Population genetics and distribution of the invasive mosquito *Aedes japonicus japonicus* (Diptera: Culicidae) in Germany and Europe

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1. INTRODUCTION

1.1 THE IMPORTANCE OF MOSQUITO RESEARCH IN GERMANY

Malaria has dominated the field of mosquito-borne diseases in Europe for centuries. Mosquitoes as vectors of malaria parasites had been identified at the end of the 19th century by the English medical scientist Ronald Ross, who was later honoured for his work with the Nobel prize. However, the connection between the fever and the presence of swamps had been known long before. The disease was believed to be caused by fumes produced by the swamps, a fact also reflected in the name "malaria", from Italian "mala aria" meaning "bad air". To avoid these fumes, people began to settle away from swamps and started draining them (DOBSON 1994). With these procedures, they unknowingly limited *Anopheles* breeding places and so confined the spread of malaria pathogens. The separation of cattle sheds and human dwellings led to a reduced contact between mosquitoes and humans. The development and administration of synthetic malaria drugs (DOBSON 1994) as well as the application of DDT (dichlorodiphenyltrichloroethane) for mosquito control in the early 20th century contributed to a further decrease in malaria cases (DE ZULUETA 1994). The last malaria epidemic in Germany occurred in 1953 in Berlin. Returning World War II soldiers obviously carried malaria pathogens which were then transmitted by indigenous *Anopheles* mosquitoes (EICHENLAUB 1979).

Due to successful control by the above mentioned measures and improved hygienic standards, malaria parasites (*Plasmodium* spec.) do not circulate in Germany anymore although local cases of malaria, such as airport malaria where infected mosquitoes are introduced by airplanes, are possible (e.g. SIGNORELLI & MESSINEO 1990, CASTELLI et al. 1993). By contrast, the vectors of the malaria parasites, mosquitoes of the genus *Anopheles*, are still widely distributed in Germany (BECKER et al. 2010). In Africa and parts of Asia, malaria is still the deadliest vector-borne disease with an estimated 198 million cases and 584,000 deaths in 2013, closely followed by another mosquito-borne disease, dengue fever, with an estimated 50-100 million infections per year (WHO 2012, 2014).

With the disappearance of malaria from Germany, mosquito research had been eclipsed in this country. In public awareness, indigenous blood-sucking insects had been degraded from pathogen vectors to mere nuisances (KAMPEN & SCHAFFNER 2008). This attitude has only changed with the outbreak of bluetongue, a viral

disease of ruminants, in Central Europe in 2006 (SAEGERMAN et al. 2008), the etiological agent of which is transmitted by the bite of *Culicoides* spp. midges (MEHLHORN et al. 2007). The epidemic cost the lives of tens of thousands of ruminants (directly and indirectly by culling) and caused enormous economic loss to farmers and the livestock industry (GETHMANN et al. 2010). In 2011, Schmallenberg, another biting midge-borne ruminant disease, emerged in Germany and struck livestock and farmers again, although less dramatically (CONRATHS et al. 2013).

The recent cases and outbreaks of biting midge-borne diseases in Central Europe and of mosquito-borne diseases in southern Europe (see below) as well as the emergence of exotic pathogens drew mosquitoes back into the focus of entomological research. Because of the long neglect of this group of insects, however, the data on the German mosquito fauna and the knowledge of the occurrence and distribution of species was largely outdated.

Today, 50 mosquito species are supposed to occur in Germany. These differ from each other in many biological and ecological characteristics like distribution, population dynamics, host preferences, choice of breeding sites, vector competences, modes of hibernation etc. Not only might the mosquito fauna composition in Germany have changed during the past decades of limited scientific attention, but also do pathogens nowadays appear in regions where they were not known before.

In order to prevent disease outbreaks or react properly in the case of a mosquito-borne disease, a profound knowledge of the mosquito fauna and the distribution of potential vector species are crucial.

1.2 MOSQUITOES AND PATHOGENS IN PRESENT-DAY EUROPE

In Germany, mosquitoes currently make an appearance primarily as nuisance pests, because their aggressive biting behaviour and their mass development in some regions spoil people's leisure time outdoors (BECKER 1997). However, in other parts of the world, and even of Europe, they are well-known vectors of pathogens. Mosquitoes transmit pathogens in a biological way, meaning that the pathogen obligatorily proliferates or completes part of its life cycle within the mosquito host before being transmitted to another host during blood feeding (CLEMENTS 2012).

In large parts of Europe, several outbreaks of mosquito-borne diseases occurred within the last two decades. These diseases are referred to as "emerging mosquito-borne diseases" which have gained high attention in Europe lately (WEISSENBÖCK et al. 2010, ZELLER et al. 2013, SCHAFFNER et al. 2013). "Emerging" means that the diseases were previously unrecognized or have reappeared, are rapidly increasing in incidence or geographic range or are supposed to become a threat in the near future (GRATZ 1999).

Malaria

Malaria is caused by protozoans of the genus *Plasmodium* which live as parasites in the human liver and blood cells. They complete their life cycle within *Anopheles* mosquitoes able to transmit them to a new human host during their next blood meal. Five species of the parasite are human-pathogenic: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale, Plasmodium malariae* and, only recently recognised, *Plasmodium knowlesi* (WHO 2014). Infection with these pathogens leads to various forms of malaria. Malaria tropica, or *P. falciparum* malaria, is characterised by high fever, anaemia and may have neurological complications which are often fatal. Malaria tertiana (*P. vivax*, *P. ovale*) and quartana (*P. malariae*) go along with a milder symptomatology and are characterised by recurrent fever episodes (WARRELL & GILLES 2002). *Plasmodium knowlesi* is a parasite of macaques, but was recently found infecting humans as well (WHO 2014). It had previously been mixed with *P. malariae* but can lead to a much severer pathology including death of the patient (COX-SINGH et al. 2008).

Local and autochthonous cases of malaria are reported from time to time from European countries with members of the *Anopheles maculipennis* complex (*An. maculipennis* s.l.) as vectors. In 2011, an epidemic broke out in Greece with 20 diseased persons without travel background (DANIS et al. 2011a). Further cases occurred in Germany (PRAETORIUS et al. 1999, KRÜGER et al. 2001, ZOLLER et al. 2009), Spain (CUADROS et al. 2002, SANTA-OLALLA PERALTA et al. 2010), France (ARMENGAUD et al. 2006, DOUDIER et al. 2007) and Italy (SARTORI et al. 1989, BALDARI et al. 1998).

Dengue fever

The causative agent of dengue fever is the dengue virus of the family Flaviviridae. An infection with dengue virus causes severe feverish illness with unspecific, flu-like symptoms, but can also lead to the so-called dengue haemorrhagic fever or dengue shock syndrome which can be fatal (WHO 2009). The virus is transmitted by aedine mosquitoes such as *Aedes aegypti* or *Aedes albopictus* (WHO 2009).

Several cases and one epidemic of dengue fever occurred within the last decades in European countries: 2010, 2013 and 2014 in France (LA RUCHE et al. 2010, MARCHAND et al. 2013, SCHAFFNER et al. 2014), 2010 in Croatia (GJENERO-MARGAN et al. 2011) and 2012 in Portugal (Madeira) (SOUSA et al. 2012). On the Island of Madeira, the main vector was *Ae. aegypti*, the yellow fever mosquito, which had established only a few years prior to the outbreak. By contrast, *Ae. albopictus* was made responsible for the other European cases (SCHAFFNER & MATHIS 2014). Worldwide, dengue is the most important vector-borne viral disease of humans and, with this, has probably overrun malaria globally in terms of morbidity and economic impact (GUBLER 2012).

Chikungunya

In 2007, a tourist from India brought the chikungunya virus to North Italy, where a population of *Ae. albopictus* had established, with the result of an epidemic with about 200 disease cases and one fatality – a proof for the effectiveness of pathogen transmission by this mosquito species (REZZA et al. 2007, CASOLARI et al. 2008). Chikungunya virus is an alphavirus belonging to the family Togaviridae. It causes an illness with fever and massive joint pain. Further recent autochthonous cases of chikungunya were reported from France in 2010 and 2014 (GRANDADAM et al. 2011, DELISLE et al. 2015).

West Nile fever

Infection with West Nile virus, which belongs to the family Flaviviridae, normally takes an asymptomatic to mild course or induces flu-like symptoms with fever, but may also cause fatal encephalitis or meningitis (HAYES 2001). This virus led to an outbreak in Greece in 2011-2013 (DANIS et al. 2011b) and has caused epidemics from time to time in other South European countries (HUBÁLEK & HALOUZKA 1999). In late summer 2008, a large epidemic of West Nile fever occurred in northeastern Italy. Horses and

humans were affected (CALISTRI et al. 2010). Further epidemics were reported from Romania (TSAI et al. 1998) and France (MURGUE et al. 2001). In the USA, West Nile virus has caused numerous human fatalities within the last decade (ROEHRIG 2013).

Filariasis

Mosquitoes are also competent vectors of several species of filariae, nematode worms infecting tissues or the circulatory system of vertebrate hosts. In Germany, mosquitoes of the species *Culex pipiens* s.l., *Aedes vexans*, *Culiseta annulata* and *An. maculipennis* s.l. infected with *Dirofilaria immitis* and *Dirofilaria repens* have recently been found (KRONEFELD et al. 2014, CZAJKA et al. 2014). Infection with these filarial species induce cardiovascular symptoms known as heartworm disease (*D. immitis*) or subcutaneous nodules and allergic dermatitis (*D. repens*) in canines, felines and other carnivores (MCCALL et al. 2008, ROCCONI et al. 2012, SIMÓN et al. 2012). Humans can also become infected, in rare cases even severely (POPPERT et al. 2009), and a first presumably autochthonous case of human infection has been documented from Germany (TAPPE et al. 2014).

Setaria tundra which has also recently been demonstrated in German mosquitoes (CZAJKA et al. 2012, KRONEFELD et al. 2014) only affects ruminants and may induce peritonitis and perihepatitis (SERVICE 2001, LAAKSONEN et al. 2007).

In Germany, the present situation concerning mosquito-borne diseases is not alarming. Mosquito-borne pathogens causing severe illnesses in humans are absent. However, some arboviruses circulate that may lead to mild flu-like illnesses in infected humans. Without any surveillance, the causative agents of such disease cases are likely to remain undetected and spread.

Sindbis virus, an alphavirus of the family Togaviridae, is endemic in northern Europe with mosquito species such as *Aedes cinereus* and *Cx. pipiens* as vectors (LUNDSTRÖM 1999). The virus is pathogenic to humans and animals, and infections of humans may present with mild febrile illness. The virus has already been found in German mosquitoes of the species *Culex torrentium*, *Cx. pipiens* and *An. maculipennis* s.l. (JÖST et al. 2010). Similar symptoms are caused by Tahyna and Batai viruses. Both belong to the family Bunyaviridae and have been isolated from German mosquitoes of the species *Ae. vexans* (PILASKI & MACKENSTEIN 1985, JÖST et

al. 2011). In 2011, the Usutu virus (Flaviviridae), detected in indigenous mosquito species of the *Cx. pipiens* complex and some *Aedes* species, caused mass mortality among blackbirds in southwestern Germany (BECKER et al. 2012, BOSCH et al. 2012). Although neuroinvasive infections in non-immunocompromised humans have been described (SANTINI et al. 2015), this virus is thought to be primarily bird-pathogenic.

1.3 INVASIVE MOSQUITO SPECIES

The displacement of plant and animal species is a well-known phenomenon since people have started travelling around the world. However, it took a long time to understand the whole range of impacts invasive species can have to ecosystems and health. Some species have been introduced on purpose to new living spaces, because they were thought to be useful in some way, e.g. as predators to control pests or as productive livestock for early settlers. Others travel with humans and goods around the world accidentally and unnoticed, such as eggs or seeds. Sometimes, these bring forth established populations in the newly infested area.

In the case of mosquitoes, the term "invasive" is generally negatively flawed, because exotic mosquitoes may come along with a very good adaptability, a competence to transmit pathogens and a high resilience concerning the competition with indigenous species. Invasive mosquito species often have an impact on the endemic mosquito fauna in the infested area (LOUNIBOS et al. 2007, ANDREADIS & WOLFE 2010, MEDLOCK et al. 2012) and sometimes implicate economic costs in the animal or public health sector. However, they provide an opportunity to observe processes that are difficult to see in long established populations (FONSECA et al. 2010). This has basic and practical relevance because understanding how species proliferate and migrate in a new habitat is critical to predicting future movements and customising control efforts (MYERS et al. 1998).

Within the last decades, various mosquito species have increased their distribution area by displacement through passive transportation, among them potential vectors of arboviruses (Figure 1). As vector species are imported continuously, the likelihood of them adapting to new environmental conditions and establishing populations increases steadily (MEDLOCK et al. 2012).

pathogen			aegypti	albopictus	atropalpus	japonicus	koreicus	triseriatus
Viruses	Alphavirus	Chikungunya	■	■	□	□	□	□
		Eastern Equine encephalitis	□	▨	□	▥	□	▥
		Venezuelan Equine encephalitis	□	▨	□	□	□	▥
		Western equine encephalitis	□	□	□	□	□	▥
	Flavivirus	Dengue	■	■	□	□	□	▥
		Japanese encephalitis	□	▨	□	▥	▨	□
		St Louis encephalitis	□	□	□	▥	□	▥
		West Nile	□	▨	▨	▨	□	▨
		Yellow fever	■	□	□	□	□	▥
		Zika	▨	□	□	□	□	□
	Bunyavirus	Jamestown Canyon	□	□	□	□	□	▨
		La Crosse	□	▨	▥	▥	□	■
Nematodes	Dirofilaria	D. immitis and D. repens	□	■	□	□	▥	□

■ Proven vector in the field

▨ Found infected in field and laboratory competence studies having potential role as vector, but no proven vector in the field

▥ Only laboratory competence studies having showed potential involvement in transmission

□ No vector or not known

Figure 1 Overview of the exotic and invasive aedine mosquito species in Europe (figure source: MEDLOCK et al. 2012, modified).

Aedes albopictus is probably the most expansive mosquito species in the world (MEDLOCK et al. 2012). Its original distribution range is Southeast Asia, but to date, it has conquered wide parts of the world and is also well established in Europe (MEDLOCK et al. 2012). In northern parts of the US, this tropical species has obviously adapted to temperate climates and managed to hibernate by producing diapausing eggs (HAWLEY 1988).

Aedes albopictus is regularly introduced into Germany from South European countries like Italy and Spain each summer, and was shown to reproduce over weeks and months for the first time north of the Alps in 2014 (WERNER & KAMPEN 2015). As a competent vector of 26 viruses, *Ae. albopictus* is considered a significant threat to public health (PAUPY et al. 2009). Even without transmitting pathogens, the aggressively biting females are a serious nuisance, particularly in urban areas (PAUPY et al. 2009).

Another important invasive mosquito species is *Ae. aegypti*, the yellow fever mosquito. It has never been found in Germany so far, but is established on the Island of Madeira and in Transcaucasian areas (ALMEIDA et al. 2007, YUNICHEVA et al., 2008; SCHAFFNER & MATHIS 2014). Similar to *Ae. albopictus, Ae. aegypti* is a dreaded vector of disease agents. Its behaviour to feed multiple times during one gonotrophic cycle with a preference for human blood makes it an efficient vector of human pathogens (MEDLOCK et al. 2012).

The Asian bush mosquito *Aedes j. japonicus* is the first invasive mosquito species in Germany having established large and stable populations. First individuals were found in 2008 in South Germany, near the Swiss border (SCHAFFNER et al. 2009).

Further invasive aedine mosquito species detected in Europe are *Aedes atropalpus*, *Aedes triseriatus* and *Aedes koreicus*. In addition, members of the genus *Culex*, such as *Culex vishnui* or *Culex tritaeniorhynchus*, with the latter being described to be a competent vector of Japanese encephalitis virus (SUCHARIT et al. 1989), show invasive tendencies (MEDLOCK et al. 2012).

All invasive mosquitoes of the genus *Aedes* are container-breeding species. Their global displacement is strongly associated with international trade and travel (MEDLOCK et al. 2012). Particularly critical is the worldwide trade with used tyres and lucky bamboo. The tyres get shipped from the original distribution ranges to other parts of the world and with them attached drought-resistant mosquito eggs. As the tyres are normally stored under the open sky, rainfall will cause the mosquito eggs to fall below the waterline and the larvae to hatch. In the case of water-based plants, such as lucky bamboo, larvae of exotic mosquitoes are dispersed. Within the continent, private and public ground transport contributes to the spread of invasive mosquitoes along motorways (MEDLOCK et al. 2012).

1.4 SYSTEMATICS AND ECOLOGY OF *AEDES JAPONICUS JAPONICUS*

The Asian bush mosquito *Aedes* (*Finlaya*) *japonicus japonicus* (THEOBALD, 1901) belongs to the *Ae. japonicus* species complex, which consists of four subspecies: *Aedes japonicus japonicus, Aedes japonicus amamiensis* TANAKA, MIZUSAWA & SAUGSTAD, 1979, *Aedes japonicus shintienensis* TSAI & LIEN, 1950, and *Aedes japonicus yaeyamensis* TANAKA, MIZUSAWA & SAUGSTAD, 1979 (TANAKA et al. 1979).

These subspecies are difficult to distinguish morphologically, because differences between them are only apparent in the scale patterns of their hindfemora (Figure 2). Curiously, *Aedes j. japonicus* is the only one of the four subspecies that has been found outside its native range. The four subspecies are genetically distinct and form a monophyletic group together with *Aedes koreicus* (CAMERON et al. 2010).
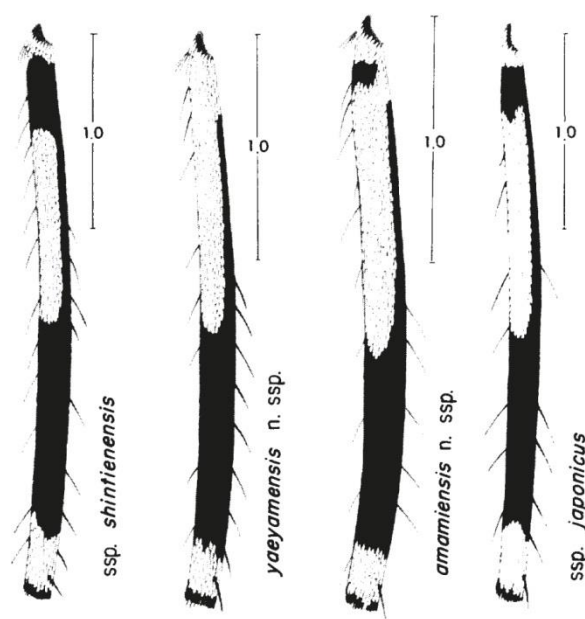
Figure 2 Hindfemora scale patterns of subspecies of the *Ae. japonicus* complex (figure source: TANAKA et al. 1979).

Following systematic work by REINERT (2000), the subgenus *Ochlerotatus* has been elevated to generic rank in the year 2000 and the name *Ochlerotatus japonicus japonicus* has been introduced for the species. After another revision, the name *Hulecoeteomyia japonica japonica* was suggested in 2006 (REINERT et al. 2006). In the present work, *Aedes japonicus japonicus* is used, following the annotation by EDMAN (2005).

The species' native distribution range is East Asia where it occurs in Japan, Korea, Taiwan, southeastern Russia and eastern China. *Aedes j. japonicus* is a tree-hole or rock pool breeder (American name: Asian rock pool mosquito) but also accepts a huge variety of small artificial water containers like buckets or flower vases as breeding habitats (MIYAGI 1971, TANAKA et al. 1979). It is adapted to temperate climates, and the overwintering eggs can endure temperatures as low as -18 °C

(BEVINS 2007). This explains the more northerly distribution of *Ae. j. japonicus* compared to the thermophilic invasive species *Ae. aegypti* and *Ae. albopictus*. EGIZI et al. (2014) suggest interspecific benefits between larvae of *Ae. j. japonicus* and *Culex quinquefasciatus*, enabling *Ae. j. japonicus* to also breed in warmer waters and thus colonise tropical regions like the Hawaiian islands (LARISH & SAVAGE 2005). During oviposition, the eggs are attached singularly to humid substrates above the water line. This can be the rough wall of the breeding container or wooden materials like ovipositioning sticks. Given adequate conditions in terms of temperature and water supply, larvae hatch after a few days.

Aedes j. japonicus larvae have a dark brown head and a body of a light yellowish brown (KAMPEN et al. 2012). In comparison to most indigenous mosquito species, they wiggle even more worm-like when moving and hide for longer periods of time in the ground debris of the water container (KAMPEN et al. 2012). The larvae are often found living together with indigenous mosquito larvae like those of *Cx. pipiens* or *Anopheles plumbeus* (SCOTT 2003, own observations). They can develop in relatively cold water which may be below 10 °C. Adult *Ae. j. japonicus* are blackish-brown with distinguished, bright white markings on their bodies and legs. The scutum shows bronze-coloured longitudinal stripes on a brown ground. The adults are associated with bushy landscapes providing shelter and shade for daytime resting. Bloodmeal analyses have shown that *Ae. j. japonicus* females readily feed on mammal blood (DAMIENS et al. 2014).

