Distribution and population genetics of the invasive Asian bush mosquito *Aedes japonicus* (Diptera, Culicidae) in Germany and eastern Europe

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Content

Introduction

Mosquitoes

Insects make up more than 74 % of all existing animal species and are the most evolutionarily successful group on our planet (Prothero 2013). In the class “Insecta”, mosquitoes represent the taxonomic family Culicidae which occurs all over the world except in polar regions (Mullen & Durden 2009). A total of 3,579 mosquito species have been described, subdivided into two subfamilies (Anophelinae, Culicinae) with 122 genera (Harbach 2020). Mosquitoes can biologically transmit viruses, protozoa and nematodes (Reiter 2001). More than half of the world's population lives at risk of mosquito-borne diseases, and regarding morbidity and mortality of vector-borne diseases, mosquitoes represent the most dangerous animals for humans (Becker et al. 2010). The World Health Organization lists mosquitoes among the deadliest animals in the world (World Health Organization 2019).

Mosquitoes are periodic ectoparasites, belonging to the “capillary feeders”. With their proboscis, the females of most species penetrate the skin of the host and feed on blood. In Germany, 51 mosquito species are considered established (Werner et al. 2020). In addition to native species, eight invasive species have recently been recognized, including five having established (*Aedes albopictus*, *Ae. japonicus*, *Ae. koreicus*, *Anopheles petragnani*, *Culiseta longiareolata*) and three not established (*Ae. aegypti*, *Ae. berlandi*, *Ae. pulcritarsis*) (Kampen et al. 2017). Every species is characterised by its particular morphology (e.g. size, colouration, wing veins, scales, setae) and ecology (e.g. distribution, breeding places, bloodmeal preference). Particularly relevant is their vector competence for pathogens. For prevention and reactive measures in case of a mosquito-borne disease outbreak, knowledge about the distribution and host-feeding behaviour of potential vectors are essential. Until the 19th century, malaria was widespread in Europe (Bruce-Chwatt & de Zulueta 1980), but since 1955, autochthonous malaria infections have been registered in Germany only occasionally (Krüger et al. 2001, Zoller et al. 2009). Linked to this and other developments (e.g. of insecticides and therapeutics), blood-sucking insects slowly disappeared from the focus of medical and scientific interest and were demoted from dangerous vectors to annoying nuisance pests. This changed beginning in the early 20th century when a considerable number of cases of dengue and chikungunya fever occurred in South Europe (Italy, France, Croatia, Madeira), following the introduction and establishment of the Asian tiger mosquito *Aedes albopictus* and the yellow fever mosquito *Aedes aegypti* (Tomasello & Schlagenhauf 2013). In the form of eggs, *Ae. albopictus* was also detected in South Germany in 2007 for the first time (Pluskota et al. 2008). About half a century after malaria had been eliminated from Germany and a shadowy existence of mosquito research, this finding coincided with a situation of basic up-to-date data on the indigenous mosquito fauna largely missing. After such a long time, characterised by fundamental environmental and ecological changes mainly caused by globalisation and climate change, the composition, the geographical spread and the phenology of the various mosquito species occurring in Germany were likely to have changed. Likewise, mosquito-borne pathogens might have established or spread.

Mosquito monitoring

After endemic malaria had been eradicated from Europe, native mosquitoes were no longer considered dangerous vectors. For this reason, the occurrence and distribution of mosquitoes had only been investigated sporadically in Germany. Although several native mosquito species are known to be able to transmit pathogens in laboratory studies (Kampen & Walther 2018), some invasive species, particular *Aedes* species, which – due to global movement and human activities – have been increasingly imported since the late 1990s, have proven their vector competence under natural conditions in the field (Medlock et al. 2015). In Europe, four invasive *Aedes* species have recently established: *Ae. albopictus*, *Ae. aegypti*, *Ae. japonicus*, *Ae. koreicus*.

Due to the outbreaks of mosquito-borne diseases in southern Europe and the detection of isolated specimens of invasive mosquito species in southern Germany, a nationwide mosquito monitoring programme was implemented in Germany in 2011 (Kampen et al. 2017). Aquatic stages and adults are collected actively in the field by dipping (larval stages), trapping, netting and aspirating (adults). Dipping for larvae and pupae is done irregularly in natural habitats like ponds and ditches as well as in artificial containers. Trapping by various kinds of traps equipped with attractants (BG Lure®, CO_2) is carried out systematically from April to October for 24 h per week throughout Germany to collect host-seeking or gravid females (Werner et al. 2020). Netting and aspirating at resting sites, for example in zoological gardens (Heym et al. 2018), complement adult collections. In addition, the citizen science project "Mückenatlas" was launched in Germany in 2012 (Werner & Kampen 2017), where private people are asked to collect mosquitoes and submit them for scientific analysis (passive monitoring).

The aim of the monitoring programme was to investigate the occurrence and the spatiotemporal distribution of mosquito species in Germany. In addition to native species, the monitoring programme detected several invasive mosquito species in Germany: *Ae.*

albopictus (Werner & Kampen 2014), *Ae. japonicus* (Kampen et al. 2012, Werner & Kampen 2013), *Ae. koreicus* (Werner et al. 2016, Hohmeister et al. 2021), *An. petragnani* (Becker et al. 2016) and *Culiseta longiareolata* (Becker & Hoffmann 2011).

Among others, a population of *Ae. japonicus* was detected in western Germany in 2012 after submission of specimens to the "Mückenatlas" (Walther & Kampen 2017). Common traps with CO_2 are not very attractive to *Ae. japonicus*, and only in areas with high densities this species can be easily caught by traps (Scott et al. 2001). The best way to demonstrate the presence of a container-breeder like *Ae. japonicus* is the use of ovitraps, where a wooden stick or a piece of styrofoam, acting as a support for egg deposition, is placed in a dark plastic cup containing water. The sticks with the eggs are brought to the laboratory, where the larvae are allowed to hatch in water. Another option often used to demonstrate and collect *Ae. japonicus* is dipping artificial habitats (e.g., flower vases, rain water barrels) for aquatic stages.

Despite two guidelines on mosquito surveillance for invasive and native species in Europe (ECDC 2012, 2014), there is no standardisation for surveillance of *Ae. japonicus* (traps to be used, size of area sampled, annual frequency of sampling, number of visits, criteria for the declaration of negative/positive sites). The monitoring programmes realised in Europe show a wide variation in methodological implementation, making different programmes difficult to compare.

Invasive species

Biological invasions occur when species settle outside their original habitat (Williamson 1997). This can take place as a natural area expansion by active migration, which is relatively slow and usually ends with natural dispersal barriers (e.g., mountains, oceans, deserts, rivers). Due to the low speed of natural active migration, native ecosystems and species have plenty of time to adapt to the invasive species. These invasions take place without human influence and the effects are moderate.

Expansions mediated by humans often have another dimension: wide range, high speed and deep impact. On the one hand, humans break down natural barriers, so natural active migration is not anymore limited by natural barriers (D´Antonio & Vitousek 1992). On the other hand, humans enable the global spread of species through international trade and travel (unnatural passive displacement). More and more plants (neophytes) and animals (neozoa) are transported around the world by humans. As of 2014, more than 600 neophytes and over 260 neozoa were reported in Germany (WWF 2014).

The impact of invasive species on the natural fauna is widely recognized, and the success of invasion depends on appropriate environmental conditions and the interaction with ecologically similar species and mutual competition. Neozoa can have a repressive effect on endemic species and decrease the biological diversity in the area of invasion (Vitousek et al. 1996). Admixture of previously isolated species due to human activities has considerably increased during the last years (Olden et al. 2004).

Mosquitoes spread actively by flying and passively by wind (drift distance up to 50 km) or by humans (Bidlingmayer & Evans 1987, Becker et al. 2010). The inadvertent transport of immature stages (eggs and larvae) caused by humans is the main reason for successful invasions by mosquitoes, especially by container-breeders whose larvae occupy small water habitats (Lounibos 2002). The displacement of invasive mosquitoes of the genus *Aedes* between continents is closely connected with international trade and travel (Medlock et al. 2012). Within continents, *Aedes* stages often move by vehicles along highways (Medlock el al. 2012). The Asian tiger mosquito, *Ae. albopictus*, native to Southeast Asia and now distributed in many parts of the world, is the most prominent example for the global spread of a mosquito caused by humans (Lounibos 2002). Most of the introductions were caused by eggs transported in used tyres (Gratz 2004). The same is true for *Ae. atropalpus*, a North American species imported to Europe (Romi et al. 1997).

Outside its native distribution area, *Ae. japonicus* co-occurs with ecologically similar species in the same breeding niche. Competition (e.g., by overlapping breeding habitats) of *Ae. japonicus* is known with *Aedes albopictus* (Armistead et al. 2012), *Aedes atropalpus* (Bevins 2007), *Aedes triseriatus* (Alto 2011), *Culex pipiens* (Hardstone & Andreadis 2012) and *Culex restuans* (Murell & Juliano 2013), with a competitive disadvantage documented for *Ae. triseriatus* (Alto 2011), *Ae. atropalpus* (Andreadis & Wolfe 2010), *Cx. pipiens* (Lorenz 2012) and *Cx. restuans* (Andreadis & Wolfe 2010).

Aedes japonicus

Ecology and distribution in Europe

The native distribution area of the Asian bush mosquito *Aedes japonicus* covers large parts of East Asia (Japan, Korea, China and southeastern Russia) (Tanaka et al. 1979). The species is one of the most expansive ones in the world and probably one of the first invasive mosquito species from overseas having established in Germany (Figure 1). A competitive advantage of *Ae. japonicus* is tolerating lower water temperatures than co-occurring container-breeders, so larval development starts earlier in the season than in many other mosquito species (Bartlett-Healy et al. 2012). Larvae may hatch at water temperatures of 4 °C and are present until the next freezing period in late autumn (Burger & Davis 2008). Furthermore, *Ae. japonicus* is active from early spring to late fall, with several generations per year (multivoltine) and considerable population sizes possible (Kaufman & Fonseca 2014). The successful establishment and spread of *Ae. japonicus* in North America and Europe was facilitated by the species' adaption to moderate temperatures and its breeding behaviour. As a container-breeder, *Ae. japonicus* exploits a wide range of small water containers (natural and artificial) for oviposition and larval development. Furthermore, the eggs are very resistant against drought, which enables surviving long travels between the continents (Medlock et al. 2012).

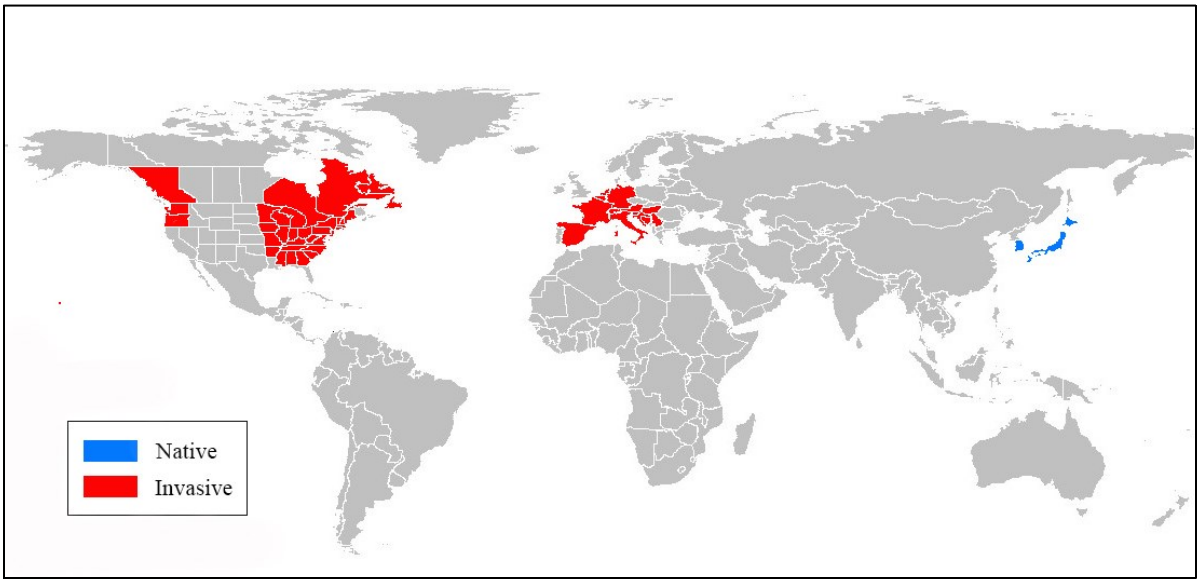

Figure 1: Native and invasive distribution areas of *Ae. japonicus* (Kaufman & Fonseca 2014, modified).

The worldwide expansion of *Ae. japonicus* started more than 25 years ago. The first time the species was detected outside its native distribution area was in 1993, when it was intercepted in New Zealand (Laird et al. 1994). The first established populations were found in New York, New Jersey and Connecticut (USA) in 1998 (Peyton et al. 1999, Andreadis et al.

2001). *Aedes japonicus* rapidly colonised the southern and eastern states of the USA and also invaded southern Canada (Kaufman & Fonseca 2014).

Today, Ae. japonicus has established in more than 50 states/countries. As of 2019, at least 33 US states (Kampen & Werner 2014), five states in Canada (Kampen & Werner 2014, Fielden et al. 2015, Jackson et al. 2016) and 15 countries in Europe (Medlock et al. 2015, Kavran et al. 2018, 2019, Eritja et al. 2019, Schaffner & Ries 2019) were affected.

The first evidence of established *Ae. japonicus* in Europe was found by Schaffner et al. (2003) in France in 2000, but it was possible to eliminate the breeding source (Schaffner et al. 2009). In the following years, *Ae. japonicus* established in Belgium (Versteirt et al. 2009) and Switzerland (Schaffner et al. 2009). The first evidence of *Ae. japonicus* occurrence in Germany was made by Schaffner et al. (2009) in 2008. Furthermore, a population of *Ae. japonicus* was detected on both sides of the Austrian-Slovenian border in 2011 (Seidel et al. 2012). In 2012, larvae of *Ae. japonicus* were recorded in western parts of Hungary (Seidel et al. 2016a). In 2015, the westward expansion from established populations in Austria crossed the Alps, and the first specimens were recorded in North Italy (Seidel et al. 2016b).

In 2013 and 2015, a nationwide monitoring programme to determine the distribution of *Ae. japonicus* was implemented in Slovenia (Kalan et al. 2017), which detected a dissemination to northeastern regions of the country since its first detection in 2011. Until 2015, *Ae. japonicus* continued to spread, and the distribution area extended throughout the country except the region bordering Italy (Kalan et al 2017).

From 2013 to 2015, Klobučar et al. (2018) carried out a study in northwestern Croatia to investigate the presence and absence of *Ae. japonicus*. In 2013, the first record of *Ae. japonicus* was reported from the border to Slovenia where *Ae. japonicus* developed from early May to October. In 2015, nearly 100 km east of the initially examined region, several counties were found colonised.

In early 2017, four isolated populations of *Ae. japonicus* occurred in Europe. Apparently, the largest one spread over Southwest Germany, France, Liechtenstein, Switzerland and western Austria. Further populations were present in northern Germany and the Netherlands. Another large population covered parts of Southeast Austria, Northeast Italy, Slovenia, Croatia and western Hungary.

In the course of 2017, a survey was conducted in northeastern parts of Bosnia and Herzegovina with *Ae. japonicus* being collected at two sites (Janssen et al. 2020).

Furthermore, specimens of Ae. japonicus were detected in Spain (Eritja et al. 2019) and Luxembourg (Schaffner & Ries) in 2018 and in Romania (Horváth et al. 2021) in 2020. In the Czech Republic, *Ae. japonicus* emerged in 2021 (Vojtíšek et al. 2022).

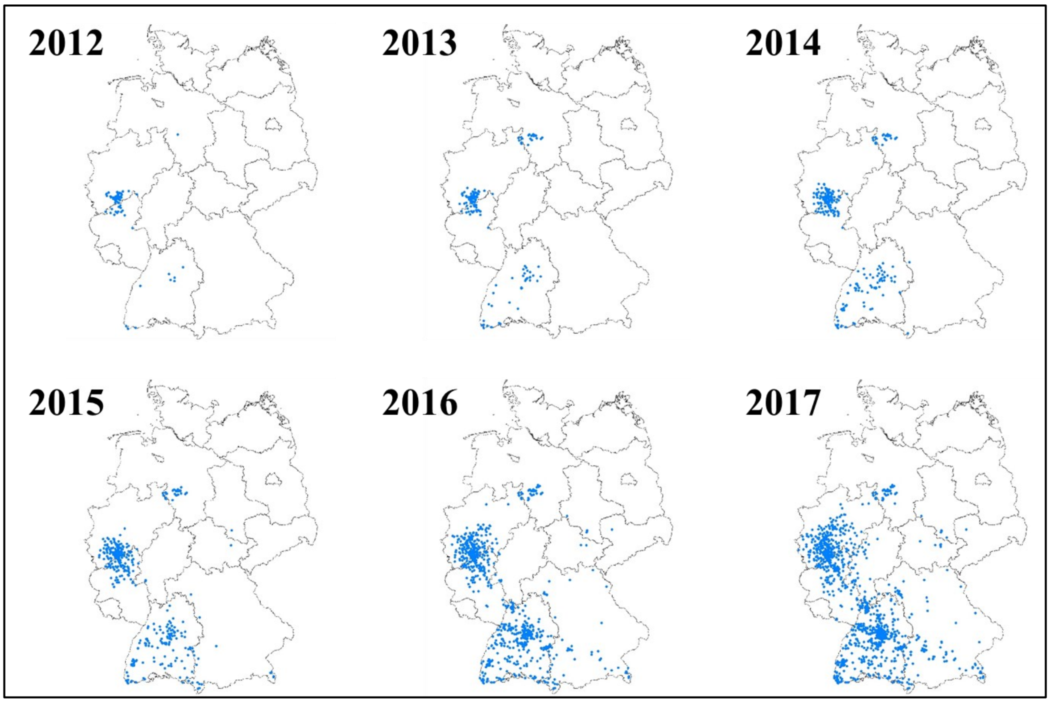

Figure 2: Distribution of *Ae. japonicus* in Germany between 2012 and 2017 (cumulative data based on "Mückenatlas" submissions and larval collections). Source: CulBase ZALF/FLI.

In Germany, *Ae. japonicus* larvae were first detected close to the German-Swiss border in the federal state of Baden-Wurttemberg in 2008 (Schaffner et al. 2009). A subsequent monitoring programme determined an area of 2,200 km² to be colonised along the border (Becker et al. 2011). In 2010, *Ae. japonicus* was found close to the airport of Stuttgart, 80 km north of the previously detected population (Schneider 2011). Later, Huber et al. (2014) documented a considerable enlargement of the colonised area in Baden-Wurttemberg. Furthermore, additional populations were discovered in western Germany (Kampen et al. 2012), close to Hanover (Werner & Kampen 2013) and in Upper Bavaria/Austria (Zielke et al 2016).

In 2012, the distribution of *Ae. japonicus* in Germany was limited to the southwest (Figure 2). Over the years, submissions to the "Mückenatlas" project and larval collections produced detections of *Ae. japonicus* in eastern parts of Germany, too. The population around Hanover did not appear to spread, but in 2017, the corridor between the Southwest and West German populations seemed to be closing.

Vector potential

In addition to the ecological consequences of mosquito invasion on biodiversity, an increasing risk for animal and human health must be expected in the case of the invasive species being potential vectors of disease agents. In its native Asian range, *Ae. japonicus* is not considered an important vector, but experimental studies suggest a vector potential for several viruses (Table 1). Under experimental conditions, *Ae. japonicus* is able to transmit West Nile virus (Turell et al. 2001), Japanese encephalitis virus (Takashima and Rosen 1989), eastern equine encephalitis virus (Sardelis et al. 2002a), St. Louis encephalitis virus (Sardelis et al. 2003), La Crosse virus (Sardelis et al. 2002b), Rift Valley fever virus (Turell et al. 2013) and Zika virus (Jansen et al. 2018). Although there is no evidence for transmission of pathogens in the field, specimens of field-collected *Ae. japonicus* have been found infected with West Nile virus (Novello et al. 2000), Japanese encephalitis virus (Chagin & Kondratiev 1943), Cache Valley virus (Yang et al. 2018) and La Crosse virus (Harris et al. 2015). Based on the laboratory experiments, *Ae. japonicus* must therefore be considered a potential vector. Its spreading in urban areas, vector potential and indiscriminative feeding require particular attention (Medlock et al. 2012).

Table 1: Pathogens linked with *Ae. japonicus* (Kampen & Walther 2014, Silaghi et al. 2017, Martinet et al. 2019).

		Field transmission	**Field infection**	**Transmission in the laboratory**	**Infection in the laboratory**
Virus	West Nile virus	?	+	+	+
	Jap. encephalitis virus	?	+	+	+
	La Crosse encephalitis virus	?	+	+	+
	Cache Valley virus	?	+	+	+
	Eastern equine encephalitis virus	?		+	+

	Rift Valley fever virus	?		+	+
	St. Louis encephalitis virus	?		+	+
	Chikungunya virus	?			+
	Dengue virus	?			+
	Getah virus	?			+
	Zika virus	?		+	+
Nematodes	*Dirofilaria immitis*	?			+
	Dirofilaria repens	?			+

Population genetics

Population genetics examines the genetic differences within and between different populations of a species with the aim to detect a population origin, separation of subpopulations and gene flow between geographically isolated populations. The frequency of certain alleles (or genotypes) in a population and the change of allele frequency in time is investigated. Changes in allele frequency are caused by mutation, selection and gene drift. The frequency of a certain genotype may be different in geographically isolated populations. Following the invasion of a species, its genetic make-up will change due to a new selection pressure (Tsutsui et al. 2000).

For the first time, studies on the population genetics of *Ae. japonicus* were carried out in Europe by genotyping seven polymorphic microsatellite loci and sequencing parts of the *nad*4 gene by Zielke et al. (2014), finding evidence for at least two genetically different populations. The data suggested a closer relationship between the populations from Belgium, Swiss and Austria/Slovenia than with the population from West Germany. Later, Zielke et al. (2015) demonstrated that the Swiss/Southwest German population was closely related to the populations found in the Netherlands and in West and North Germany. Also, a close relationship of the Southeast German/Austrian and Austrian/Slovenian populations was demonstrated (Zielke et al. 2016).

Smitz et al. (2021) investigated *Ae. japonicus* from Belgium, West Germany, North Germany and South West Germany. The results suggested multiple introductions in Belgium (Smitz et al. 2021). Specimens from the German-Belgium border showed a close relatedness to the population from West Germany, while the Belgian collection site Natoye showed a different genetic make-up, indicating additional introductions. Otherwise, the individuals from North Germany and South West Germany showed a high genetic relatedness.

Further studies on the population genetics of *Ae. japonicus* are scarce. Since the work of Zielke et al. (2014, 2015, 2016), *Ae. japonicus* has continued spreading (Kampen et al. 2016, 2017) and admixture of specimens from other populations or by new introductions has probably enlarged its genetic make-up, thus leading to a higher adaption and stronger expansion potential.

