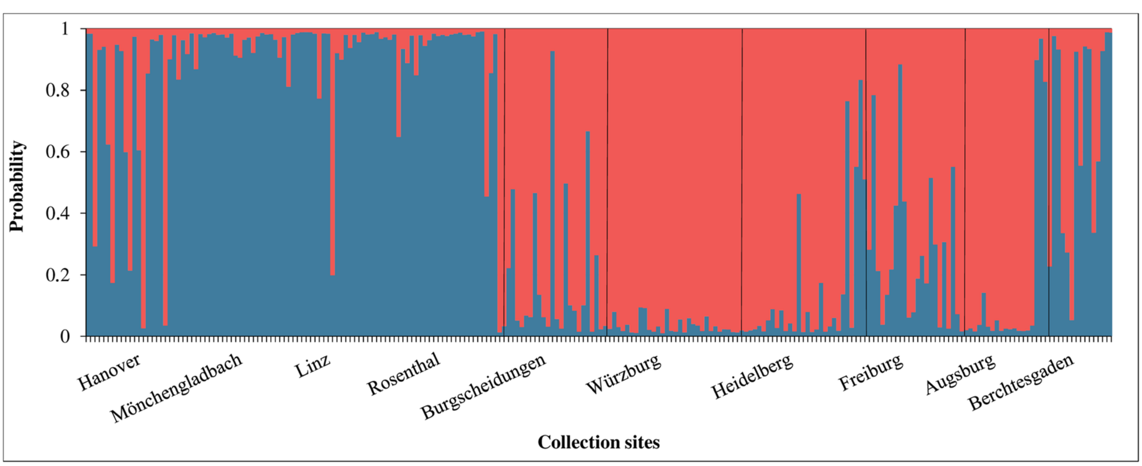


Fig. 5 Results of microsatellite multilocus genotyping. Each line represents one individual (Δk = 2). The colours show the probability of belonging to one of the two genetic clusters (blue = genotype 1; red = genotype 2). The order of the bars (left to right) corresponds to the geographical location of the populations (north to west, to east, to south)

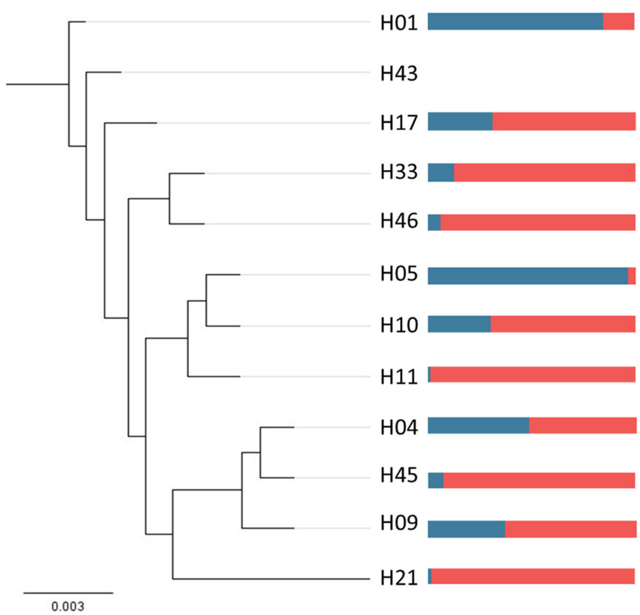


Fig. 6 Phylogenetic tree of *nad*4 haplotypes. The colours of the bars represent the probability of belonging to one of the two microsatellite genotypes (blue = genotype 1; red = genotype 2). H43 was detected in one single individual only for whom no result could be obtained in the microsatellite analysis. The scale shows the genetic distance

Thus, the PCoA seems to show a change in the genetic makeup of the mosquitoes more precisely and probably earlier than the cluster analysis.

The occurrence of haplotypes H9, H10 and H17 in 2017 in Freiburg might also be the result of a carry-over of specimens from southeastern Germany. Freiburg had not been included in *Ae. japonicus* analyses in previous studies but is located in the same densely colonized Southwest German population area from where two other sites had been sampled by Zielke et al. (2015) in 2013 and examined without evidence of the three haplotypes. Similarities in the cluster analysis between Freiburg, Berchtesgaden and Hanover seem to confirm this relatedness, although it is apparently less close between Freiburg and Berchtesgaden than between Hanover and Berchtesgaden, according to the PCoA.

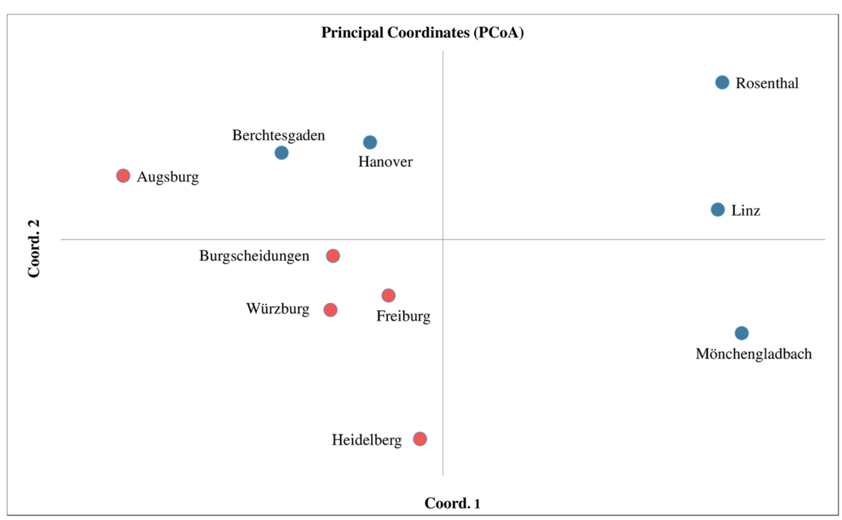
The specimens from Freiburg, Heidelberg, Würzburg and Augsburg, all of which were located in the southwestern distribution area of *Ae. japonicus* in 2017, are characterized by some identical *nad*4 haplotypes. Obviously, no admixture has occurred by mosquitoes from the western population.