1.5 MEDICAL IMPORTANCE OF *AEDES JAPONICUS JAPONICUS*

Although *Ae. j. japonicus* is not considered an important vector in its native range and evidence for a major role in field transmission of disease agents is generally absent, it has been shown to be able to transmit several arboviruses in the laboratory (KAMPEN & WERNER 2014). Under experimental conditions, six different viruses have been successfully transmitted (Table 1): West Nile virus (TURELL et al. 2005), Japanese encephalitis virus (TAKASHIMA & 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) and Rift Valley fever virus (TURELL et al. 2008). *Aedes j. japonicus* is also capable of transmitting Japanese encephalitis virus

transovarially, meaning that infected females pass the virus on to their offspring (TAKASHIMA & ROSEN 1989). The species is also susceptible to infection with chikungunya, dengue and Getah viruses (SCHAFFNER et al. 2011, TAKASHIMA & HASHIMOTO 1985). However, *Ae. j. japonicus* has never been proven to be a vector in the field so far although it has been found infected with Japanese encephalitis virus in Russia (CHAGIN & KONDRATIEV 1943) and with West Nile and LaCrosse viruses in the USA (MOLAEI et al. 2009, WESTBY et al. 2011, HARRIS et al. 2015). Its frequent habitation in urban and suburban surroundings and its preference for mammalian and avian blood make *Ae. j. japonicus* a likely candidate to act as a bridge vector for zoonotic viruses like West Nile virus (KAMPEN & WERNER 2014).

Table 1 Pathogens linked with *Ae. j. japonicus* (figure source: KAMPEN & WERNER 2014, modified).

	Transmission in the field	Infection in the field	Transmission in the lab	Infection in the lab
West Nile virus	?	+	+	+
Jap. encephalitis virus	?	+	+	+
La Crosse virus	?	+	+	+
St. Louis encephalitis virus	?		+	+
Eastern equine encephalitis virus	?		+	+
Rift Valley fever virus	?		+	+
Chikungunya virus	?			+
Dengue virus	?			+
Getah virus	?			+

1.6 INTRODUCTION AND DISTRIBUTION OF *AEDES JAPONICUS JAPONICUS*

Aedes j. japonicus has been found for the first time outside its natural distribution range in the early 1990s in New Zealand (LAIRD et al. 1994). These and other individuals intercepted later in New Zealand could be eradicated immediately upon detection (DERRAIK et al. 2003), and to date, there is no population of *Ae. j. japonicus* known from New Zealand (KAMPEN & WERNER 2014). The first established

populations outside East Asia were reported in 1998 from the eastern United States of America, where the three states Connecticut, New Jersey and New York had been found infested (PEYTON et al. 1999, MUNSTERMANN & ANDREADIS 1999). During the next ten years, *Ae. j. japonicus* was found in 30 additional US states. Today, it is also present in Canada and on the Hawaiian islands (LARISH & SAVAGE 2005, THIELMAN & HUNTER 2006). The latest detected established population in North America was reported from New Foundland in 2013 (FIELDEN et al. 2015).

In 2000, the first individuals of *Ae. j. japonicus* were reported from France (SCHAFFNER et al. 2003). As in New Zealand, these were immediately eradicated (SCHAFFNER et al. 2009). The first established population in Europe was documented in 2002 in Belgium (VERSTEIRT et al. 2009). This population is the only European population whose mode of introduction is known: so far, all individuals have been collected on the premises of two close-by used tyre trading companies and their surroundings. In the years to come, further populations of *Ae. j. japonicus* emerged in Europe. In 2008, a population was reported from the border region of Germany and Switzerland (SCHAFFNER et al. 2009). Three years later, in 2011, local reproduction was demonstrated in the border region of Austria and Slovenia (SEIDEL et al. 2012). Two more populations were found in Germany in 2012 (West Germany) and 2013 (North Germany) (KAMPEN et al. 2012, WERNER & KAMPEN 2013). The Austrian-Slovenian population spread into parts of Hungary and Croatia and significantly increased its distribution range in Slovenia (KALAN et al. 2014). The Swiss-German population spread into eastern France (KREBS et al. 2014), while a further population was detected in the Netherlands in 2013 (IBÁÑEZ-JUSTICIA et al. 2014).

The most recent European population of the Asian bush mosquito so far was detected in southeastern Germany/Austria in summer 2015 (ZIELKE et al. 2015).

In summary, there are seven geographically separated populations of *Ae. j. japonicus* in Europe (Figure 3). To unveil sources, routes and modes of introduction, as well as underlying migration processes, kinship analyses among the European populations and between these and populations abroad must be addressed.
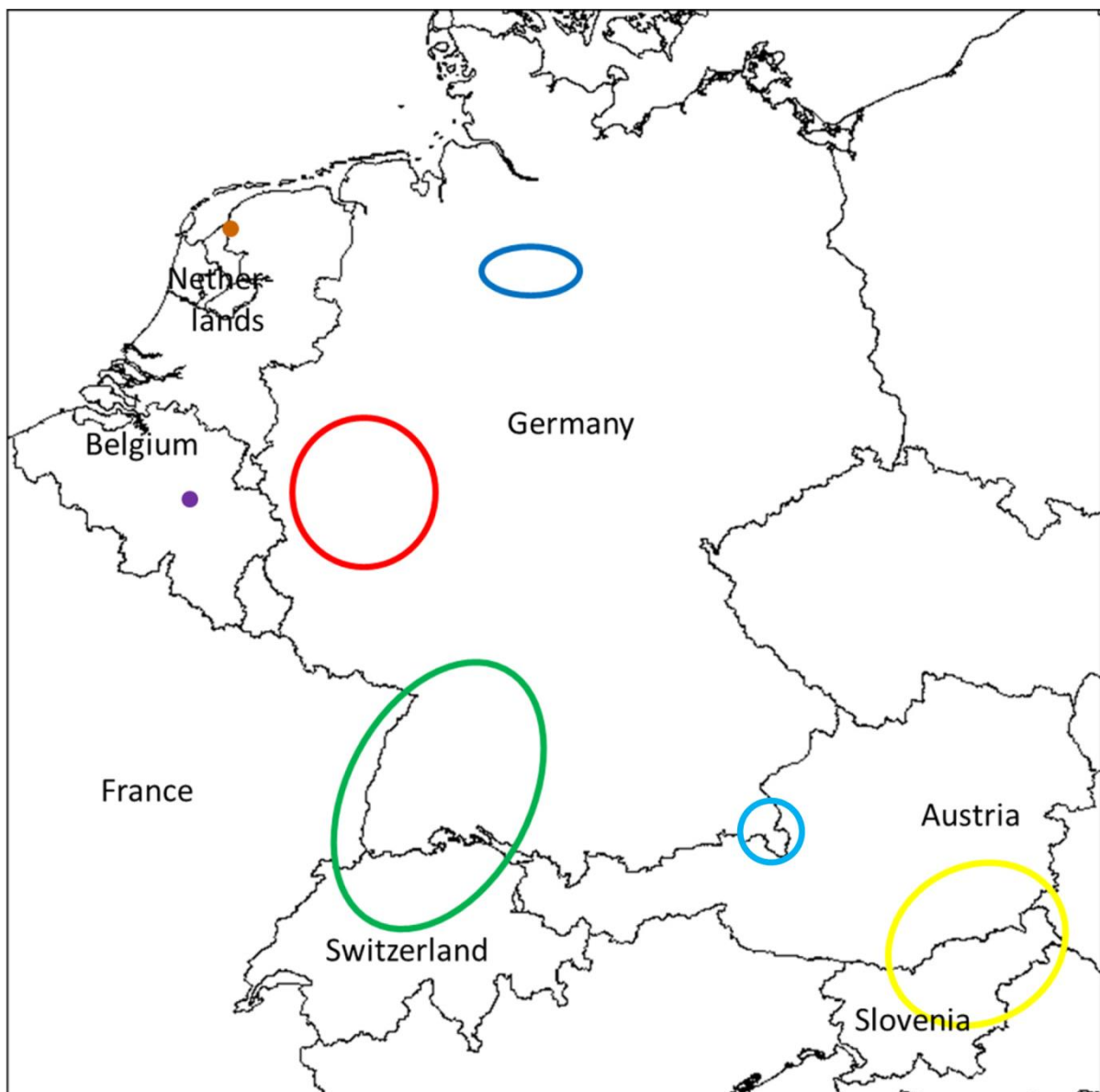
Figure 3 European populations of *Ae. j. japonicus* (figure source: KAMPEN & WERNER 2014, modified).

1.7 POPULATION GENETICS OF *AEDES JAPONICUS JAPONICUS*

Population genetic studies allow an insight into a species' displacement and spread. They may help to uncover relationships between imported populations and to identify their source populations and introduction ways.

The examination of microsatellites is an adequate approach to draw frequency-based conclusions on the relatedness between populations. Microsatellites are short DNA sequence repeats (short tandem repeats = STRs) of 2-5 bp length. Among or within populations the frequency of repetitions might be different and produce different

alleles on a certain locus. The exact length of the microsatellite can be measured by fragment length analysis. Thus, the alleles existing in the examined population can be identified. The same alleles refer to a close relationship between populations or individuals. The more alleles exist in a population, the higher is its genetic diversity. A high genetic diversity, in turn, may correlate with high adaptability and a high likelihood of this population to establish and persist. Seven microsatellite loci useful for population genetic studies are currently known for *Ae. j. japonicus* (WIDDEL et al. 2005, Figure 4).

Locus	Primer sequences (5′–3′)	Repeat motif	No. of alleles	Allele size range (bp)
OJ5	F: CACGAAGTCTGGAAGATCTGG R: fam-ATTCGTGCAGCGAAATCTG	$(GTT)_6(GCT)_3(GTT)$	5	144–157
OJ10	F: GCTTGTCCTGGCTAAGTACTGC R: ned-CGGTAATGTCCACCTGATTG	$(GTT)(GTG)(GTT)_8$	8	114–137
OJ70	F: CGTTGACAAAGCTCATCTGC R: ned-TGATCTCCAACGGAAGTATGC	$(GCT)_4(GTT)_2(GCT)_2(GTT)$ $(GCT)_2(GTT)_2(GCT)_7$	6	186–212
OJ85	F: ned-CATAAAGCAGCAAGCACAGC R: TGTCTTCCGGATTGATTTCC	$(CAG)_6$	4	161–173
OJ100	F: fam-CGCATTCCTCAAACCCTAAC R: TCGGTCCGAGGGAAAAAC	$(GT)_5$	3	180–189
OJ187	F: hex-AAATCAGCTGCCAGTGCAAG R: TGTGTACTTTGCGGTGAAGG	$(CGA)_{11}$	5	135–157
OJ338	F: ned-TCTCCTGATCCTGAAGAAGC R: AGGGAGCAGAGCAACACTTG	$(CAA)_{10}$	9	134–179

Figure 4 Microsatellite loci of *Ae. j. japonicus* (figure source: WIDDEL et al. 2005, modified).

A further way to examine a population's origin and its relatedness to other populations is the sequencing of the *nad*4 (NADH dehydrogenase subunit 4) gene of the mitochondrial DNA that has shown to be sufficiently variable and informative for population level analyses (FONSECA et al. 2001). Mitochondrial DNA is maternally inherited and evolves rapidly. The additional examination of the *nad*4 region allows better conclusions on the genetic makeup of a population because the results may complement and refine microsatellite analyses. The respective PCR amplifies a 424 bp segment of the *nad*4 gene showing single nucleotide polymorphisms (Figure 5) which are unique for a genetic lineage/population. To date, 45 *nad*4 haplotypes have been described for *Ae. j. japonicus* (ZIELKE et al. 2016).

	0	0	0	0	0	1	1	1	2	2	3	3	3	4	4
	0	5	7	8	9	3	6	9	0	9	0	7	7	0	1
	1	5	6	2	1	5	3	6	2	0	0	6	3	0	5
H1	A	C	C	C	T	A	T	A	C	T	A	A	C	C	C
H2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T
H4	.	.	.	.	C	.	.	.	.	.	.	.	T	.	
H6	.	.	.	T	.	G	.	.	.	.	.	.	.	.	.
H10	.	.	T	.	.	.	.	.	.	.	G	.	.	.	.
H11	.	T	T	.	.	.	.	.	.	.	.	.	.	.	.
H9	.	.	.	.	C	.	.	.	.	.	.	.	T	T	.
H12	.	.	T	.	.	.	.	.	.	.	G	.	.	.	.
H3	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H7	.	.	.	.	.	.	.	.	T	.	.	.	.	T	.
H8	.	.	.	.	.	G	C	.	.	.	.	.	.	.	.
H14	.	.	.	.	C	.	.	.	.	C	.	.	T	T	.
H15	G	.	.	.	.	.	G	.	.	.	.	.	.	.	.
H5	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.
H13	.	.	.	.	.	.	.	.	.	.	.	.	T	T	.

Figure 5 15 out of 45 known nucleotide haplotypes of the 424 bp segment of the *nad*4 gene mitochondrial DNA of *Ae. j. japonicus* (figure source: FONSECA et al. 2001, modified).

*Nad*4 haplotype determination can be complicated by heteroplasmy, the coexistence of multiple mitochondrial haplotypes in a single organism (MAGNACCA & BROWN 2010). Heteroplasmy can be caused by somatic mutations or paternal leakage, the latter term describing the non-elimination of the paternal mitochondrium during fertilisation of an egg (KVIST et al. 2003). The affected individuals cannot be used for haplotype analyses, because assigning them to one single haplotype is impossible.

1.8 OBJECTIVES

The invasive mosquito species Europe had to deal with recently show vector competences for numerous pathogens, including some not endemic ones. Presently, mosquito-borne pathogens are scarce in Germany, and viruses that appear to circulate generally cause mild febrile illness only. Pathogens having the potency to trigger severe and even deadly diseases, like dengue virus, chikungunya virus or West Nile virus have not yet been reported from Germany. Malaria parasites are an exception but are not regarded a general threat to public health anymore (KAMPEN & WERNER 2015).

Aedes j. japonicus has been shown to be a competent vector of some dangerous pathogens in the laboratory, and this species must now be considered belonging to the indigenous German mosquito fauna, as it has established here at least seven

years ago. Further, a species that is new to a region can be a strong competitor to endemic species and sometimes crowd them out and replace them. In the case of *Ae. j. japonicus* where there are hints for suppression and replacement of indigenous species (ANDREADIS & WOLFE 2010, ROCHLIN et al. 2013), a change would take place from the presence of a species that was probably not vector-competent to one that can efficiently transmit pathogens to humans and livestock.

The object of the present dissertation work was to understand migration patterns, distribution ways and putative sources of the known European *Ae. j. japonicus* populations and to unveil relationships between the various geographic populations of *Ae. j. japonicus* in Europe. Further, the distribution of *Ae. j. japonicus* in Germany was to be examined.

Therefore, the following studies were conducted:

I.   Examination of the distribution range of *Ae. j. japonicus* in West Germany after the detection of the population in August 2012 (chapter 2.1).

II.   Population genetic analysis of the West German *Ae. j. japonicus* population (chapter 2.2).

III.   Population genetic analysis of newly detected populations of *Ae. j. japonicus* from North Germany and the Netherlands (chapter 2.3).

IV.   Examination of the distribution range of *Ae. j. japonicus* in southeastern Germany and population genetic analyses (chapter 2.4).

V.   Surveillance of the *Ae. j. japonicus* populations in West and North Germany from 2012 until 2015 (chapter 2.5).

2. RESULTS

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

KAMPEN H, **ZIELKE D**, WERNER D (2012). A new focus of *Aedes japonicus japonicus* (THEOBALD, 1901) (Diptera: Culicidae) distribution in western Germany: rapid spread or a further introduction event? Parasites & Vectors 5: 284-290.

I was involved in collecting and identifying the mosquitoes, analysing the data and writing the manuscript.

ZIELKE DE, WERNER D, KAMPEN H, SCHAFFNER F, FONSECA DM (2014). Unexpected patterns of admixture in German populations of *Aedes japonicus japonicus* (Diptera: Culicidae) underscore the importance of human intervention. PLoS One 9: e99093.

I was involved in collecting the mosquitoes and identifying them. I performed all laboratory work and was major contributor to data analysis and writing of the manuscript.

ZIELKE DE, IBÁÑEZ-JUSTICIA A, KALAN K, MERDIC E, KAMPEN H, WERNER D (2015). Recently discovered *Aedes japonicus japonicus* (Diptera: Culicidae) populations in The Netherlands and northern Germany resulted from a new introduction event and a split from an existing population. Parasites & Vectors 8: 40.

I was involved in collecting the mosquitoes and identifying them. I performed all laboratory work and was major contributor to data analysis and writing of the manuscript.

ZIELKE DE, WERNER D, KAMPEN H (2016). Newly discovered population of *Aedes japonicus japonicus* (Diptera: Culicidae) in Upper Bavaria, Germany, and Salzburg, Austria, is closely related to the Austrian/Slovenian bush mosquito population. Parasites & Vectors 9: 163.

I analysed the data and was involved in writing the manuscript.

KAMPEN H, **ZIELKE DE**, KUHLISCH C, WERNER D (2015). Occurrence and spread of invasive Asian bush mosquito *Aedes japonicus japonicus* (Diptera: Culicidae) in West and North Germany since detection in 2012 and 2013, respectively. PLoS One, submitted.

I was involved in collecting and identifying the mosquitoes, designed the figures and contributed to writing the manuscript.

Dorothee E. Zielke   PD Dr. Helge Kampen

Parasites
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A new focus of *Aedes japonicus japonicus* (Theobald, 1901) (Diptera, Culicidae) distribution in Western Germany: rapid spread or a further introduction event?

Helge Kampen[1*], Dorothee Zielke[2] and Doreen Werner[2]

Abstract

Background: The Asian bush mosquito, *Aedes japonicus japonicus*, a potential vector of several viruses, was first detected in Germany in 2008 on the Swiss-German border. In the following years, this invasive species apparently succeeded in establishing populations in southern Germany and in spreading northwards. In 2011, its distribution area already covered large areas of the federal state of Baden-Wurttemberg, and its northernmost German collection point was reported to be close to Stuttgart. Several independent submissions to our laboratories of *Ae. j. japonicus* specimens in July 2012, originating from the same area in the federal state of North Rhine-Westphalia, western Germany, prompted us to carry out an immediate surveillance in this region in the expectation of finding a further distribution focus of *Ae. j. japonicus* in Germany.

Methods: After inspecting the places of residence of the collectors of the submitted mosquito specimens, all kinds of water containers in 123 cemeteries in surrounding towns and villages were checked for mosquito developmental stages. These were collected and kept to produce adults for morphological species identification. One specimen per collection site was identified genetically by COI sequence analysis.

Results: *Aedes j. japonicus* adults and immature stages were found in 36 towns/villages that were checked (29%) over an area of approximately 2,000 km^2 in southern North Rhine-Westphalia and northern Rhineland Palatinate. The species could not be demonstrated further south when monitoring towards the northernmost previous collection sites in southern Germany. It therefore remains to be elucidated whether the species has entered western Germany from the south, from Belgium in the west where it has been demonstrated to occur locally since 2002, or through a new introduction.

Conclusions: *Aedes j. japonicus* is obviously much more widely distributed in Germany than previously thought. It appears to be well adapted, to have a strong expansion tendency and to replace indigenous mosquito species. Thus, a further spread is anticipated and elimination seems hardly possible anymore. The vector potency of the species should be reason enough to thoroughly monitor its future development in Germany.

Keywords: *Aedes japonicus japonicus*, Cemeteries, Distribution focus, Spread, Western Germany

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BioMed Central

Background

Next to the Asian tiger mosquito *Aedes albopictus*, the Asian bush mosquito, *Aedes japonicus japonicus* (Diptera, Culicidae), is one of the most expansive invasive mosquito species in the world [1]. After the brief interception of *Ae. j. japonicus*, together with *Ae. albopictus*, in New Zealand in the early 1990s [2], the species was first reported to have established outside its native distribution area in 1998 in the eastern US [3]. It was probably introduced by the international used tyre trade some years before that, and several times independently [4,5]. *Aedes j. japonicus* has spread considerably since then and is now widely distributed in the eastern part of North America, including Canada [6]. In central Europe, larvae were found in 2000 on the premises of a used tyre trading company in France, but these were quickly eliminated [7]. In 2002, specimens were also collected at two sites in a town in central Belgium, again associated with tyre trading companies. The species still colonized that area in 2003 and 2004 and was present in 2007 and 2008 during a national Belgian mosquito survey [8]. As specimens were never collected elsewhere in Belgium during this survey, the species was considered to be confined to the surroundings of the two companies and control did not take place until 2012. In 2008, *Ae. j. japonicus* appeared in northern Switzerland, and immature stages were also discovered at several sites on German territory along the Swiss-German border [9]. Monitoring carried out in the German federal state of Baden-Wurttemberg in 2009 and 2010 showed the mosquito to occur in a broader region of southern Germany along the Swiss border [10]. Another small study restricted to a limited area south of Stuttgart demonstrated *Ae. j. japonicus* in mid-2010 some 80 km north of the South German distribution area [11]. Three hitherto unpublished findings from 2011 of five females outside the described distribution areas were made in the cities of Stuttgart, Freiburg and Breisach, approx. 20 km west of Freiburg (own results).