Use of mtDNA for population genetics

The mitochondria of eukaryotes represent essential cell organelles. They occur in all eukaryotes except for the protozoan genus *Monocercomonoides* (Karnkowska et al. 2016). Mitochondria generate most of the chemical energy for the cell's supply through the respiratory chain (Campbell et al. 2006). The mitochondrial DNA (mtDNA) of insects is circular and encodes for 37 genes (Cameron 2014). Due to free oxygen and less repair function than in nuclear DNA, mtDNA has a high mutation rate (Richter et al. 1988). Furthermore, rapid evolution, maternal inheritance and intraspecific polymorphism make mtDNA a suitable and common target in population genetics (Krzywinski et al. 2006). One gene of the mtDNA, encoding for subunit 4 of the enzyme NADH dehydrogenase (*nad*4), has proven suitable in population genetic studies on mosquitoes (Fonseca et al. 2001, 2009; Zielke et al. 2014, 2015, 2016, Campos et al. 2020).

Using mtDNA for population genetic analyses may be problematic in cases of heteroplasmy (Rubinoff et al. 2006). Heteroplasmy is the presence of several different mtDNAs (haplotypes) in one organism (Magnacca & Brown 2010), while, classically, mtDNA is haploid and exists in the form of numerous identical copies. So far, heteroplasmy has been detected in humans (Grzybowski et al. 2003), bats (Petri et al. 1996), birds (Crochet & Desmarais 2000), fish (Hilsdorf & Krieger 2004), insects (Nardi et al. 2001) and nematodes (Tsang & Lemire 2002). Induced by somatic mutation, heteroplasmy of the oocyst can lead to length (Lunt et al. 1998) or site variations (Hauswirth & Laipis 1982).

The 424 base pairs of the *nad*4 gene show single nucleotide polymorphisms (SNPs). These yield different *nad*4 haplotypes by gene polymorphism, which are unique for a population.

For *Ae. japonicus*, 45 different *nad*4 haplotypes have been described (Fonseca et al. 2001, Zielke et al. 2015). They are different in 37 positions of the nucleotide sequence.

Use of polymorphic microsatellite loci for population genetics

To complement *nad*4 sequence analysis and get a more detailed insight into the genetic make-up of populations, microsatellites can be used. Microsatellites are currently the marker system of choice for population genetics. These are short tandem repeats (STRs) of the DNA sequence with lengths of 2-5 bp. Due to their small size, high degree of polymorphisms and easy analysis, microsatellites are widely used in fundamental and applied science (Christiakov et al. 2006). Mutations (insertions and deletions) will cause a change in the repetitive motif, resulting in a change of motif lengths. The mutation rate in microsatellites is higher than in other DNA regions (Brinkmann et al. 1998) and caused by DNA polymerase slippage (Klintschar et al. 2004). Changes in the repetitive motif can be measured by fragment length analysis. In this way, alleles existing in the investigated population can be identified. Identical alleles indicate a close relationship, and many different alleles refer to a high genetic diversity. For *Ae. japonicus*, seven informative microsatellite markers have been identified (Widdel et al. 2005). Unlike mitochondrial DNA, microsatellites are very species-specific, so that the amplification primers usually do not work with different species. The *Ae. japonicus* primers do work with the closely related species *Ae. koreicus*, but applied to *Ae. albopictus* and *Ae. aegypti*, no amplification is possible (Widdel et al. 2005). The genetic signature of the microsatellites can be determined by a Bayesian algorithm calculating the number of genetic clusters (microsatellite genotypes). Based on the genetic signature, the genetic distance can be pictured by a principal coordinate analysis (PCoA).

Aim of the dissertation and research questions

The aim of this dissertation was to follow up on the spatial distribution of *Ae. japonicus* in Europe and the relationship of the different populations. As a potential vector of several disease agents, knowledge about dissemination and genetic diversity of the populations is essential. Since its first detection in France (2000), *Ae. japonicus* has established in several countries in Europe. This dissertation was meant to update the distribution status of *Ae. japonicus* in Europe and examine the monitoring methods used to determine its presence. For a better comparability of collection data, suggestions for a more standardized monitoring strategy should be developed.

Since the last population genetic investigation by Zielke et al. (2014, 2015, 2016), the German *Ae. japonicus* populations have spread considerably (Kampen et al. 2016, 2017). Therefore, the relationship between the different German populations of *Ae. japonicus* should be re-examined regarding their genetic make-up in order to check whether previously separated populations have merged already and to what extent an admixture had taken place.

In addition, new data on the occurrence of *Ae. japonicus* in eastern and southeastern Europe (Croatia, Serbia, Bosnia and Herzegovina) should be used to shed light on the origin of populations popping up in eastern and southeastern Europe and their genetic relatedness to more western European populations.

The following studies were conducted:

I. The present distribution of *Ae. japonicus* in Europe was summarised and supplemented by own collection data. The monitoring strategies implemented in affected European countries were critically reviewed. Suggestions to harmonise monitoring methods to improve data comparability were developed. The study results are summarised in Publication 1.
II. The relatedness of *Ae. japonicus* populations in Germany was analysed using published and established population genetic approaches. It was examined whether the genetic make-up of the different populations had changed since investigations carried out from 2012 to 2015, i.e. whether a carry-over of individuals between isolated populations had taken place? The study results are summarised in Publication 2.
III. The relatedness of *Ae. japonicus* populations found in southeastern Europe were investigated and compared with West European populations. Attempts were made to elucidate the origins of the southeastern European populations

and their relatedness to previously investigated populations from Germany/Austria/Slovenia. The study results are summarised in Publication 3.

IV. Population genetic studies were performed on *Ae. japonicus* specimens recently emerged in the Czech Republic. The question was addressed whether this new population originated from populations in Germany or from southeastern Europe. The study results are summarised in Publication 4.

Publications

Major parts of this doctoral thesis have been published in peer-reviewed journals:

Publication 1: The Asian bush mosquito Aedes japonicus japonicus (Diptera: Culicidae) in Europe, 17 years after its first detection, with a focus on monitoring methods

Koban *et al. Parasites Vectors (2019) 12:109*
https://doi.org/10.1186/s13071-019-3349-3

Parasites & Vectors

REVIEW **Open Access**

The Asian bush mosquito *Aedes japonicus japonicus* (Diptera: Culicidae) in Europe, 17 years after its first detection, with a focus on monitoring methods

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Abstract

After the first detection of the Asian bush mosquito *Aedes japonicus japonicus* in the year 2000 in France, its invasive nature was revealed in 2008 in Switzerland and Germany. In the following years, accumulating reports have shown that *Ae. j. japonicus* succeeded in establishing in several European countries. Surveillance efforts suggest that there are currently four populations in Europe, with the largest one, formed by the recent fusion of several smaller populations, ranging from West Germany, with extensions to Luxembourg and French Alsace, southwards to Switzerland and continuing westwards through Liechtenstein to western Austria. This paper summarises the present distribution of *Ae. j. japonicus* in Europe, based on published literature and hitherto unpublished findings by the authors, and critically reviews the monitoring strategies applied. A proposal for a more standardised monitoring approach is provided, aiming at the harmonisation of future data collections for improving the comparability between studies and the suitability of collected data for further research purposes, e.g. predictive modelling approaches.

Keywords: *Aedes japonicus japonicus*, Asian bush mosquito, Asian rock pool mosquito, Europe, Spread, Invasive species, Monitoring, Surveillance

Background

The Asian bush mosquito or Asian rock pool mosquito *Aedes* (*Hulecoeteomyia*) *japonicus japonicus* (Theobald, 1901) is a highly invasive culicid species originating from East Asia (Japan, Korea, southern China, southeastern Russia) [1]. Outside its native distribution area, it was first reported from New Zealand in 1993, where larvae of *Ae. j. japonicus* were found in used tyres imported from Japan [2]. Although additional introductions were reported until 2003 (no pertinent data are available from 2003 onwards), there is no evidence that this species has become established in New Zealand [3].

In contrast, the species has successfully invaded North America and is now considered established in 33 US states and parts of Canada [4–8], presumably following repeated importations with used tyres and subsequent continental spread since 1999 [9, 10].

In Europe, *Ae. j. japonicus* was first detected in 2000 [11] and has since emerged in numerous countries, either through continental spread or additional introduction events from overseas [6]. While the Asian tiger mosquito *Aedes* (*Stegomyia*) *albopictus* (Skuse, 1895) and the yellow fever mosquito *Aedes* (*Stegomyia*) *aegypti* (Linnaeus, 1762) were considered responsible for several disease outbreaks in Europe after their establishment [12], there are no confirmed reports thus far of pathogen transmission through *Ae. j. japonicus* in the field, although it is a competent vector of several disease agents in the laboratory [13–19]. In addition, the isolation of La Crosse, Cache Valley and West Nile viruses or their respective

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RNAs from field-collected adults and the detection of La Crosse virus RNA in *Ae. j. japonicus* eggs suggests a possible role as a vector [20–22]. Knowing its geographical distribution is therefore essential from both a public and an animal health point of view, although eradication from Europe is no longer considered possible [6].

Several methods are available to detect and track *Ae. j. japonicus* populations, targeting all life stages of the species. In addition to adult trapping and ovitrapping [23–26], the surveillance of larval habitats is an appropriate cost-effective method [27].

This contribution will update the distribution of *Ae. j. japonicus* in Europe by the end of the mosquito season 2017, provide an overview over the genetic relationship of European populations and review the methods used to monitor this invasive species. Finally, a more standardised monitoring approach is proposed, aiming at the harmonisation of future data collections for improving the comparability between studies and the suitability of collected data for further research purposes, e.g. predictive modelling approaches.

Methods

Criteria for inclusion of reports

Articles, abstracts and presentations were analysed if findings of *Ae. j. japonicus* in previously non-infested areas in Europe were presented. In these cases, studies carried out until December 2017 were included. Studies on methodological evaluations conducted in areas already known to be infested were not considered.

Sources

To find pertinent studies, PubMed, Google Scholar and Web of Science were the main search engines for the terms "*Aedes japonicus*", "*Ochlerotatus japonicus*", "*Ae. japonicus*", "*Oc. japonicus*" and "*Aedes*". In addition, the working group's reference collection was searched for relevant information. The search results were manually scanned for studies concerning findings in new geographical areas of European countries.

Data extraction

Coordinates provided for *Ae. j. japonicus* collection sites were copied to a CSV file and imported as a layer in QGIS. If GPS coordinates of collection sites were not provided in a study, data points were extracted by overlaying the included maps, using the "GDAL Georeferencer plugin" for QGIS and marking the dots manually. Further data extraction, e.g. collection dates or periods were extracted manually and collected in a separate CSV file.

Detection, spread and current distribution of *Ae. j. japonicus* in Europe

After the first detection of *Ae. j. japonicus* in Europe (northwestern France [11]), observations have been published from numerous European countries suggesting continuous importation, e.g. through the used tyre trade, or quick dispersal of the species.

Despite two guidelines on mosquito surveillance, published by the ECDC [28, 29] and aiming at standardisation, monitoring efforts in Europe show a wide variation of methodological approaches. Differences can be found in the trigger of monitoring efforts, life stages targeted, traps used, structures searched, size of the area monitored and annual frequency of monitoring activities (Table 1). This section reviews the approaches and circumstances of initial local or regional findings of *Ae. j. japonicus* in Europe. Each subsection refers to originally detected populations and their subsequent development, ending in the delineation of the current populations in Europe.

France/Switzerland/southwestern Germany/Liechtenstein/western Austria

In 2000, two larvae of *Ae. j. japonicus* were found in Normandy, northwestern France, in used tyres [11]. The larvae and breeding source were successfully eliminated [30].

Schaffner et al. [30] reported the first finding of *Ae. j. japonicus* in northern Switzerland and southwestern Germany in July 2008. Monitoring activities included the inspection of almost 3550 possible breeding habitats in Switzerland and bordering Germany and France. A source of introduction of this population was not identified [30].

Following this first detection of *Ae. j. japonicus* in Germany, Becker et al. [31] started a monitoring programme in southwestern Germany in 2009 to check for further distribution. Flower vases in cemeteries, used tyres and other water-holding containers in 86 villages were examined, and an infested area of approximately 2200 km^2 was found. Locations were chosen due to their proximity to the Swiss border and the infested areas described by Schaffner et al. [30]. In 2010, the surveyed area was extended to 155 municipalities (villages visited in 2009 included) to account for the already well-established population.

Shortly thereafter, Schneider [32] found immature stages of *Ae. j. japonicus* in water-holding containers, e.g. vases, stone basins and rain barrels, in four cemeteries (of five inspected) and on one camping site in 2011. The southernmost site was located 80 km north of the *Ae. j. japonicus* distribution area previously reported by Becker

Table 1 Specifics of the reviewed studies, including monitoring trigger, scope and methods used. A "dynamically adjusted" area is given if monitoring efforts were expanded while the field work was conducted. In the column "Year of first detection", if the presence of *Ae. j. japonicus* in the monitored country was already reported, the corresponding study is referenced

Monitored country	Year of first detection	Trigger of monitoring	Monitoring area	Monitoring scope	Monitoring locations	Monitoring methods	No. of visits at the same sites	Population size (km^2)	Additional information	Reference
France	2000	Passive monitoring	Fixed	Local	Tyre trading company	Larval sampling, adult trapping	6	Not stated	Eradicated [41]	[11]
Switzerland, Germany, France	2008 Switzerland, Germany	Submitted specimen	Dynamically adjusted	Regional	Cemeteries	Larval sampling	1	1400		[30]
Germany	2008 [30]	Report [30]	Not stated	Regional	Cemeteries	Larval sampling	2 (1 per year)	2200	Monitoring area expanded in second year	[31]
Germany	2008 [30]	Not stated	Fixed	Regional	Cemeteries, camping site	Larval sampling	1	Not stated		[32]
Germany	2008 [30]	Report [30, 31]	Fixed	Local	Along motorways	Adult trapping	8 (1 per week)	Not stated		[33]
Germany	2008 [30]	Report [31]	Fixed	Regional (federal state)	Cemeteries (mainly)	Larval sampling	1	1200 and 4000	Grid overlay, cell size: 135 km^2; sites of [31] were included	[34]
Germany	2008 [30]	Report [34]	Fixed	Regional (federal state)	Cemeteries (mainly)	Larval sampling	1	Not stated	Revisited locations from [34]	[35]
France	2013	Report [30, 31]	Fixed	Regional	Cemeteries, others	Larval sampling, adult trapping	Multiple (not further specified)	Not stated		[36]
Austria, Germany, Hungary, Liechtenstein, Switzerland	2012 Hungary; 2015 Liechtenstein	Report [30, 31, 49]	Dynamically adjusted	Regional	Human settlements, forests, cemeteries	Ovitraps, larval sampling	Multiple (not further specified)	Not stated		[37]
Belgium	2002	Passive monitoring	Fixed	Local	Tyre trading company	Hand catching (human bait), larval sampling, adult trapping	Multiple (not further specified)	Not stated	No evidence for spread of population	[39]
Belgium	2002 [39]	Passive monitoring	Fixed	Local	Tyre trading company	Larval sampling, adult trapping	Multiple (not further specified)	Not stated	No evidence for spread of population; eradicated [41]	[40]
Germany	2008 [30]	Citizen science project "Mueckenatlas"	Dynamically adjusted	Regional	Cemeteries, private garden	Larval sampling	1	2000		[42]
Germany	2008 [30]	Report [42]	Dynamically adjusted	Regional	Cemeteries	Larval sampling	Multiple (1 per year)	8900	Grid overlay, cell size: 100 km^2	[44, 60]

Table 1 (continued)

Monitored country	Year of first detection	Trigger of monitoring	Monitoring area	Monitoring scope	Monitoring locations	Monitoring methods	No. of visits at the same sites	Population size (km^2)	Additional information	Reference
Germany	2008 [30]	Citizen science project "Mueckenatlas"	Dynamically adjusted	Regional	Cemeteries	Larval sampling	1	–	Only central water basins inspected	[45]
Germany	2008 [30]	Report [45]	Dynamically adjusted	Regional	Cemeteries	Larval sampling	Multiple (1 per year)	800	Grid overlay, cell size: 100 km^2	[44, 60]
The Netherlands	2012	National framework for mosquito surveys	Dynamically adjusted	Local	Tyre trading company, allotment gardens, cemeteries, forests	Larval sampling, adult trapping	Multiple (not further specified)	Not stated	Population reduced but not eradicated [48]	[47]
Austria, Slovenia	2011	Not stated	Not stated	Local	"Kneipp" site, human settlements	Larval sampling	1	Not stated		[49]
Austria, Germany, Hungary, Liechtenstein, Switzerland	2012 Hungary, 2015 Liechtenstein	Report [30, 31, 49]	Dynamically adjusted	Regional	Human settlements, forests, cemeteries	Ovitraps, larval sampling	Multiple (not further specified)	Not stated		[37]
Austria, Italy	2015 Italy	Report [49]	Dynamically adjusted	Regional	Human settlements, forests, cemeteries	Larval sampling	–	Not stated		[50]
Slovenia	2011 [49]	Report [49]	Fixed	National	Human settlements, cemeteries, tire trading company	Larval sampling	Multiple (2 per year)	Not stated		[52]
Croatia	2013	Unknown	Fixed	Regional	Urban areas, cemeteries	Ovitraps, larval sampling	Multiple (not further specified)	Not stated		[53]
Austria, Germany	2008 Germany [30]; 2011 Austria [49]	Citizen science project "Mueckenatlas"	Dynamically adjusted	Regional	Cemeteries, private garden	Larval sampling	1	900	Grid overlay, cell size: 100 km^2	[54]

et al. [31]. It was speculated that the species had reached the studied area by passive transportation rather than by active expansion [32] since it was thought to have a low dispersal range [9]. Interestingly, the northernmost location examined was about 10 km south of the airport of Stuttgart, which, according to Schneider [32], could be a possible introduction site, although no evidence exists of *Ae. j. japonicus* introductions via airports [30].

Coincidentally to the findings of larvae by Schneider [32], Werner et al. [33] reported the first trapping of adult females in southwestern Germany in the summer of 2011.

The studies of Schneider [32] and Werner et al. [33] led to the expansion of the monitoring activities in South Germany, with Huber et al. [34] conducting field investigations encompassing the entire federal state of Baden-Wuerttemberg (almost 35,000 km^2) in 2011. Remarkably, the results suggested that the distribution area near the border to Switzerland had decreased, but at the same time, another, much larger infested area was discovered between the city of Stuttgart and the Swabian Mountains [34]. One year later, in 2012, Huber et al. [35] revisited the previously inspected sites and registered an increase from 54 to 124 positive municipalities. Genetic analyses indicated that the new population was likely to be the result of a northward spread of the southwestern population [35].

In 2013, Krebs et al. [36] found *Ae. j. japonicus* larvae only in one of nine deliberately selected cemeteries in French Alsace. Yet, immature stages were collected from additional random locations in a 6 km radius around the positive cemetery. Krebs et al. [36] concluded that the Swiss/German population had expanded to France, declared the establishment of *Ae. j. japonicus* in France, and considered eradication on the French territory unrealistic.

In 2011, Seidel et al. [37] investigated natural and artificial *Ae. j. japonicus* breeding sites in settlements and forests in western Austria. The study was expanded once developmental stages were detected in April 2015, resulting in a substantial increase in the survey area coverage. Eventually, the Asian bush mosquito was not only found in parts of Austria but also in Liechtenstein, Switzerland and southwestern Germany [37].

Data collected in Germany in 2016 and 2017 indicate that *Ae. j. japonicus* continued to expand eastwards from the federal state of Baden-Wuerttemberg into the federal state of Bavaria, and northwards into the federal states of Rhineland-Palatinate and Hesse (Fig. 1). In addition, *Ae. j. japonicus* was found in the southeastern part of Rhineland-Palatinate in 2017, close to the German federal state of Saarland (Fig. 1), suggesting that a far larger than known region of French Alsace might be colonised. Unfortunately, except for one finding by Seidel et al. [37], no *Ae. j. japonicus* distribution data have been published from Switzerland after 2009 [30] although the spread has continued, preventing precise mapping (Fig. 1).

Belgium

In 2002, a surveillance programme targeting *Ae. albopictus* in France [38] led to the first detection of *Ae. j. japonicus* in Belgium. Potential larval breeding sites examined on and around the premises of a tyre-trading company in 2002, 2003, 2004, 2007 and 2008, demonstrated its establishment. A second company ground in the vicinity of the survey area was found to be infested in 2008. Surprisingly, there was no evidence of this population spreading to the surrounding area [39].

The lack of dispersal was confirmed by a follow-up study conducted in 2009. Damiens et al. [40] repeatedly visited the previously affected premises and reported the finding of several larvae on the companies' grounds while only one larva was found in a puddle 100 m away. Control was initiated in 2012, based on larvicidal Bti (*Bacillus thuringiensis israelensis*)-toxin application and reduction of potential breeding habitats. The Belgian population has been considered eliminated since 2015 [41] but reinvasion from Germany seems probable.

Western Germany

A third European *Ae. j. japonicus* population was detected in western Germany in 2012 [42], after specimens had been submitted for identification to the German 'Mueckenatlas' passive surveillance scheme, a citizen science project [43]. The collection sites and their surroundings, e.g. the gardens of the submitters, were searched for potential breeding sites and developmental stages. After it had become clear that a larger area was colonised, the surveillance was expanded to cemeteries in the villages surrounding the positive localities. Finally, a colonised area of about 2000 km^2 was found. Three possible origins of this second German population were discussed: (i) a northward spread of the southwestern population; (ii) an eastward spread of the Belgian population; and (iii) an additional introduction. So far, no clarification was possible.

Since its detection, Kampen et al. [44] annually monitored the geographic expansion of this population and found a tremendous increase of the colonised area in 2015. The spread continued in all directions in 2016 and 2017, with branches of the population reaching into central Germany and to, possibly across, the Belgian border in the west (Fig. 1). Additionally, a further expansion to the south took place, which, together with the northward spread of the southwest German population, resulted in the merging of these two populations in 2017 (Fig. 1).