The *Ae. japonicus* specimens from the West German collection sites (Mönchengladbach, Linz, Rosenthal) are very homogeneous in their microsatellite setup. In addition, the PCoA indicates close relatedness and genetic isolation from specimens collected at the other sites. This is again supported by the *nad*4 haplotyping results. Samples from locations in the western part of Germany show similar haplotypes, suggesting that they were still closely related in 2017.

The newly detected haplotype H46 (27 specimens) is most closely related to haplotype H33 (4 specimens), only differing in one nucleotide position. It is possible, therefore, that H46 is a mutation of H33. Both were detected sympatrically at three collection sites (Würzburg, Heidelberg, Freiburg), with H46 being less frequent than H33 only in Freiburg. In Burgscheidungen and Augsburg, the other two sites with H46, no H33 was found. In previous studies, H33 had been detected in Switzerland and South Germany (Zielke et al. 2014, 2015). Obviously, H46 has replaced H33 at some locations.

The number of *nad*4 haplotypes detected increased from three in 2012 (Zielke et al. 2014) to 12 in the present study. In principle, both introduction and mutation can cause a rise in the number of haplotypes, with mutation being most plausible when differences consist of one nucleotide only. New introductions of *Ae. japonicus* to Germany cannot be excluded but are impossible to verify without including specimens in the analysis of populations from abroad. The higher number of haplotypes in this study as compared to previous studies can, however, also be attributed to the fact that a higher number of collection sites was investigated in 2017 (ten as opposed to

Fig. 7 Principal coordinates analysis (PCoA) plot for the ten *Ae. japonicus* collection sites. Populations marked by blue dots belong to microsatellite genotype 1, by red dots to genotype 2



five in 2012). In addition, the collection sites were distributed over a much larger area and different regions of Germany, concordant with the continuing spread of *Ae. japonicus*.

H9 and H10 had not been detected in Germany in 2012 and 2013, but H9 had been identified in Belgium (2012) and the Netherlands (2013) (Zielke et al. 2014, 2015). In 2015, Zielke et al. (2016) detected H9 and H10 in Upper Bavaria and the Austrian federal state of Salzburg. In the present study, haplotype H9 was detected at seven collection sites in Germany (Hanover, Linz, Burgscheidungen, Würzburg, Heidelberg, Freiburg, Berchtesgaden). Moreover, haplotype H10 was found in Berchtesgaden (Upper Bavaria), Freiburg, Burgscheidungen and Hanover.

The first study dealing with population genetics of *Ae. japonicus* was performed on individuals from USA, Japan and New Zealand (Fonseca et al. 2001). The most frequent haplotype H1, which has been suggested by Fonseca et al. (2001) to be an ancestral common source of all *Ae. japonicus* populations, has the highest degree of homology with all other haplotypes. Often, only a single base substitution distinguishes the different haplotypes. In 2001, most of the *Ae. japonicus* haplotypes described were unique for the USA or Japan (Fonseca et al. 2001), with the exception of H1. This haplotype was present in almost all populations examined (USA, Japan, New Zealand), indicating a common origin. Previous to the study presented here, H4, H9, H10, H11 and H21 had only been documented in populations from the USA (Fonseca et al. 2001, 2010). By contrast, H5 had been determined in Japan and New Zealand (Fonseca et al. 2001). H33, which was found in Germany in 2013, had never been reported from the USA, wherefore Zielke et al. (2015) suggested an introduction into Germany from Asia.

In summary, an increasing haplotype diversity became apparent for 2017. With respect to one of the major modes of displacement of *Ae. japonicus*, ground vehicular travel, this seems to be a result of admixture of specimens with different genetic setup originating from other populations, possibly in other countries. The haplotypes H4, H11, H17 and H46 were demonstrated in Europe for the first time in the present study (c.f. Zielke et al. 2014, 2015, 2016).

Conclusion

At this point (about 10 years after the first detection), it seems impossible to reconstruct original point(s) of entry of *Ae. japonicus* into Germany. Population genetic studies should have been carried out in the beginning of Germany's colonisation and be continued on a regular basis. In addition to active spread, introductions and carry-overs may continuously take place, due to increasing trade and travel, adding to genetic mixing of populations. The results of the present study suggest admixture of specimens from southeastern Germany to the North and Southwest German populations and a coinciding decreased relatedness between the western and northern populations as compared to previous studies. By contrast, the West German population has genetically remained relatively uniform.

As the spread of *Ae. japonicus* is going on, it is just a question of time when *Ae. japonicus* can be found nationwide in Germany. With the geographical spread, growth of population densities and climate warming, the risk of pathogen transmission keeps increasing simultaneously.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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