Aedes j. japonicus is not only an aggressive biter but a suspected or known vector of various viruses. It has repeatedly been found infected in the field with West Nile virus in the US [12] and efficiently transmits this virus in the laboratory [13,14]; it was shown to be able to transmit Japanese encephalitis virus horizontally and vertically [15] and to be vector-competent for LaCrosse [16], eastern equine encephalitis [17] and St. Louis encephalitis viruses [18] under experimental conditions. Only recently it has also been demonstrated to be vector-competent for chikungunya and dengue viruses in the laboratory, two disease agents that have emerged and re-emerged in southern Europe [19].

In July 2012, seven specimens of *Ae. j. japonicus* were submitted to our laboratories for species identification. In four cases, they had been collected indoors while attacking humans. Three of the mosquitoes were sent by one and the same person, the other four by different persons independently, but all of them from the larger Bonn area in the federal state of North Rhine-Westphalia, West Germany. Taking these coincidences to be a strong hint that there could be a local *Ae. j. japonicus* population, a small monitoring programme was promptly initiated including an inspection of the mosquito collection sites and the screening of cemeteries in that area for immature stages.

Methods

The field work of the study was carried out in August 2012 within less than two weeks. To start with, the immediate surroundings of all five sites where the submitted *Ae. j. japonicus* adults had been collected were inspected for mosquito breeding sites. Water containers of every kind, such as flower-pot saucers, rain water barrels and paddling pools, were checked in the collectors' gardens as well as in the neighbourhood and in the nearest cemeteries, similar to the procedure described by Schaffner *et al.* [9]. To check for further distribution areas, flower vases, watering cans, stone basins and other potential breeding sites were examined in the cemeteries of towns and villages in all geographic directions from the initial detection sites. Once the bush mosquito was found in an inspected cemetery, further villages were checked in distances between 5 and 20 km, following the direction of larval presence. All visited sites were geo-referenced.

Mosquito larvae and pupae were collected by sieves and pipettes and transferred to labelled glas jars with screw tops filled with water from their breeding sites, where they were kept until adult emergence. For the purpose of collecting the emerged adults, a jar was put into a mesh insect cage where its top was removed. The mosquitoes were then collected from the cage by an aspirator and killed by deep freezing (−20°C) for 15 to 20 min. They were identified morphologically using the determination keys by Tanaka *et al.* [20], Schaffner *et al.* [21] and Becker *et al.* [22]. For genetic species confirmation, the cytochrome c oxidase subunit I (COI) gene of one specimen per collection site was PCR-amplified by primers PanCuli-COX1-211 F (5'-ATCATAATTGGTGGG TTTGGWAAYTGA-3') and PanCuli-COX1-727R (5'-TAT AAACTTCDGGRTGNCCAAARAATC-3') and sequenced according to standard protocols [23].

Results and discussion

Mosquito larvae were found on all premises inspected. *Aedes j. japonicus* could be detected in flower vases, flower-pot saucers, watering cans, rain water barrels and a baby paddling pool, either as immature stages or as freshly emerged adults, in the gardens of four of the five
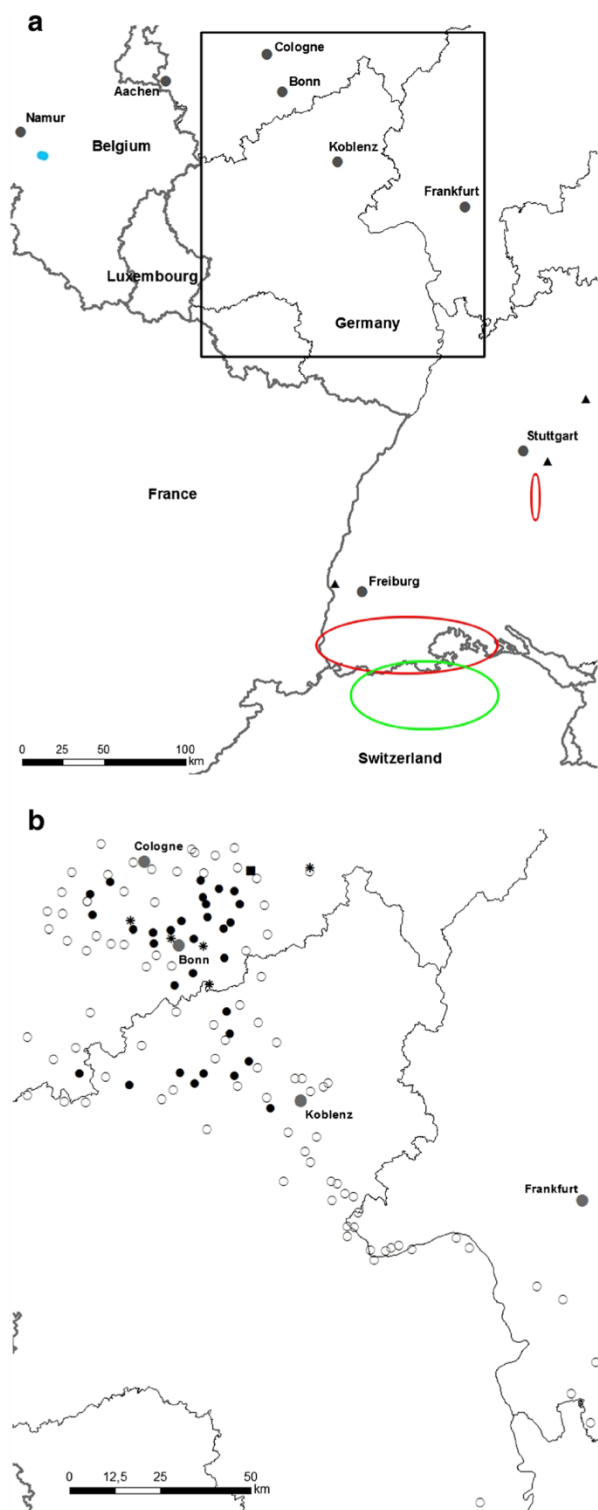
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Figure 1 (See legend on next page.)

(See figure on previous page.)
Figure 1 Distribution areas of *Ae. j. japonicus* in central Europe. a: Overview map: area encircled red: distribution areas in South Germany according to Becker *et al.* [10] and Schneider [11]; area encircled green: distribution area in northern Switzerland according to Schaffner *et al.* [9]; blue dots: local occurrence in Belgium according to Versteirt *et al.* [8]. ▲: Own unpublished accidental findings of *Ae. j. japonicus* outside the previously described German distribution area. Box: see Figure 1b. **b**: Detailed map with collection sites of this study. ✳: Places of residence of *Ae. j. japonicus* senders, ●: cemeteries positive for *Ae. j. japonicus,* ○: cemeteries negative for *Ae. j. japonicus,* ■: rain water barrel positive for *Ae. j. japonicus.*

senders of the original mosquitoes or in the immediate surroundings of their homes. Only at the place of residence of the fifth sender, corresponding to the easternmost collection site, no bush mosquitoes were discovered during a site inspection.

Whilst *Ae. j. japonicus* adults can be easily identified in the field, the larvae can also be differentiated from those of indigenous species with some reliability. They are brownish-yellow or darker, have a quite slender appearance and a moderately long siphon. Even in an advanced larval stage, their head capsule has no bright areas on the dorsal side giving the impression of a dark, more or less round shield. Eyes and mouthparts are not distinctly visible with the naked eye. The larvae spend long periods of time on the ground of their breeding site with little movement, often between debris if present, but if they move they wiggle more intensely and worm-like than the larvae of most indigenous mosquito species ([20,24], own observation).

Using these identification criteria, the distribution area of *Ae. j. japonicus* was provisionally defined whilst in the field, by checking vases and other water containers in cemeteries, which had proved to be a suitable approach in other studies [9,25]. Only after the collecting trips, which took several days each, the emerged mosquitoes were properly identified to species in the laboratory using a dissecting microscope. The GenBank accession numbers for the *Ae. j. japonicus* COI sequences generated for confirmation of the morphological determination are JX888952-94.

In total, out of 123 cemeteries inspected, 35 were found to be infested by *Ae. j. japonicus*. In some towns/villages where cemeteries were positive, larvae of the bush mosquito were also found in water containers away from the cemeteries. In one village without a cemetery, larvae were detected in a rain water barrel. Thus, *Ae. j. japonicus* could be demonstrated in 36 of 124 towns/villages checked, accounting for 29%. The area where *Ae. j. japonicus* occurred in western Germany covered approximately 2,000 km^2 (Figure 1).

Considering the area of distribution, it can be assumed that the introduction of *Ae. j. japonicus* into western Germany dates back to earlier years although complaints only started in 2012 and immature stages could not be found in the infested area during occasional inspections of cemeteries in 2011 and in May 2012.

The origin of the infestation could not be resolved. Of the most obvious possibilities, a northward spread from infested areas in southern Germany (shortest direct line distance between the newly discovered distribution area and the northernmost point of previously known occurrence: approx. 250 km) or an eastward spread from the infestation area in Belgium (shortest direct line distance between the newly discovered distribution area and the Belgian town with the two infested tyre trading companies: approx. 150 km), neither could be verified under the hypothesis that a continuous, more or less linear distribution had taken place. Regions with no documentation of *Ae. j. japonicus* appear to exist between all three distribution areas, although distribution zones with low population densities might have been missed. Prevailing modes of dispersal, active or passive, and routes, e.g. along valleys or rivers, are unknown for this mosquito species. An accidental finding of *Ae. j. japonicus* larvae in 2011, however, close to the town of Schwaebisch-Hall in northeastern Baden-Wurttemberg rather suggests an active invasion from the south.

A third possibility is an additional importation event of the bush mosquito into central Europe, with subsequent establishment. The used tyre trade is no big business in Germany, but it does exist on a limited scale. Efforts to identify tyre trading companies in the newly recognized infested area are under way as well as molecular (microsatellite DNA) analyses of the mosquitoes collected in western Germany to elucidate their genetic relationships to populations from other European and non-European distribution areas.

Although the mosquito collection data from western Germany are not appropriate for ecological and epidemiological analyses, the strong impression arose that *Ae. j. japonicus* tends to displace and replace indigenous mosquito species, just as it appears to do in the US [26]. Except for the peripheral sites of the infested area, where sometimes only one or two water containers with *Ae. j. japonicus* larvae were discovered in a cemetery, often only very few water containers, or no container at all, were occupied by larvae of other mosquito species when *Ae. j. japonicus* immature stages were present. This subject needs further investigation.

A more detailed and systematic monitoring for *Ae. j. japonicus* in western Germany, addressing questions including indigenous mosquito species displacement

and speed of spread, will be carried out in 2013, taking into account the strategies suggested by the ECDC in their guidelines for the surveillance of invasive mosquitoes in Europe [27].

Conclusion

Aedes j. japonicus is not considered an important vector of disease agents under natural conditions in its native geographic distribution area, Japan, Korea, Taiwan and southern China, but has been shown to be vector-competent for several highly pathogenic viruses in the laboratory. Thorough observation of the species in its new distribution areas in Europe is therefore recommended, the more so as it feeds on both birds and humans and thus might serve as a bridge vector for zoonotic disease agents reservoired by birds [28].

Taking into account the considerable distribution areas of *Ae. j. japonicus* in Switzerland and Germany, it is hardly conceivable that this invasive mosquito species could again be eliminated from central Europe. On the contrary, it seems to be very well adapted to European conditions, and a further spread is likely.

Abbreviations
COI: Cytochrome c oxidase subunit I.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
HK designed the study, collected the mosquitoes, carried out the molecular mosquito identification and drafted the manuscript. As part of her doctoral thesis, DZ collected the mosquitoes, performed the morphological identifications and was involved in writing the manuscript. DW designed the study, collected the mosquitoes, conducted a significant part of the morphological mosquito identification and contributed to the drafting of the manuscript. All authors read and approved the final version of the manuscript.

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References
1. Medlock JM, Hansford KM, Schaffner F, Versteirt V, Hendrickx G, Zeller H, van Bortel W: **A review of the invasive mosquitoes in Europe: ecology, public health risks, and control options.** *Vector Borne Zoon Dis* 2012, **12**:435–447.
2. Laird M, Calder L, Thornton RC, Syme R, Holder PW, Mogi M: **Japanese** *Aedes albopictus* **among four mosquito species reaching New Zealand in used tires.** *J Am Mosq Control Assoc* 1994, **10**:14–23.
3. Peyton EL, Campbell SR, Candeletti TM, Romanowski M, Crans WJ: *Aedes* (*Finlaya*) *japonicus japonicus* (Theobald), **a new introduction into the United States.** *J Am Mosq Control Assoc* 1999, **15**:238–241.
4. Scott JJ, Carle FL, Crans WJ: *Ochlerotatus japonicus* **collected from natural rock pools in New Jersey.** *J Am Mosq Contr Assoc* 2001, **17**:91–92.
5. Fonseca DM, Widdel AK, Hutchinson M, Spichiger S-E, Kramer LD: **Fine scale spatial and temporal population genetics of** *Aedes japonicus*, **an new US mosquito, reveal multiple introductions.** *Mol Ecol* 2010, **19**:1559–1572.
6. Morris JA, Lampman RL, Ballmes G, Funes J, Halvorsen J, Novak RJ: **First record of** *Aedes japonicus japonicus* **in Illinois: defining its spatial distribution and associated mosquito species.** *J Am Mosq Control Assoc* 2007, **23**:243–251.
7. Schaffner F, Chouin S, Guilloteau J: **First record of** *Ochlerotatus* (*Finlaya*) *japonicus japonicus* (Theobald, 1901) **in metropolitan France.** *J Am Mosq Control Assoc* 2003, **19**:1–5.
8. Versteirt V, Schaffner F, Garros C, Dekoninck W, Coosemans M, van Bortel W: **Introduction and establishment of the exotic mosquito species** *Aedes japonicus japonicus* **in Belgium.** *J Med Entomol* 2009, **46**:1464–1467.
9. Schaffner F, Kaufmann C, Hegglin D, Mathis A: **The invasive mosquito** *Aedes japonicus* **in central Europe.** *Med Vet Entomol* 2009, **23**:448–451.
10. Becker N, Huber K, Pluskota B, Kaiser A: *Ochlerotatus japonicus japonicus* – **a neozoon in Germany and a revised list of the German mosquito fauna.** *Eur Mosq Bull* 2011, **9**:88–102.
11. Schneider K: **Breeding of** *Ochlerotatus japonicus japonicus* (Diptera: Culicidae) **80 km north of its known range in southern Germany.** *Eur Mosq Bull* 2011, **29**:129–132.
12. Centers for Disease Control: *Mosquito species producing WNV positives by year.* http://www.cdc.gov/ncidod/dvbid/westnile/mosquitospecies.htm. Accessed 30 September 2012.
13. Sardelis MR, Turell MJ: *Ochlerotatus japonicus japonicus* **in Frederick county, Maryland: discovery, distribution and vector competence for West Nile virus.** *J Am Mosq Control Assoc* 2001, **17**:137–141.
14. Turell MJ, O'Guinn ML, Dohm DJ, Jones JW: **Vector competence of North American mosquitoes for West Nile virus.** *J Med Entomol* 2001, **38**:130–134.
15. Takashima I, Rosen L: **Horizontal and vertical transmission of Japanese encephalitis virus by** *Aedes japonicus*. *J Med Entomol* 1989, **26**:454–458.
16. Sardelis MR, Turell MJ, Andre RG: **Laboratory transmission of La Crosse virus by** *Ochlerotatus j. japonicus*. *J Med Entomol* 2002, **39**:635–639.
17. Sardelis MR, Dohm DJ, Pagac B, Andre RG, Turell MJ: **Experimental transmission of eastern equine encephalitis virus by** *Ochlerotatus japonicus japonicus*. *J Med Entomol* 2002, **39**:480–484.
18. Sardelis MR, Turell MJ, Andre RG: **Experimental transmission of St. Louis encephalitis virus by** *Ochlerotatus japonicus japonicus*. *J Am Mosq Control Assoc* 2003, **19**:159–162.
19. Schaffner F, Vazeille M, Kaufmann C, Failloux A-B, Mathis A: **Vector competence of** *Aedes japonicus* **for chikungunya and dengue viruses.** *Eur Mosq Bull* 2011, **29**:141–142.
20. Tanaka K, Mizusawa K, Saugstad ES: **A revision of the adult and larval mosquitoes of Japan (including the Ryukyu Archipelago and the Ogasawara Islands) and Korea (Diptera: Culicidae).** *Contrib Am Ent Inst* 1979, **16**:1–987.
21. Schaffner F, Angel G, Geoffrey B, Hervy JP, Rhaiem A, Brunhes J: *The mosquitoes of Europe. An identification and training programme (CD Rom)*. Montpellier: IRD Éditions & EID Méditerrannée; 2001.
22. Becker N, Petrić D, Zgomba M, Boase C, Madon M, Dahl C, Kaiser A: *Mosquitoes and their control*. 2nd edition. Heidelberg: Springer; 2010.
23. Lehmann K, Werner D, Hoffmann B, Kampen H: **PCR identification of culicoid biting midges (Diptera, Ceratopogonidae) of the Obsoletus complex including putative vectors of bluetongue and Schmallenberg viruses.** *Parasit Vectors* 2012, **5**:213.
24. Miyagi I: **Notes on the** *Aedes* (*Finlaya*) *chrysolineatus* **subgroup in Japan and Korea (Diptera: Culicidae).** *Trop Med* 1971, **13**:141–151.
25. Vezzani D: **Artificial container-breeding mosquitoes and cemeteries: a perfect match.** *Trop Med Int Health* 2007, **12**:199–313.
26. Andreadis TG, Wolfe RJ: **Evidence for reduction of native mosquitoes with increased expansion of invasive** *Ochlerotatus japonicus japonicus* (Diptera: Culicidae) **in the northeastern United States.** *J Med Entomol* 2010, **47**:43–52.

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27. ECDC (European Centre for Disease Prevention and Control): *Guidelines for the surveillance of invasive mosquitoes in Europe*. ECDC Technical Report. Stockholm; 2012.
28. Molaei G, Farajollahi A, Scott JJ, Gaugler R, Andreadis TG: **Human bloodfeeding by the recently introduced mosquito,** *Aedes japonicus japonicus*, **and public health implications.** *J Am Mosq Control Assoc* 2009, **25**:210–214.

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Unexpected Patterns of Admixture in German Populations of *Aedes japonicus japonicus* (Diptera: Culicidae) Underscore the Importance of Human Intervention

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Abstract

The mosquito *Aedes japonicus japonicus*, originally restricted to temperate East Asia, is now widespread in North America and more recently has become established in Europe. To ascertain the putative number of separate introductions to Europe and examine patterns of expansion we analyzed the genetic makeup of *Ae. j. japonicus* populations from five cemeteries in North Rhine-Westphalia and Rhineland-Palatinate, two western German federal states, as well as of specimens from populations in Belgium, Switzerland, and Austria/Slovenia. To do so, we genotyped individual specimens at seven pre-existing polymorphic microsatellite loci and sequenced part of the *nad*4 mitochondrial locus. We found evidence of two different genotypic signatures associated with different *nad*4 mitochondrial haplotypes, indicating at least two genetically differentiated populations of *Ae. j. japonicus* in Europe (i.e. two distinct genotypes). Belgian, Swiss, and Austrian/Slovenian populations all share the same genotypic signature although they have become differentiated since isolation. Contrary to expectations, the German *Ae. j. japonicus* are not closely related to those in Belgium which are geographically nearest but are also highly inbred. German populations have a unique genotype but also evidence of mixing between the two genotypes. Also unexpectedly, the populations closest to the center of the German infestation had the highest levels of admixture indicating that separate introductions did not expand and merge but instead their expansion was driven by punctuated human-mediated transport. Critically, the resulting admixed populations have higher genetic diversity and appear invasive as indicated by their increased abundance and recent spread across western Germany.

Competing Interests: One of the authors, F. Schaffner, is employed by the commercial company AVIA-GIS. This does not alter the authors' adherence to PLOS ONE policies on sharing data and materials.

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Introduction

Biological invasions of potential disease vectors such as mosquitoes (Diptera: Culicidae) inflict more direct threat to human health than those of many other species because they can radically alter the frequency of local or exotic pathogen transmission to humans and wildlife [1]. Notably, invasive mosquitoes are the leading drivers of historic epidemics of yellow fever and of contemporary epidemics of West Nile fever, dengue, and chikungunya [2,3]. The contemporaneous worldwide movement of humans and goods has increased the rate of introductions and establishment of exotic mosquitoes [4], especially those with desiccation resistant eggs, such as many in the genus *Aedes* [5].

A very recent expansion is that of *Aedes japonicus japonicus* Theobald, 1901, also known as the Asian bush mosquito. Although intercepted a few times in the early nineties in New Zealand's ports [6], the first established populations outside the original distribution range were detected in two eastern states of the United States of America (US) in 1998 [7] from where they have quickly spread to over 30 eastern US states [8–10]. Today, the species is present in both US coasts and Canada [11,12] and although this subspecies of *Ae. japonicus*, one of four, is originally restricted to climates with cold snowy winters in Japan and Korea [13,14] surprisingly it became established in the Hawaiian Islands in 2003 [15].

Of note, the current generic name of this species is controversial as it is often called *Ochlerotatus japonicus* and, more recently, *Hulecoeteomyia japonica* [16]. We are using *Aedes japonicus* following the guidelines of Edman and colleagues [17].

In Europe, the first detection of *Ae. j. japonicus* occurred in 2000 when larvae in water in tires at a used tire import platform in Normandy, France, were eliminated before adult emergence [18]. However, in 2002, the species was detected at one location in Belgium, again on a used tire import platform, in Namur province.