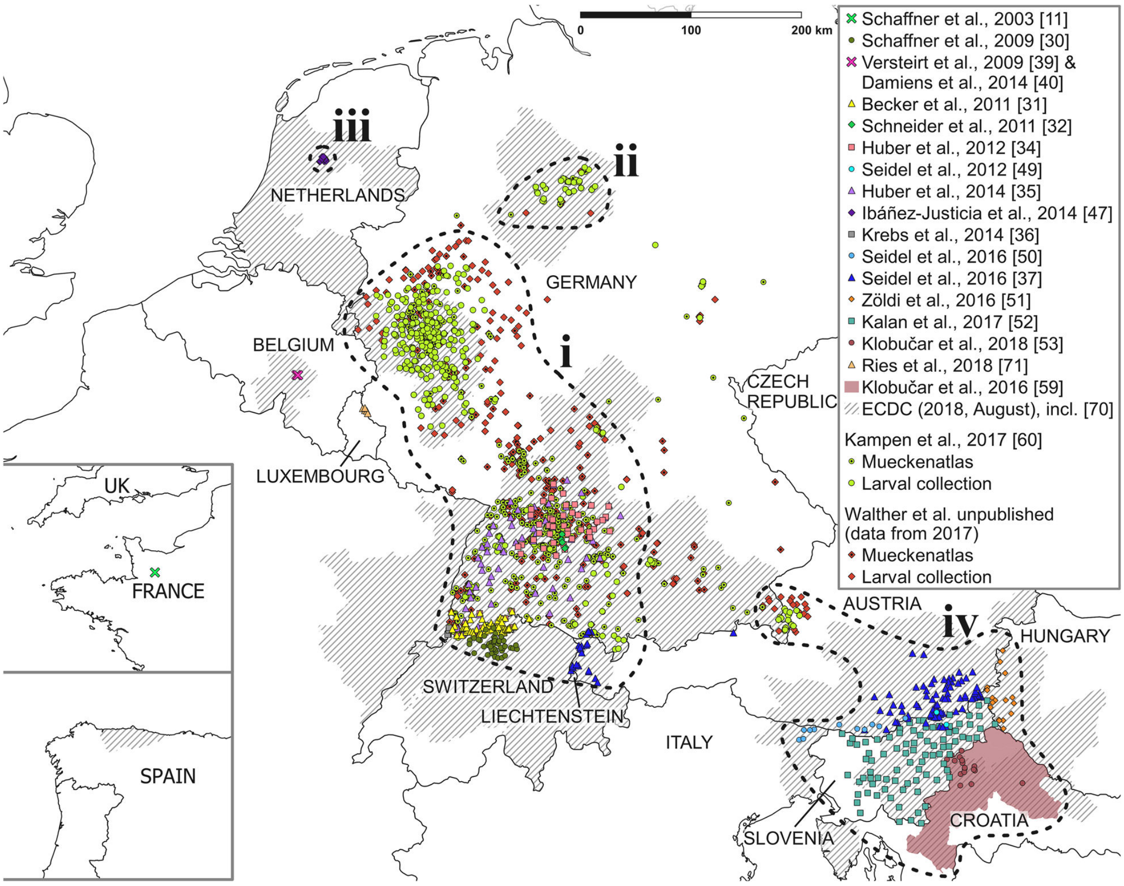

Fig. 1 *Aedes j. japonicus* occurrence reported in Europe according to studies published until the end of 2017 plus two online reports from 2018 (coloured symbols; crosses indicate reports of eradication). Dashed outlines and their respective numbers refer to the present populations as mentioned in section "Summary of the European distribution as of 2017". *Aedes j. japonicus* findings between 2012 and 2016 from studies by Kampen et al. [6, 42, 44], Walther et al. [43], Werner et al. [33, 45] and Zielke et al. [54] are summarised in Kampen et al. [60]; unpublished data from 2017 are referred to as 'Walther et al., unpublished'. Hatched areas display *Ae. j. japonicus* reporting (introduction and establishment) based on NUTS3 level, according to the ECDC *Ae. j. japonicus* vector map from August 2018 (https://ecdc.europa.eu/en/publications-data/aedes-japonicus-current-known-distribution-august-2018). Hatched areas may be misleading considering the size of the putative distribution areas but are particularly important for following-up affected areas when no recent original data are available, e.g. for Switzerland. The map was created with QGIS, v.2.18.14 (QGIS Development Team, 2018). The base map of Europe and respective administrative areas were downloaded from http://www.naturalearthdata.com

Northern Germany

In late summer 2012, an *Ae. j. japonicus* female was submitted to the 'Mueckenatlas' scheme from northern Germany [45]. Due to some delay in processing and the end of the mosquito season approaching, this case was only followed up in 2013. The survey produced *Ae. j. japonicus* larvae in 25 of 129 monitored cemeteries [45]. It was noted that the infested cemeteries seemed to concentrate along two motorways, indicating that this new population could be an offshoot of the West German population that arose through passive displacement of specimens by cars [45]. This was later confirmed by genetic analyses [46]. During the following years, Kampen et al. [44] continued the survey and showed that, contrary to the West German population, the infested area in northern Germany did not expand but even appeared to have decreased by 30% in terms of area coverage until 2015. Such a decrease could not be confirmed in 2016 and 2017 when additional findings rather suggested stagnancy, with some annual fluctuations (Walther et al., unpublished).

The Netherlands

In 2012, a female *Ae. j. japonicus* specimen was collected in the municipality of Lelystad [47], the Netherlands, leading to intensified monitoring efforts. In 2013, several adult females were trapped at almost the same site where the specimen had been collected in 2012 and a female was caught in the vicinity of a tyre-trading company. After a larva had been found some 7 km from the first location, the survey area was expanded to the whole municipality in late 2013. The extension brought forth *Ae. j. japonicus* eggs, larvae, pupae and adults in allotment gardens, forested areas and the cemetery of Lelystad. It was not possible to identify the point of entry to the Netherlands [47]. In 2016, control was initiated using source reduction and application of Bti-toxin. The population could be reduced but not eliminated [48].

Eastern Austria/Slovenia/Hungary/Croatia/Italy

For southeastern Austria and northeastern Slovenia, *Ae. j. japonicus* was recorded for the first time in 2011, when larvae were found some 50–60 km apart [49]. Successive investigations of suitable breeding sites, in human settlements or forests, taking place in 2011 and 2012, demonstrated a large area of infestation in southeastern Austria [37]. Seidel et al. [50] assumed that a westward expansion was quite likely and hypothesised that *Ae. j. japonicus* might soon cross the border to Italy. The area was monitored during the following years, starting in 2013, and *Ae. j. japonicus* was indeed found to have expanded westwards and southwards into North Italy between 2013 and 2015, representing its first detection in Italy [50].

Additionally, Seidel et al. [37] reported an expansion of the Asian bush mosquito to the east. In the summer of 2012, *Ae. j. japonicus* larvae were detected in Hungary, depicting the first detection of an invasive mosquito species in this country. Further specimens were identified in Hungary in 2014 and 2015 along the border to Austria [51].

During the first large-scale study targeting invasive mosquitoes in Slovenia, the whole national territory was surveyed to determine the distribution of *Ae. j. japonicus*, among other invasive species. In total, Kalan et al. [52] monitored 141 municipalities throughout Slovenia in 2013 and 2015, with emphasis on municipalities along major traffic axes. The results showed that since the first detection of *Ae. j. japonicus* close to the Austrian border in 2011 [49], the population had spread over most of northeastern Slovenia. By 2015, *Ae. j. japonicus* was present throughout Slovenia except for a small strip of land adjacent to the border to Italy [52].

In Croatia, Klobučar et al. [53] monitored *Ae. j. japonicus* from 2013 to 2015. In 2013, several *Ae. j. japonicus* larvae were collected in a cemetery, while in 2015, 12 of 369 water-filled vases inspected in four cemeteries were inhabited by *Ae. j. japonicus*. In the city of Zagreb, the species was found for the first time as larvae in a wooden container in 2015. A further spread to several northwestern counties was shown by 2016 [53].

Southeastern Germany/Austria (federal state of Salzburg)

In 2015, Zielke et al. [54] found another *Aedes j. japonicus* population spanning from southeastern Germany across the border into Austria. According to data collected in 2016 and 2017, this population has since also expanded considerably, both into Germany and into Austria (Fig. 1). As of 2017, the typical character of a population, isolation, was no longer given on the German side as a loose corridor of *Ae. j. japonicus* larval collection sites connected this population with that of southwest Germany (Fig. 1).

Summary of the European distribution as of 2017

In summary, of the seven populations of *Ae. j. japonicus* that came to attention in Europe, only four still exist as of 2017 due to the elimination of one population (Belgium) and the merging of three others (Fig. 1):

(i) The largest population covered western Germany (parts of the federal states of North Rhine-Westphalia, Rhineland-Palatinate and Hesse), the whole federal state of Baden-Wuerttemberg, from where it crossed the border to France (Alsace) in the west and to Bavaria in the east, interlinking with the southeast German/Austrian (Salzburg) population, and a significant part of northern Switzerland from where it extends to the east through Liechtenstein into western Austria. Regionally, considerable population densities occur.

(ii) A relatively small population which had not spread since its detection in 2013 exists in north Germany in parts of the federal states of Lower Saxony and North Rhine-Westphalia. Due to the ongoing expansion of the West German population to the north, it is expected that both populations will merge in the near future.

(iii) The very small Dutch population remained restricted to the municipality of Lelystad.

(iv) Probably the second largest, the population covered southeastern Austria, northern Italy, almost the whole of Slovenia (except for the most western part) and parts of Croatia and Hungary.

In addition to the *Ae. j. japonicus* reports from Germany allocated to the various populations and federal states, there are scattered findings from the central part of the country (northern Bavaria, Thuringia, Saxony and Saxony-Anhalt) (Fig. 1).

Genetic relationship of European *Ae. j. japonicus* populations

Population genetic studies support the assumption that at least two separate introductions of *Ae. j. japonicus* mosquitoes into Europe took place since 2000 [35, 55], when the first evidence of *Ae. j. japonicus* was reported from France [11]. Individuals from the Belgian population collected in 2008 and 2012 and subjected to microsatellite analysis rather resembled the subsequently found German/Swiss population than the populations from western and northern Germany regarding their genetic makeup [55].

Cluster analyses based on microsatellite data from all European populations detected until 2015 (except samples from France [36]) clearly show two genotypes of *Ae. j. japonicus* [35, 46, 55]. One cluster, called 'genotype 1', includes samples from the early detected populations in Belgium and southwestern Germany/Switzerland as well as from those from eastern Austria/Slovenia and southeastern Germany/northwestern Austria [46, 54, 55]. By contrast, mosquitoes from western and northern Germany represent 'genotype 2' [46, 55]. The quite admixed Dutch population shows both genotypes and is thus most likely based on at least two introductions of mosquitoes from different parts of Europe or from overseas [46]. Specimens from the distribution areas in northern and western Germany also share one *nad*4 (NADH dehydrogenase subunit 4 gene) mitochondrial haplotype ("H5") which was unique in Europe at the time of examination, underlining the assumed origin from different source populations [55]. This haplotype was also detected in Slovenian mosquitoes [55].

The Belgian population is the only one among the European populations for which the introduction pathway could be hypothesised. Individuals were only found on the premises and in the close vicinity of one intercontinentally operating used tyre-trading company [39], indicating its introduction by the international tyre-trade. As for the other European populations, means of introduction and transportation are quite obscure. Active migration of the mosquitoes certainly plays a minor role over long distances [56]. It must be assumed that in some cases (e.g. western and northern Germany, Slovenia and southeastern Germany), motorways, connecting the distribution areas, serve as routes of passive transportation of all life stages [45, 54, 56, 57].

Methodological appraisal

The review by Vezzani [58], who stressed the importance of cemeteries as highly suitable and readily accepted breeding habitats for container-breeding mosquitoes, considerably influenced the methodology of invasive mosquito surveillance in recent years. This is particularly obvious in large-scale studies. Almost all published surveillance activities used cemeteries, alone or in combination with other landscape structures, e.g. allotment gardens, to monitor infestation and delimit areas inhabited by *Ae. j. japonicus*. Furthermore, most studies are similar considering the season in which the surveillance work was conducted (Fig. 2).

All reviewed studies (except [33]) deal with immature stages, and only few groups used additional adult or oviposition traps. Unfortunately, many reviewed studies are short or rapid communications, thus missing detailed methodological descriptions. For two studies [51, 59], only findings are presented, as the available sources (abstracts from conference talks) provide insufficient methodological information.

Furthermore, the reviewed studies exhibit crucial differences in methodologies applied and details of presentation. First of all, type and quantity of examined containers frequently remain unmentioned. In addition, recurring visits in the same year have been done almost exclusively in the scope of small-scale studies (except for the large-scale study by Kalan et al. [52]), which is likely attributable to the workload. In some cases, the number of visits is not specified. Most importantly, the criteria regarding the declaration of negative sites differ significantly and must be questioned in some cases. While Schaffner et al. [30] used the exclusive presence of culicid larvae other than *Ae. j. japonicus* in one container or the complete lack of culicids in five containers as a negative indicator, other groups used the complete absence of *Ae. j. japonicus* larvae after a certain number of containers checked as an indicator. Furthermore, the number of containers or sites which were screened and found negative differs between the various approaches.

Recommendations to harmonise monitoring

To standardise and harmonise large-scale surveillance activities (when the species is already found established in an area larger than 25 km^2), we suggest a halo approach, creating a circle of negative sites around positive sites to define the boundaries of a population, in combination with the use of a grid cell pattern. The principle of this method is based on the studies by Schaffner et al. [30], Huber et al. [34], Kampen et al. [42, 44], Werner & Kampen [45] and Zielke et al. [54]. As a first step, a virtual grid with a defined cell size is generated, as done by Huber et al. [34], Kampen et al. [44] and

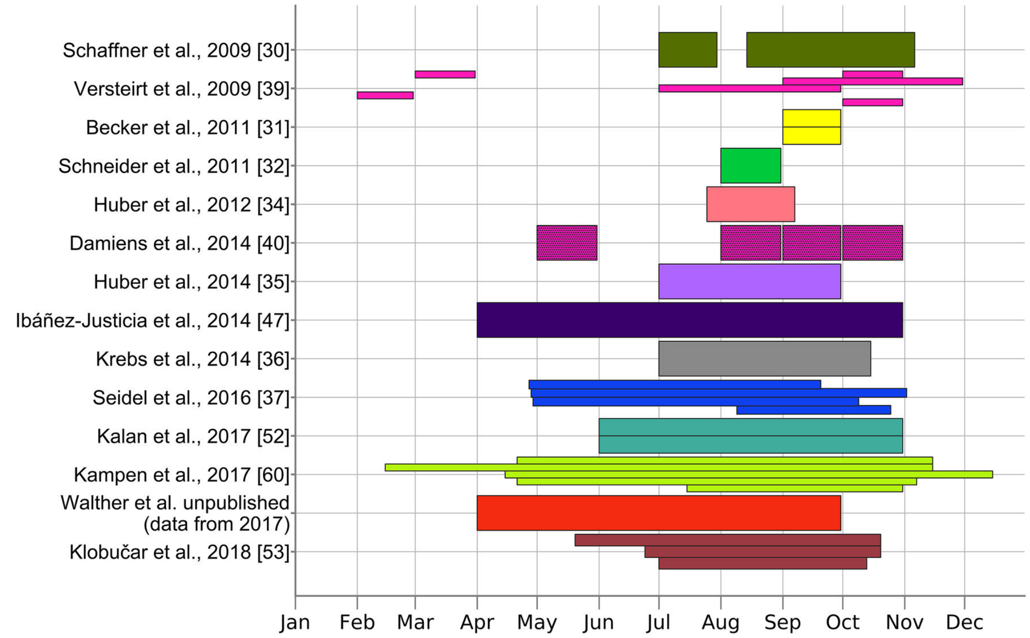

Fig. 2 Timelines showing the seasonal periods in which monitoring was carried out. Studies describing monitoring efforts over two or more years are shown with multiple, horizontally separated bars. Studies with imprecisely defined monitoring periods are not included [11, 50, 51]. Graphs were generated with Matplotlib v.2.1.0 for Python v.3.6.4

Zielke et al. [54]. To our knowledge, there are no studies comparing the effect of different grid resolutions; thus, specifying an evidence-based cell size is not feasible. Studies reviewed in this paper either used a cell size of 11 × 12.5 km = 137.5 km^2 [34] or 10 × 10 km = 100 km^2 [44, 54, 60], while the ECDC 'Guidelines for the surveillance of invasive mosquitoes in Europe' [29] suggest to inspect 40 containers in an area with a maximum size of 25 km^2 at the very beginning of the colonisation phase. The latter depicts a small-scale approach and would, if projected on a large scale, correspond to 160 containers per 100 km^2 and 220 containers per 137.5 km^2.

As mentioned earlier, the review by Vezzani [58] strongly pushed the selection of study sites towards cemeteries. Yet, allotment gardens were also shown to be suitable [48]. We therefore suggest that the search for developmental stages be performed in cemeteries or allotment gardens, owing to several advantages such as time efficiency, a high density of potential breeding sites and a high acceptance by *Ae. j. japonicus* and other invasive *Aedes* species [48, 58]. Cemeteries offer the additional advantage of being public property and therefore easily accessible, which usually is not the case with allotment gardens as these are private property. If neither suitable cemeteries nor allotment gardens can be found or accessed, alternative structures to be searched for developmental stages may include used tyre storages, farms and other locations where small water-holding containers can be found. If possible, the surveyed structures should be located in the vicinity of forested areas, as several studies indicate that *Ae. j. japonicus* uses forest edges to spread [61, 62].

Unless *Ae. j. japonicus* stages are quickly found, all water containers in a suitable structure should be inspected when the structure is small or has a low number of water containers available. In huge structures with a high number of water containers, a pre-determined time limit for inspection (e.g. one hour per location) or number of water containers to be inspected ensures a time-efficient compromise. Huber et al. [34], for example, inspected a minimum number of 30 containers per cell, while Kampen et al. [44] checked at least 80 containers per cemetery if one hour was not enough to inspect all containers. We propose to inspect a minimum of 150 containers per 100 km^2, which approximately corresponds to the above ECDC recommendations [29]. If available, at least three structures per cell should be inspected, which are to be selected in a way that the imaginary triangular area between them covers an area as large as possible. Especially in rural and mountainous areas it may happen, however, that a cell contains only one or two of the desired structures. In this case, all water bodies in the structures should be searched, ignoring a time limit to compensate for the loss of area coverage. Alternatively, deciduous forests (with tree-holes instead of artificial water containers) can be screened. A grid cell should be rated positive as soon as one single larva or pupa is unambiguously identified. It should be rated negative if no *Ae. j. japonicus* immature stages are found following the aforementioned criteria.

As realised in most of the reviewed studies (Fig. 2), the survey should be conducted during the seasonal activity peak of mosquitoes (August to September), when optimal developmental conditions are provided. In this case, a single areal inspection per year is considered sufficient. Although *Ae. j. japonicus* has been shown to be active from early spring until late autumn [27], population densities might be extremely low in a situation of initial colonisation and cause false negative results outside the activity optimum.

If the density of a population was high (numerous water containers colonised with plenty of larvae) in a previous survey and the workload needed for surveillance turns out to increase significantly, due to a continuous expansion of the population, consecutive monitoring efforts may start with the outermost positive cells of the previous study, assuming that by verifying infestation in the margins of the colonised area determined before, the centre of that area is also still infested. If the border area cell checked first is found negative, the cell adjacent in the direction to the centre of the previously colonised area should be examined before continuing away from the centre.

The suggested approach needs relatively little preparation time as only GIS-software is needed to generate a grid overlay. Furthermore, cemeteries, allotment gardens and land use (e.g. forested areas) can be found as features on open-source GIS layers (e.g. OSM data). Alternatively, web searches can help in detecting suitable locations. Additionally, the costs of this approach are manageable, as the biggest cost factor is travel expenses, and only basic equipment such as small sieves, dippers, pipettes and sample containers are required. Other cost factors, such as training and labour need to be taken in account, although these are always incurred, independent of the applied monitoring technique.

Furthermore, the resulting distribution map is easily understandable (c.f. [44]), hence usable to quickly inform the broader scientific community, and easily comparable regarding the colonised area between different monitoring years and different monitoring regions. Relative to the size of the survey area, the required workforce is low. Two researchers experienced in identifying *Ae. j. japonicus* larvae under field conditions, were able to trace the area of the western Germany population in 2016 within a time period of two weeks, by starting with the outermost positive sites of the distribution area (approx. 8900 km^2) as found in 2015 [44].

A gradual adoption of the suggested method in surveying *Ae. j. japonicus* in the years to come may pave the way for large-scale collaborations regarding data analysis and the design of predictive models, while the standardisation will generally support future mosquito research. However, collecting data is only one part of large-scale cooperation, as it could be beneficial to establish a database which explicitly contains presence and absence data. Our proposed method could also prove useful for other invasive *Aedes* species, since no standardised approach for large-scale larval surveillance can be found in the pertinent European literature for either *Ae. albopictus* [63–67] or *Ae. koreicus* [68, 69].

Conclusions

By late 2017, 17 years after the first detection of *Ae. j. japonicus* in Europe, this invasive mosquito species was demonstrated to be established in ten countries, in most of which it continues to spread. Only in Belgium could it be eliminated after several years of restricted local occurrence. In June and July 2018, *Aedes j. japonicus* was detected for the first time in northern Spain [70] and Luxembourg [71], respectively, increasing the number of infested European countries to 12. As these two recent reports were short online notifications without any details, they are mentioned here for completeness only (Fig. 1). Even if eradication of *Ae. j. japonicus* no longer seems feasible, further surveillance may add important information for mosquito-borne disease risk assessments and is an opportunity to study the spread and the occupation of an ecological niche by a newly emerging climatically adapted species. Additionally, monitoring data of several years could reveal environmental conditions, such as specific landscape structures, which support or impede the spread. To improve future research and harmonisation of data collection, we propose a methodological approach for the continuous surveillance of populations which infest an area larger than 25 km^2 (c.f. [29]), that pose a high risk of further spread. This methodological approach could be further used as a framework for more detailed data collections including key figures, such as container indices, larval counts, occurrence of species-coexistence, exact counts of positive containers per container type, environmental data, etc. Recording such detailed data will of course require more time and increase costs both directly (labour and training) and indirectly (possible additional tools), yet it could prove useful for meta-studies and modelling approaches, as most existent studies only work with presence/absence data and climate data [52, 72–75]. The use of other strategies, e.g. trapping of eggs and adults, can be suitable in situations where the efforts aim to determine initial establishment, population density, small-scale distribution or presence of pathogens (e.g. [23, 24, 45]). Utilisation of stationary traps in large-scale surveys (e.g. [60]) does not seem feasible, or will at least prove expensive and be linked to a wide range of problems, e.g. malfunctions, theft or demolition. Ovitrap networks are also cost-efficient but increase travel expenses as at least two visits per site are needed (setup and removal) and usually do not allow species identification on the spot.

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Availability of data and materials
The majority of data supporting the conclusions of this study are included in the reviewed articles. Recent, hitherto unpublished data by the authors are available from the corresponding author upon reasonable request.

Authors' contributions
MK did the literature research, analysed the data from the reviewed studies, drafted the initial manuscript and prepared the final manuscript. MK, LF, CK, NJ, DW and HK contributed unpublished collection data. LF, CK, GS, JS, NJ, DW and HK critically revised the manuscript. DS provided molecular data, contributed to the manuscript and critically revised the manuscript. HK and DW designed the study and provided scientific guidance. All authors revised, read and approved the final manuscript.

Ethics approval and consent to participate
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Competing interests
The authors declare that they have no competing interests.

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Publication 2: 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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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.