It was still there in 2003 and 2004 as well as in 2008 during a national mosquito monitoring campaign when it was also documented on a second used tire platform 2 km away [19]. Because *Ae. j. japonicus* has never been caught outside these two locations, the populations were considered to be not expanding. Nonetheless, in 2012 a control campaign was started aiming at eliminating the species, although to our knowledge it is unclear if it succeeded. In 2008, larvae of *Ae. j. japonicus* were also found in northern Switzerland and in several locations near the border on the German side [20]. A monitoring program carried out in 2009 and 2010 in the affected German federal state of Baden-Württemberg showed the species already occurring over a large area along the border with Switzerland [21]. Another study in 2010 demonstrated a punctual appearance near the city of Stuttgart, approximately 80 km north of the previously known northern distribution border of the species [22]. In the same year, *Ae. j. japonicus* was also found distributed across a 50 km area around the Austrian-Slovenian border [23]. The origins of all these discontinuous populations have remained a mystery so far, but in Germany further *Ae. j. japonicus* infestations have been recognized and the species is now prevalent in the northern part of the federal state of Rhineland-Palatinate and the southern part of the state of North Rhine-Westphalia up to the city of Cologne [24].

Because *Ae. j. japonicus* is a potential vector of several human encephalitis viruses as well as of dengue, chikungunya and Rift Valley fever viruses [20,25,26], it is important to understand the routes of introduction and expansion of the species in Europe. Compared to the Asian tiger mosquito *Aedes albopictus*, *Ae. j. japonicus* is adapted to colder climate, which explains why the latter occurs in northern Europe while the former remains restricted to southern Europe and so far is only found occasionally in Southwest Germany [27,28]. Although the introduction of a new species to a region is always a risk, it also provides an excellent opportunity to observe important drivers of population growth and dispersion that are difficult to detect in existing populations [9]. Knowledge about the number of introductions and modes of spreading after introduction of an invasive species is important to make predictions on future movements and to decide on appropriate control measures [29].

Highly polymorphic DNA regions such as those associated with simple sequence repeats (SSR, also called microsatellites) are powerful tools for studying populations of introduced species both by revealing putative origins as well as changes in allelic frequencies through space and time [30]. Shifts in genetic makeup associated with introductions can be measured by comparison of populations across the exotic range or with those in the original range. These genetic changes can be substantial and have unexpected behavioral or physiological consequences [31–33]. Additionally, analyzing the genetic makeup of a newcomer may help us predict the invader's ability to become established successfully, a trait that can be greatly influenced by genetic variability [34].

The objective of the present study was to examine the patterns of expansion of *Ae. j. japonicus* in North Rhine-Westphalia and Rhineland-Palatinate in western Germany. Specifically, we aimed to answer the following questions: Did the West German mosquitoes originate from nearby existing populations in Belgium, or from populations farther away in Switzerland or Austria/Slovenia, or did other introduction events take place? If multiple introductions occurred are they remaining separate or are they mixing? How are German *Ae. j. japonicus* populations expanding?

Materials and Methods

Mosquito collections

Because adult male or female *Ae. j. japonicus* are not efficiently attracted to standard traps used in mosquito surveillance [35], to the best of our knowledge the most reliable way to collect this species is as larvae from small water holding containers, which was the strategy used across all locations included in this study. Since cemeteries generally have many small water containers such as flowerpot pans and vases, they are ideal habitats for mosquitoes that utilize containers for immature development such as many *Aedes* and *Culex* species [36]. Cemeteries are also hotspots for mosquito production because adults find shelter in those park-like sites, where a profusion of plants provide protective moist habitats as well as sugary nutrition to both males and females [36].

In August 2012, after several specimens of *Ae. j. japonicus* had been sent to us at ZALF in the framework of the online project "Mückenatlas" (http://www.mueckenatlas.de), we started a monitoring program focusing on cemeteries in the states of North Rhine-Westphalia and Rhineland-Palatinate (western Germany). In every cemetery sampled, *Ae. j. japonicus* larvae were collected from at least seven water containers to avoid over-sampling across siblings which would potentially bias the genetic analyses. Larvae were similarly sampled in Switzerland, while in Belgium they were collected from several used tires. Specimens from Austria/Slovenia were obtained from various containers scattered on the side of roads. The mosquitoes were processed as described in Kampen and colleagues [24]. All collection locations are shown in Figure 1 with more details in Table 1.

Mosquito identification and DNA extraction

Larvae from German sites were brought to the laboratory and reared to adults, then killed by exposing them to −20°C for a few minutes, and identified morphologically to species using the key developed by Schaffner and colleagues [37]. Identified adults were stored frozen at −20°C. To confirm the morphological identification, DNA from at least one mosquito from every location was extracted and the CO1 region was sequenced following the protocols of Kampen and colleagues [38] and compared to *Aedes j. japonicus* CO1 sequences in GenBank. Specimens from Belgium, Switzerland and Austria/Slovenia were killed as larvae, stored in alcohol (70%) and identified using the dichotomous key from Schaffner [39] and the multiple-entry key from Schaffner and colleagues [37].

We examined individuals from five cemeteries in western Germany, one tire-recycling platform in Belgium, five locations in Switzerland and eight locations in Austria/Slovenia (Fig. 1, Table 1). We extracted DNA from individual whole adult or larval mosquitoes using either a QIAamp DNA Mini Kit or a DNeasy Blood & Tissue Kit (both from Qiagen) and re-suspended the DNA in 80 µl of buffer EB (Qiagen).

Nad4 sequencing

We sequenced a 424-bp fragment in the sodium dehydrogenase subunit 4 (*nad*4) region of the mosquito mitochondrial DNA (between positions 8398 and 8821 in the *Anopheles gambiae* complete mitochondrial genome sequence, GenBank accession #L20934, [8]) that has shown to be variable and informative for population level analyses [8]. We used primers ND4F 5′-CGTAGGGAG-GAGCAGCTATATT-3′ and ND4R1X 5′-TGATTGCC-TAAGGCTCATGT-3′ [40]. Amplifications were performed in a Bio-Rad C1000 thermal cycler (Bio-Rad Laboratories). Amplifications were preceded by a five minute denaturation at 96°C and consisted of 35 cycles of 40 s at 94°C, 40 s at 56°C and 40 s at

Table 1. Sampling spots in Belgium, Germany, Switzerland, Austria and Slovenia, listed in order of decreasing latitude.

Federal state or province/canton or statistical region	Location	Latitude	Longitude	Date	N_S	N_m
Namur, **Belgium**	Natoye, Hamois	50.3389 N	05.0447 E	08/14/2008	6	6
				08/31/2010	12	12
North Rhine-Westphalia, **Germany**	Altenrath	50.8597 N	07.1959 E	08/29/2012	25	35
	Walberberg	50.7933 N	06.9094 E	08/23/2012	32	40
	Bonn-Hoholz	50.7365 N	07.1979 E	08/23/2012	38	40
Rhineland-Palatinate, **Germany**	Linz	50.5748 N	07.2962 E	08/23/2012	32	41
	Bad Hönningen	50.5197 N	07.3104 E	08/24/2012	27	41
Aargau, **Switzerland**	Möhlin	47.5696 N	07.8256 E	06/29/2012	7	7
	Laufenburg	47.5561 N	08.0611 E	09/04/2008	3	3
	Gebenstorf	47.4842 N	08.2386 E	08/28/2008	6	6
Zurich, **Switzerland**	Dietikon	47.3979 N	08.4060 E	09/02/2008	2	6
Südweststeiermark, **Austria**	Graz Straβgang	47.0219 N	15.3984 E	09/23/2011	2	2
	Dietersdorf	46.9197 N	15.4021 E	09/10/2011	2	2
	Kitzeck	46.7814 N	15.4541 E	09/10/2011	4	4
	Arnfels	46.6762 N	15.4037 E	09/24/2011	5	5
	Glanz	46.6620 N	15.5355 E	09/10/2011	2	2
Steiermark, **Austria**	Haag	46.8477 N	15.9046 E	08/30/2011	5	5
Kärnten, **Austria**	Lavamünd	46.6358 N	14.9539 E	09/24/2011	6	6
Koroška, **Slovenia**	Brezno	46.5961 N	15.3169 E	09/24/2011	5	4
Drava, **Slovenia**	Kamnica	46.5718 N	15.5965 E	09/24/2011	6	6

A total of 227 individuals were sequenced at *nad*4 (N_S) and 273 were genotyped at 7 microsatellite loci (N_m). "Date" refers to the day of collection from the field. The same order of specimens was used in the Bayesian multilocus genotype analysis (Fig. 2).
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72°C, followed by a final extension step of five minutes at 72°C. PCR products were gel-electrophoresed, excised from the gels and recovered with a QIAamp Gel Extraction Kit (Qiagen). They were then cycle-sequenced in both directions with a BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems/Life Technologies) using one of the amplification primers, then purified with SigmaSpin Sequencing Reaction Clean-Up Columns (Sigma-Aldrich) and run on a 3130 Genetic Analyzer (Applied Biosystems/Life Technologies).

Microsatellite analysis

We amplified seven microsatellite loci currently available for *Ae. j. japonicus* [41] using a re-designed OJ5F primer [9]. PCR amplifications were performed in Veriti 96-Well Thermal Cyclers (Applied Biosystems/Life Technologies). The PCR profile was comprised of 30 cycles of 30 s at 94°C, 30 s at 56°C and 30 s at 72°C, preceded by a 5 minute denaturation at 96°C and followed by a 10 minute extension at 72°C. The PCR products were run in an ABI3130XL Genetic Analyzer (Life Technologies) and binned and sized with GeneMapper 3.7 (Applied Biosystems/Life Technologies) using bins optimized on worldwide populations of *Ae. j. japonicus* (Fonseca, unpublished data).

Statistical analysis

We examined departures from Hardy-Weinberg and obtained Shannon's information index (I), mean number of alleles (N_a), observed heterozygosity (Ho), and unbiased expected heterozygosity (uH_e) for each population in GenAlEx 6.5 [42]. Shannon's information index is a diversity measure that takes into consideration the frequency of each allele in addition to the total number of alleles [43,44]. We also assigned individuals to putative

populations based on the expected frequencies of their genotypes in those populations using a "leave one out" option in GenAlEx 6.5 [45,46].

To uncover genetic discontinuities among specimens, we examined the relationships between individual multi-locus signatures using a Bayesian approach in STRUCTURE [47] and determined the optimal number of clusters (K) using the method of Evanno et al. [48] implemented in STRUCTURE HARVESTER [49]. We also performed a factorial correspondence analysis in Genetix 4.05 [50]. We used the GenAlEx software to calculate population based F_{ST} values and Nei's index of divergence (both biased and unbiased), that formed the distance matrices analyzed with a principal coordinate analysis and were used in Mantel tests to examine the relationship between genetic and geographic distances. Pairwise F_{ST} values were also calculated with FSTAT 1.2 in order to check for significance using Fisher exact tests [51].

The distribution of *nad4* haplotypes matches to some extent the distribution of the two genotypes. Specifically, haplotype H5 occurs exclusively and is highly abundant in populations with a predominant genotype 2 signature (Fig. 1).

Results

We genotyped a total of 273 specimens from four European countries and obtained 227 *nad*4 sequences (Table 1). We identified six *nad*4 haplotypes in our samples: H1, H5, H6, H9, H10, H33 (Fig. 1) that can be reconstructed from GenBank accession no. AF305879 and [8], and accession no. KJ958405 (Fonseca, unpublished data). Strikingly, H5 and H6 occurred only in German populations and H6 was restricted to Linz. In contrast, haplotype H1 was found broadly across all populations except in Belgium where H9 was the only haplotype detected. Haplotype
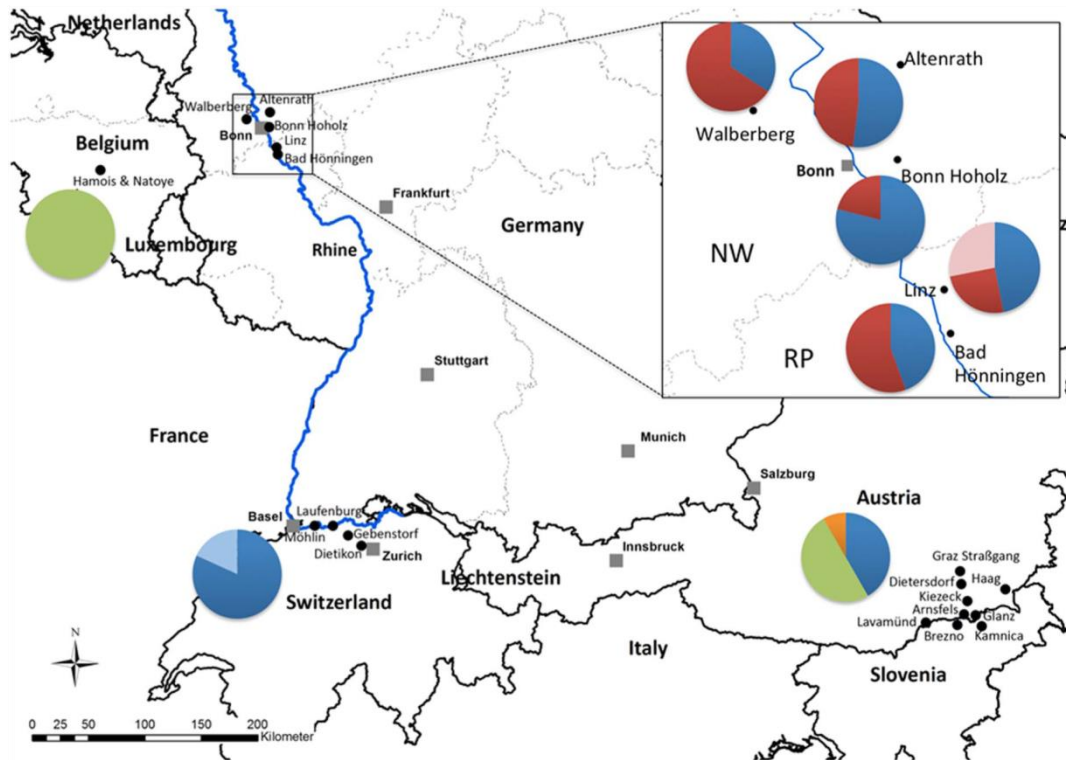
Figure 1. Sampling spots in North Rhine-Westphalia and Rhineland-Palatinate in Germany (box) as well as in Belgium, Switzerland, and Austria/Slovenia. Pie charts show the relative frequency of the six *nad*4 mitochondrial DNA haplotypes. NW = North Rhine-Westphalia, RP = Rhineland-Palatinate. Haplotypes: blue = H1, red = H5, pink = H6, green = H9, orange = H10, light blue = H33. The numbers of specimens sequenced at the *nad*4 locus in each population are listed in Table 1.
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H9 also occurred in Austria/Slovenia together with H10, and H33 occurred in Swiss specimens only (Fig. 1).

The microsatellite multilocus genotype signatures of the specimens fell into two groups (Fig. 2). Specimens from Belgium and Austria/Slovenia had almost exclusively a signature from one group, henceforth named genotype 1, since they were the first found in Europe. Specimens from Bad Hönningen, the southern-most location examined in Germany (Table 1) had almost

exclusively a signature from a second group, henceforth named genotype 2. Specimens from the remaining four German populations showed predominantly a signature from genotype 2 but with clear evidence of admixture with genotype 1. Conversely, specimens from Switzerland showed predominantly signatures from genotype 1 but with evidence of some admixture with genotype 2. Microsatellite raw data generated within this study can be obtained from DF.
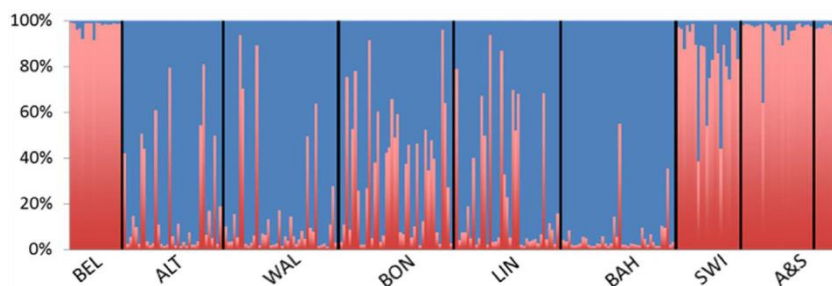
Figure 2. Results of a Bayesian cluster analysis of multilocus microsatellite genotypes. Each individual included in the analysis is represented by a thin vertical line, partitioned into colored segments that represent the individual's probability of belonging to one of the two most likely genetic clusters. The origin of each specimen was not used in the analysis. Red = genotype 1, green = genotype 2, BEL = Belgium; ALT = Altenrath; WAL = Walberberg; BON = Bonn-Hoholz; LIN = Linz; BAH = Bad Hönningen; SWI = Switzerland; A&S = Austria/Slovenia. One specimen from Bad Hönningen has a genotype indicating it is likely the result of a cross between genotype 1 and genotype 2 (an F1).
doi:10.1371/journal.pone.0099093.g002

Table 2. Results of the assignment tests.

Population	BEL	WAL	ALT	BON	LIN	BAH	SWI	A&S	SELF%
Belgium (BEL)	18								100
Walberberg (WAL)		26	5	2	6	1			65
Altenrath (ALT)		4	12	6	7	5	1		34
Bonn Hoholz (BON)		7	8	6	8	8	3		15
Linz (LIN)		5	3	8	9	8	4		24
Bad Hönningen (BAH)				2	2	36			90
Switzerland (SWI)		3				1	16	2	73
Austria/Slovenia (A&S)		1	1			1	3	29	83

"SELF%" is the rate of self-assignment to the population of origin. The actual numbers of specimens self-assigned are highlighted with small boxes on the diagonal. Populations within the center box are from western Germany.
doi:10.1371/journal.pone.0099093.t002

To increase statistical power [52] in non-individual based microsatellite analyses we combined the specimens from Austria and Slovenia and those from Switzerland into single populations although they were collected from a variety of locations ranging from 10 to 50 km from each other (Fig. 1). We checked for departures from Hardy-Weinberg (H&W) frequencies and found significant departures at OJ10 in Austria/Slovenia and at OJ10 and OJ85 in the Swiss population. However, we found similar levels of incidence of significant departures from H&W frequencies at some loci in the German populations and in Belgium that were derived from specimens collected in the same cemetery or tire platform indicating a lack of biological significance to this small number of random departures from H&W. Departures were both due to significantly lower than expected and higher than expected heterozygosity values, and close inspection did not indicate a significant effect of null alleles [53].

Two genetic groups were also identified by assignment tests based on allelic frequency, likelihood, and genetic distance although there was evidence of strong differentiation among locations with a genetic signature from genotype 1 as evidenced by the lack of assignment of specimens to locations outside their own (Table 2). This differentiation among Belgian, Austrian/Slovenian and Swiss populations is also evident from the results of the principal coordinate analyses (Fig. 3) based on pairwise F_{ST} values. As would be expected from their proximity, most German populations are closely related although overall the relationship between genetic distance and geographic distance is not significant (Mantel tests, $P>0.05$). Instead, the two populations, Linz and Bonn-Hoholz, closer to the geographic "middle" of the five German sites, have more signs of admixture with genotype 1 and are therefore more similar to Swiss specimens than to the Bad Hönningen population, which is further south and therefore geographically closer to Switzerland (Figs. 2 and 3). Of note, equivalent results were obtained with other measures of pair-wise genetic distance/similarity such as Nei's indices of divergence (data not shown) as well as from the factorial correspondence analysis based on individual genotypes (Fig. S1).

Discussion

The expanding populations in Germany show a signature of admixture reminiscent of the mixing across Pennsylvania of two separate introductions to the US [9]. Unlike in Pennsylvania where the mixing appeared to occur as the introductions abutted, in western Germany it appears the admixture between the two introductions occurred from the center of the sampled infestation. The most admixed population is Bonn-Hoholz, followed by Linz and Altenrath, instead of those populations closest to Belgium (Walberberg) or Switzerland (Bad Hönningen). It therefore seems that genotype 1 specimens were transported from Belgium, Switzerland and/or Austria/Slovenia (or even possibly other locations where genotype 1 may have established) into Bonn, a medium sized city on the margins of the Rhine River in the German federal state of North Rhine-Westphalia, where they admixed with a local population of *Ae. j. japonicus*, which originally had a very distinct signature both in its *nad*4 composition and genotypic makeup (genotype 2). The population with a genotype 2 signature with the least admixed specimens and a low genetic diversity is Bad Hönningen, which of the five locations studied is the farthest from Bonn (Fig. 1). This indicates that introductions of both genotypes (first genotype 2 and subsequently genotype 1) may have occurred into the Bonn area.

Clearly our data show that western German *Ae. j. japonicus* are not simply an expansion of the Belgium population, as we first
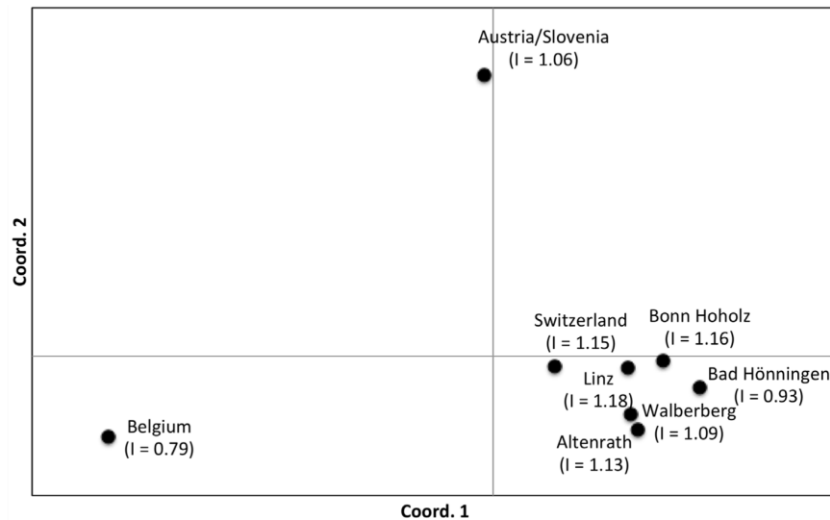
Figure 3. Principal coordinate analysis plot of pairwise F_{ST} genetic distances for the five German populations and samples from Belgium, Switzerland, and Austria/Slovenia. For sample sizes please refer to Table 1. Values in parentheses are Shannon diversity indices (I). Coordinates 1 and 2 account for 86.6% of the variation.
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thought possible because of the geographical adjacency. Instead our results indicate that the population of *Ae. j. japonicus* that was first detected in Belgium in 2002 has not expanded on its own, possibly due to low genetic diversity (this population had the lowest genetic diversity of all populations examined, Fig. 3). However, the presence of genotype 1 specimens, with similar genotypic and haplotypic signatures in Belgium, Switzerland and Austria/Slovenia opens the possibility that all three introductions were derived from the same source of *Ae. j. japonicus*. At this point it is unclear if the Belgium populations were really "the first" or just "the first found". In any case, following human-mediated transport from its source in northeast Asia to somewhere in Europe where it established, genotype 1 was moved to multiple locations in Europe. Alternatively, there could have been multiple introductions and establishments from the same source in Asia over the years. In either scenario the fact that the distribution of genotype 1 is very discontinuous indicates that the expansion of *Ae. j. japonicus* in Europe has been predominantly human-mediated.

Critically, we found undisputed evidence of a second genotype of *Ae. j. japonicus* in Europe, possibly introduced to or near Bonn in western Germany. And we found evidence that in areas where specimens from genotype 1 encountered specimens from genotype 2, admixed populations with increased genetic diversity were produced. These appear to be invasive, i.e. capable of spreading unaided, since the species now occurs continuously in a broad region in Germany from the state of North Rhine-Westphalia into Rhineland-Palatinate and potentially into Baden-Württemberg, although a detailed analysis of specimens from that state will be required to test this hypothesis. In conclusion, we found evidence of at least two different introductions of *Ae. j. japonicus* to Europe resulting in two unique genetic signatures and an expanding population with clear signs of admixture of the two (Figs. 2 and 3).

These analyses, of course, do not reveal the origin of European *Ae. j. japonicus*. They may have arrived from sources in Asia or from the USA where the species is now relatively abundant [11], although haplotype H5, commonly associated with the European genotype 2, is very rare in the US and H33, associated with

genotype 1, has not been detected there [9], which indicates that Asia may be the more likely source of the European specimens. Assessing origin will require a thorough comparative analysis of representative populations of *Ae. j. japonicus* across the world.

Regarding the expansion across Europe, however, and especially considering the known distribution of genotype 1, we speculate that the Rhine River may have played an important role because it provides a traffic artery between industrial sites in the Netherlands in the north all the way across western Germany to the Swiss border (Fig. 1). Importantly, our results also indicate that for the last decade human-mediated transport has been the main driver of the expansion of *Ae. j. japonicus* across Europe. However, if the recent seemingly fast expansion across Germany is a true event and not just the result of increased surveillance, then the evident mixing between the two genotypes may have changed something important in the characteristics of the European populations. After a decade of relatively slow expansion, *Ae. j. japonicus* abundance and continuous occurrence from Zurich in Switzerland to Bonn in Germany and even potentially into Hanover in northern Germany [54] and the Netherlands where the species was just detected [55] indicate populations that are expanding unaided, which may complicate control measures considerably.

Although in Japan this mosquito is not considered an important nuisance or disease vector [14], US populations of *Ae. j. japonicus* have risen to nuisance levels especially in more northern states such as Michigan [11], which are too cold for urban vectors such as *Ae. albopictus* and *Culex pipiens*. Their willingness to bite humans is underscored by the fact that 30% of the blood meals identified from *Ae. j. japonicus* from New Jersey suburbs were human with predominance of blood meals from large vertebrates such as deer [56]. Their preference for large vertebrates is especially worrisome due to the proximity and extensive trade between Europe and Africa where Rift Valley fever, a disease of ruminants that can be deadly to humans, is endemic [57]. Because US populations of *Ae. j. japonicus* have been shown to be highly competent vectors of Rift Valley fever virus [26] the presence of large populations of *Ae. j. japonicus* in northern Europe increases the likelihood of Rift Valley

fever epidemics, similar to the danger created by the presence of large populations of *Ae. albopictus* in southern Europe. Indeed, the abundance of *Ae. albopictus* in Italy has already resulted in local transmission of chikungunya virus [58]. "Human intervention" regarding *Ae. j. japonicus* needs to cease to be accidental and instead become deliberate and organized. Our results indicate that a first and critical step towards managing *Ae. j. japonicus* is increased surveillance and active control to identify and stop further introductions, establishments and mixing of differentiated populations.

Supporting Information

Figure S1 Results of a factorial correspondence analysis performed on individual genotypes in Genetix 4.05. Yellow squares and burgundy squares correspond to individuals from Belgium and Austria/Slovenia populations, respectively. Swiss specimens are shown in dark blue, the remaining colors (light blue, pink, green, grey and white) are from German populations. These results mirror the results of the principal coordinate analysis on populations although it is hard to separate

German populations, which is not surprising since they all have some degree of admixture between two introductions. Of note the green squares correspond to specimens from Bad Hönningen, which have the lowest genetic diversity (lowest levels of admixture). (JPG)

References

1. Crowl TA, Crist TO, Parmenter RR, Belovsky G, Lugo AE (2008) The spread of invasive species and infectious disease as drivers of ecosystem change. Front Ecol Environ 6: 238–246.
2. Lounibos LP (2002) Invasions by insect vectors of human disease. Annu Rev Entomol 47: 233–266.
3. Tsetsarkin KA, Weaver SC (2011) Sequential adaptive mutations enhance efficient vector switching by chikungunya virus and its epidemic emergence. PLoS Pathog 7: e1002412.
4. Medlock JM, Hansford KM, Schaffner F, Versteirt V, Hendrickx G, et al. (2012) A review of the invasive mosquitoes in Europe: ecology, public health risks, and control options. Vector Borne Zoonotic Dis 12: 435–447.
5. Hawley WA (1988) The biology of *Aedes albopictus*. J Am Mosq Control Assoc Suppl 1: 1–39.
6. Laird M, Calder L, Thornton RC, Syme R, Holder PW, et al. (1994) Japanese *Aedes albopictus* among four mosquito species reaching New Zealand in used tires. J Am Mosq Control Assoc 10: 14–23.
7. Peyton EL, Campbell SR, Candeletti TM, Romanowski M, Crans WJ (1999) *Aedes (Finlaya) japonicus japonicus* (Theobald), a new introduction into the United States. J Am Mosq Control Assoc 15: 238–241.
8. Fonseca DM, Campbell S, Crans WJ, Mogi M, Miyagi I, et al. (2001) *Aedes (Finlaya) japonicus* (Diptera: Culicidae), a newly recognized mosquito in the United States: analyses of genetic variation in the United States and putative source populations. J Med Entomol 38: 135–146.
9. Fonseca DM, Widdel AK, Hutchinson M, Spichiger SE, Kramer LD (2010) Fine-scale spatial and temporal population genetics of *Aedes j. japonicus*, a new US mosquito, reveal multiple introductions. Mol Ecol 19: 1559–1572.
10. Neitzel DF, Johnson KA, Brogren S, Kemperman MM (2009) First collection records of *Aedes japonicus* in Minnesota. J Am Mosq Control Assoc 25: 367–369.
11. Kaufman MG, Fonseca DM (2014) Invasion biology of *Aedes japonicus japonicus*. Annu Rev Entomol 59: 31–49.
12. Thielman A, Hunter FF (2006) Establishment of *Ochlerotatus japonicus* (Diptera: Culicidae) in Ontario, Canada. J Med Entomol 43: 138–142.
13. Cameron EC, Wilkerson RC, Mogi M, Miyagi I, Toma T, et al. (2010) Molecular phylogenetics of *Aedes japonicus*, a disease vector that recently invaded western Europe, North America, and the Hawaiian islands. J Med Entomol 47, 527–535.
14. Tanaka K, Mizusawa K, Saugstad ES (1979) A revision of the adult and larval mosquitoes of Japan (including the Ryukyu archipelago and the Ogasawore islands) and Korea (Diptera: Culicidae). Contrib Amer Ent Inst 16: 1–987.
15. Larish LB, Savage HM (2005) Introduction and establishment of *Aedes (Finlaya) japonicus japonicus* (Theobald) on the island of Hawaii: implications for arbovirus transmission. J Am Mosq Control Assoc 21: 318–321.
16. Reinert JF, Harbach RE, Kitching IJ (2009) Phylogeny and classification of tribe Aedini (Diptera: Culicidae). Zool J Linn Soc 157: 700–794.
17. Edman JD (2005) Journal policy on names of Aedine mosquito genera and subgenera. J Med Entomol 42: 511.
18. Schaffner F, Chouin S, Guilloteau J (2003) First record of *Ochlerotatus (Finlaya) japonicus japonicus* (Theobald, 1901) in metropolitan France. J Am Mosq Control Assoc 19: 1–5.
19. Versteirt V, Schaffner F, Garros C, Dekoninck W, Coosemans M, et al. (2009) Introduction and establishment of the exotic mosquito species *Aedes japonicus japonicus* (Diptera: Culicidae) in Belgium. J Med Entomol 46: 1464–1467.

20. Schaffner F, Kaufmann C, Hegglin D, Mathis A (2009) The invasive mosquito *Aedes japonicus* in Central Europe. Med Vet Entomol 23: 448–451.
21. Becker N, Huber K, Pluskota B, Kaiser A (2011) *Ochlerotatus japonicus japonicus* – a newly established neozoan in Germany and a revised list of the German mosquito fauna. Eur Mosq Bull 29: 88–102.
22. Schneider K (2011) Breeding of *Ochlerotatus japonicus japonicus* (Diptera: Culicidae) 80 km north of its known range in southern Germany. Eur Mosq Bull 29: 129–132.
23. Seidel B, Duh D, Nowotny N, Allerberger F (2012) First record of the mosquitoes *Aedes (Ochlerotatus) japonicus japonicus* (Theobald, 1901) in Austria and Slovenia 2011 and for *Aedes (Stegomyia) albopictus* (Skuse, 1895) in Austria. Entomol Z 122: 223–226.
24. Kampen H, Zielke D, Werner D (2012) A new focus of *Aedes japonicus japonicus* (Theobald 1901) (Diptera, Culicidae) distribution in western Germany: rapid spread or a further introduction event? Parasit Vectors 5: 284.
25. Schaffner F, Medlock JM, Van Bortel W (2013) Public health significance of invasive mosquitoes in Europe. Clin Microbiol Infect 19: 685–692.
26. Turell MJ, Byrd BD, Harrison BA (2013) Potential for populations of *Aedes j. japonicus* to transmit Rift Valley fever virus in the USA. J Am Mosq Control Assoc 29: 133–137.
27. Becker N, Geier M, Balczun C, Bradersen U, Huber K, et al. (2013) Repeated introduction of *Aedes albopictus* into Germany, July to October 2012. Parasitol Res 112: 1787–1790.
28. Kampen H, Kronefeld M, Zielke D, Werner D (2013) Further specimens of the Asian tiger mosquito *Aedes albopictus* (Diptera, Culicidae) trapped in southwest Germany. Parasitol Res 112: 905–907.
29. Myers JH, Savoie A, van Randen E (1998) Eradication and pest management. Annu Rev Entomol 43: 471–491.
30. Lee CE (2002) Evolutionary genetics of invasive species. Trends Ecol Evol 17: 386–391.
31. Ross KG, Vargo EL, Keller L, Trager JC (1993) Effect of a founder event on variation in the genetic sex-determining system of the fire ant *Solenopsis invicta*. Genetics 135: 843–854.
32. Tsutsui ND, Suarez AV, Holway DA, Case TJ (2000) Reduced genetic variation and the success of an invasive species. Proc Natl Acad Sci USA 97: 5948–5953.
33. Tsutsui ND, Suarez AV, Holway DA, Case TJ (2001) Relationships among native and introduced populations of the Argentine ant (*Linepithema humile*) and the source of introduced populations. Mol Ecol 10: 2151–2161.
34. Kolbe JJ, Glor RE, Rodriguez Schettino L, Lara AC, Larson A, et al. (2004) Genetic variation increases during biological invasion by a Cuban lizard. Nature 431: 177–181.
35. Schaffner F, Bellini R, Petrić D, Scholte EJ, Zeller H, et al. (2013) Development of guidelines for the surveillance of invasive mosquitoes in Europe. Parasit Vectors 6: 209.
36. Vezzani D (2007) Artificial container-breeding mosquitoes and cemeteries: a perfect match. Trop Med Int Health 12: 299–313.
37. Schaffner F, Angel G, Geoffrey B, Hervy JP, Rhaiem A, et al. (2001) The mosquitoes of Europe. An identification and training programme (CD-Rom). IRD Éditions & EID Méditerrannée, Montpellier.
38. Kampen H, Kronefeld M, Zielke D, Werner D (2014) Three rarely encountered and one new *Culiseta* species (Diptera: Culicidae) in Germany. J Eur Mosq Control Assoc 31: 36–39.

39. Schaffner F (2003) Mosquitoes in used tyres in Europe: species list and larval key. Eur Mosq Bull 16: 7–12.

40. Egizi A, Fonseca DM (2014) Ecological limits can obscure expansion history: patterns of genetic diversity in a temperate mosquito in Hawaii. Biol Invasions, in press.

41. Widdel AK, McCuiston LJ, Crans WJ, Kramer LD, Fonseca DM (2005) Finding needles in the haystack: single copy microsatellite loci for *Aedes japonicus* (Diptera: Culicidae). Am J Trop Med Hyg 73: 744–748.

42. Peakall R, Smouse PE (2012) GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research–an update. Bioinformatics 28: 2537–2539.

43. Sherwin WB (2010) Entropy and information approaches to genetic diversity and its expression: genomic geography. Entropy 12: 1765–1798.

44. Sherwin WB, Jabot F, Rush R, Rossetto M (2006) Measurement of biological information with applications from genes to landscapes. Mol Ecol 15: 2857–2869.

45. Manel S, Gaggiotti OE, Waples RS (2005) Assignment methods: matching biological questions with appropriate techniques. Trends Ecol Evol 20: 136–142.

46. Waser PM, Strobeck C (1998) Genetic signatures of interpopulation dispersal. Trends Ecol Evol 13: 43–44.

47. Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155: 945–959.

48. Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software structure: a simulation study. Mol Ecol 14: 2611–2620.

49. Earl DA, vonHoldt BM (2012) STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conserv Genet Resour 4: 359–361.

50. Belkhir K, Borsa P, Chikhi L, Raufaste N, Catch F (2004) GENETIX 4.05.2 software under Windows TM for the genetics of the populations. Laboratory genome, populations, interactions. CNRS UMR 5000, University of Montpellier II, Montpellier, France.

51. Goudet J (1995) FSTAT (Version 1.2): A computer program to calculate F-statistics. J Hered 86: 485–486.

52. Cohen J (1988) Statistical Power Analysis for the Behavioral Sciences, 2nd ed. Lawrence Erlbaum Associates, Inc., Hillsdale, New Jersey.

53. Dakin EE, Avise JC (2004) Microsatellite null alleles in parentage analysis. Heredity 93: 504–509.

54. Werner D, Kampen H (2013) The further spread of *Aedes japonicus japonicus* (Diptera, Culicidae) towards northern Germany. Parasitol Res 112: 3665–3668.

55. Ibáñez-Justicia A, Kampen H, Braks M, Schaffner F, Steeghs M, et al. (2014) First report of *Aedes japonicus japonicus* (Theobald, 1901) (Diptera, Culicidae) population in the Netherlands. J Eur Mosq Control Assoc 32: 9–13.

56. Molaei G, Farajollahi A, Scott JJ, Gaugler R, Andreadis TG (2009) Human blood feeding by the recently introduced mosquito, *Aedes japonicus japonicus*, and public health implications. J Am Mosq Control Assoc 25: 210–214.

57. Woods CW, Karpati AM, Grein T, McCarthy N, Gaturuku P, et al. (2002) An outbreak of Rift Valley fever in Northeastern Kenya, 1997–98. Emerg Infect Dis 8: 138–144.

58. Rezza G, Nicoletti L, Angelini R, Romi R, Finarelli AC, et al. (2007) Infection with chikungunya virus in Italy: an outbreak in a temperate region. Lancet 370: 1840–1846.

Parasites & Vectors

RESEARCH   **Open Access**

Recently discovered *Aedes japonicus japonicus* (Diptera: Culicidae) populations in The Netherlands and northern Germany resulted from a new introduction event and from a split from an existing population

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Abstract

Background: Originally native to East Asia, *Aedes japonicus japonicus*, a potential vector of several arboviruses, has become one of the most invasive mosquito species in the world. After having established in the USA, it is now spreading in Europe, with new populations emerging. In contrast to the USA, the introduction pathways and modes of dispersal in Europe are largely obscure.

Methods: To find out if two recently detected populations of *Ae. j. japonicus* in The Netherlands and northern Germany go back to new importations or to movements within Europe, the genetic makeup of mosquito specimens from all known European populations was compared. For this purpose, seven microsatellite loci from a representative number of mosquito specimens were genotyped and part of their mitochondrial *nad*4 gene sequenced.

Results: A novel *nad*4 haplotype found in the newly discovered Dutch population of *Ae. j. japonicus* suggests that this population is not closely related to the other European populations but has emanated from a further introduction event. With five *nad*4 haplotypes, the Dutch population also shows a very high genetic diversity indicating that either the founder population was very large or multiple introductions took place. By contrast, the recently detected North German population could be clearly assigned to one of the two previously determined European *Ae. j. japonicus* microsatellite genotypes and shows *nad*4 haplotypes that are known from West Germany.

Conclusion: As the European populations of *Ae. j. japonicus* are geographically separated but genetically mixed, their establishment must be attributed to passive transportation. In addition to intercontinental shipment, it can be assumed that human activities are also responsible for medium- and short-distance overland spread. A better understanding of the processes underlying the introduction and spread of this invasive species will help to increase public awareness of the human-mediated displacement of mosquitoes and to find strategies to avoid it.

Keywords: *Aedes japonicus japonicus*, Asian bush mosquito, Europe, Microsatellites, Population genetics, *nad*4 haplotypes

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Background

The Asian bush mosquito *Aedes* (*Finlaya*) *japonicus japonicus* (Theobald, 1901) (*Hulecoeteomyia japonica japonica* sensu Reinert *et al.* [1]) is one of the most expansive mosquito species in the world [2]. After repeated interceptions in New Zealand in the early 1990s [3], the first established populations outside the original distribution range were detected in the eastern USA [4,5]. From three states that had initially been invaded, it spread in only a few years into 30 further states, including Hawaii [6-8]. Today, the species is also present in Canada [9].

In Europe, larvae of *Ae. j. japonicus* were first detected in the year 2000 in a used tyre trade company in France, but were eradicated [10]. In 2002, the mosquito was found in Belgium, again in the context of a used tyre platform. The species was still present in 2003 and 2004 and was found during a national mosquito monitoring programme in 2007 and 2008, when a second company trading with used tyres was affected in the same Belgian town [11]. As *Ae. j. japonicus* was never caught more than 2 km away from these two locations, it was concluded that the population was not expanding.

Also in 2008, larvae of *Ae. j. japonicus* were discovered in northern Switzerland and in several places on the German side of the Swiss-German border [12]. A monitoring programme carried out in 2009 and 2010 in the German federal state of Baden-Wurttemberg showed that the Asian bush mosquito had already infested a large area along the border with Switzerland [13]. Another study from 2010 detected its presence near the city of Stuttgart, approximately 80 km north of what had been assumed to be the northern distribution limit of the species [14]. Several findings of *Ae. j. japonicus* individuals made in 2011 at various places in Baden-Wurttemberg (Werner & Kampen, unpublished) indicated a much greater distribution area at that time. In the same year, a population was found widely distributed on both sides of the Austrian-Slovenian border [15]. By 2013, this population had expanded over the entire country of Slovenia, even reaching northern Croatia (Kalan, Merdić, unpublished).

Since its first detection in Baden-Wurttemberg in 2008, *Ae. j. japonicus* seems to have been spreading continuously across Germany. Populations have been shown to exist in the federal states of North Rhine-Westphalia and Rhineland-Palatinate [16] and, more recently, as far north as in Lower Saxony [17]. In January 2013, Dutch researchers detected a single *Ae. j. japonicus* female already trapped in July 2012 during routine monitoring in the municipality of Lelystad (province of Flevoland). Extensive surveillance from April to October 2013 in the surroundings of the first finding identified numerous breeding sites over large parts of the municipality [18].