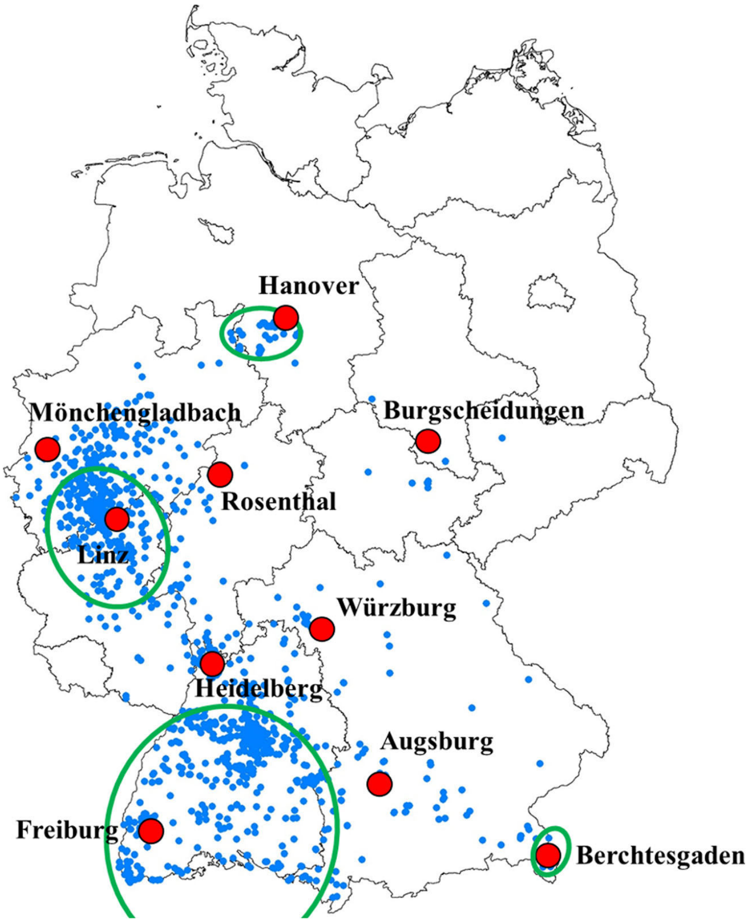


Fig. 1 Geographic distribution of *Ae. japonicus* collection sites analysed in this study (red dots). Blue dots mark *Ae. japonicus* detection sites from 2012 to 2017, and areas encircled in green colour represent approximate population distributions as of 2015

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.

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

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).

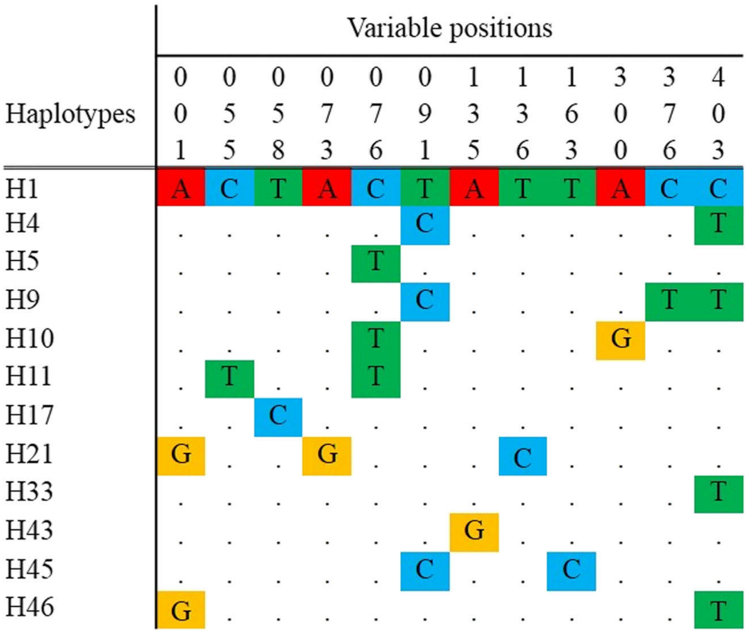
The most common *nad*4-haplotype found was H1, detected 69 times at eight collection sites. Haplotype H21 was detected exclusively in Heidelberg, and the haplotypes H11 and H43 were identified only in Burgscheidungen (Fig. 3). The number of haplotypes per site varied from one (Rosenthal) to seven (Burgscheidungen, 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.



Haplotypes	Variable positions											
	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

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

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

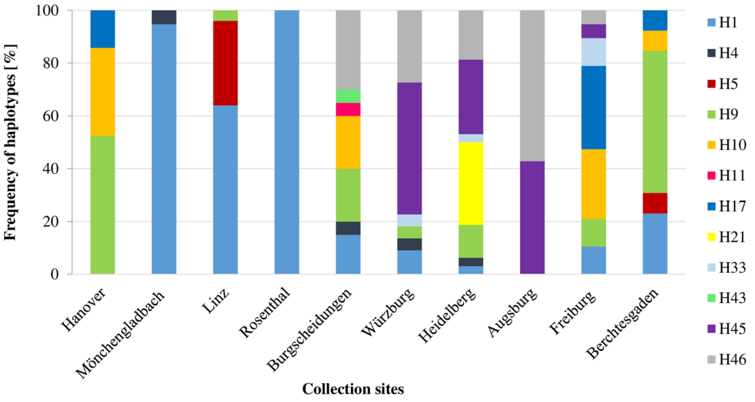


Fig. 3 Frequency of *nad*4 haplotypes detected

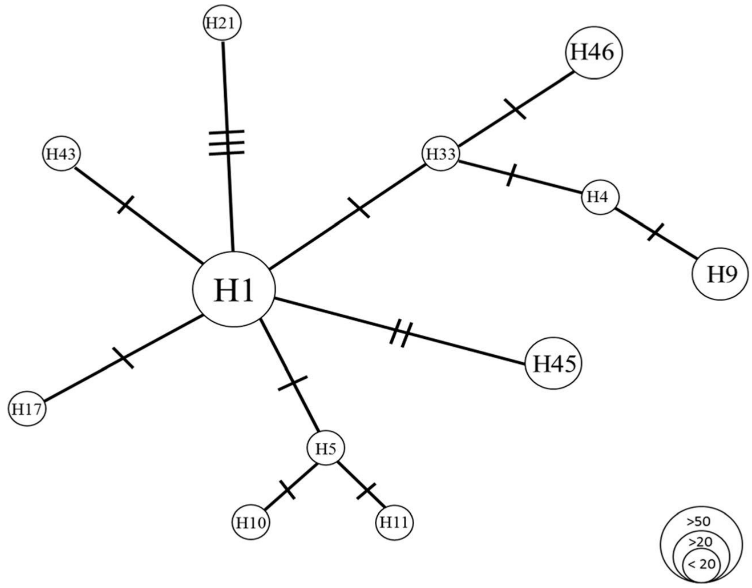


Fig. 4 Spanning tree of *nad*4 haplotypes detected. Every cross line represents one transition. The size of the circles represents the number of detected specimens with the respective haplotypes

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).

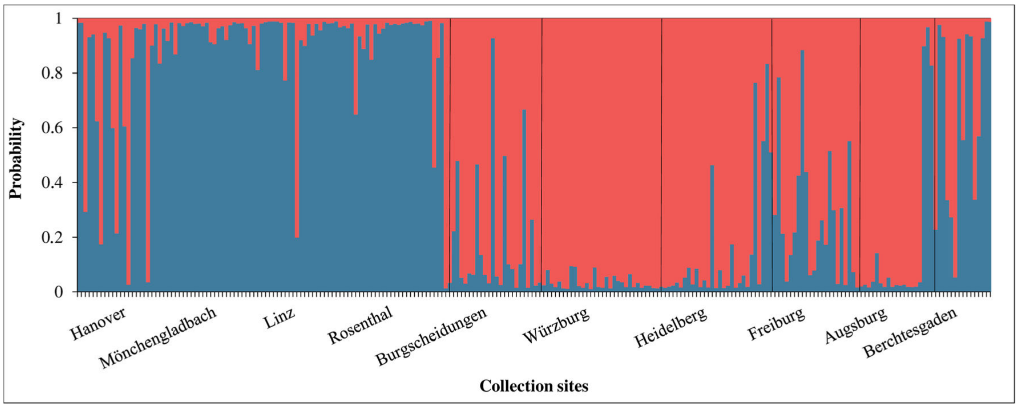


Fig. 5 Results of microsatellite multilocus genotyping. Each line represents one individual (Δk = 2). The colours show the probability of belonging to one of the two genetic clusters (blue = genotype 1; red = genotype 2). The order of the bars (left to right) corresponds to the geographical location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

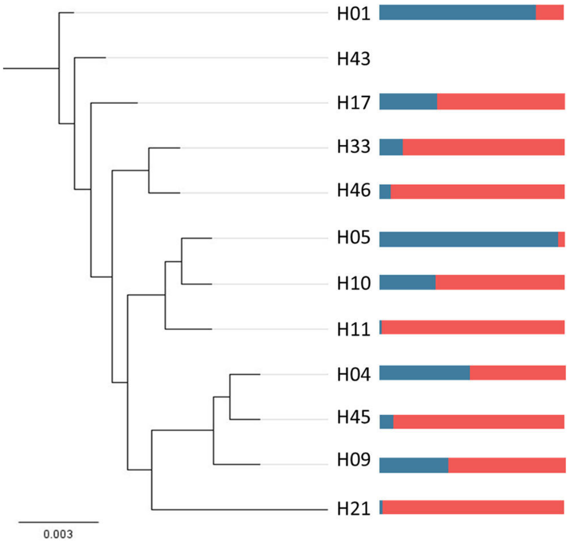


Fig. 6 Phylogenetic tree of *nad*4 haplotypes. The colours of the bars represent the probability of belonging to one of the two microsatellite genotypes (blue = genotype 1; red = genotype 2). H43 was detected in one single individual only for whom no result could be obtained in the microsatellite analysis.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

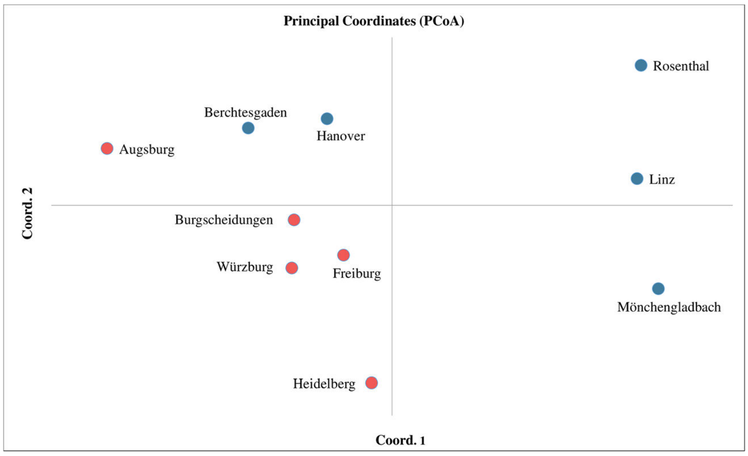
The specimens from Freiburg, Heidelberg, Würzburg and Augsburg, all of which were located in the southwestern distribution area of *Ae. japonicus* in 2017, are characterized by some identical *nad*4 haplotypes. Obviously, no admixture has occurred by mosquitoes from the western population.

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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Publication 3: Rapid spread and population genetics of Aedes japonicus japonicus (Diptera: Culicidae) in southeastern Europe (Croatia, Bosnia and Herzegovina, Serbia)

PLOS ONE

RESEARCH ARTICLE

Rapid spread and population genetics of *Aedes japonicus japonicus* (Diptera: Culicidae) in southeastern Europe (Croatia, Bosnia and Herzegovina, Serbia)

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Abstract

The Asian bush mosquito, *Aedes japonicus japonicus* (Theobald, 1901), a potential vector of several pathogens, has recently established in North America and Central Europe. In 2013, it was found on the Slovenian-Croatian border, and during the following years, it emerged in more and more counties of northwestern Croatia. Surveillance of *Ae. j. japonicus* and other invasive mosquito species was subsequently extended both spatially and temporally in Croatia and neighbouring Bosnia and Herzegovina and Serbia. Mosquito collections were conducted in 2017 and 2018, based on adult trapping through dry ice-baited CDC traps and BG-Lure-baited BG-Sentinel traps, larval sampling through dippers and nets, and ovitrapping. *Aedes j. japonicus* specimens from collected samples were subjected to population genetic analysis by comparing microsatellite signatures and *nad*4 DNA sequences between sampled locations and with data previously obtained from more western European distribution areas. *Aedes j. japonicus* immature stages were found at 19 sites in Croatia, two sites in Bosnia and Herzegovina and one site in Serbia. In Croatia, four new counties were found colonised, two in the east and two in the south of the previously known distribution area. A spread of 250 km could thus be documented within five years. The findings in Bosnia and Herzegovina and Serbia represent the first records of *Ae. j. japonicus* in these countries. Genetic analysis suggests at least two introduction events into the surveyed area. Among the locations analysed, Orahovica can be considered a genetic border. The individuals collected west of this point were found to be similar to samples previously collected in the border regions of Southeast Germany/Austria and Austria/Slovenia, while the specimens from more eastern Croatian localities, together with those from Bosnia and Herzegovina and Serbia, were genetically different and could not be assigned to a probable origin. Thus, introduction from Central Europe, possibly by vehicular traffic, into the study area is likely, but other origins, transportation routes and modes of entry appear to contribute. Further dispersal of *Ae. j. japonicus* to other parts of southeastern Europe is anticipated.

Data Availability Statement: All relevant data are within the manuscript and its Supporting Information files.

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Abbreviations: ECDC, European Centre for Disease Prevention and Control; EFSA, European Food Safety Authority; WNV, West Nile virus.

Introduction

The invasive Asian bush or rock pool mosquito *Aedes* (*Hulecoeteomyia*) *japonicus japonicus* (Theobald, 1901) originates from East Asia and the Far East, where it is widespread and even colonises regions with harsh winters [1]. In its native range, the species has a preference for forested and bushy areas, where it is essentially a rock pool breeder [1]. In invaded areas of North America and Europe, larvae develop in rock pools and tree-holes, too, but more frequently and more easily to find, they use artificial containers such as used tyres, rain-water barrels, catch basins, tin cans, bird-baths, roof gutters, flower vases, buckets, etc. [2]. The developmental stages can withstand a wide range of water temperatures but are absent from warm water constantly exposed to sunlight [3, 4]. These facts explain the geographical and altitudinal distribution of the species in its invaded territories, which are usually characterised by moderate climates. Thus, *Ae. j. japonicus* has become widely distributed in North America and Central Europe where it can be found from lowlands to mountainous areas higher than 1,000 m a.s.l. (meters above sea level; e.g., the US Appalachian Mountains and the German Black Forest) [5, 6]. The species is usually absent from areas with temperatures regularly exceeding 30–35˚C [7], although it has succeeded in establishing on Hawaii and in Florida [8, 9].

So far, *Ae. j. japonicus* has not presented itself as an important vector in the field although it has been found infected with West Nile virus (WNV), Japanese encephalitis virus, Cache Valley virus and La Crosse virus [10–13]. Under experimental conditions, it has shown vector-competent for West Nile, Japanese encephalitis, eastern equine encephalitis, La Crosse, Rift Valley, chikungunya and dengue viruses [14–18]. It can be assumed that its actual vector role might temporarily or permanently increase with further spread, rising population densities and numbers of infection sources available as well as climate warming. After all, in some laboratory studies it transmitted WNV even more efficiently than *Culex pipiens* [19] which is particularly alarming before the background of the unprecedented 2018 WNV outbreak in Europe [20] as this suggests widespread and intense virus circulation in natural cycles and, thus, a multitude of infection sources.

Aedes j. japonicus is one of the most expansive mosquito species of the world. It is assumed that it is regularly displaced to overseas territories via used tyres in which the females lay their eggs when stored under the open sky and filled with water [21]. Its first evidence outside its native region was in the early 1990s in New Zealand, where it was intercepted but did not succeed in establishing [22]. In 1998, established populations were reported from the eastern USA, and until 2014, this species spread across 34 states in the USA and four states in Canada [2, 8, 23, 24]. In the year 2000, developmental stages were registered for the first time in Europe, in a storage yard of imported used tyres in northwestern France, but were promptly eliminated [25]. Establishment was documented for a population that had been detected in Belgium in 2002 but was not controlled until 2012 as it did not propagate [26]. In Europe, *Ae. j. japonicus* has to date established in Belgium, Switzerland, Germany, the Netherlands, Austria and Slovenia and, more recently, in Hungary, Liechtenstein, Italy, Croatia and Spain [27–29].

The first detection of *Ae. j.japonicus* in Croatia was made in 2013 when eggs were found during a survey for invasive mosquitoes in Krapinsko-Zagorska county, bordering Slovenia

[28] where the species was widely distributed in 2015 [30]. The survey in Croatia included ovitrapping at possible points of entry and in house yards, occasionally complemented by larval collections from cemetery vases. The investigation was continued for another two years and extended to further Croatian counties. In 2014, *Ae. j. japonicus* was detected in northwestern Croatia and in 2015 approximately 100 km eastward of the area of its first record [28]. Within three years, *Ae. j. japonicus* colonised four regional units of northwestern Croatia: Krapinsko-Zagorska, Zagrebačka and Bjelovarsko-Bilogorska counties as well as the city of Zagreb.

Prompted by these findings, a nationwide monitoring programme for invasive mosquito species was initiated in Croatia in early 2016, covering all counties except one in the North-East (Požeško-slavonska). By using ovitraps checked weekly from May to November, new presence data of *Ae. j. japonicus* were obtained from the northern counties Međimurska, Varaždinska, Koprivničko-Križevačka and Virovitičko-Podravska, as well as from the southern counties Karlovačka and Istarska [31]. The continuation of these activities in 2017 produced further distribution data from the eastern county Brodska Posavska [32]. By contrast, no *Ae. j. japonicus* occurrence had been documented before 2017 from Bosnia and Herzegovina and from Serbia, despite recent sporadic (Bosnia and Herzegovina) or extensive invasive mosquito surveillance activities (Serbia).

The present study was meant to follow up on the further spread of *Ae. j. japonicus* in Croatia and check for its occurrence at selected sites close to the Croatian border in neighbouring Serbia and Bosnia and Herzegovina. By genetic analyses of found specimens we attempted to track relationships, origins and transportation routes of populations.

Materials and methods

Study areas

The surveillance was carried out in three different areas of Croatia as well as on Bosnia and Herzegovinian and Serbian territories in the border triangle of these three countries (Fig 1).

Croatia. Collections were performed in the Slavonian Mountains, Gorski Kotar and the Central Velebit, in the east and south of the known *Ae. j. japonicus* distribution area (Fig 1). All three regions represent mountainous areas characterised by forest vegetation and high precipitation, but are interspersed by villages. Hence, both natural and urban settings were sampled where tree-holes, rock pools and man-made containers were available as suitable breeding sites for *Ae. j. japonicus* in considerable numbers.

The first area is located in Slavonia, northeastern Croatia, in the mountains surrounding Požega Valley. Three transects, along which mosquitoes were collected, crossed Papuk and Požeška Gora mountains, and had a minimum altitude of 177 m and a maximum altitude of 515 m. Papuk is distinguished by its forest richness, dominated by beech and oak, along with maple and ash. The mean annual temperature in this region is 11˚C and the mean annual precipitation 782 mm.

The second area is located in Gorski Kotar, western Croatia, at the foot of the Samarske Stijene rocks (maximum altitude 1,011 m). Gorski Kotar is a plateau with an average elevation of 700–900 m from which mountain peaks rise to up to 1,500 m. Above 1,200 m, the climate is subarctic with a lot of snow, while the lower areas belong to the warm and wet, moderate climate zone. Of particular relevance to the climate are winds, which sometimes reach fierce intensities at higher altitudes. Short and fresh summers and long and harsh winters with lots of snow are characteristic for the sharp mountain climate. This part of Croatia is known for large amounts of precipitation (2,150 mm mean annual rainfall), which are caused by the proximity to the Adriatic Sea and the influence of the high relief. The mean annual temperature is 5˚C. Beech and fir predominate the tree populations.

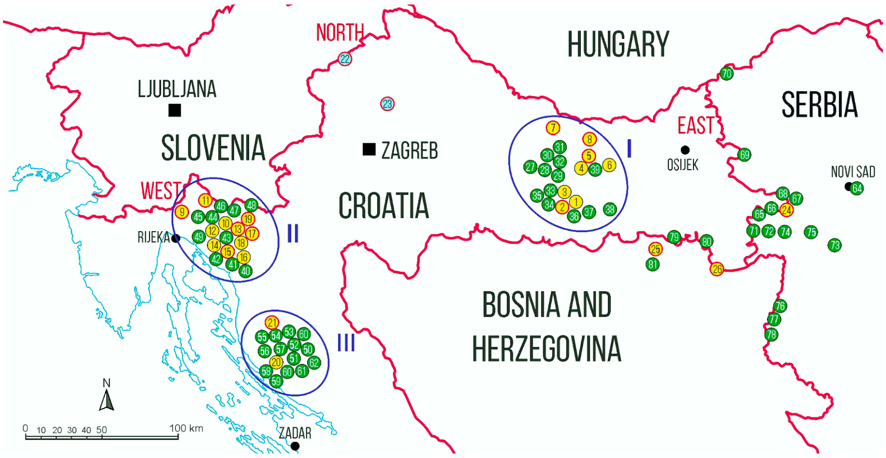

Fig 1. Study area with monitored locations: Yellow and blue dots mark locations with, green dots locations without *Ae. j. japonicus* findings. Collection locations considered in the population genetic analysis are marked by dots with red rims. These include two locations, marked by blue dots, found positive for *Ae. j. japonicus* already in the framework of a previous study [28], whereas locations found colonised in the present study and included in the population genetic analyses are marked by yellow dots. Numbers in the dots refer to the compilation of sampled locations in S1 Table, numbers of encirclements to the Croatian study areas (I = Slavonian Mountains, II = Gorski Kotar, III = Central Velebit).

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The third area is located in Central Velebit, also in the western part of Croatia and not far from the second study area. Altitudes reach 530 m to 1,000 m. The Velebit mountains build a natural barrier between continental and Mediterranean Croatia. On their peaks, two different climates clash, causing unpredictable weather conditions. The mean annual precipitation in this region is 1,870 mm, while the mean annual temperature is 6.2˚C. The Velebit area is a mosaic of diverse habitats (forests, meadows, rocks, rivers).

Bosnia and Herzegovina. Monitoring of invasive mosquito species was carried out in the Posavina region in the northeastern part of the country (average altitude: 90 m a.s.l.), adjacent to Croatian Slavonia. The selected area is located on the banks of two rivers, Sava and Bosna, and close to the Croatian border. The regional climate is generally temperate continental, but often shows extremes. The mean annual temperature in Posavina region is 10˚C and the mean annual precipitation 800 mm. Its vegetation is characterised by deciduous forest, dominated by oak and European hornbeam, but willow and poplar are also quite common. Five municipalities were sampled: Bosanski Šamac, Odžak, Orašje, Brčko and Modriča (Fig 1).

Serbia. Invasive mosquitoes were searched for in the northern province of Vojvodina (13 locations) and the western Mačva District (3 locations). Vojvodina is located in the lowest part of the Pannonian Plain (average altitude: 107 m a.s.l.). The mountains surrounding this lowland have a significant impact on its climate, which is continental. Cold winters alternate with hot and humid summers with a huge range of extreme temperatures, featuring inconsistent amounts of rainfall over months, which leads to different levels of aridity. The mean annual temperature is 11˚C and the mean annual precipitation 602 mm.

Mačva is located in the southern edge of the Pannonian basin, between the Cer and Fruška Gora mountains. It has humid subtropical climate with cold winters and warm to hot summers. The altitude ranges from 75 to 95 m and the mean annual precipitation is 774 mm.