The first finding was about 7 km from a tyre trading company, but no individuals could be found on the company's premises.

Of the six *Ae. j. japonicus* populations detected in Europe, only the Belgian one is known to be due to an importation in used tyres. For the other five populations there is no information on their mode of introduction nor on their origin or relatedness.

Aedes j. japonicus is a potential vector of several arboviruses including West Nile virus (WNV) and Japanese encephalitis virus [19,20]. In the USA, it has been found infected with WNV in the field [21]. In addition, the species is able to transmit La Crosse encephalitis, St. Louis encephalitis, eastern equine encephalitis and Rift Valley fever viruses under laboratory conditions [22-24] and is susceptible to infection with chikungunya and dengue viruses [25]. Its spread and behaviour therefore merit close observation.

The objective of the present study was (i) to learn more about the relationships between the various European populations of *Ae. j. japonicus* and, in particular, (ii) to assign the newly discovered populations in Lelystad, The Netherlands, and in Lower Saxony, Germany, to already known genotypes, in order to detect genetic proofs for introduction or migration events. For this purpose we analysed highly polymorphic simple sequence repeats (microsatellites) and maternally inherited, rapidly evolving mitochondrial *nad*4 sequences which are characterised by variable numbers of repeats or nucleotide sequences, making them appropriate targets for population genetics and the identification of source populations [26,27].

Methods

Mosquitoes

Mosquito larvae and eggs were collected between May and October 2013 from flower vases and other small artificial water containers and from ovitraps in cemeteries and gardens [28].

Individuals central to the study were collected from two sites, about 60 km apart, in the North German federal state of Lower Saxony (NG) and from six sites within the municipality of Lelystad, The Netherlands. In addition, specimens from two towns in the South German federal state of Baden-Wurttemberg (SG) and from 18 sites in Slovenia as well as two individuals from two close collection sites in Croatia were examined (for details see Table 1 and Figure 1). For comparison, previously analysed specimens from West Germany (WG), Belgium and Switzerland [29] were included.

Larvae from NG and SG were sampled in cemeteries, taken to the laboratory in their original water and reared to adults. These were killed by exposing them to -20°C for at least 1 h, identified morphologically to species level according to the key by Schaffner *et al.* [30] and

Table 1 Origin of *Ae. j. japonicus* specimens included in the study

Country	Federal state/province/region	Location	No. of mosquitoes analysed		I
			Microsatellites	***nad*4**	
Germany	Baden-Wurttemberg (SG)	Korntal (1)	39	22	1.26
		Waldshut-Tiengen (2)	41	26	1.21
	Lower Saxony (NG)	Bad Eilsen (3)	20	20	1.1
		Sarstedt (4)	12	10	1.15
The Netherlands	Flevoland	Lelystad (5)	43	37	1.06
Slovenia	Pomurska(6)	Ljutomer	5	2	0.67
	Podravska (7)	Selnica ob Dravi	5	4	1.16
		Pesnica pri Mariboru	2	0	
		Sentilj	5	2	
		Lovrenc na Dravskem Polju	2	1	
		Ptuj	3	2	
		Makole	5	5	
		Ormoz	2	2	
	Koroska (8)	Lovrenc na Pohorju	5	4	1.12
		Ribnica na Pohorju	2	2	
		Muta	3	3	
		Slovenj Gradec	5	4	
	Savinjska (9)	Rogatec	2	1	1.23
		Smarje pri Jelsah	2	0	
		Bistrica ob Sotli	5	4	
	Osrednjeslovenska (10)	Ig	3	1	0.74
		Grosuplje	2	2	
		Ivancna Gorica	1	0	
Croatia	Krapina-Zagorje (11)	Djurmanec	1	0	n. a.
		Macelj	0	0	

Out of a total of 215 individuals subjected to microsatellite analysis, 154 produced analysable *nad*4 sequence data. Numbers in parentheses refer to the geographic location of the collection sites as shown in Figure 1. NG = North Germany, SG = South Germany, I = Shannon's information index, n. a. = not applicable.

kept frozen at -20°C until molecular examination. Dutch individuals were collected from rain water barrels and buckets in allotment gardens and from a flower vase in a cemetery. They were placed as larvae into 80% ethanol immediately after collection in the field and were also identified using the key by Schaffner *et al.* [30]. Slovenian specimens were also collected as larvae from cemeteries and gardens and preserved in 80% ethanol. Identification was made using the key in the ECDC's guidelines for the surveillance of invasive mosquitoes in Europe [28]. The specimens from Croatia were reared to adults from eggs found in cemeteries and were determined to species using the key by Gutsevich *et al.* [31].

DNA extraction

DNA extraction was performed on complete adult mosquitoes or larvae using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. In the case of the Croatian individuals, DNA was extracted from single legs using the same kit. DNA was eluted in 80 μl EB buffer (Qiagen) and kept frozen until use.

Microsatellite analysis

PCR amplification was performed in a C1000™ 96 well thermal cycler (BioRad). The thermoprofile consisted of a 3 min denaturation step at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 56°C and 30 s at 72°C, and a final 10 min elongation step at 72°C. For each of the seven targeted microsatellite loci (OJ5, OJ10, OJ70, OJ85, OJ100, OJ187 and OJ338) one pair of primers was used as previously described [32]. Only the forward primer for locus OJ5 was redesigned [33]. PCR products were sized in a 3130xl Genetic Analyzer (Applied Biosystems/Hitachi), and the obtained fragment length analysis data were visualised and verified with GeneMapper 3.7 (Applied Biosystems).

Because frequency-based microsatellite analysis requires a minimum population size that was not given for every
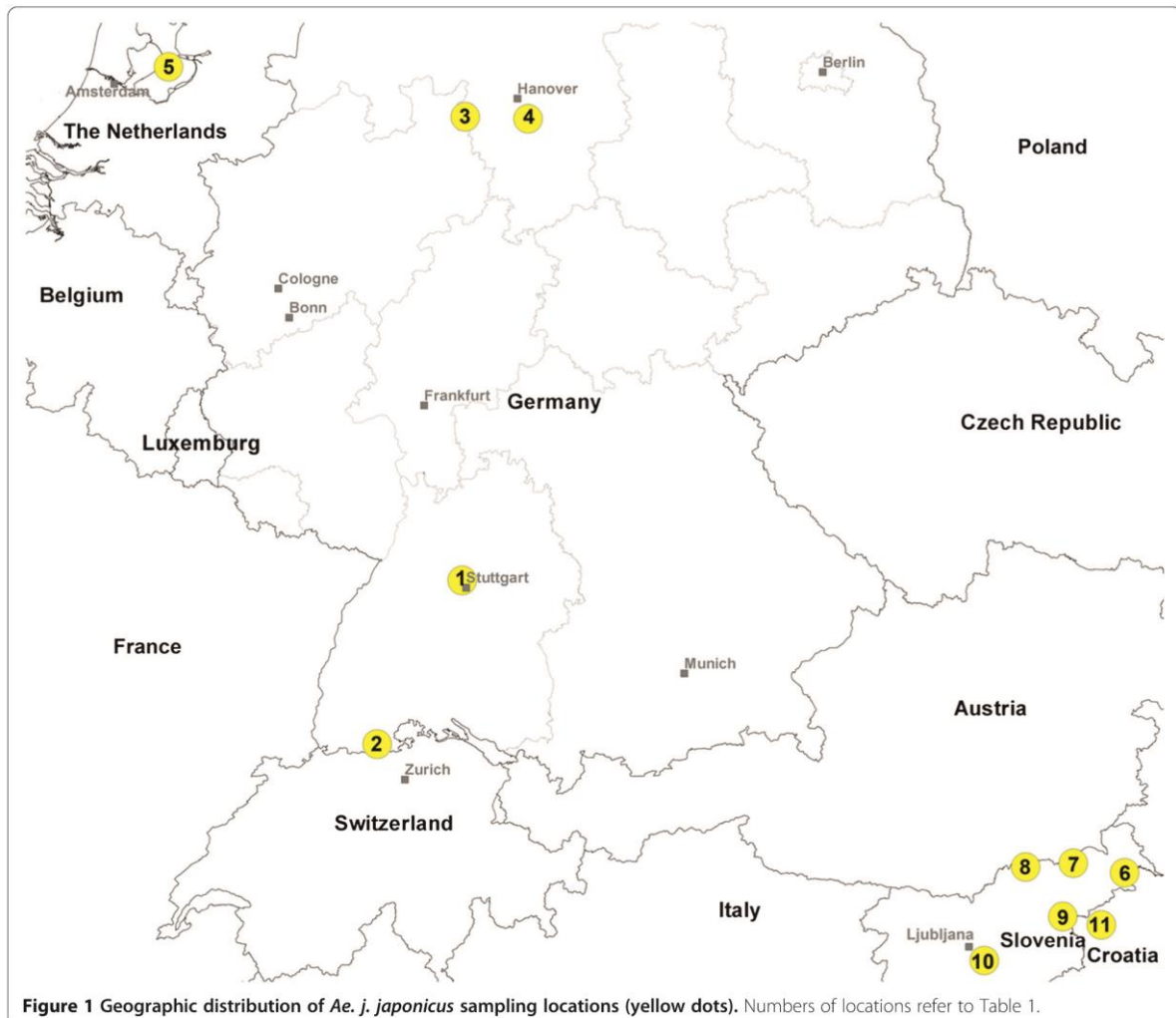
Figure 1 Geographic distribution of *Ae. j. japonicus* sampling locations (yellow dots). Numbers of locations refer to Table 1.

sampling site in Slovenia, individuals from there were assigned to five groups according to the geographic regions where they were sampled.

nad4 sequencing

Additionally, part of the sodium dehydrogenase subunit 4 (*nad*4) gene of the mitochondrial DNA of the sampled specimens was sequenced using a modification of the protocol of Fonseca et al. [32]. The primers used, ND4F (5′-CGTAGGAGGAGCAGCTATATT-3′) and ND4R1X (5′-TGATTGCCTAAGGCTCATGT-3′) [33], amplify a 424 bp fragment between positions 8398 and 8821 in the *Anopheles gambiae* genomic sequence (GenBank accession no. L20934). DNA amplification was preceded by a 10 min denaturation step at 96°C and consisted of 35 cycles of 40 s at 94°C, 40 s at 56°C and 60 s at 72°C. A final extension step of 7 min at 72°C was added. PCR products were checked by electrophoresis on a 1.5%

agarose gel run for one hour and visualised by ethidium-bromide staining. DNA bands were excised and recovered with the QIAamp Gel Extraction Kit (Qiagen). Afterwards, they were cycle-sequenced in both directions with the BigDye Terminator v1.1 Cycle Sequencing Kit (Life Technologies). PCR products were cleaned with SigmaSpin Sequencing Reaction Clean-Up Columns (Sigma-Aldrich) before being run on a 3130xl Genetic Analyzer. FASTA files of the obtained sequences were aligned with MultAlin [34] to detect nucleotide polymorphisms.

Statistical analysis

Microsatellite signatures were subjected to Bayesian cluster analysis of multilocus microsatellite genotypes implemented in the software STRUCTURE 2.0 [35]. Following the method of Evanno [36], the optimal number of clusters was determined using the web-based software STRUCTURE HARVESTER [37].

Nei's genetic distance and pairwise population F_{ST} values were calculated with GenAlEx to perform a principal coordinate analysis (PCoA) [38]. Furthermore, departures from the Hardy-Weinberg equilibrium were examined. Shannon's information index (I), the mean number of alleles and the observed and unbiased expected heterozygosity were identified using GenAlEx [39]. Shannon's information index which is used to calculate genetic diversity is a quantity that shows the frequency of each allele in addition to the total number of alleles [39,40]. The higher the Shannon's information index, the higher the population diversity. As the Shannon's information index is frequency-based, it is not applicable to single individuals.

Results

Microsatellites of a total of 215 *Ae. j. japonicus* specimens from the Dutch, German and Slovenian/Croatian populations were analysed. In some cases, previously obtained data from West German, Swiss and Belgian samples [29] were included in the analyses for comparative purposes.

The obtained microsatellite signatures enabled the examined specimens to be assigned to one of two genotypes described for Germany (Figure 2). The NG population displayed exactly the same genotype 2 as the individuals from WG that had been previously analysed [29]. All other European populations were to be assigned to genotype 1, although the Dutch population showed clear signs of admixture. Only one of the two Croatian individuals available provided analysable microsatellite data. For the statistical analyses, this individual was added to the geographically closest Slovenian region of Podravska. It showed a probability of more than 95% to belong to genotype 1.

A Hardy-Weinberg equilibrium check showed significant deviations at most microsatellite loci of specimens from the two SG sites, Korntal and Waldshut-Tiengen. A few more departures from the Hardy-Weinberg equilibrium, mostly at loci OJ5, OJ10 and OJ187, were found across all populations. A closer inspection of these did not produce considerable effects of null alleles [41]. Deviations were due both to higher than expected and to lower than expected heterozygosity.

Of the 215 genotyped individuals, 154 examined for their *nad*4 gene sequence provided analysable results. Nine different *nad*4 haplotypes were obtained: H1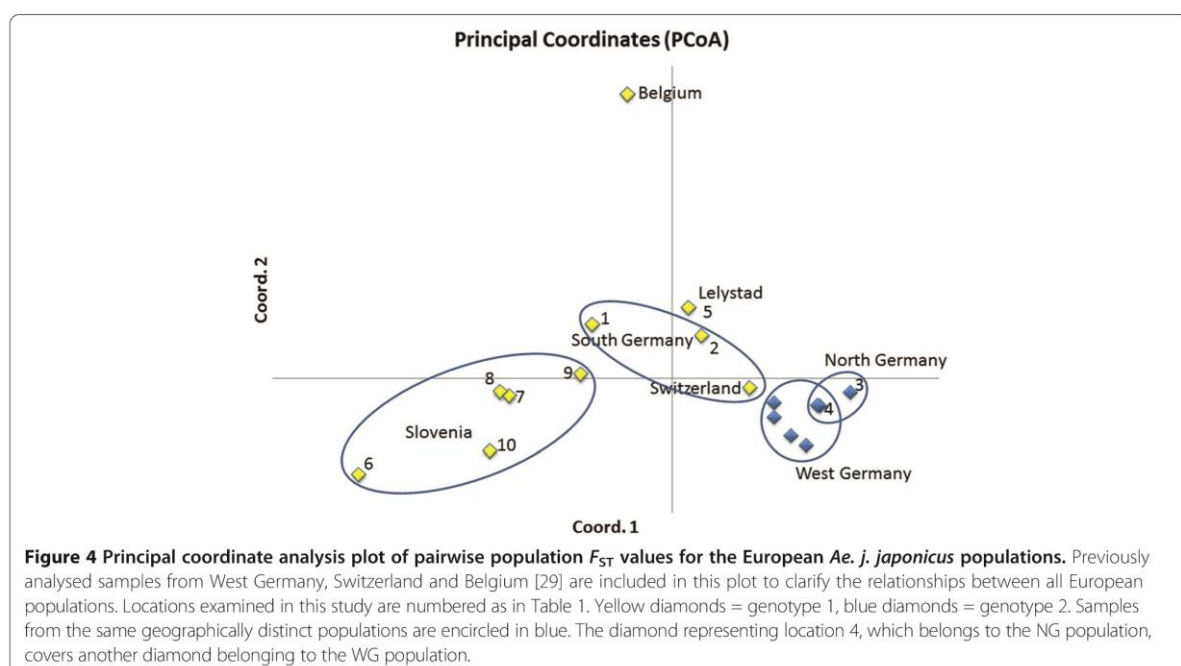, H3, H5, H9, H12, H21, H33, and two haplotypes that have not previously been described (Figure 3). As there are currently 43 *nad*4 haplotypes known for *Ae. j. japonicus* (Fonseca, pers. comm.), we suggest naming the newly found haplotypes H44 and H45. Haplotype sequences are available in [27] and GenBank (accession nos. KJ958405, DQ470159, KM610232 and KM610233, with the latter two being the new ones). H21 and H45 occurred exclusively in Korntal (SG) near the city of Stuttgart in the federal state of Baden-Wurttemberg, while the newly discovered NG population only showed haplotypes H1 and H5. In the Dutch municipality of Lelystad, the most frequent haplotype was H12, which is unique in Europe. Displaying four further haplotypes, the Dutch population was the most heterogeneous one. This observation underlines the admixed microsatellite signature of this population. Among the haplotypes found in The Netherlands, H3 (a single individual) was shared with the Korntal (SG) samples, while H9 (five individuals) was a common haplotype in Slovenia. Surprisingly, the samples from the two SG locations did not share any *nad*4 haplotype, and their microsatellite signatures were different too. The Croatian mosquitoes could not be assigned to a defined haplotype

Figure 2 Results of Bayesian cluster analysis showing the two *Ae. j. japonicus* genotypes in Europe (yellow = genotype 1, blue = genotype 2). As the Slovenian and Croatian samples are considered to belong to one and the same population, the Croatian specimen has been added to the samples from Podravska (Slovenia) for the purpose of this presentation. WG, Swiss and Belgian individuals had previously been analysed [29] but are included for comparison.

Figure 3 nad4 haplotypes found at the different sampling locations. Croatian individuals were excluded due to heteroplasmy. WG, Swiss and Belgian individuals had previously been analysed [29] but are included for comparison. Black = haplotypes described from Europe only, blue = haplotypes also described from the USA, red = haplotypes also described from the USA and Japan.

as both specimens were characterised by mitochondrial heteroplasmy, the coexistence of multiple mitochondrial haplotypes in a single organism [42] which is a common phenomenon in *Ae. j. japonicus*.

A principal coordinate analysis based on pairwise population F_{ST} values (Figure 4) suggests that the NG and WG samples are very closely related, but stand separate from the SG/Switzerland population. Although belonging to the same genotype 1, samples from the other populations were admixed and displayed a greater genetic distance. Interestingly, the Dutch samples seem to be closely related to those from Waldshut-Tiengen (SG). These, in turn, are more closely related to the previously examined and geographically closest Swiss individuals than to those from Korntal (SG) which are most similar to the Slovenian/Croatian ones. The previously analysed Belgian population [29] stands far apart from all other populations, underlining its geographic and genetic isolation.

Figure 4 Principal coordinate analysis plot of pairwise population F_{ST} values for the European *Ae. j. japonicus* populations. Previously analysed samples from West Germany, Switzerland and Belgium [29] are included in this plot to clarify the relationships between all European populations. Locations examined in this study are numbered as in Table 1. Yellow diamonds = genotype 1, blue diamonds = genotype 2. Samples from the same geographically distinct populations are encircled in blue. The diamond representing location 4, which belongs to the NG population, covers another diamond belonging to the WG population.

Discussion

A previous study focussing on the West German *Ae. j. japonicus* population established the existence of two distinct but mixed genetic strains of this species in Europe [29]. During that study, mosquito samples from the most recently discovered geographic populations in The Netherlands and northern Germany were not yet available. In addition, only a few specimens from the Slovenian population were included. In the present study, representative numbers of all European populations not previously analysed were considered and, based on Bayesian cluster analysis of their microsatellite data, could be clearly assigned to one of the two genetic strains previously identified in Europe.

The results of Zielke et al. [29] led to the conclusion that the Asian bush mosquito was introduced into Europe on at least two occasions. Samples from the Belgian population [11] show a genotype 1 genetic signature, equal to most of the individuals from the SG and Slovenian/Croatian populations. By contrast, the population detected in western Germany in 2012 appears to be the result of a second introduction of *Ae. j. japonicus* into Europe [29]. Due to microsatellite signatures identical to those of samples from WG, the NG population must be supposed to be an offshoot of the WG population. In summary, the microsatellite analysis (Figures 2 and 4) mirrors the geographic separation of the various European populations.

The mitochondrial haplotypes H1 and H5 found in North Germany are also common in the previously described WG population and support a close genetic relationship. H1 is a common haplotype which can be found in most *Ae. j. japonicus* populations all over the world. Haplotype H5 is also common on the northern Japanese island of Hokkaido [27] but was very rarely found in the USA, suggesting that genotype 2 German *Ae. j. japonicus* might have been introduced from Japan. As adults or larvae, individuals could then have been transported by vehicles along the motorways between the German federal states. This assumption is supported by the fact that the initial findings of *Ae. j. japonicus* in northern Germany were concentrated in cemeteries of towns close to a motorway running northwards from West Germany [17]. An active migration of the species from West to North Germany can be excluded because the two populations are geographically separated.

Samples from South Germany differ significantly from each other in their genetic makeup although forming one geographic population. Individuals from Waldshut-Tiengen (SG) are apparently more closely related to the Swiss samples of the same geographic population than to the Korntal (SG) samples, as only the first two of them show haplotype H33. This haplotype has not been reported from the USA, and in Europe has so far only

been found in Swiss specimens [29]. This suggests an introduction of at least a few individuals from Asia. By contrast, individuals from Korntal (SG) are characterised by haplotypes H3, H21 and H45. The latter two haplotypes have been found exclusively in the Korntal (SG) samples. This might indicate a separate introduction of mosquitoes into this area. H3 was first detected in individuals from the southern Japanese islands of Honshu and Kyushu [27] whereas H21 was previously found in some Pennsylvanian individuals [32]. Furthermore, the Korntal (SG) samples show a very clear microsatellite signature of genotype 1 with almost no admixture of genotype 2 (Figure 2).