The Serbian surveillance activities included eight border crossings to Croatia, three sites close to a border crossing to Bosnia and Herzegovina and one site in Novi Sad, the second-largest city in the country. Two toll and two gas stations located on the motorway E70, which connects Zagreb (Croatia) and Belgrade (Serbia), were also included (Fig 1).

Mosquito collection

Mosquito collections in Croatia were conducted during July and August 2017 at 58 locations (Fig 1 and S1 Table): 21 in the Slavonian Mountains, 21 in Gorski Kotar and 16 in Central Velebit, once per locality. Collections of developmental stages (larvae, pupae) were carried out utilising plastic dippers or a net, depending on the size of the water bodies which included natural ones, such as ponds, ditches and rock pools, as well as man-made containers, such as tyres, bathtubs, barrels etc. In addition, ovitraps were used at two sites in 2017, and flower vases were checked in a cemetery in 2018. Part of the collected larvae were stored in 100% ethanol for molecular analyses, another part transferred to the laboratory and mounted on slides for a reference collection as described by Merdić et al. [33]. Adult mosquitoes were to be caught using dry ice-baited CDC traps (Bioquip, Rancho Dominguez CA, USA) at 30, and BG-Sentinel traps equipped with BG-Lure (Biogents, Regensburg, Germany) and dry ice as attractants at six of the 58 locations. All traps were operated for at least 12 hours over night, including dusk and dawn. Both larvae and adults were morphologically identified according to Gutsevich et al. [34].

In Bosnia and Herzegovina and Serbia, the surveillance was carried out by ovitrapping (using standard ovitraps of 0.5 l volume and masonite strips as oviposition supports) and larval dipping according to the ECDC (European Centre for Disease Prevention and Control) guidelines for the surveillance of invasive mosquitoes in Europe [35] (Fig 1 and S1 Table). Ovitraps in Bosnia and Herzegovina were positioned from early June to early October 2017 on private properties (n = 15), in cemeteries (n = 4), on border crossings (n = 2), a church garden (n = 1), and on the premises of a petrol station (n = 1), a tyre repair service (n = 1) and a carwash service (n = 1). In Serbia, 95 ovitraps were set up from mid-May to late October (at one location to early December) 2018 in a technical car service in the urban area of Novi Sad (n = 5), on eight border crossings (n = 45), at three petrol stations (n = 13), in a church garden and its surroundings (n = 10), in a forest (n = 5), in a private ethno-village (n = 10) and at two toll stations (n = 7). Distances between the traps were at least 500 m. Fortnightly, the traps were checked and strips with eggs collected. If larvae had already hatched, the water was collected and transferred to the laboratory. On each occasion of checking ovitraps in the five sampled municipalities of Bosnia and Herzegovina, discarded tyres were inspected, too (7 sites). Also, other potential natural and artificial breeding sites close to the ovitraps (up to 20 m) were regularly checked. Both eggs and larvae were reared to adult stages which were then identified according to the key by Gutsevich et al. [34].

Altitude was measured for all locations, as this was meant to be correlated to occurrence of *Ae. j. japonicus*.

The selected study locations (S1 Table) had never been checked for mosquitoes in general or the presence of invasive mosquito species in particular before the present survey. However, mosquito collections carried out sporadically or regularly elsewhere in the study regions (Croatia: since 2016; Serbia: since 2009; Bosnia and Herzegovina: since 2015) did not produce *Ae. j. japonicus* specimens, suggesting its previous absence.

Population genetic analysis

Aedes j. japonicus samples from 16 locations in Croatia, Bosnia and Herzegovina and Serbia (Fig 1) were subjected to population genetic analysis. These included 14 new sites containing *Ae. j. japonicus* as described here and specimens collected during the present study from two sites previously known to be colonised by the species [28]: Macelj and Konjščina (blue dots in Fig 1).

Following Fonseca et al. [36, 37], the genetic analysis was based on two different approaches: the DNA sequence of the mitochondrial NADH dehydrogenase gene subunit 4 (*nad*4) gene was examined for nucleotide polymorphisms, and microsatellite loci (nuclear DNA) demonstrated to be informative for *Ae. j. japonicus* [38] were analysed for differences in length/number of repetitive motifs.

However, due to poor DNA quality, heteroplasmy (simultaneous presence of two or more haplotypes in the same individual) or microsatellite analysis of certain loci failing in some specimens, not all individuals available could be considered. For that reason and because of a limited number of water containers sampled and of specimens collected per locality, several localities produced only few data.

DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Germany), following the manufacturer's instructions. Two approaches were applied in parallel: on the one hand, a segment of the mitochondrial *nad*4 gene was sequenced for each specimen, as described by Fonseca et al. [37], Zielke et al. [39] and Janssen et al. [40]. On the other hand, the fragment lengths of seven microsatellite loci (OJ5, OJ10, OJ70, OJ85, OJ100, OJ187, OJ338) were determined, following the protocol of Widdel et al. [38], as modified by Fonseca et al. [36] and Egizi and Fonseca [41]. The results were edited with Geneious 10.2.3 (Biomatters), analysed in STRUCTURE with a Bayesian algorithm [42] (length of burn-in period: 50,000; number of Markov-Chain-Monte-Carlo repetitions after burn-in: 100,000) and evaluated with the software STRUCTURE HARVESTER [43]. Furthermore, a principal coordinate analysis (PCoA) was performed on the microsatellite data using Nei's genetic distance and pairwise F_{ST} values (only localities with more than one specimen) [44].

To get indications on origins and introduction routes, the genetic data were compared to data previously produced for the closest western European *Ae. j. japonicus* populations known, the Southeast German/Austrian and Austrian/Slovenian populations [39, 45].

Results

Mosquito collection and identification

Aedes j. japonicus was only collected as larvae or eggs. These were obtained from 21 Croatian locations (Fig 1 and S1 Table), where barrels (6 out of 31 checked), tyres (5/20), bathtubs (3/5), ponds (2/9), rock pools (2/2), ovitraps (2/6) and flower vases (1/9) were colonised. The detection sites were located at altitudes from 184 m in Slatinski Drenovac (Slavonian Mountains) to 921 m in Baške Oštarije (Central Velebit). Approximately 52% (11/21) of them were around or above 700 m (Fig 2).

In Bosnia and Herzegovina, *Ae. j. japonicus* eggs were found at two locations close to the Croatian border, Odžak (7 July 2017) and Brčko (9 August 2017), once each (Fig 1 and S1 Table). In Serbia, one single ovitrap positioned on the Serbian-Croatian border in Ljuba was positive for *Ae. j. japonicus* eggs twice (21 August and 15 September 2018) (Fig 1 and S1 Table).

The findings reveal a continuing expansion of the distribution area of *Ae. j. japonicus* in southeastern Europe (Fig 3), with 10 new collection sites in Primorsko-Goranska county, two new sites in Ličko-Senjska county, four new sites in Požeško-Slavonska county and one new site in Osijek Baranja county in Croatia, two new collection sites in Bosnia and Herzegovina and one new collection site in Serbia.

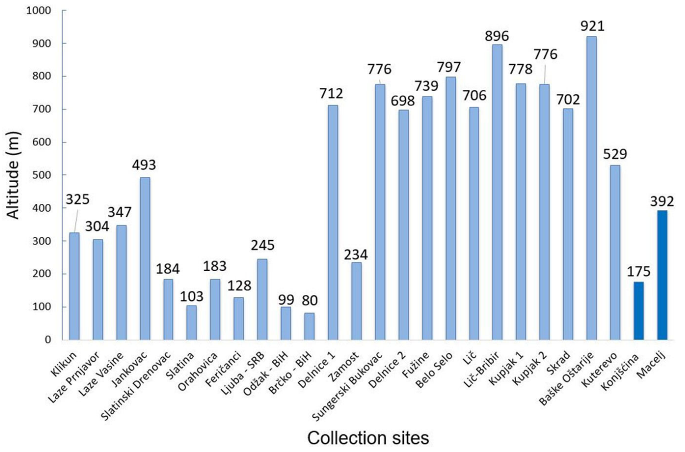

Fig 2. Altitude of *Ae. j. japonicus* collection locations (SRB = Serbia, BiH = Bosnia and Herzegovina; locations without country code are in Croatia). Locations with bars coloured in light blue were found positive for *Ae. j. japonicus* in the present study whereas those coloured in dark blue had been found colonised already in the framework of a previous study [28].

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Population genetics

A total of 142 *Ae. j. japonicus* specimens from 16 locations in Croatia, Bosnia and Herzegovina and Serbia were analysed genetically (Table 1).

Analysable microsatellite signatures were produced for 130 individuals, which were grouped into two different clusters according to a Bayesian cluster analysis using the programmes STRUCTURE and STRUCTURE HARVESTER (k = 2, Δk = 24.950; Fig 4). Most of the individuals from Macelj and Orahovica had a relatively high probability of belonging to one cluster, named genotype 1. By contrast, specimens from Zamost, Slatintinski Drenovac, Skrad, Laze Prnjavor, Ljuba and Brčko had a high probability for the second cluster, genotype 2. The other localities were more admixed with intermediate probabilities to belong to one of the two genotypes.

In a second Bayesian analysis (Fig 5), the previously investigated populations from Southeast Germany/Austria and Austria/Slovenia [39, 45] and the populations from southeastern Europe as examined in this study, were jointly analysed. This time, the Bayesian algorithm yielded the highest probability for 13 different clusters (k = 13, Δk = 38,149; Fig 5). The populations from SE-G/AU and AU/SLO as well as those from Delnice 1, Lič, Belo Selo, Skrad, Zamost, Kupjak 1 and Kuterevo turned out to have no preponderate probability of individuals to belong to one or another genotype. Due to this similarity and their geographic origin, they are summarised under the microsatellite signature group 'West Croatia' for the purpose of assessing their genetic makeup with regard to relatedness to specimens from other locations in a PCoA and relating them to haplotype configurations. By contrast, specimens from other locations show a high probability for only one genotype: Konjščina (dark blue), Macelj (light blue), Laze Prnjavor (light green), Orahovica (dark green) and Ljuba (grey). The single specimen from Brčko shows a similar microsatellite signature as the specimens from Odzak, so both are summarised under 'Bosnia and Herzegovina'. The microsatellite signature of the mosquitoes from Slatinski Drenovac and Slatina looks similar to that of 'West Croatia', but is

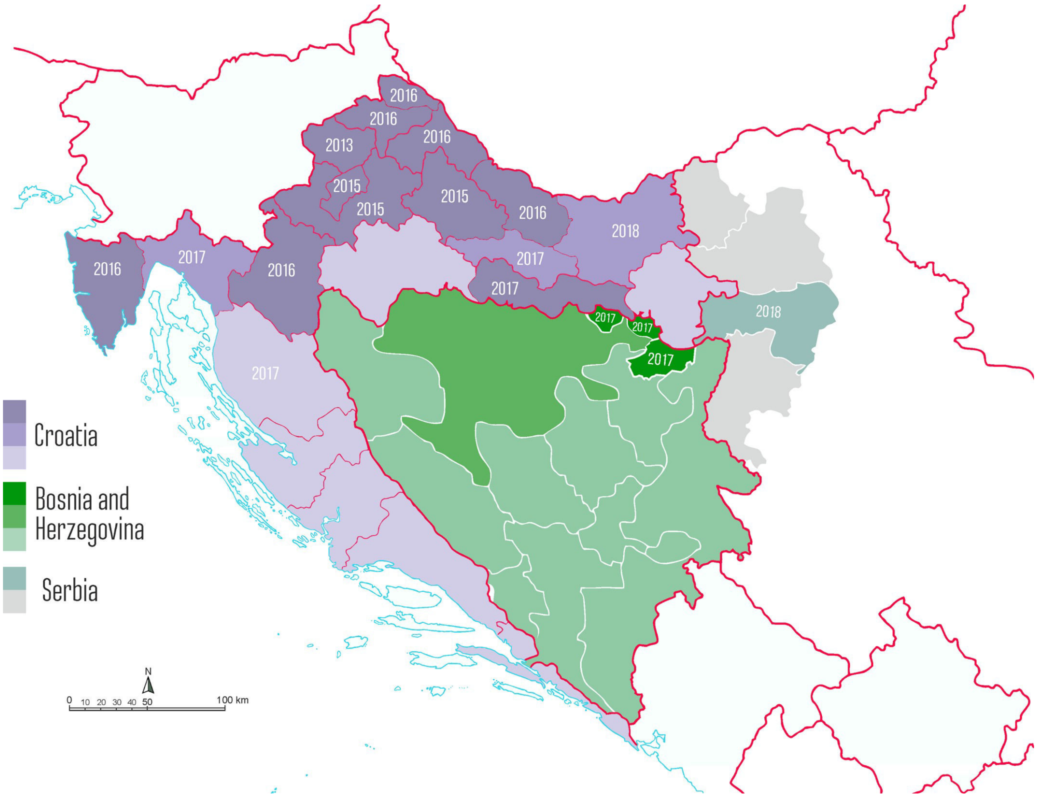

Fig 3. Results of *Ae j. japonicus* monitoring in Croatia, Bosnia and Herzegovina and Serbia, related to province. Numbers of years refer to the first detection of *Ae. j. japonicus* (Croatia: dark red–presence of *Ae. japonicus* according to previous studies, light red–presence of *Ae. japonicus* according to this study, orange–monitored, *Ae. j. japonicus* not found; Bosnia and Herzegovina: dark green–presence of *Ae. j. japonicus* according to this study, green–monitored, *Ae. j. japonicus* not found, light green–not monitored; Serbia: dark grey–presence of *Ae. j. japonicus* according to this study, grey–monitored, *Ae. j. japonicus* not found, light grey–not monitored).

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clearly influenced by the light green genotype predominating at Laze Prnjavor. These two locations are summarised under the microsatellite group 'East Croatia'. The genetic make-up of the remaining collection sites (Macelj, Konjščina, Orahovica, Laze Prnjavor, Ljuba) showed no similarity and was analysed individually.

The results of the PCoA (Fig 6) confirm the close relatedness of the *Ae. j. japonicus* populations from the Austrian/Slovenian and Southeast German/Austrian border regions with the 'West Croatia' group. The individuals from Konjšćina and Ljuba do not seem to be closely related to the other populations.

For 127 samples, *nad*4 sequences could be determined, showing variable base pairs in eight different positions. According to Fonseca et al. [36, 37, pers. comm.] and Zielke et al. [39], these single nucleotide polymorphisms could be assigned to nine *nad*4 haplotypes: H1, H3, H4, H9, H10, H12, H19, H33 and H35 (Fig 7).

Table 1. ***Aedes j. japonicus*-positive locations from which specimens were subjected to population genetic analysis.**

Country	County	Location	N_{Total}	N_S	N_M
Croatia	Primorje-Gorski Kotar	Zamost	1	1	1
		Delnice 1	1	1	1
		Skrad	13	13	13
		Kupjak 1	4	4	4
		Belo Selo	1	1	1
		Lič	11	11	11
	Lika-Senj	Kuterevo	5	1	5
	Krapina-Zagorje	Macelj	19	15	19
		Konjščina	12	10	12
	Virovitica-Podravina	Slatina	4	4	4
		Slatinski Drenovac	4	4	4
		Orahovica	19	18	18
	Požega-Slavonia	Laze Prnjavor	20	18	20
Bosnia and Herzegovina	Posavina	Odzak	20	19	9
		Brčko	7	6	7
Serbia	Vojvodina	Ljuba	7	1	1
Total			142	127	130

N_{Total} is the number of specimens processed, independent of successful analysis, N_S the number of individuals examined for *nad*4 sequences and N_M of individuals investigated by microsatellite analysis.

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Most common and widespread was haplotype H1 (n = 62), followed by H12 (n = 32). In most of the locations (n = 11), *nad*4 haplotype H1 was dominant, while H12 was dominant in the 'Bosnia and Herzegovina'-group and in Ljuba. In Orahovica, H9 was the dominant *nad*4 haplotype. Orahovica was the only locality with haplotypes H4 and H33 and Macelj the only

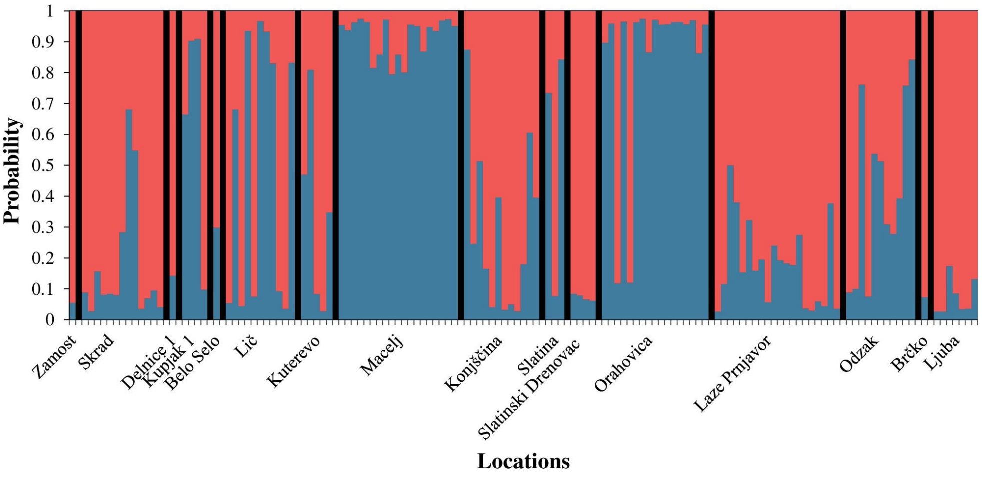

Fig 4. Bayesian cluster analysis of multilocus microsatellite genotyping (Δk = 2; optimal number of genetic clusters). Bars represent single individuals, colours the average probabilities of those to belong to one of the two most likely genetic clusters (blue = genotype 1; red = genotype 2).

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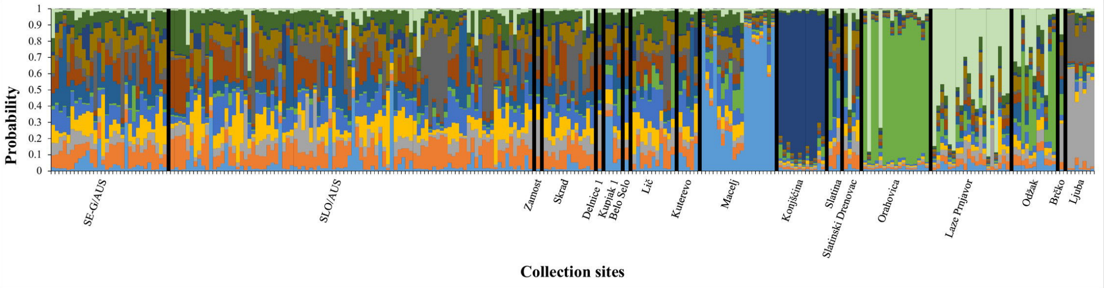

Fig 5. Bayesian cluster analysis of multilocus microsatellite genotyping (Δk = 13; optimal number of genetic clusters). Bars represent single individuals, colours the average probabilities of those to belong to one of the 13 most likely genetic clusters as calculated from previously investigated populations from Southeast Germany/Austria (SE-G/AU) and Austria/Slovenia (AUS/SLO) [42, 43] and of the populations from southeastern Europe as examined in this study.

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locality with haplotype H19. At five places, the haplotypes of one single individual could be identified only (Zamost, Delnice 1 and Belo Selo: H1, Kuterevo: H10, Brčko: H12). Fig 8 shows the relative relatedness of the different *nad*4 haplotypes found.

Fig 9 displays the geographic distribution of the most common haplotypes (H1, H5, H9, H10, H12), as related to the microsatellite make-up of *Ae. j. japonicus* at the various

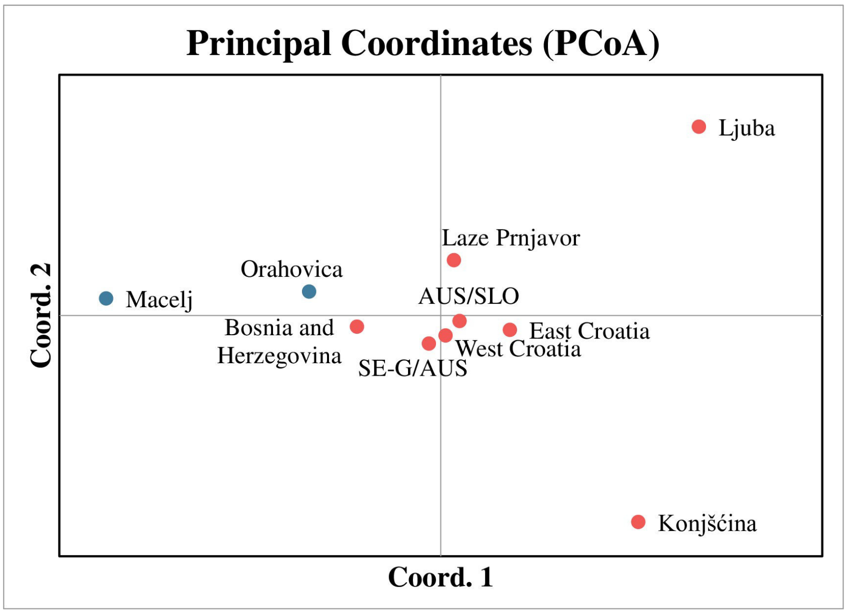

Fig 6. Principal coordinates analysis (PCoA) plot of pairwise population F_{ST} values for the locations sampled in southeastern Europe in this study and for previously investigated populations from Southeast Germany/Austria (SE-G/AU) and Austria/Slovenia (AU/SLO) [39, 45].

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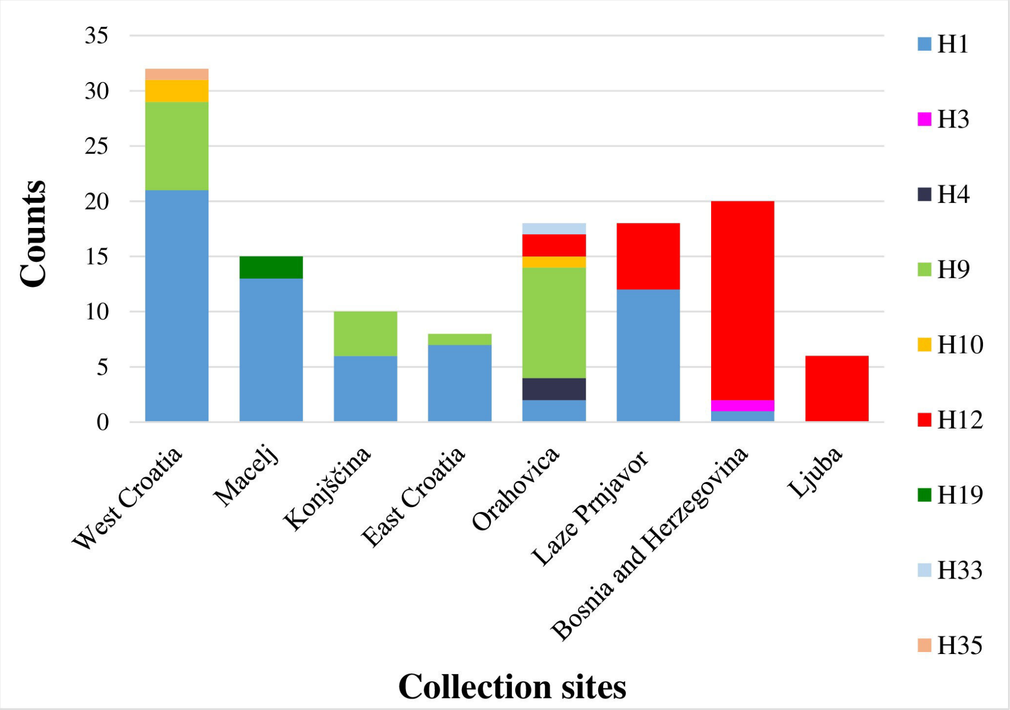

Fig 7. Frequencies of *Ae. j. japonicus* haplotypes found, based on presorting of populations according to microsatellite clusters.