To date, Lelystad in The Netherlands is considered to be the northernmost location infested by *Ae. j. japonicus* in Europe. The Dutch population shows an admixed microsatellite signature as displayed by Bayesian cluster analysis. Its genetic makeup looks like a mixture between Slovenian/SG and WG/NG individuals, which is supported by PCoA, suggesting that at least two introductions of mosquitoes into The Netherlands have taken place. However, the fact that the Dutch population shows a relatively large number of five *nad*4 haplotypes including one novel haplotype suggests a different conclusion: a large number of individuals with high genetic diversity could have been introduced recently from overseas. The genetic diversity may in this case be a measure of the time that has passed since the introduction. With time passing, founder populations normally experience a loss of genetic diversity, known as the founder effect [43]. If present, it should be possible to see this effect in future analyses of the Dutch *Ae. j. japonicus* population. Haplotypes H9 and H12, which account for 42% of the Dutch individuals, are the only haplotypes described by Fonseca et al. [27] for populations in Pennsylvania and Maryland.

Of the 18 Slovenian sampling localities examined, seven were located close to the Austrian border, eight close to the Croatian border and three in the centre of the country (cf. Figure 1). According to the Bayesian cluster analysis, all Slovenian/Croatian individuals show the same microsatellite signature of genotype 1 with only a little admixture of genotype 2 (Figure 2). Because the species is not yet widely distributed in Croatia, only two individuals were available. As with some specimens from other locations, these unfortunately could not be analysed due to their being heteroplasmic. Together with the Dutch samples, the Slovenian samples were the only ones in Europe to show *nad*4 haplotype H9. Additionally, some Slovenian individuals displayed haplotype H5, which is widely distributed in the NG and WG populations, indicating a possible link between these two and the Slovenian population.

In summary, the genetic information is not sufficient to decide whether the European *Ae. j. japonicus* have

been introduced from the USA or from Japan, or from both countries. As the worldwide expansion of the species started almost two decades ago, exact source determination is becoming more and more difficult. Mitochondrial haplotypes are widespread, and in many cases it is impossible to say whether the species has found its way to Europe from Japan or from the USA.

In spite of this, all known *Ae. j. japonicus* populations in Europe must be assumed to have reached their infestation areas through human-mediated transport. Their apparent geographic separation does not permit any other conclusion. Individuals reach new regions passively and initially may manage to establish a relatively small population, depending on the number of founder individuals. When such populations merge, they can increase their genetic diversity and, accordingly, their adaptability. It is the mixed populations with high genetic diversity that are adaptable and likely to establish [44]. So far, six populations of *Ae. j. japonicus* have been found in Europe. Their genetic makeup shows a mixture of two genotypes, and most of the populations are expanding. The exception is the Belgian population, which seems to be very inbred and has a low genetic diversity [29]. At the same time, this population is the only one that has not expanded the area of infestation over the years since its introduction [45].

Conclusions

Regarding intercontinental trade and travel as well as the transport of mosquitoes and pathogens between countries, it is to be expected that further populations of *Ae. j. japonicus* will appear in Europe and that the risk of pathogen transmission will increase. *Aedes j. japonicus* is known to feed on both birds and mammals, and bloodmeal analyses have shown that a high percentage (up to 60 %) of the identified blood sources were human [45]. Whilst *Ae. j. japonicus* has not attracted attention as an important vector in its native distribution area in East Asia, it was susceptible to several arboviruses in the laboratory, including WNV. A new study claims that German *Ae. j. japonicus* are refractory to WNV [46]. However, all the mosquitoes tested originated from a limited area in southern Germany, suggesting that a restricted and relatively homogeneous gene pool was included in the study. Other populations or subpopulations may very well be able to transmit WNV [19,47] or other pathogenic viruses.

There is consent that the eradication of *Ae. j. japonicus* from Europe is no longer possible, but efforts should be made to eliminate sources of introduction and to prevent the present populations from spreading further.

Abbreviations

SG: South German(y); NG: North German(y); WG: West German(y); WNV: West Nile virus; ECDC: European Centre for Disease Prevention and Control; PCR: Polymerase chain reaction; nad4: Sodium dehydrogenase subunit 4; PCoA: Principal coordinate analysis.

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References

1. Reinert JF, Harbach RE, Kitching IJ. Phylogeny and classification of *Finlaya* and allied taxa (Diptera: Culicidae: Aedini) based on morphological data from all life stages. Zool J Linn Soc. 2006;148:1–101.
2. GISD (Global invasive species database). http://www.issg.org/database/welcome, accessed 19 November 2014.
3. Laird M, Calder L, Thornton R, Syme R, Holder P, Mogi M. Japanese *Aedes albopictus* among four mosquito species reaching New Zealand in used tires. J Am Mosq Control Assoc. 1994;10:14–23.
4. Peyton E, Campbell SR, Candeletti TM, Romanowski M, Crans WJ. *Aedes (Finlaya) japonicus japonicus* (Theobald), a new introduction into the United States. J Am Mosq Control Assoc. 1999;15:238–41.
5. Munstermann L, Andreadis T. *Aedes japonicus* in Connecticut. Vector Ecol Newsl. 1999;30:7–8.
6. Widdel AK, McCuiston LJ, Crans WJ, Kramer LD, Fonseca DM. Finding needles in the haystack: single copy microsatellite loci for *Aedes japonicus* (Diptera: Culicidae). Am J Trop Med Hyg. 2005;73:744–8.
7. Neitzel DF, Johnson KA, Brogren S, Kemperman MM. First collection records of *Aedes japonicus* in Minnesota. J Am Mosq Control Assoc. 2009;25:367–9.
8. Kampen H, Werner D. Out of the bush: the Asian bush mosquito *Aedes japonicus japonicus* (Theobald, 1901)(Diptera: Culicidae) becomes invasive. Parasit Vectors. 2014;7:59.
9. Thielman A, Hunter FF. Establishment of *Ochlerotatus japonicus* (Diptera: Culicidae) in Ontario, Canada. J Med Entomol. 2006;43:138–42.
10. Schaffner F, Chouin S, Guilloteau J. First record of *Ochlerotatus (Finlaya) japonicus japonicus* (Theobald, 1901) in metropolitan France. J Am Mosq Control Assoc. 2003;19:1–5.
11. Versteirt V, Schaffner F, Garros C, Dekoninck W, Coosemans M, Van Bortel W. Introduction and establishment of the exotic mosquito species *Aedes japonicus japonicus* (Diptera: Culicidae) in Belgium. J Med Entomol. 2009;46:1464–7.
12. Schaffner F, Kaufmann C, Hegglin D, Mathis A. The invasive mosquito *Aedes japonicus* in Central Europe. Med Vet Entomol. 2009;23:448–51.
13. Becker N, Huber K, Pluskota B, Kaiser A. *Ochlerotatus japonicus japonicus* – a newly established neozoan in Germany and a revised list of the German mosquito fauna. Eur Mosq Bull. 2011;29:88–102.
14. Schneider K. Breeding of *Ochlerotatus japonicus japonicus* (Diptera: Culicidae) 80 km north of its known range in southern Germany. Eur Mosq Bull. 2011;29:129–32.
15. Seidel B, Duh D, Nowotny N, Allerberger F. First record of the mosquitoes *Aedes (Ochlerotatus) japonicus japonicus* (Theobald, 1901) in Austria and

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Slovenia in 2011 and for *Aedes* (*Stegomyia*) *albopictus* (Skuse, 1895) in Austria 2012. Entomol Zeitschr. 2012;122:223–6 [in German].

16. Kampen H, Zielke D, Werner D. A new focus of *Aedes japonicus japonicus* (Theobald, 1901) (Diptera: Culicidae) distribution in Western Germany: rapid spread or a further introduction event? Parasit Vectors. 2012;5:284.

17. Werner D, Kampen H. The further spread of *Aedes japonicus japonicus* (Diptera: Culicidae) towards northern Germany. Parasitol Res. 2013;112:3665–8.

18. Ibáñez-Justicia A, Kampen H, Braks M, Schaffner F, Steeghs M, Werner D, et al. First report of established population of *Aedes japonicus japonicus* (Theobald, 1901) (Diptera: Culicidae) in the Netherlands. J Eur Mosq Control Assoc. 2014;32:9–13.

19. Sardelis MR, Turell MJ. *Ochlerotatus j. japonicus* in Frederick County, Maryland: discovery, distribution, and vector competence for West Nile virus. J Am Mosq Control Assoc. 2001;17:137–41.

20. Takashima I, Rosen L. Horizontal and vertical transmission of Japanese encephalitis virus by *Aedes japonicus* (Diptera: Culicidae). J Med Entomol. 1989;26:454–8.

21. Molaei G, Farajollahi A, Scott JJ, Gaugler R, Andreadis TG. Human bloodfeeding by the recently introduced mosquito, *Aedes japonicus japonicus*, and public health implications. J Am Mosq Control Assoc. 2009;25:210–4.

22. Sardelis MR, Dohm DJ, Pagac B, Andre RG, Turell MJ. Experimental transmission of eastern equine encephalitis virus by *Ochlerotatus j. japonicus* (Diptera: Culicidae). J Med Entomol. 2002;39:480–4.

23. Sardelis MR, Turell MJ, Andre RG. Laboratory transmission of La Crosse virus by *Ochlerotatus j. japonicus* (Diptera: Culicidae). J Med Entomol. 2002;39:635–9.

24. Turell MJ, Byrd BD, Harrison BA. Potential for populations of *Aedes j. japonicus* to transmit Rift Valley fever virus in the USA. J Am Mosq Control Assoc. 2013;29:133–7.

25. Schaffner F, Vazeille M, Kaufmann C, Failloux A-B, Mathis A. Vector competence of *Aedes japonicus* for chikungunya and dengue viruses. Eur Mosq Bull. 2011;29:141–2.

26. Brown WM, George M, Wilson AC. Rapid evolution of animal mitochondrial DNA. Proc Natl Acad Sci U S A. 1979;76:1967–71.

27. Fonseca DM, Campbell S, Crans WJ, Mogi M, Miyagi I, Toma T, et al. *Aedes* (*Finlaya*) *japonicus* (Diptera: Culicidae), a newly recognized mosquito in the United States: analyses of genetic variation in the United States and putative source populations. J Med Entomol. 2001;38:135–46.

28. ECDC (European Centre for Disease Prevention and Control). Guidelines for the surveillance of invasive mosquito species in Europe. Stockholm, Sweden: ECDC Technical Report; 2012.

29. Zielke DE, Werner D, Schaffner F, Kampen H, Fonseca DM. Unexpected patterns of admixture in German populations of *Aedes japonicus japonicus* (Diptera: Culicidae) underscore the importance of human intervention. PLoS One. 2014;9:e99093.

30. Schaffner F, Angel G, Geoffroy B, Hervy JP, Rhaiem A, Brunhes J. *The mosquitoes of Europe* (CD-Rom). Montpellier, France: IRD Éditions & EID Méditerrannée; 2001.

31. Gutsevich A, Monchadskii A, Shtakelberg A. Fauna of the U.S.S.R. Diptera. Vol. 3, No. 4. Mosquitoes, family Culicidae. Jerusalem, Israel: Keter Publishing House Ltd; 1974.

32. Fonseca DM, Widdel AK, Hutchinson M, Spichiger SE, Kramer LD. Fine-scale spatial and temporal population genetics of *Aedes japonicus*, a new US mosquito, reveal multiple introductions. Mol Ecol. 2010;19:1559–72.

33. Egizi A, Fonseca D. Ecological limits can obscure expansion history: patterns of genetic diversity in a temperate mosquito in Hawaii. Biol Invasions. 2015;17:123–32.

34. Corpet F. Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res. 1988;16:10881–90.

35. Pritchard JK, Stephens M, Donnelly P. Inference of population structure using multilocus genotype data. Genetics. 2000;155:945–59.

36. Evanno G, Regnaut S, Goudet J. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol Ecol. 2005;14:2611–20.

37. Earl DA, Vonholdt BM. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conserv Genet. 2012;4:359–61.

38. Peakall R, Smouse PE. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research—an update. Bioinformatics. 2012;28:2537–9.

39. Sherwin WB. Entropy and information approaches to genetic diversity and its expression: genomic geography. Entropy. 2010;12:1765–98.

40. Sherwin WB, Jabot F, Rush R, Rossetto M. Measurement of biological information with applications from genes to landscapes. Mol Ecol. 2006;15:2857–69.

41. Dakin E, Avise J. Microsatellite null alleles in parentage analysis. Heredity. 2004;93:504–9.

42. Magnacca KN, Brown MJ. Mitochondrial heteroplasmy and DNA barcoding in Hawaiian *Hylaeus* (*Nesoprosopis*) bees (Hymenoptera: Colletidae). BMC Evol Biol. 2010;10:174.

43. Mayr E. Systematics and the origin of species, from the viewpoint of a zoologist. Boston, MA, USA: Harvard University Press; 1942.

44. Kolbe JJ, Glor RE, Schettino LR, Lara AC, Larson A, Losos JB. Genetic variation increases during biological invasion by a Cuban lizard. Nature. 2004;431:177–81.

45. Damiens D, Ayrinhac A, Van Bortel W, Versteirt V, Dekoninck W, Hance T. Invasive process and repeated cross-sectional surveys of the mosquito *Aedes japonicus japonicus* establishment in Belgium. PLoS One. 2014;9:e89358.

46. Huber K, Jansen S, Leggewie M, Badusche M, Schmidt-Chanasit J, Becker N, et al. *Aedes japonicus japonicus* (Diptera: Culicidae) from Germany have vector competence for Japan encephalitis virus but are refractory to infection with West Nile virus. Parasitol Res. 2014;113:1–5.

47. Turell MJ, Sardelis MR, Dohm DJ, O'Guinn ML. Potential North American vectors of West Nile virus. Ann N Y Acad Sci. 2001;951:317–24.

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CrossMark

Newly discovered population of *Aedes japonicus japonicus* (Diptera: Culicidae) in Upper Bavaria, Germany, and Salzburg, Austria, is closely related to the Austrian/Slovenian bush mosquito population

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Abstract

Background: The German mosquito surveillance instrument 'Mueckenatlas' requests the general public to collect and submit mosquito specimens. Among these, increasing numbers of individuals of invasive species have been registered. Specimens of the Asian bush mosquito *Aedes japonicus japonicus* submitted from German Upper Bavaria, where this species had not previously been recorded, triggered regional monitoring in mid-2015.

Methods: The search for *Ae. j. japonicus* breeding sites and developmental stages concentrated on cemeteries in the municipality of origin of the submitted specimens and, subsequently, in the whole region. A virtual grid consisting of 10×10 km^2 cells in which up to three cemeteries were checked, was laid over the region. A cell was considered positive as soon as *Ae. j. japonicus* larvae were detected, and regarded negative when no larvae could be found in any of the cemeteries inspected. All cells surrounding a positive cell were screened accordingly. A subset of collected *Aedes j. japonicus* specimens was subjected to microsatellite and *nad*4 sequence analyses, and obtained data were compared to individuals from previously discovered European populations.

Results: Based on the grid cells, an area of approximately 900 km^2 was populated by *Ae. j. japonicus* in Upper Bavaria and neighbouring Austria. Genetic analyses of microsatellites and *nad*4 gene sequences generated one genotype out of two previously described for Europe and three haplotypes, one of which had previously been found in Europe only in *Ae. j. japonicus* samples from a population in East Austria and Slovenia. The genetic analysis suggests the new population is closely related to the Austrian/Slovenian population.

Conclusion: As *Ae. j. japonicus* is well adapted to temperate climates, it has a strong tendency to expand and to colonise new territories in Central Europe, which is facilitated by human-mediated, passive transportation. The new population in Upper Bavaria/Austria is the seventh separate population described in Europe. According to our data, it originated from a previously detected population in eastern Austria/Slovenia and not from an introduction event from abroad. The dispersal and population dynamics of *Ae. j. japonicus* should be thoroughly surveyed, as this species is a potential vector of disease agents.

Keywords: *Aedes japonicus japonicus*, Asian bush mosquito, Austria, Germany, Human-mediated displacement, Microsatellites, *nad*4 haplotypes, Population genetics, Spread

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Background

The invasive Asian bush mosquito *Aedes* (*Hulecoeteomyia*) *japonicus japonicus* (Theobald, 1901) (taxonomic nomenclature according to [1]) was first reported from Europe in 2000 when it was detected in Normandy (Orne Department) in northern France [2]. While the species was later eliminated there [3], another population detected in 2002 in Belgium succeeded in establishing [4]. After more than a decade of remaining locally restricted, it was eventually controlled and is now also considered eliminated (pers. comm. Versteirt 2016). Several additional populated areas were discovered in Europe between 2008 and 2014 (Fig. 1): Switzerland/Southwest Germany/France [3, 5, 6], west Germany [7], north Germany [8], Austria/Slovenia/Croatia/Hungary [9–11] and The Netherlands [12].

Aedes j. japonicus is native to east Asia (Japan, Korea, southern China, southeastern Russia) where it colonises regions climatically similar to central Europe [13]. It has been transported intercontinentally by air and sea since the early 1990s and was demonstrated as invasive in the late 1990s in the US where it is now widely distributed [14].

Aedes j. japonicus is a potential vector of disease agents, able to transmit Getah, Japanese encephalitis, West Nile, dengue, chikungunya and Rift Valley fever viruses in the laboratory [15–19]. In the field, it has been found infected with Japanese encephalitis, West Nile and La Crosse viruses [20–22].

After decades of neglect, the occurrence and spatio-temporal distribution of culicid species, including invasive ones such as *Ae. j. japonicus*, have been thoroughly monitored in Germany since 2011. In addition to active data sampling by trapping, a passive approach was implemented in 2012 in the form of a citizen-science project. The 'Mueckenatlas' (mosquito atlas) calls upon private persons to collect mosquitoes and submit them for mapping spatiotemporal mosquito occurrence [23]. Since its launch, it has contributed thousands of distribution records and has been particularly valuable regarding invasive mosquito species. It enabled the detection of the west and north German populations of *Ae. j. japonicus* in 2012 and 2013 [7, 8], of a reproductive population of the Asian tiger mosquito *Ae. albopictus* in south Germany in 2014 [24] and of the introduction of *Ae. koreicus* in 2015 [25].

In early July 2015, three adult specimens of *Ae. j. japonicus* were submitted to the 'Mueckenatlas' team from Berchtesgaden, a town in Upper Bavaria, southeastern Germany, immediately on the border with Austria. Since this region was far from areas of previous *Ae. j. japonicus* documentation, the submissions prompted immediate local monitoring.

Methods

Mosquito collection

On the occasion of a visit to Upper Bavaria (inset in Fig. 1) in August 2015, water containers on the premises
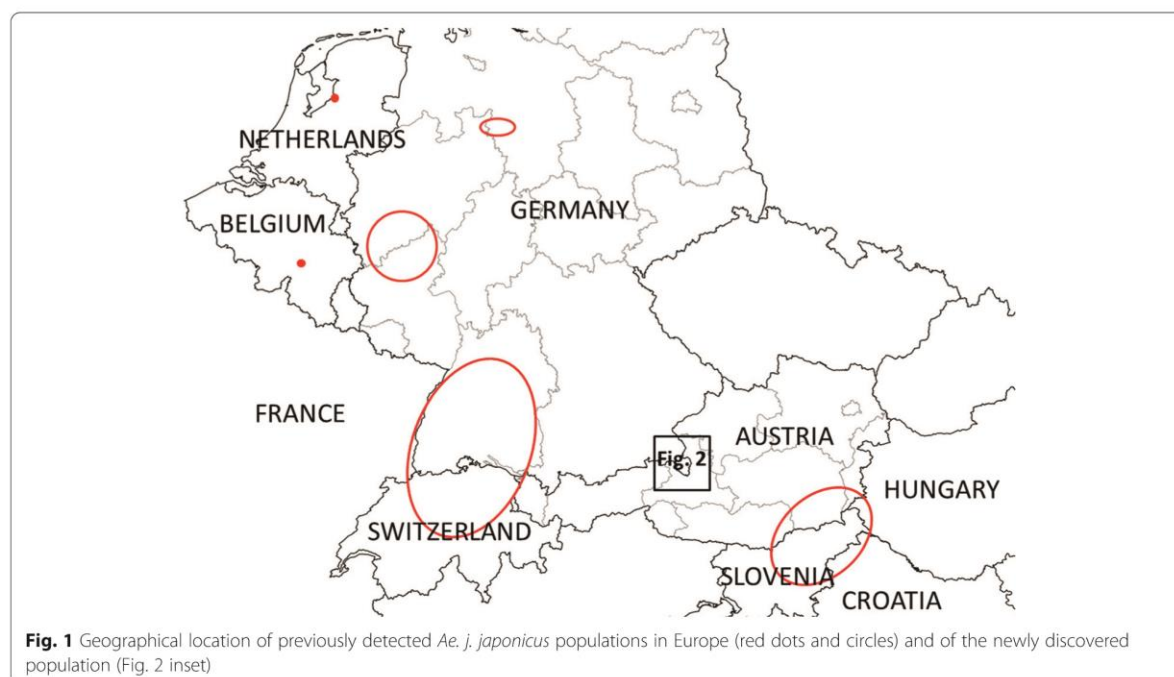

Fig. 1 Geographical location of previously detected *Ae. j. japonicus* populations in Europe (red dots and circles) and of the newly discovered population (Fig. 2 inset)

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and surroundings of the submitter were checked for *Ae. j. japonicus* immature stages. Larvae were found in water accumulated in the inverted top of a rain-water barrel in the garden of the submitter and in a bucket filled with debris and water in the neighbour's garden. An inspection of the municipality's cemetery, the distance to which was 1.3 km (direct line), also revealed *Ae. j. japonicus* larvae in many of the numerous water containers.