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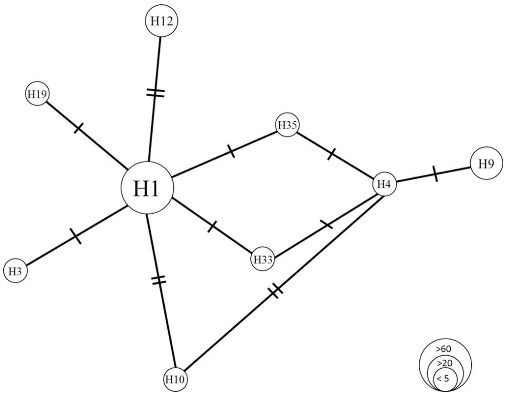

Fig 8. Relative relatedness of determined *nad*4 haplotypes. The circle sizes represent the number of detected haplotypes, every crossed line represents one transition.

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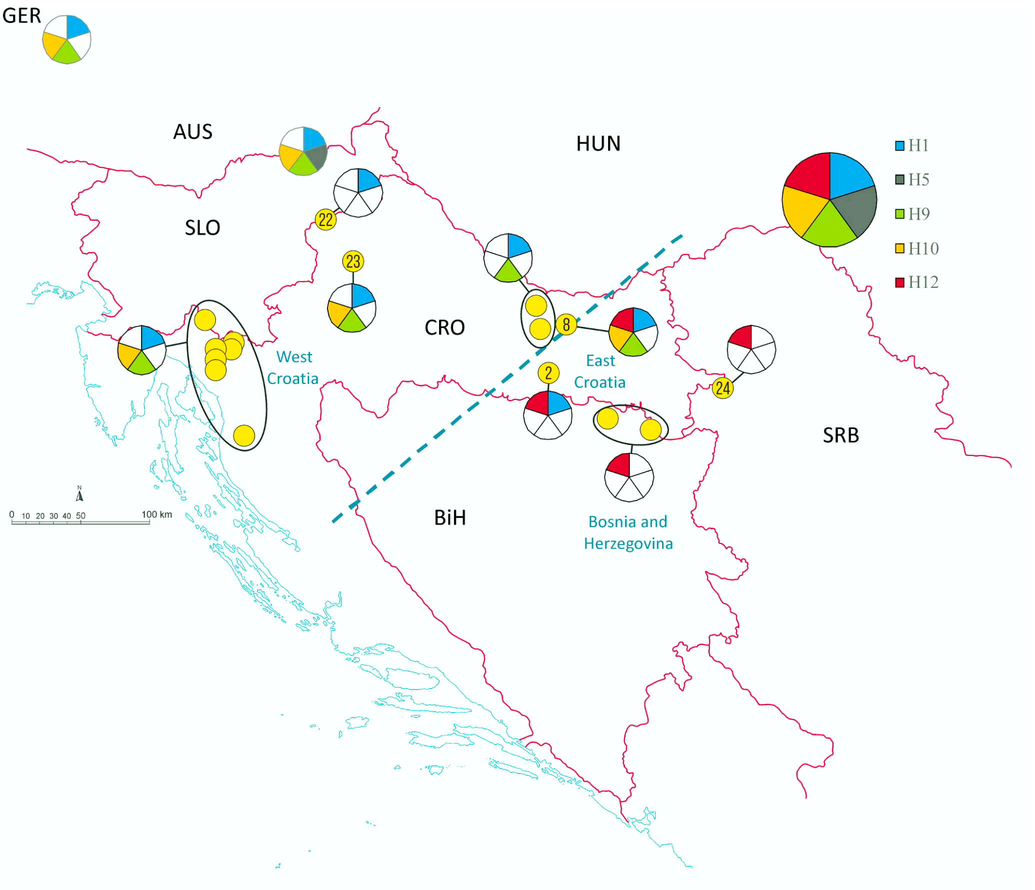

Fig 9. Geographic distribution of the most common *nad*4 haplotypes H1, H5, H9, H10 and H12 as related to the microsatellite make-up of *Ae. j. japonicus* at the various locations. In case of absent *nad*4 haplotypes, the pie chart field is white. Numbering of collection sites: 2 = Laze Prnjavor, 8 = Orahovica, 22 = Macelj, 23 = Konjšćina, 24 = Ljuba. Encircled collection locations mark the microsatellite groups 'West Croatia', 'East Croatia' and 'Bosnia and Herzegovina', the dashed blue line may represent a "genetic border".

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locations. In the western regions, haplotypes H1, H9 and H10 are very common. Towards the east, haplotypes H9 and H10 become rarer and are finally absent. In the very east, only H12 occurs. The distribution of the *nad*4-haplotyes shows a division of the collection sites into two areas, with Orahovica, displaying the highest diversity of haplotypes, in between. All collection sites east of Orahovica are characterised by a high number of *nad*4-haplotype H12. By contrast, the locations to the west are dominated by H1 and H9. The *nad*4 haplotypes detected in 'West Croatia', 'East Croatia' and 'Bosnia and Herzegovina' do not disagree to the microsatellite signature grouping, but a direct correlation of *nad*4-haplotype and genotype of the microsatellite analysis is not possible.

Discussion

According to the ECDC, ovitrapping is the method of choice for checking points of entry or small-size areas for presence and absence of invasive mosquito species [35]. To follow up on the spread of *Ae. j. japonicus* on a wider scale, Koban et al. [27] suggest larval sampling, e.g. in cemeteries, allotment gardens or deciduous forest with plenty of tree-holes. Thus, ovitrapping and inspection of cemeteries in urban areas has constituted the main method of monitoring in Croatia since 2013, after *Ae. j. japonicus* had first been recorded in western Croatia [28]. The same is true for Serbia regarding surveillance of *Ae. albopictus* (unpublished data), while invasive mosquito monitoring in Bosnia and Herzegovina was carried out only in 2015 and 2016 in the framework of the ECDC/EFSA VectorNet project. While focusing on higher altitudes, various approaches (adult trapping, larval sampling targeting both natural and artificial habitats, ovitrapping) were used in this study to find out about the further spread of *Ae. j. japonicus* in Croatia and adjacent regions.

The study confirms that trapping of adult *Ae. j. japonicus* is not a sensitive detection method. Experience shows that adults of this species are caught by commonly used mosquito traps only in areas with high population densities, and even in such areas only occasionally [46]. Therefore, adult trapping does not seem to be an appropriate approach when trying to track spreading, since newly occupied areas may be characterised by low population densities for a long time [7]. Larval sampling and ovitrapping are usually more successful [27, 47]. In our study, *Ae. j. japonicus* could be detected by larval sampling in the Croatian collection areas both in natural habitats such as ponds and rock pools, and artificial containers such as barrels, tyres and bathtubs, and by ovitrapping in all collection areas.

Due to previous sporadic or systematic sampling in the study area with no *Ae. j. japonicus* specimens being found, it must be assumed that the findings indicate a relatively new presence of the species. However, detection of invasive species certainly depends on sampling effort which is hard to measure and compare when different methodologies are applied in different regions or at different times. One must be aware that not finding a species does not necessarily mean that the species is absent, particularly at the beginning of its colonisation when population densities are low.

Obviously, population densities in the studied area were high enough for *Ae. j. japonicus* to be detected in numerous places. A high proportion of sampled locations (21 out of 58) turned out positive for *Ae. j. japonicus* larvae in Croatia. Particularly many new findings (11 out of 20) were made in Gorski Kotar area, south of the place where *Ae. j. japonicus* had been recorded first in Croatia. Although the new findings represent the first records in this area, the high number of positive sites suggests that the species had probably arrived much earlier and remained undetected. Population densities, however, that could give hints on the duration of establishment, have not been assessed.

Aedes j. japonicus is well adapted to temperate climates, and its eggs are capable of enduring cold and snowy winters as occurring in its endemic home range in northern Japan [34]. In Germany, larvae were found in water as cold as 4°C [47]. This cold tolerance allows the species to occur at higher elevations. In the southern Appalachians, USA, larvae were detected at altitudes of up to 1,500 m a.s.l. where winter temperatures can reach -18°C [5], while the species is also prevalent at 1,200 m a.s.l. in the German Black Forest [6]. Higher altitudes in southeastern Europe provide comparable conditions and seem to be readily accepted by *Ae. j. japonicus*, as indicated by the high percentage of breeding sites found around or above 700 m a.s.l. This may indicate that mountains are not necessarily barriers to the spread of *Ae. j. japonicus* and enable establishment and survival of this species, which is adapted to moderate climates, in relatively warm (or even subtropical) regions, e.g. of the Mediterranean.

After *Ae. j. japonicus* had been found in Krapinsko-Zagorska county in 2013 and again in 2014, a continuous spread was observed in Croatia: three surrounding counties were found positive in 2015, and another six, mostly in the north, in 2016. According to the present study, four more Croatian counties are now colonised, two counties in Bosnia and Herzegovina and one in Serbia. Only two counties of inland Croatia plus four coastal ones remain without documentation of *Ae. j. japonicus* (Fig 3). One of the inland counties is Sisačko-Moslavačka county in Central Croatia where *Ae. j. japonicus* is most probably present but has not been demonstrated due to insufficient monitoring. The second inland county is Vukovar-Srijem county in eastern Croatia, which has been included in the national monitoring since 2016 but without findings so far. The negative results are interesting considering the findings in Posavina county in Bosnia and Herzegovina in 2017 and Srem county in Serbia in 2018, which border Vukovar-Srijem county. Most likely, population densities in the latter area are rather low, with specimens escaping detection at many places.

In summary, *Ae. j. japonicus* appears to have quickly spread through Croatia from 2013 to 2018, with evidence of presence from all but six counties and even passing the borders to riparian countries in the east. Precisely, the species was found 250 km east and south of the place of its first record within five years, corresponding to an average dispersal of 50 km per year.

In the USA, three states notified collections of *Ae. j. japonicus* in 1998, New Jersey, New York and Connecticut [48, 49]. It is not clear when the introduction had taken place but, supposedly, several years before. In 2005, *Ae. j. japonicus* emerged in Missouri, 1,800 km away from New York [50]. By 2011, the species was noted in 33 US states, including Hawaii [8]. Thus, the speed of spreading was extremely high in the US, approximately 200 km per year, probably due to the tyre trade business [7, 51]. As this business is not well developed in Croatia, spread might essentially be active there [c.f. 47], supported by passive displacement by trade with, and vehicular transport of, horticulture equipment [c.f. 27].

To obtain clues about migration routes and pathways, individuals of *Ae. j. japonicus* collected from 16 locations in Croatia, Bosnia and Herzegovina and Serbia were genetically analysed for *nad*4 region DNA sequences and microsatellite loci signatures. The most common and widespread *nad*4 haplotype H1 is considered the haplotype from which most of the other existent haplotypes have evolved [36]. Because of its high abundance in the study area, H1 had probably been introduced first. The second most frequent *nad*4 haplotype, H12, differs by two bases from other haplotypes and only occurred in the eastern sampling area (Ljuba, Laze Prnjavor, Orahovica and Bosnia and Herzegovina). In 2013, Zielke et al. [52] had detected this haplotype in the Netherlands. Presumably, it must be attributed to a second introduction into the eastern study area. Haplotype H9 (third most common) was identified 23 times. This haplotype had been found by Zielke at al. [39, 45] in Belgium, the Netherlands and Slovenia. Due to its geographic distribution, which is similar to that of H1, it is likely that these haplotypes were imported to the study area simultaneously. In addition to H12, the haplotypes H3, H4 and H33 were also exclusively found in the eastern locations (Ljuba, Brčko, Odzak, Laze Prnjavor, Orahovica). These must therefore be attributed to introductions from unknown or non-investigated populations rather than from the known populations in Southeast Germany/Austria and Austria/Slovenia.

The collection locality Orahovica shows the highest *nad*4 diversity (six haplotypes). All locations east of this point are dominated by haplotype H12 (in addition to H1), and almost all locations (with more than one individual analysed) west of this point show a high number of H9. Therefore, Orahovica can be considered a *nad*4 haplotype border region, influenced by both eastern and western populations.

Furthermore, the cluster analysis shows a high probability of the eastern locations (Brčko, Odzak, Laze Prnjavor, Ljuba) to belong to genotype 2, just like specimens from Konjščina.

However, the PCoA suggests that these two groups are genetically isolated from each other, which may have been caused by gene flow between these and other, unknown populations. All other haplotypes found in the study area are likely to have evolved from H1, H9 and H12. The genotype signatures of the microsatellites also indicate at least two independent introductions.

Most of the results of the PCoA and the Bayesian analyses correspond well to each other. Accordingly, the microsatellite group 'West Croatia' seems to be more closely related to the populations from SE-G/AUS and AUS/SLO, although comparative analysis of the microsatellite dataset of these populations shows a high number of probable genetic clusters and does not allow substantiated conclusions. Nevertheless, a genetic similarity between these three and the genetic group 'East Croatia' is given, suggesting gene flow may have taken place. Genetically separated from the other populations and of unknown origin are the collection sites Ljuba, Macelj and Konjšćina.

Aedes j. japonicus is not considered an important vector in its native distribution area, and evidence for a substantial role in the transmission of disease agents in the field is generally missing. By contrast, experimental data from the laboratory do suggest a vector potential for several viruses of medical and veterinary relevance, including WNV [53]. *Aedes j. japonicus* was found infected with WNV in the field [11], although this does not allow any conclusions on its vector competence since complete homogenised mosquitoes had been examined. In the last decade, mosquito-borne diseases broke out almost every year in Croatia. Human cases of dengue [54], West Nile fever [55] and Usutu fever [56] infections were registered. The highest prevalence is attributed to human neuroinvasive disease caused by WNV, which has emerged in Croatia every year since 2012. While 38 cases of human West Nile fever were noted until 2017, the number of cases accumulated to 51 in 2018 alone [57], and for the first time eight people died (Vilibić-Čavlek, pers. comm). During the same year, Serbia even recorded 415 human cases [57] with 36 deaths [58]. Therefore, the occurrence of another potential vector in this endemic area, such as *Ae. j. japonicus*, may increase the potential risk of WNV transmission.

Conclusion

The ongoing quick spread through Croatia and the first records of *Ae. j. japonicus* in neighbouring Bosnia and Herzegovina and Serbia underline the strong expansion drive of this invasive mosquito species and its high adaptation to temperate conditions, irrespective of geography. It appears that *Ae. j. japonicus* prefers higher altitudes in Mediterranean countries to find such conditions, but this has to be further elucidated. The close relatedness of the samples collected at localities northwest of Orahovica to remote populations from more western European countries confirm that introduction and spread are mainly mediated by humans, although on a regional scale, active migration does certainly contribute. Given all these supporting factors, it cannot be expected that the spread of *Ae. j. japonicus* will soon come to an end in Europe. In southeastern Europe, a further spread is anticipated at least in temperate climate areas such as mountainous ones.

Supporting information

S1 Table. Study locations, collection approaches and outcome.
(DOCX)

Acknowledgments

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Publication 4: Emergence of the invasive Asian bush mosquito Aedes (Hulecoeteomyia) japonicus (Theobald, 1901) in the Czech Republic

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Parasites & Vectors

RESEARCH

Emergence of the invasive Asian bush mosquito *Aedes* (*Hulecoeteomyia*) *japonicus* (Theobald, 1901) in the Czech Republic

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Abstract

Background: *Aedes japonicus* is a mosquito species native to North-East Asia that was first found established outside its original geographic distribution range in 1998 and has since spread massively through North America and Europe. In the Czech Republic, the species was not reported before 2021.

Methods: *Aedes* invasive mosquitoes (AIM) are routinely surveyed in the Czech Republic by ovitrapping at potential entry ports. This surveillance is supported by appeals to the population to report uncommon mosquitoes. The submission of an *Ae. japonicus* specimen by a citizen in 2021 was followed by local search for aquatic mosquito stages in the submitter's garden and short-term adult monitoring with encephalitis virus surveillance (EVS) traps in its surroundings. Collected *Ae. japonicus* specimens were subjected to *nad*4 haplotype and microsatellite analyses.

Results: *Aedes japonicus* was detected for the first time in the Czech Republic in 2021. Aquatic stages and adults were collected in Prachatice, close to the Czech-German border, and eggs in Mikulov, on the Czech-Austrian border. Morphological identification was confirmed by molecular taxonomy. Genetic analysis of specimens and comparison of genetic data with those of other European populations, particularly from Germany, showed the Prachatice specimens to be most closely related to a German population. The Mikulov specimens were more distantly related to those, with no close relatives identifiable.

Conclusions: *Aedes japonicus* is already widely distributed in Germany and Austria, two countries neighbouring the Czech Republic, and continues to spread rapidly in Central Europe. It must therefore be assumed that the species is already present at more than the two described localities in the Czech Republic and will further spread in this country. These findings highlight the need for more comprehensive AIM surveillance in the Czech Republic.

Keywords: *Aedes japonicus*, Central Europe, Introduction, Invasive species, Surveillance, Vector

Background

The presence of four *Aedes* invasive mosquito (AIM) species in Europe is consistently noted: *Aedes aegypti*, *Aedes albopictus*, *Aedes japonicus* and *Aedes koreicus* have been introduced into many European countries and have succeeded in establishing in several of them. Rather than being a nuisance, these mosquito species may pose a serious threat to public health by their capability of transmitting arboviruses such as dengue, chikungunya, yellow fever and Zika viruses. The intercontinental spread of these AIMs is facilitated by global trade [1] and is attributable to their drought-resistant eggs [2]. Establishment and further dispersal in the new geographical regions are possible when climatic and environmental conditions are adequate. In Central Europe, *Ae. japonicus* and *Ae. koreicus* find similar (moderate) climatic conditions as in their

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original Asian distribution range, while the thermophilic species *Ae. albopictus* increasingly adapts to moderate temperatures and takes advantage of climate warming.

Aedes (*Hulecoeteomyia*) *japonicus* (Theobald 1901), also known as the Japanese bush mosquito or Asian rock pool mosquito, is native to North-East Asia [3]. Its first finding in Europe was reported in 2000 from north-western France, where two larvae were collected in car tyres [4]. Subsequently, the species was found in Belgium in 2002 as larvae and in 2004 as adults [5]. Later reports describe occurrences in Switzerland and Germany in 2008, Austria and Slovenia in 2011, and the Netherlands and Hungary in 2012 [6–8]. In 2013, the establishment of *Ae. japonicus* was confirmed in France [9], and in the same year, the emergence of this mosquito was reported from Croatia [10]. Its spread continued in 2015 to Liechtenstein and Italy, in 2017 to Bosnia and Herzegovina, and in 2018 to Serbia, Spain and Luxembourg [11–14]. The most recent report of *Ae. japonicus* introduction was added from Romania for 2020 [15].

Unlike *Ae. albopictus*, which is exclusively spread by human mediation, *Ae. japonicus* spreads to new areas by active dispersal, too [16, 17], with spreading corridors mainly being the borders between rural and urban habitats, such as suburban gardens, vineyards, small patches of forest adjacent to fields or forest edges with streams [12, 18, 19]. Adults usually live in forests but migrate to gardens or cemeteries to lay eggs, where they find more potential breeding sites, i.e. artificial containers with stagnant water [20]. In the United States, larvae of *Ae. japonicus* are commonly found in rock pools, similar to their native range, but in Europe they have also been collected from tree holes and a variety of man-made containers such as barrels, tyres, bathtubs or flower vases [12, 13, 21]. *Aedes japonicus* typically seeks larger containers than other container-breeding AIMs [19, 21]. In Switzerland and Germany, the species *Aedes geniculatus* and *Anopheles plumbeus* use similar breeding sites as *Ae. japonicus* [18, 20].

Moreover, *Ae. japonicus* is well adapted to cold winters and snow. Its larvae have been found in water as cold as 4 °C [16]. Its occurrence above 700 m above sea level (a.s.l.) in south-eastern Europe [12] and at 1200 m a.s.l. in the German Black Forest [22] suggests that crossing mountains should not be very problematic for this species. In the southern Appalachians in the United States, *Ae. japonicus* was the only container-breeding mosquito species found above 1400 m a.s.l. [21].

Aedes japonicus feeds on a variety of hosts, but appears to be less anthropophilic than other AIMs [23].

There is no evidence of pathogen transmission by *Ae. japonicus* under natural conditions. Therefore, this species is considered less important as a public health vector than *Ae. aegypti* or *Ae. albopictus*. However, genetic material of West Nile virus (WNV) has been demonstrated several times in *Ae. japonicus* collected in the field in the USA [24, 25]. Furthermore, in laboratory studies, this mosquito shows vector competence for eastern equine encephalitis, La Crosse, St. Louis encephalitis, Rift Valley fever, Usutu, chikungunya, dengue and Zika viruses [26–31] in addition to WNV [25], as well as for the nematodes *Dirofilaria repens* and *Dirofilaria immitis* [32].

We here describe the first encounters with *Ae. japonicus* mosquitoes in the Czech Republic, which took place in 2021, independently in two areas more than 190 km apart. One finding locality was localised close to the border with Germany and the second on the border with Austria.

Methods

Study sites and mosquito collection

In the evening hours of 15 June 2021, a citizen of the town of Prachatice in South Bohemia caught a strangely coloured adult female mosquito in his house. He sent five pictures of this mosquito to our laboratory for preliminary identification. Since it was not possible on these to distinguish between *Ae. japonicus* and *Ae. koreicus*, the pictures were followed by the physical specimen for accurate identification. On 9 July, two additional adult mosquitoes of the same appearance (one male, one female) were captured by the citizen at the same location.

From 11 to 13 August, the garden of the reporting resident and its surroundings were checked for AIMs by dipping water containers for aquatic mosquito stages and trapping adult mosquitoes by encephalitis virus surveillance (EVS) traps equipped with dry ice as an attractant. Sampling of aquatic mosquito stages was done repeatedly on 11 and 12 August from three dark green plastic rainwater collection barrels, that stood immediately against the house wall, each holding approximately 300–400 L of water and having a water surface area of about 0.8 m^2. From 11 to 12 August, eight EVS traps were operated within 100 m and another seven traps within 1.5 km from the submitter's house. Two EVS traps were placed even further away (about 3.5 km). The traps were located at shady places near the gardens of family houses, in an allotment area and on the edge of a forest. Three of the traps in the 100 m diameter were placed about 20 m from the rainwater barrels, another two about 50 m and three about 80 m. One of the three traps within 20 m distance was equipped with sweaty socks as an additional attractant. All traps were run overnight. On 12 August, one trap was kept working all day next to the rainwater barrels. From the evening of 12 August to the morning of 13 August, a total of 14 EVS traps were operated in close vicinity (100 m) of the rainwater barrels in the submitter's garden.

The city of Prachatice (Fig. 1) is situated in the foothills of the Bohemian Forest and borders its protected landscape area, which includes the Bohemian Forest National Park. The town is located in a valley basin with a flowing stream at an altitude of 561 m a.s.l. In the surrounding area, there are many suburbs with gardens and gardening colonies. The landscape is hilly and consists of forest and meadows rather than agricultural land. About 25 km to the south-east runs the German-Czech border, with *Ae. japonicus* occurrence reported not far from the border on the German side (Kampen & Werner, unpublished data).

Ovitrapping was done in the framework of a long-term surveillance programme focusing on the occurrence of *Ae. albopictus* at the border between the Czech Republic and Austria [33, 34]. The site 'Mikulov 2' (Fig. 1) is one of three parking lots in the outskirts of the town of Mikulov included in the monitoring programme. This place is located between the motorway A5 on the Austrian side and the motorway 52 to Brno on the Czech side and is used by lorry drivers as a stopover. For a detailed description of the surveillance methodology by ovitrapping, see Rudolf et al. [34].

Rearing and identification of mosquitoes

Oviposition supports with eggs from ovitraps were brought to the laboratory where the eggs were flooded in jars with stale tap water for the larvae to hatch. Hatched larvae were fed ground fish food (Tetra GmbH, Melle, Germany). Larvae from the rainwater barrels were transferred to jars together with original barrel water, the organic compounds of which served as larval food during further development. Jars were covered with nets until emergence of adults, which were collected with a battery-powered aspirator (Hausherr's Machine Works, Toms River, NJ, USA) and killed by freezing at −20 °C for at least 24 h.

Adults were morphologically identified using the determination key provided in Becker et al. [35]. Morphological determination was confirmed genetically on one specimen from Prachatice and two specimens from Mikulov (adults). For this purpose, one leg per mosquito specimen was individually homogenised in 150 µl sterile demineralised water with sterile 5 mm steel beads at 30 Hz for 60 s in a TissueLyser II (Qiagen, Hilden, Germany). Genomic DNA was extracted from

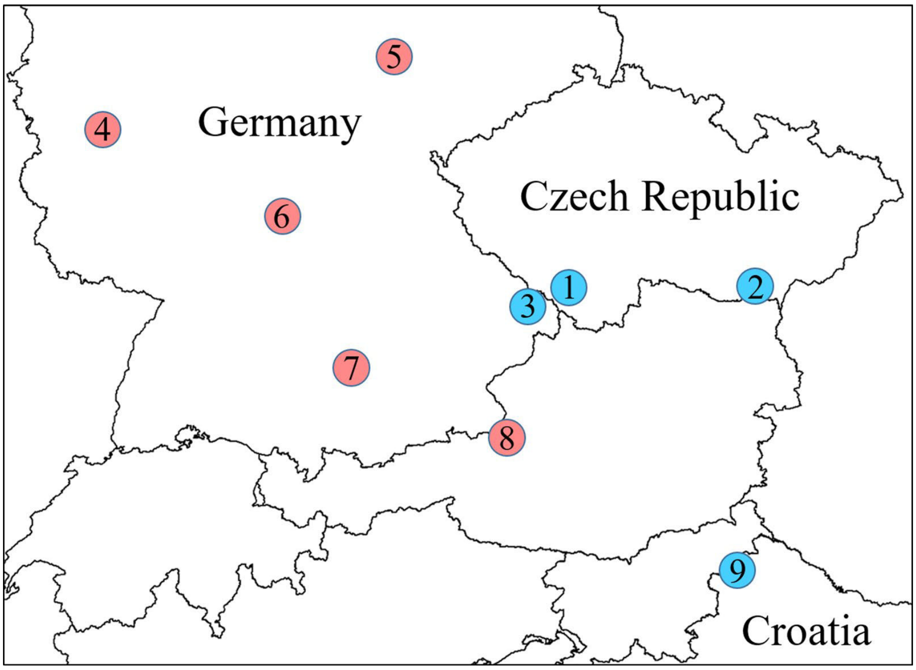

Fig. 1 Location of *Ae. japonicus* collection sites in the Czech Republic (nos. 1, 2) and of *Ae. japonicus* populations genetically compared with them (nos. 3–9). Names of locations are provided in Table 1; colours of dots refer to the two genetic clusters as shown in Fig. 3

100 µl of the homogenates using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's protocol. The DNA was eluted in 80 µl elution buffer and stored at −20 °C until further processing.

Partial segments of the mitochondrial cytochrome c oxidase subunit 1 (*cox*1) [36, 37] and of the NADH dehydrogenase subunit 4 (*nad*4) genes were amplified for mosquito identification [38] using Combi PPP Master Mix (Top-Bio, Vestec, Czech Republic) following published protocols [39]. Polymerase chain reaction (PCR) products were sequenced bidirectionally according to Janssen et al. [40] and the sequences obtained blasted to the GenBank sequence library (https://blast.ncbi.nlm.nih.gov).

Population genetic analysis

To obtain clues on the origin of the Czech *Ae. japonicus* samples and their relationships to conspecific populations in Europe, population genetic analyses were performed on 20 individuals from Prachatice, 14 individuals from Mikulov and 20 individuals from Grafenau, the closest German place to the Czech border and Prachatice known to be colonised by *Ae. japonicus* (beeline distance between Grafenau and Prachatice ca. 50 km). The data obtained from these three locations were compared with data from previously investigated *Ae. japonicus* populations from other areas in Germany and one location in Croatia (Table 1). Analysis was performed on *nad*4 haplotypes and microsatellites. For the latter, seven polymorphic loci (OJ5, OJ10, OJ70, OJ85, OJ100, OJ187, OJ338) were genotyped as described by Janssen et al. (2019). The results of the microsatellite analysis were interpreted with Geneious Prime version 2021.0.1 (Geneious Biomatters, Auckland, New Zealand), subjected to a Bayesian cluster analysis using STRUCTURE [41] and evaluated with STRUCTURE HARVESTER [42]. Furthermore, based on Nei's genetic distance and pairwise F_{ST} values, a principal coordinate analysis (PCoA) was performed.

Results

Mosquito collection and identification

The first adult female mosquito from the Prachatice locality was identified as *Ae. japonicus* on the basis of characteristic morphological features—scutal stripes, scales on the sides of the thorax, tergite scale pattern and hind leg colouration (Fig. 2). Molecular analysis of that specimen revealed 99.86% similarity of the *cox*1 partial sequence (GenBank accession no. OM307664) to *Ae. japonicus* GenBank accession no. KF211505 from Germany and 99.35% similarity of the obtained *nad*4 partial sequence (GenBank accession no. OM307666) to *Ae. japonicus* GenBank entry AF305879 from Germany.

Adults developed from several dozen larvae and pupae collected from the three rainwater barrels (out of hundreds present) were also morphologically identified as *Ae. japonicus*. Other mosquito species detected in the barrels belonged to the *Culex pipiens* and *Anopheles maculipennis* complexes.

No *Ae. japonicus* was collected in the EVS traps during the first night of trapping in Prachatice, but one *Ae. japonicus* female was captured during the second night with the EVS trap equipped with sweaty socks. All other EVS traps remained negative for *Ae. japonicus*. However, *Cx. pipiens* complex females and some *Aedes vexans* specimens were collected by the EVS traps within the 100 m radius, and several individuals of *Ae. geniculatus* and *An. plumbeus* were collected from EVS traps placed on the forest edge. One EVS trap placed in a garden colony contained an *An. maculipennis* complex female.

Thirty-six morphologically identified adult *Ae. japonicus* (25 females, 11 males) emerged between 28 and 30 August 2021 from ovitrap egg collections carried out from 10 to 18 August 2021 at 'Mikulov 2'. The sequences of the two tested *Ae. japonicus* individuals were

Table 1 Origin and number of processed specimens from current and previously investigated populations of *Ae. japonicus* in Europe

Population	Country	Collection year	Number of specimens processed	Reference
(1) Prachatice	Czech Republic	2021	20	This study
(2) Mikulov	Czech Republic	2021	14	This study
(3) Grafenau	Germany	2019	20	This study
(4) Linz	Germany	2017	24	[40]
(5) Burgscheidungen	Germany	2017	20	[40]
(6) Wurzburg	Germany	2017	30	[40]
(7) Augsburg	Germany	2017	19	[40]
(8) Berchtesgaden	Germany	2017	13	[40]
(9) Macelj	Croatia	2017	15	[40]

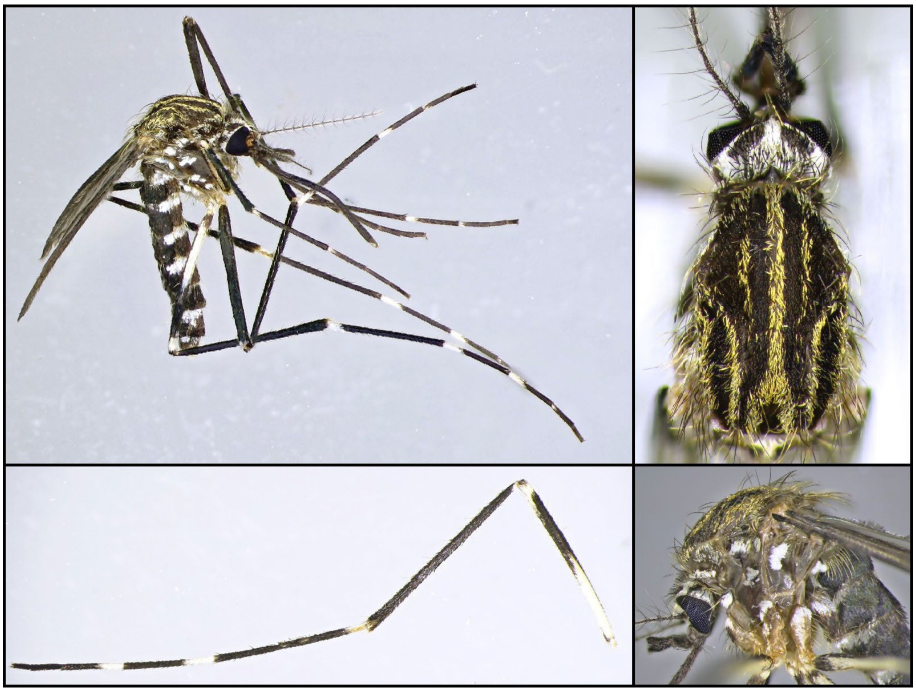

Fig. 2 The very first *Ae. japonicus* mosquito detected in the Czech Republic, submitted from Prachatice. **a** Overall view of the specimen. **b** Five stripes of golden scales on the scutum. **c** Hind leg with no white rings on tarsal segments IV and V (as opposed to *Ae. koreicus*). **d** Lateral view of thorax

identical (GenBank accession nos. OM307665 for *cox*1 and OM307667 for *nad*4) and shared 99.58% nucleotide homology with a *cox*1 sequence from an isolate from Germany (GenBank accession number KF211505) and 99.14% nucleotide homology with a *nad*4 sequence from an isolate from the USA (GenBank accession number AF305879).

Population genetic analysis

*Nad*4 haplotype analysis was performed on 54 specimens from Prachatice, Mikulov and Grafenau (south-eastern Germany). The alignment indicated 21 variable nucleotide positions, leading to four different *nad*4 haplotypes: H1, H3, H9 and H21 (Table 2). Six individuals were characterised by heteroplasmy, the presence of different mitochondrial DNA (mtDNA) variants in one organism, as indicated by peaks for two different nucleotides at the same position in the sequencing electropherogram.

Analysable microsatellite data were obtained for 53 samples from the two Czech populations and the German population from Grafenau. For comparison, data available from previous studies on German and Croatian *Ae. japonicus* populations were included in the analysis (Additional file 1: Table S1). The Bayesian cluster analysis showed the highest probability for the existence of two genetic clusters among the tested populations ($k=2$; $\Delta=160.08$). According to this analysis, the populations

Table 2 *Nad*4 haplotypes detected in *Ae. japonicus*

Population	Number of individuals examined	No. of heteroplasmic individuals	*nad*4 haplotypes			
			H1	H3	H9	H21
Prachatice	20	5	5	2	3	5
Mikulov	14	0	0	0	13	1
Grafenau	20	1	14	0	5	0

from Prachatice, Mikulov, Grafenau and Macelj (northwestern Croatia) have a high probability of belonging to the same microsatellite genetic cluster 1 (blue colour), whereas the previously investigated populations from Germany rather appear to belong to genetic cluster 2 (red colour) (Fig. 3). Despite the same principal genetic cluster 1, the Mikulov population seems to be somewhat different from the Prachatice, Grafenau and Macelj populations.

The results of the PCoA, based on F_{ST} values and Nei's genetic distance of the microsatellite locus data, show a close genetic relatedness of the Prachatice and Grafenau populations. The other included populations are much more distantly related (Fig. 4).

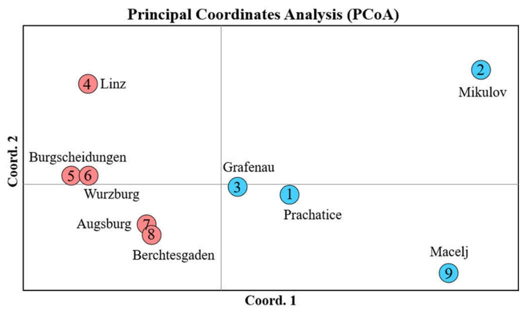

Fig. 4 Principal coordinates analysis (PCoA) plot of pairwise population F_{ST} values for the investigated *Ae. japonicus* populations from the Czech Republic, Germany and Croatia. The colours of the dots (blue and red) represent the principal microsatellite genotype according to Fig. 1

Discussion

Emergence of *Ae. japonicus* in the Czech Republic

The Bohemian Forest region in which the town of Prachatice is embedded provides appropriate landscape structures and paths for the spread of *Ae. japonicus* (e.g. more rural than urban area, presence of many deciduous forest patches and occurrence of transition zones between forest and local settlements) [18, 20]. Based on the demonstrated presence in close-by Germany and the genetic data, an origin of the *Ae. japonicus* specimens from Prachatice and a (possibly active) spread of *Ae. japonicus* from Germany can be assumed. This, however, suggests that *Ae. japonicus* is likely to have already been established between Prachatice and the Czech-German border as well and would explain the high abundance of larvae (hundreds) found in the rainwater barrels in the submitter's garden, which were hardly produced by a single female. Amazingly, no adult specimens were trapped in Prachatice but this could be due to poor trappability of this species by EVS traps [43]. Larval sampling in Prachatice and the area west of it is planned for 2022 to check for further *Ae. japonicus* occurrence.

Locality 'Mikulov 2' is a parking lot with the first gas station on the Czech side of the Czech-Austrian border. A few family houses are about 250 m away. The surrounding landscape on both sides of the border consists mainly of open fields with a few small forest patches and vineyards. Although shady transects are missing, it is not clear whether the emergence of *Ae. japonicus* eggs in

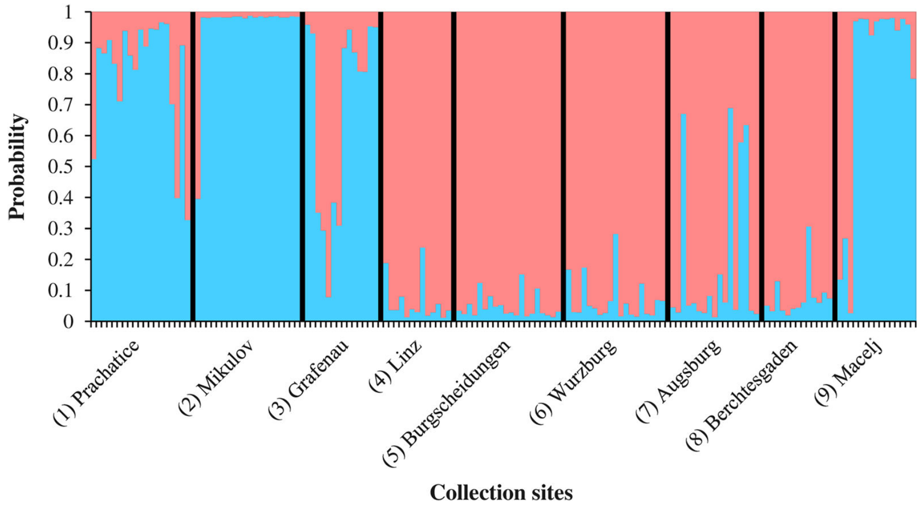

Fig. 3 Results of microsatellite multilocus genotyping for $k = 2$ ($\Delta = 160.08$), with each bar representing a single individual and the different colours representing the probability of that individual belonging to the corresponding genetic cluster

'Mikulov 2' is attributable to passive transport and introduction via long-distance traffic of a single gravid female along the highway from the south or to active dispersal. The latter is assumed to have taken place with specimens of *Ae. japonicus* found in the framework of the Austrian mosquito monitoring programme in the Lower Austrian district of Gmünd [44], about 10 km from the Czech border and far from an international traffic route.

Population genetic analysis

The most frequent *nad*4 haplotype found in the *Ae. japonicus* populations from Prachatice, Mikulov and Grafenau was H9. This *nad*4 haplotype is known from several populations in Europe: Belgium, Austria, The Netherlands, Slovenia and Croatia [12, 39, 40, 45–47]. *Nad*4 haplotype H3 was exclusively detected in Prachatice. This haplotype has been found in populations in South Germany, The Netherlands and Bosnia and Herzegovina [12, 45]. A carry-over by mosquito displacement from those populations to the Czech Republic is possible. The same is true for *nad*4 haplotype H21, as found in both Czech populations in this study, which had previously been detected in South Germany and Bosnia and Herzegovina [12, 45]. In summary, since Prachatice is represented by all four haplotypes found in this study and both Mikulov and Grafenau by two each, but different ones (Table 2), the haplotype analysis is not informative enough to deduce relatedness of the tested populations and displacement/migration routes.

With regard to the results of the microsatellite analysis and the PCoA (Figs. 3, 4), the spatially close collection sites Prachatice and Grafenau show high genetic relatedness, with the second next closest relationship to the populations from Macelj (microsatellite multilocus genotype) or Augsburg and Berchtesgaden (PCoA). A common origin of these populations could be in Austria or Slovenia, where *Ae. japonicus* is widely distributed [48]. The Mikulov population seems to be genetically more different from the other populations tested. A new introduction from a European population not included in the analysis or even from overseas could be the reason.

Conclusions

Invasive *Ae. japonicus* mosquitoes were reported for the first time in the Czech Republic. Due to the low diversity of *nad*4 haplotypes, the two populations from the Czech Republic as well as that from Grafenau, south-eastern Germany, can be assumed to be relatively young. However, owing to the already wide distribution of *Ae. japonicus* in Europe with ongoing mixture of populations, haplotypes cannot be assigned to certain populations anymore as was the case during the first years of invasion of the western world (e.g. [38, 49, 50]). According to microsatellite and PCoA analyses, the Prachatice population was most likely introduced to the Czech Republic from Germany, while the Mikulov population is at least admixed with genetic material from one (or several) other population(s) of unknown origin.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13071-022-05332-5.

Additional file 1: Table S1. Microsatellite fragment lengths of *Ae. japonicus* populations studied (0: not analysable).

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Author contributions

IR, HK and JV designed the study, JV, IR, OŠ and SŠ participated in mosquito sampling and morphological determination, JV carried out the basic molecular taxonomy, NJ and HK performed the population genetic work and the corresponding data interpretation. JV, IR, NJ and HK wrote the manuscript. All authors read and approved the final manuscript version.

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Availability of data and materials

Data supporting the conclusions of this article are included within the article. Representative DNA sequences have been deposited in the GenBank database under the accession numbers OM307664–OM307667.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Publikation 4: Vojtíšek J, Janssen N, Šikutová S, Šebesta O, Kampen H, Rudolf I (2022) Emergence of the invasive Asian bush mosquito *Aedes* (*Hulecoeteomyia*) *japonicus* (Theobald, 1901) in the Czech Republic. Parasit Vectors. 15:250.

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Ivo Rudolf:	Konzept, Datenanalyse und -interpretation, Fördermittelakquise, Projektadministration, Erstellung des Manuskripts und der Abbildungen

Bestätigung der Angaben:

PD Dr. Helge Kampen: Nele Janssen:

___________________ ___________________

Results and conclusion of the published studies

The main results of the conducted studies and published publications are summarized in the following points.

I. Current distribution of *Ae. japonicus* in Europe and critical review of the monitoring strategies and recommendations to harmonise monitoring:
    (i) Surveillance studies suggest four populations of *Ae. japonicus* to occur in Europe (southeastern Germany/Austria, northern Germany, The Netherlands and eastern Austria/Slovenia/Hungary/Croatia/Italy).
    (ii) For standardisation of monitoring strategies, the use of a grid cell pattern is advisable.
    (iii) At least three sites (cemeteries, allotment gardens) per cell should be investigated.
    (iv) All water containers in a suitable site should be investigated (minimum of 150 containers per 100 km²).
    (v) To define boundaries of colonisation, a circle of negative sampling sites around every positive sampling site should be created.
    (vi) Investigation should be conducted during the seasonal activity peak of *Ae. japonicus* (August to September).

II. Populations genetics of *Ae. japonicus* in Germany:
    (i) The German populations of *Ae. japonicus* still fall in two genetic clusters (microsatellite genotypes).
    (ii) The northern population (2015: split from the western population) was genetically closer to the populations from Central and South Germany than to the western population.
    (iii) The genetic diversity increased from three *nad*4 haplotypes in 2012 to 12 in 2017.
    (iv) Since the previous population genetic studies by Zielke et al., new introductions are likely.

III. Populations genetics of *Ae. japonicus* in southeastern Europe:
    (i) At least two introduction events of *Ae. japonicus* into southeastern Europe took place.
    (ii) The western populations showed a high genetic similarity with populations from Austria/Slovenia (2014).

    (iii) The eastern populations were genetically different and could not be assigned to a probable origin.

IV. Population genetics of *Ae. japonicus* in the Czech Republic:
    (i) A common origin of these populations could be in Austria or Slovenia.
    (ii) A carry-over from South Germany or Bosnia and Herzegovina is possible.
    (iii) The Mikulov specimens are more distantly related than the Prachatice population and the other populations investigated.

Discussion

Since 2000, when *Ae. japonicus* was detected in Europe for the first time, the species has successfully established in 15 European countries (2019), and its spread is still ongoing. Although populations have been successfully eliminated (Schaffner et al. 2009) or have remained locally restricted (Versteirt et al. 2009), it is not conceivable anymore to eradicate *Ae. japonicus* from Europe. However, even though *Ae. japonicus* must nowadays be considered part of the European fauna, efforts should be made to keep population densities at a possible minimum. In this context, large-scale surveillance programmes are still important, especially regarding the species' role as a potential vector of several diseases.

For container-breeding mosquitoes, cemeteries are preferred habitats, due to the high density of artificial containers (flower vases, wells, birth baths, etc.), and most of the published monitoring studies investigate cemeteries to deduce the distribution of *Ae. japonicus* (Vezzani 2007). Almost all of the published *Ae. japonicus* monitoring activities focus on larval stages, since adults are not readily trappable and cemeteries are easily accessible. Otherwise, the studies show huge methodological differences, e.g. regarding numbers of checked containers and collection sites, type and quantity of investigated containers, number of recurring visits and declaration of negative sites. An approach to comparable and standardised large-scale monitoring could be the use of a virtual grid with defined cell size, which is generated for a given region. The borders of a populated area is defined as a circle of negative grids around every positive grid.

In 2001, Fonseca et al. (2001) determined for the first time the genetic diversity of *nad*4 haplotypes among *Ae. japonicus* populations in the US and compared them to samples of populations from Japan and to individuals collected from New Zealand. They found 15 different *nad*4 haplotypes, with only haplotype H1 being found in all populations. H12 was detected in the US and Japan and H5 in Japan and New Zealand while the other *nad*4 haplotypes were unique for the US (H2, H4, H6, H10, H11, H9), Japan (H3, H7, H8, H14, H15) or New Zealand (H13). Fonseca et al. (2001) suggested at least two different introductions into the US: into the north-eastern states (H2, H4, H6, H10; H11), and into Pennsylvania and Maryland (H9, H12). In a second study, Fonseca et al. (2009) investigated specimens from the US for differences in their genetic make-up by microsatellite analysis. Differences found indicated two separate introductions again, creating populations with increasing genetic diversity.

Aedes japonicus was collected in 2017, 2018 and 2020 at various sites in Germany (Janssen et al. 2019), Croatia, Serbia, Bosnia and Herzegovina (Janssen et al. 2020) and the Czech Republic (Vojtíšek et al. 2022). These individuals were genetically analysed concerning mitochondrial single nucleotide polymorphism (*nad*4 haplotypes) and the frequency of short DNA repeats (microsatellites). Four hundred fourty-five individuals of *Ae. japonicus* from five different countries were analysed. The *nad*4 haplotype from 384 specimens was determined, and the microsatellite analysis produced analysable data for 417 samples. In total, 16 different *nad*4 haplotypes were detected: H1, H3, H4, H5, H9, H10, H11, H12, H17, H19, H21, H33, H35, H43, H45 and H46 (Fonseca et al. 2001, 2010, Fonseca, pers. comm., Zielke et al. 2015, Janssen et al. 2019). With a high probability, the results of the microsatellite analysis fell in two genetic clusters (microsatellite genotypes) for every study.

*Nad*4 haplotype H1 is most common and widespread in all investigated collection sites (except the two collection sites in the Czech Republic). In Germany, 33 % and in southeastern Europe almost 50 % of the investigated individuals displayed haplotype H1. This *nad*4 haplotype was not detected at only 6 of the 29 collection sites (Hanover, Augsburg, Kuterevo, Ljuba, Brčko, Mikulov). H1 shows the greatest agreement regarding the number of transitions with the other *nad*4 haplotypes. For seven haplotypes, the difference from H1 is one transition, for six haplotypes two transitions, and for two haplotypes three transitions. Thus, almost 50 % of the detected haplotypes may have evolved from H1. The high frequency of *nad*4 H1 in nearly all of the collection sites examined does not necessarily represent gene flow between them, but probably a common origin of all populations (Fonseca et al. 2001). Because of its high abundance, H1 had probably been introduced first into new areas.

The second most common *nad*4 haplotype is H9 (19 % of the detected haplotypes) which was determined at 19 (from 29) collection sites in the presented studies. Only four collection sites lacked H1 or H9 (Augsburg, Kuterevo, Ljuba, Brčko). This haplotype was the only one indicated by Fonseca et al. (2001) for populations in Pennsylvania and Maryland and had been found in Belgium (Zielke et al. 2014, Smitz et al. 2021), Austria/Slovenia (Zielke et al. 2014), the Netherlands (Zielke et al. 2015), Slovenia (Zielke et al. 2015) and Southeast-Germany/Austria (Zielke et al. 2016). Because of the widespread distribution of this haplotype, it is likely introduced at an early stage of *Ae. japonicus* invasion. Due to a distribution similar to H1, a common introduction into Europe of both H1 and H9 is conceivable.

The number of *nad*4 haplotypes per collection site differed from one to seven. Most heterogeneous collection sites in Germany were Heidelberg, Burgscheidungen and Freiburg with seven different haplotypes. With six *nad*4 haplotypes, the Croatian collection site Orahovica was most heterogeneous in southeastern Europe.

Five haplotypes were found both in Germany and in southeastern Europe (H1, H4, H9, H10, H33). *Nad*4 haplotypes H5, H11, H17 H21, H43, H45 and H46 were exclusively found in Germany. and haplotypes H12, H19 and H35 were exclusively detected in southeastern Europe. Furthermore, three specimens were exclusively assigned to *nad*4 haplotype H3: one in Bosnia and Herzegovina and two in the Czech Republic. In contrast to *nad*4 haplotypes H4, H9, H10, H11 and H12 which were detected in the US, H3 and H5 were detected in Japan and New Zealand (Fonseca et al. 2001, 2009). At this point, two decades after the onset of the worldwide expansion of *Ae. japonicus*, the genetic information is not adequate anymore to determine the source of a population investigated in this studies. It is not possible to decide whether specimens were imported first from Japan or the US to Europe.

According to the new data (Janssen et al. 2019), the West German population (collection sites Monchengladbach, Linz, Rosenthal) seemed to differ from the other German populations. With 86 %, *nad*4 haplotype H1 was most common in the West German population, and with four different haplotypes these western collection sites were relatively homogeneous (H1, H4, H5, H9). Zielke et al. (2014) detected the haplotypes H1, H5 and H6 in this West German population. Even more to the west, in the Belgian population, *nad*4 haplotype H9 was identified (Zielke et al. 2014). It is possible that H9 was carried over from Belgium to Germany. Smitz et al. (2021) confirmed this possibility by detecting H9 as the most common *nad*4 haplotype in Belgium. *Nad*4 haplotype H4 could have been introduced or resulted from a mutation from H9 (1 transition) or H1 (2 transitions). These results are in accordance with the microsatellite analysis, uniformly showing a high probability of belonging to the same microsatellite genotype. According to a Principal Coordinates Analysis (PCoA), the West German collection sites are genetically different from other German populations and most remote from the East European populations. Although the corridor between western and southwestern German distribution areas of *Ae. japonicus* seems to have closed by 2017, an even genetic mixing had not taken place. Probably, these populations are attributed to various introductions. Although the results of the *nad*4 haplotypes showed a genetic increase from three haplotypes (Zielke et al. 2012) to 12 (Janssen et al. 2019), the German populations of *Ae. japonicus* still fell in two different clusters (genotypes) regarding their genetic signature of the microsatellites in 2017.

Zielke et al. (2016) found the population from Southeast Germany/Austria to be closely related to the population from Austria/Slovenia. The present study also indicated a genetic relation between the southern Bavarian collection sites (Berchtesgaden, Augsburg) to the collection sites in eastern Europe. The Bavarian collection sites, like the population from Austria/Slovenia, show a high probability of belonging to the same microsatellite genotype. Zielke and colleagues determined the haplotypes H1, H9 and H10 in the population 'Austria/Slovenia' (Zielke et al. 2014) and H1, H5 and H10 in the population 'Southeast Germany/Austria' (Zielke et al. 2016). In this study, the *nad*4 haplotypes H1, H5, H9, H10 and H17 were detected in Southeast Germany (Berchtesgaden). The result of the PCoA shows that these populations are closer related to East European collection sites (Ljuba, Skrad) than to other German populations. Presumably, a carryover of *Ae. japonicus* between these areas has taken place.

In contrast to the results by Zielke et al. (2015), the present study suggests that the population around Hanover is not closely related to the population in West Germany (Janssen et al. 2019), but that a carryover has taken place from that population to Southeast Germany (Berchtesgaden). In the present study, the West German population was characterised by the haplotypes H1, H4, H5 and H9 whereas the specimens collected around Hanover had the haplotypes H9, H10 and H17. The same *nad*4 haplotypes were detected in Southeast Germany (Berchtesgaden). These results are again confirmed by Smitz et al. (2021) by determining H9 and H10 in North Germany and H1, H5 and H6 in West Germany. This genetic constellation is in agreement with the results of the microsatellite analysis. Both populations, Hannover and West Germany, show a high probability of belonging to one and the same microsatellite genotype with a clear admixture by a second microsatellite genotype. In addition, according to the PCoA, these populations are genetically separated from the other German populations. Smitz et al. (2021) determined a close relationship of the populations around Hanover and from South West Germany (Baden Wurttemberg), caused by a carry over from South to North Germany.

The Central German collection sites (Freiburg, Heidelberg, Wurzburg, Burgscheidungen), lie genetically between the West and Southeast German collection sites (Janssen et al. 2019). They show a higher diversity for *nad*4 haplotypes (six or seven, respectively) as compared to the other German collection sites. Zielke and colleagues determined the *nad*4 haplotypes H1 and H33 in Switzerland (Zielke et al. 2014) and H1, H3, H5, H21, H33 and H45 in South Germany (Zielke et al. 2015). At every collection site, the newly detected *nad*4 haplotype H46 was found. This haplotype is most closely related to haplotype H33, only differing in one nucleotide position. Therefore, it could be possible that H46 is a mutation of H33. The collection sites in Central Germany (Freiburg, Heidelberg, Wurzburg, Burgscheidungen) are the only German collection sites with haplotypes H11, H33 and H43. Between these collection sites, a genetic exchange seems to have occurred. Furthermore, due to the high genetic diversity, several introductions must have taken place.

In southeastern Europe, the Croatian Orahovica collection site had the highest *nad*4 diversity (six haplotypes) (Janssen et al. 2020). In addition to H1, the collection sites southeast to Orahovica were dominated by H12 and those in the northwest by H9. Therefore, Orahovica can be seen as a genetic border in southeastern Europe, influenced by both eastern and western populations and genetic make-ups. This separation is also displayed by the results of the PCoA. The collection sites in the northwest of Orahovica seem to be closely related to each other and to the populations Austria/Slovenia (Zielke et al. 2014) and Southeast Germany/Austria (Zielke et al. 2016). Furthermore, most specimens from these collection sites/populations have a high probability of belonging to the same microsatellite genotype. By contrast, the southeastern collection sites (Odzak, Laze Prnjavor, Ljuba) show a high probability for another, second microsatellite genotype (except Ljuba) and form a separate group of sites in the PCoA. The collection site Macelj is the only collection site with *nad*4 haplotype H19 and, in contrast to other geographically collection sites close-by, shows a microsatellite signature similar to eastern collection sites (Orahovica, Laze Prnjavor, Odzak).

In contrast to the populations in Germany (Janssen et al. 2019) and southeastern Europe (Janssen et al. 2020), the most common haplotype detected at collection sites in the Czech Republic is H9 (Vojtíšek et al. 2022). It is the only haplotype discovered in Mikulov (except one specimen: H21), while Prachatice represented all haplotypes found in the Czech Republic (H1, H3, H9, H21). A carry-over by mosquitoes with *nad*4 haplotype H3 from populations in South Germany (Zielke et al. 2015), The Netherlands (Zielke et al. 2015) or Bosnia and Herzegovina (Janssen et al. 2020) could have taken place. Furthermore, H21 could have been carried over from South Germany (Zielke et al. 2015, Janssen et al. 2019). The results of the fragment length analysis show a close relationship of individuals from Prachatice and Grafenau. Individuals from Mikulov seem to be genetically less related to the other investigated populations from Germany and Croatia (Vojtíšek et al. 2022), so a new introduction from a population not included could have occurred.

The numbers of *Ae. japonicus* specimens investigated from the Czech Republic (n=34) and from the southeastern European populations (n=127) are much smaller than those from the

German populations (n=209). While the German specimens were targetedly collected by larval sampling, the mosquitoes from the Czech Republic and the southeastern European ones were rather captured accidentally, and a considerable part of the larvae from southeastern Europe were prepared for voucher collections and thus not available for the genetic analyses. Nevertheless, the data were sufficient to demonstrate clear differences between the genetic make-ups of the German populations, the Southeast European populations and the Czech population.

Outlook

At this point of invasion of *Ae. japonicus* into Germany and eastern Europe (about 10 years after the first detection), it becomes more and more difficult to identify the origin of the various established populations or the points of entry into Europe. *Aedes japonicus* is well adapted to European environmental and climatic conditions, allowing for a quick spread by both active migration of adults and passive displacement of eggs and larvae by trade and travel. The spread of *Ae. japonicus*, including merging and mixture of populations, will probably continue and be supported by new introductions from overseas. The increase in admixture of populations by individuals from other sources becomes apparent when comparing previous with more recent studies: the number of *nad*4 haplotypes identified for Germany was three in 2012 (Zielke et al. 2014) and 12 in 2017 (Janssen et al. 2019). Although the genetic diversity has increased on individual level, no complete homogenisation on population level has taken place yet, and sites belonging to once separated populations still display genetic differences. By all means, population genetic studies asking for origins and migration paths are the more efficient the sooner they are carried out after new invasion events.

As the spread of *Ae. japonicus* is going on, it is just a question of time when *Ae. japonicus* can be found Germany-wide. In 2021, reproduction, and maybe a new population, was found in a city on the German North Sea coast (Kampen, pers. comm.), far more to the north than the most northern European collection sites so far. On top of that, continuing climate warming will increase the risk of pathogen transmission by *Ae. japonicus* and other mosquito species.

Summary

The present study deals with the spread and population genetics of the invasive Asian bush mosquito *Ae. japonicus* in Europe and Germany. Since the first detection of *Ae. japonicus* in Europe in 2000, the species spread rapidly through Europe, either actively by flying or passively by human activities. In 2017, four confirmed populations of *Ae. japonicus* existed in Europe. The largest population covered western Germany, parts of France, Switzerland, Liechtenstein, Austria and Italy. The most northern population around Hanover, Germany, did not spread since 2013. A very small population existed in Belgium and the second largest population covered parts of Austria, Italy, Slovenia, Croatia and Hungary. By 2019, *Ae. japonicus* had established in 15 European countries.

Most of the monitoring programmes in Europe dealing with the distribution and spread of *Ae. japoncus* investigate cemeteries for juvenile stages. However, activities are not harmonised, e.g. regarding numbers of investigated collection sites and declaration of negative sites, making data comparison between different studies difficult. Therefore, suggestions for a standardised *Ae. japonicus* monitoring method have been developed and provided.

In the present study, 445 individuals of *Ae. japonicus* originating from five different European countries were investigated for population genetic analyses by sequencing parts of the *nad*4 gene and genotyping seven polymorphic microsatellite loci. In total, 16 different *nad*4 haplotypes were identified with haplotype H1 being the most common and widespread one through all populations.

Within Germany*, Ae. japonicus* has been spreading immensely over the last decade. Even though the present results (2017) demonstrate incipient genetic admixture of populations as compared to previous studies (2012-2015), no complete genetic mixture has taken place yet. The populations of *Ae. japonicus* still fall into two genetic clusters, but the genetic diversity on individual level had increased considerably (from three *nad*4 haplotypes in 2012 to 12 according to the present thesis). Both additional introductions and mutation are possible reasons, but determining the origin of the German populations is not possible anymore.

In the years following the invasion of Germany, *Ae. japonicus* spread to southeastern Europe. In 2013, it established in Croatia, in 2017 in Bosnia and Herzegovina and in 2018 in Serbia. In the current study, immature stages of *Ae. japonicus* were found at 19 sites in Croatia, two sites in Bosnia and Herzegovina and one site in Serbia. The population genetic analyses indicate at least two independent introductions in that area. *Aedes japonicus*

collected west of Orahovica (Croatia) seemed to be genetically similar to samples previously investigated from Southeast Germany/Austria and Austria/Slovenia. By contrast, samples from east of Orahovica, together with those from Serbia and Bosnia and Herzegovina, were characterised by another genetic make-up, but their origin could not be determined.

In 2021, individuals of *Ae. japonicus* were detected at two collection sites in the Czech Republic for the first time: Prachatice close to the Czech-German border and Mikulov on the Czech-Austrian border. Population genetics and comparison of genetic data showed a close relationship of the Prachatice samples to a German population, while for *Ae. japonicus* from Mikulov close relatives could not be identified.

In the future, the global spread and establishment of invasive mosquitoes through international trade and travel will increase. Potential vectors, like the Asian bush mosquito *Ae. japonicus*, can become a problem in Europe and Germany, especially in the course of global warming which supports pathogen transmission. Monitoring the known populations and identifying introduction and migration routes are therefore essential for vector managing.

Zusammenfassung

Die vorliegende Studie behandelt die Ausbreitung und populationsgenetische Untersuchungen der invasiven Asiatischen Buschmücke *Ae. japonicus* in Europa und Deutschland. Seit der erstmaligen Entdeckung von *Ae. japonicus* in Europa im Jahr 2000, hat sich diese Spezies sowohl aktiv durch Fliegen als auch passiv durch menschliche Aktivitäten sehr schnell in Europa ausgebreitet. Im Jahr 2017 existierten vier Populationen von *Ae. japonicus* in Europa. Die größte Population erstreckte sich über das westliche Deutschland, Teile von Frankreich, der Schweiz, Liechtenstein sowie Österreich und Italien. Die nördlichste Population um Hannover hat sich seit 2013 nicht ausgebreitet. Eine sehr kleine Population befindet sich in Belgien und die zweitgrößte bedeckt Teile von Österreich, Italien, Slowenien, Kroatien und Ungarn. Bis 2019 hatte sich *Ae. japonicus* in 15 europäischen Ländern etabliert.

Die meisten auf die Ver- und Ausbreitung von *Ae. japonicus* ausgerichteten Monitoringprogramme in Europa untersuchen Friedhöfe auf die Juvenilstadien. Die Studien sind jedoch nicht harmonisiert, z.B. hinsichtlich der Anzahl untersuchter Sammlungsstellen und Festlegung von negativen Stellen, so dass der Datenvergleich zwischen verschiedenen Studien schwierig ist. Aus diesem Grund wurden Vorschläge für eine standardisierte Monitoringmethode entwickelt und vorgelegt.

In der vorliegenden Studie wurden 445 Individuen von *Ae. japonicus* aus fünf verschiedenen europäischen Ländern populationsgenetisch untersucht, indem Teile des *nad*4-Gens sequenziert und sieben polymorphe Mikrosatelliten Loci untersucht wurden. Insgesamt wurden 16 verschiedenen *nad*4-Haplotypen identifiziert, wobei Haplotyp H1 in allen Populationen am häufigsten und weitesten verbreitet war.

Innerhalb Deutschlands hat sich *Ae. japonicus* in den letzten 10 Jahren stark ausgebreitet. Auch wenn die aktuellen Ergebnisse (2017) in Relation zu vorherigen Studien (2012-2015) eine beginnende genetische Vermischung der Populationen anzeigen, hat bisher keine vollständige Vermischung stattgefunden. Die *Ae. japonicus*-Populationen fallen noch immer in zwei genetische Cluster, aber die genetische Vielfalt auf individualer Ebene hat beträchtlich zugenommen (von drei *nad*4-Haplotypen in 2012 zu zwölf in der vorliegenden Studie). Sowohl neue Eintragungen als auch Mutationen könnten mögliche Erklärungen sein. Allerdings ist es nicht mehr möglich den Ursprung der deutschen Populationen zu bestimmen.

In den Jahren nach der Invasion Deutschlands breitete sich *Ae. japonicus* im südöstlichen Europa aus. Seit 2013 ist diese Art in Kroatien, seit 2017 in Bosnien und Herzegowina und seit 2018 in Serbien angesiedelt. In der vorliegenden Studie wurden juvenile Stadien an 19 Stellen in Kroatien, an zwei in Bosnien und Herzegowina und an einer in Serbien nachgewiesen. Die populationsgenetischen Untersuchungen weisen auf mindestens zwei unabhängige Einschleppungen in dieses Gebiet hin. Individuen, die westlich des Standortes Orahovica (Kroatien) gesammelt wurden, weisen genetisch eine höhere Ähnlichkeit mit vormals untersuchten Individuen aus Südostdeutschland/Österreich und Österreich/Slowenien auf. Dagegen sind westlich von Orahovica (Kroatien) gesammelte Proben, zusammen mit denen aus Serbien und Bosnien und Herzegowina, genetisch unterschiedlich und ihr Ursprung konnte nicht ermittelt werden.

Erstmals wurden 2021 Individuen von *Ae. japonicus* an zwei Sammelstellen in der Tschechischen Republik nachgewiesen: Prachatice nahe der tschechisch-deutschen Grenze und Mikulov an der tschechisch-österreichischen Grenze. Die populationsgenetischen Untersuchungen und der Vergleich der genetischen Daten weisen auf eine enge Verwandtschaft der Individuen aus Prachatice zu einer deutschen Population hin, während für *Ae. japonicus* aus Mikulov keine engen Verwandten ermittelt werden konnten.

Zukünftig werden die globale Verbreitung und Ansiedlung von invasiven Stechmücken durch internationalen Handel und Reisen zunehmen. Potentielle Vektoren, wie die Asiastische Buschmücke *Ae. japonicus*, können ein Problem in Europa und Deutschland werden, insbesondere im Zuge des Klimawandels, welcher die Transmission von Pathogenen begünstigt. Die Überwachung der bekannten Populationen sowie die Identifizierung von Eintritts- und Migrationsrouten sind essentiell für das Vektor Management.

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Eigenständigkeitserklärung

Hiermit erkläre ich, dass diese Arbeit bisher von mir weder an der Mathematisch-Naturwissenschaftlichen Fakultät der Universität Greifswald noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde.
Ferner erkläre ich, dass ich diese Arbeit selbstständig verfasst und keine anderen als die darin angegebenen Hilfsmittel und Hilfen benutzt und keine Textabschnitte eines Dritten ohne Kennzeichnung übernommen habe.

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List of publications, oral presentations and posters

Publications

Koban MB, Kampen H, Scheuch DE, Frueh L, Kuhlisch C, Janssen N, Steidle JLM, Schaub GA, Werner D (2019) The Asian bush mosquito *Aedes japonicus japonicus* (Diptera: Culicidae) in Europe, 17 years after its first detection, with a focus on monitoring methods. Parasit Vectors. 12:109.

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Vojtíšek J, Janssen N, Šikutová S, Šebesta O, Kampen H, Rudolf I (2022) Emergence of the invasive Asian bush mosquito *Aedes* (*Hulecoeteomyia*) *japonicus* (Theobald, 1901) in the Czech Republic. Parasit Vectors. 15:250.

Oral presentations

„Konkurrenzverhalten zwischen invasiven (*Aedes albopictus* und *Aedes japonicus*) und einheimischen Stechmückenarten“ – Meeting of the German Society for Medical Entomology and Acarology, 18-20 September 2017, Leipzig

„Populationsgenetische Untersuchungen an *Aedes japonicus* (Diptera: Culicidae) in Deutschland“– Meeting of the German Society for Medical Entomology and Acarology, 20-22 September 2018, Frankfurt/Main

Poster

“Population genetics of the invasive mosquito *Aedes japonicus* in Germany” – FLI Junior Scientist Symposium – 24-26 September 2018, Greifswald

Curriculum vitae

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