To gain an impression of the size of the populated area, a virtual grid with 10 × 10 km cells was laid over the region (Fig. 2). Starting in Berchtesgaden, all cells surrounding *Ae. j. japonicus*-positive cells were checked by inspecting water containers in cemeteries, as suggested by Vezzani [26] and demonstrated to be efficient by various other authors (e.g. [3, 6]). In smaller cemeteries all natural and artificial water-holding containers identified were examined, while in larger cemeteries the inspection was limited in time to one hour. Cells were considered positive as soon as *Ae. j. japonicus* larvae were found, irrespective of the number of cemeteries screened. Cells were rated negative if no larvae could be found in the cemeteries of three villages (provided three villages were present in the cell), or in one or two cemeteries in some more alpine and little-populated cells when no further cemeteries could be located.

Aedes j. japonicus larvae were identified on the spot by their habitus and behaviour (cf. [6]) but samples from each collection site were taken to the laboratory for further development until adult emergence and genetic analyses. Adults were identified morphologically according to the key by Schaffner *et al.* [27]. For confirmation, at least one specimen per site was identified genetically by CO1 DNA barcoding [12].
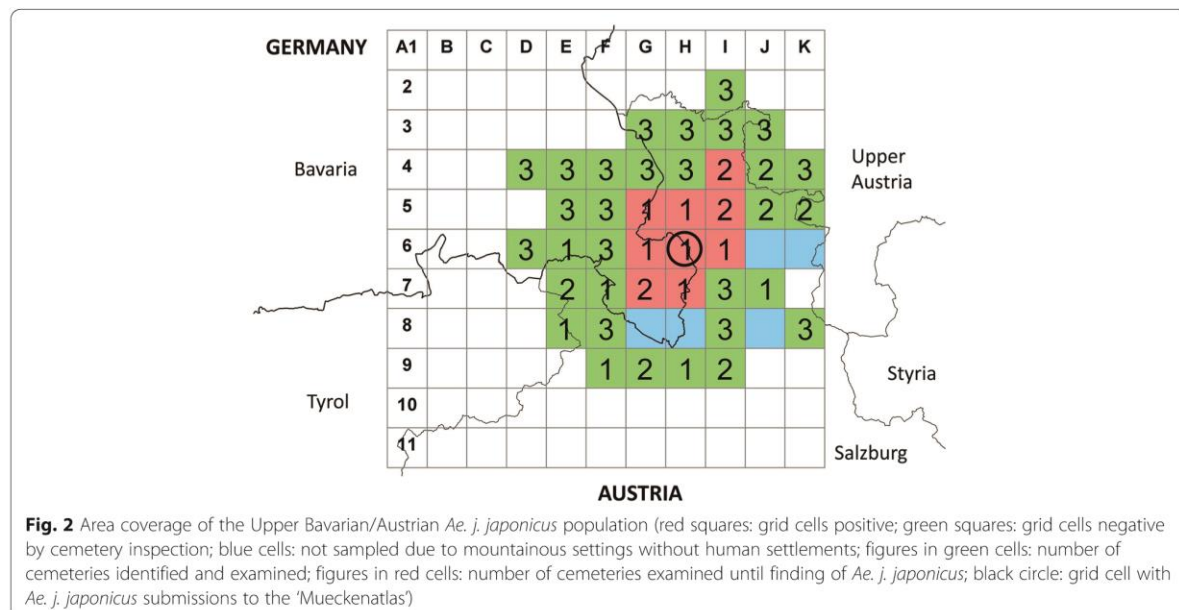
Genetic analysis

To unveil putative relationships to other populations of *Ae. j. japonicus* in Europe, and to identify possible source populations, population genetic analyses, using *nad*4 (NADH dehydrogenase subunit 4) gene polymorphisms, were carried out on 30 specimens from the colonised area, according to protocols presented by Fonseca *et al.* [28] and Zielke *et al.* [29]. The same 30 individuals were subjected to analysis of seven microsatellite loci, with the results interpreted with GeneMapper (Applied Biosystems/Thermo Fisher Scientific, Waltham, MA, USA), STRUCTURE [30], STRUCTURE HARVESTER [31] and GenAlEx [32]. This approach had been specifically adapted and optimized to *Ae. j. japonicus* population genetics in previous work and has been proven effective [28, 29, 33, 34].

For DNA extraction, legs of adult mosquitoes or three to four abdominal segments of larvae were processed using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

Results

Aedes j. japonicus immature specimens were found in nine grid cells, corresponding to an infested area of about 900 km^2 stretching from Upper Bavaria to the Austrian federal state of Salzburg (Fig. 2). Of the nine grid cells positive, *Ae. j. japonicus* was found in the first cemetery checked in six cells, while in three cells the second cemetery checked was populated.

Fig. 2 Area coverage of the Upper Bavarian/Austrian *Ae. j. japonicus* population (red squares: grid cells positive; green squares: grid cells negative by cemetery inspection; blue cells: not sampled due to mountainous settings without human settlements; figures in green cells: number of cemeteries identified and examined; figures in red cells: number of cemeteries examined until finding of *Ae. j. japonicus*; black circle: grid cell with *Ae. j. japonicus* submissions to the 'Mueckenatlas')

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Among the analysed individuals (one individual was not analysable), three *nad*4 haplotypes were found: the ubiquitous H1 (5 individuals), as well as H9 (10 individuals) and H10 (14 individuals) (naming of haplotypes according to [34]), the latter previously described only in the population occurring in southeastern Austria and Slovenia [33].

To comparatively analyse the microsatellite data of the newly discovered Upper Bavarian/Austrian population, data of formerly examined specimens from populations found in Switzerland/southwest Germany, Belgium, west Germany, north Germany, The Netherlands and Austria/Slovenia [29, 33] were included (Table 1). A check for Hardy-Weinberg equilibrium showed significant deviations on locus OJ10 in the Upper Bavarian/Austrian population, due both to higher and to lower than expected heterozygosity.

The microsatellite data were further subjected to a Bayesian cluster analysis with the programme STRUCTURE [30], demonstrating genotype 1 for the Upper Bavarian/Austrian population out of two genotypes occurring in Europe (Fig. 3), as identified as the optimal number of genetic clusters for the complete dataset by STRUCTURE HARVESTER [31].

A principal coordinates analysis, based on a pairwise population matrix of Nei's genetic distances (Additional file 1) and performed with GenAlEx [32] on the microsatellite data, shows a close genetic proximity of the newly discovered Upper Bavarian/Austrian population to the formerly analysed Austrian/Slovenian population (Fig. 4). The plot shows four groups of *Ae. j. japonicus* populations in Europe: The Belgian population is on top of a triangle with roughly the same genetic distance to the north and west German populations in one corner and to the Austrian/Slovenian and the Upper Bavarian/Austrian populations in the other corner. The Swiss/southwest German and Dutch populations, which are genetically more heterogeneous than the others (cf. [33]), are located between the north and west German populations on the one side and the Austrian/Slovenian and the Upper Bavarian/Austrian populations on the other side (Fig. 4).

Table 1 Number of specimens and determined genotypes of other European *Ae. j. japonicus* populations comparatively examined by microsatellite analyses

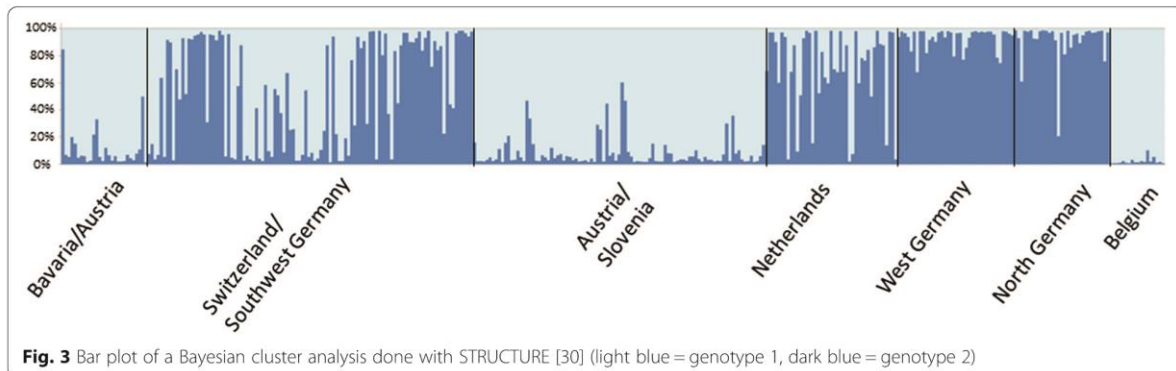| Population	Number of individuals	Principal microsatellite genotype	Reference
Austria/Slovenia	36/60	1	[29, 33]
Belgium	18	1	[29]
West Germany	197	2	[29]
North Germany	32	2	[33]
Switzerland/ Southwest Germany	22/80	1, 2	[29, 33]
Netherlands	43	1, 2	[33]

Discussion

A fourth German population of *Ae. j. japonicus*, representing the seventh European population of this species, was detected in southeastern Germany crossing the border to Austria. The distribution of this isolated population was roughly determined by a grid cell pattern with findings of immature stages of the species in some cells surrounded by cells without findings. This approach can serve merely as an indication of the extent of the population, as only cemeteries were inspected in the cells, and of these, only three per cell. Neither natural potential breeding sites, e.g. tree holes in forests, nor artificial breeding sites in gardens were checked.

No estimate is currently possible for the time of establishment of the Upper Bavarian/Austrian population. Its assumed distribution area is comparable in size to that of the north German population, which is supposed to be a relatively recent offshoot of the west German population [33] but has existed at least since 2012 [8]. Due to the area coverage, the Upper Bavarian/Austrian population might be of similar age, although a BG Sentinel trap operated in the affected area of Upper Bavaria from 2011 to 2013 (mid-April to late October each year once a week for 24 hours) never collected an *Ae. j. japonicus* specimen. The BG Sentinel is not particularly effective at collecting *Ae. j. japonicus* [35], but had already shown this species to occur in another German region previously not known to be populated (Werner & Kampen, unpubl.).

Climatic conditions are rather different in Upper Bavaria and northern Germany. German Upper Bavaria and the Austrian federal state of Salzburg are located at the northern boundaries of the Alps (minimum regional altitudes *c*. 400 m a.s.l.) and are characterised by a rather low annual average temperature (*c*. 7–8 °C) and monthly average temperatures exceeding 10 °C only from May to September. Snowfall typically occurs from early November to late April. Now that the distribution area of the *Ae. j. japonicus* population in Upper Bavaria/Austria has been determined, its future development and spread in this short-seasoned region should be directly compared with the north German population.

The *nad*4-haplotypes characterising the newly discovered Upper Bavarian/Austrian population suggest descent from the formerly detected Austrian/Slovenian population. Haplotype H9 is also known from the Belgian and the Dutch populations, but neither H9 nor H10 occur in any of the other German populations of *Ae. j. japonicus*. H10 had previously been demonstrated exclusively in the Austrian/Slovenian population in Europe. Outside of Europe, this haplotype has so far been described only from populations

Fig. 3 Bar plot of a Bayesian cluster analysis done with STRUCTURE [30] (light blue = genotype 1, dark blue = genotype 2)
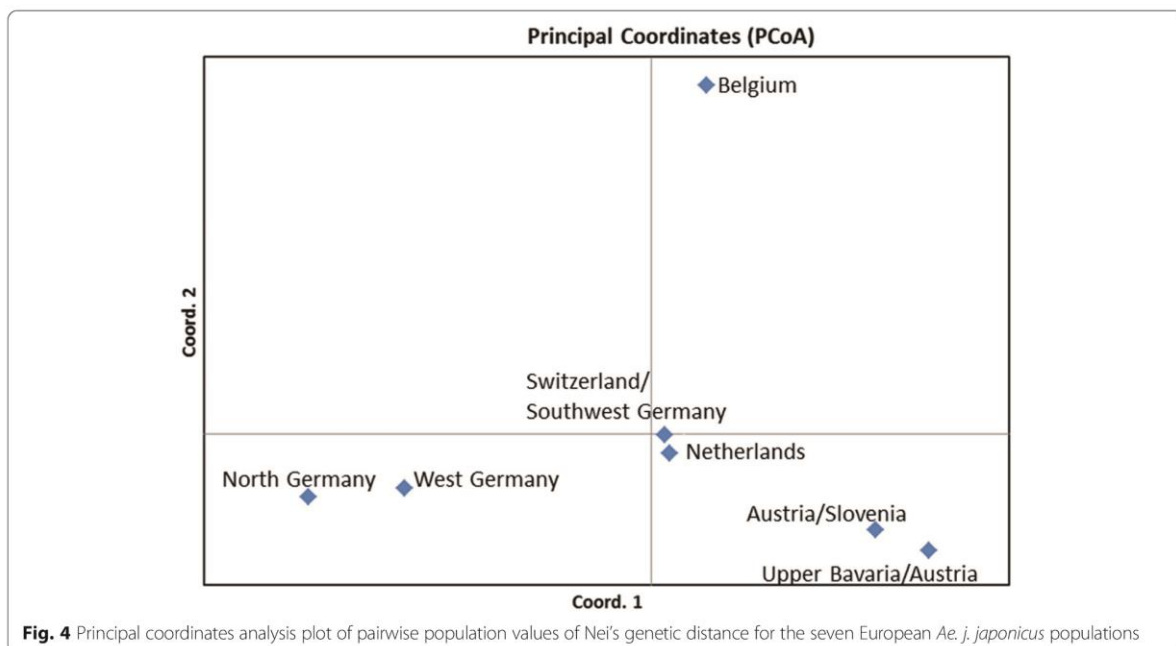
in New York and Connecticut in the USA [28], although it probably also exists in Asia where few studies have been made. With the available data, non-European source populations of the Upper Bavarian, Austrian and Slovenian individuals, therefore, cannot be narrowed down.

Microsatellite analyses show that the Upper Bavarian/Austrian population is assignable to genotype 1, the first *Ae. j. japonicus* genotype detected in Europe [29], although one individual suggests admixture with genotype 2. A close relationship with the Austrian/Slovenian population is shown, underlining the *nad*4 haplotype results. Conversely, the Upper Bavarian/Austrian population does not seem to be closely related to or to have the same origin as one of the other German populations.

Conclusions

As the distribution area of the newly detected *Ae. j. japonicus* population in Upper Bavaria/Austria is beyond the flight distance to the population in east Austria, human-mediated transport of founder individuals, such as by eggs attached to used tyres, must be postulated. The Austrian Tauern Autobahn (motorway A10), which, coming from Slovenia, passes the Upper Bavarian/Austrian distribution area of *Ae. j. japonicus*, might be a possible route for the displacement of mosquitoes from east Austria or Slovenia into the Austrian federal state of Salzburg.

Given the high ecological plasticity of *Ae. j. japonicus*, more populations should be expected to emerge in subsequent years, with spatial coverage of large parts of central Europe.

Fig. 4 Principal coordinates analysis plot of pairwise population values of Nei's genetic distance for the seven European *Ae. j. japonicus* populations

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Additional file

Additional file 1: Pairwise population matrix of Nei's genetic distances. The lower the values, the closer the genetic relatedness between the respective populations. (XLSX 9 kb)

Abbreviations
CO1: cytochrome c oxidase subunit 1; *nad*4: NADH dehydrogenase subunit 4.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
Conceived and designed the experiments: DEZ, DW, HK. Collected and identified the mosquitoes: DEZ, DW, HK. Generated and analysed the genetic data: DEZ. Contributed reagents/materials/analysis tools: DW, HK. Wrote the paper: DEZ, DW, HK. All authors read and approved the final version of the manuscript.

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References

1. Wilkerson RC, Linton YM, Fonseca DM, Schultz TR, Price DC, Strickman DA. Making mosquito taxonomy useful: a stable classification of tribe Aedini that balances utility with current knowledge of evolutionary relationships. PLoS ONE. 2015;10:e0133602.
2. Schaffner F, Chouin S, Guilloteau J. First record of *Ochlerotatus* (*Finlaya*) *japonicus japonicus* (Theobald, 1901) in metropolitan France. J Am Mosq Control Assoc. 2003;19:1–5.
3. Schaffner F, Kaufmann C, Hegglin D, Mathis A. The invasive mosquito *Aedes japonicus* in Central Europe. Med Vet Entomol. 2009;23:448–51.
4. Versteirt V, Schaffner F, Garros C, Dekoninck W, Coosemans M, van Bortel W. Introduction and establishment of the exotic mosquito species *Aedes japonicus japonicus* (Diptera: Culicidae) in Belgium. J Med Entomol. 2009;46:1464–7.
5. Huber K, Pluskota B, Jöst A, Hoffmann K, Becker N. Status of the invasive species *Aedes japonicus japonicus* (Diptera: Culicidae*)* in southwest Germany in 2011. J Vector Ecol. 2012;37:462–5.
6. Krebs T, Bindler P, L'Ambert G, Toti C, Perrin Y, Jourdain F. First establishment of *Aedes japonicus japonicus* (Theobald, 1901) (Diptera: Culicidae) in France in 2013 and its impact on public health. J Vector Ecol. 2014;39:437–40.
7. Kampen H, Zielke D, Werner D. A new focus of *Aedes japonicus japonicus* (Diptera, Culicidae) distribution in western Germany: rapid spread or a further introduction event? Parasit Vectors. 2012;5:284.
8. Werner D, Kampen H. The further spread of *Aedes japonicus japonicus* (Diptera, Culicidae) towards northern Germany. Parasitol Res. 2013;112:3665–8.
9. Seidel B, Duh D, Nowotny N, Allerberger F. First record of the mosquitoes *Aedes* (*Ochlerotatus*) *japonicus japonicus* (Theobald, 1901) in Austria and Slovenia 2011 and for *Aedes* (*Stegomyia*) *albopictus* (Skuse, 1895) in Austria 2012. Entomol Zeitschr. 2012;122:223–6 [in German].
10. Indra A. Stechmücken-Surveillance in der Steiermark. Report to the 'Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH', Vienna, Austria. 2013.
11. Kalan K, Buzan VE, Ivović V. Distribution of two invasive mosquito species in Slovenia. Parasit Vectors. 2014;7 Suppl 1:9.
12. Ibáñez-Justicia A, Kampen H, Braks M, Schaffner F, Steeghs M, Werner D, et al. First report of established population of *Aedes japonicus japonicus* (Theobald, 1901) (Diptera, Culicidae) in the Netherlands. J Eur Mosquito Contr Assoc. 2014;32:9–13.
13. Tanaka K, Mizusawa K, Saugstad ES. A revision of the adult and larval mosquitoes of Japan (including the Ryukyu Archipelago and the Ogasawara Islands) and Korea (Diptera: Culicidae). Contrib Amer Ent Inst. 1979;16:1–987.
14. Kampen H, Werner D. Out of the bush: the Asian bush mosquito *Aedes japonicus japonicus* (Theobald, 1901) (Diptera, Culicidae) becomes invasive. Parasit Vectors. 2014;7:59.
15. Takashima I, Hashimoto N. Getah virus in several species of mosquitoes. Trans R Soc Trop Med Hyg. 1985;79:546–50.
16. Takashima I, Rosen L. Horizontal and vertical transmission of Japanese encephalitis virus by *Aedes japonicus* (Diptera: Culicidae). J Med Entomol. 1989;26:454–8.
17. Sardelis MR, Turell MJ. *Ochlerotatus j. japonicus* in Frederick County, Maryland: discovery, distribution, and vector competence for West Nile virus. J Am Mosq Control Assoc. 2001;17:137–41.
18. Schaffner F, Vazeille M, Kaufmann C, Failloux A-B, Mathis A. Vector competence of *Aedes japonicus* for chikungunya and dengue viruses. Eur Mosq Bull. 2011;29:141–2.
19. Turell MJ, Byrd BD, Harrison BA. Potential for populations of *Aedes j. japonicus* to transmit Rift Valley fever virus in the USA. J Am Mosq Control Assoc. 2013;29:133–7.
20. Chagin KP, Kondratiev PI. Vectors of autumnal (Japanese) encephalitis in Primor'ye region and measures for controlling them. Med Parazitol Parazit Bolezni. 1943;12:34–44 [In Russian].
21. CDC (Centers for Disease Control and Prevention). Mosquito species in which West Nile virus has been detected, United States, 1999–2012. 2012. http://www.cdc.gov/westnile/resources/pdfs/mosquitospecies1999-2012.pdf (accessed 9 December 2015).
22. Harris MC, Dotseth EJ, Jackson BT, Zink SD, Marek PE, Kramer LD, et al. La Crosse virus in *Aedes japonicus japonicus* mosquitoes in the Appalachian Region, United States. Emerg Infect Dis. 2015;21:646–9.
23. Kampen H, Medlock JM, Vaux AG, Koenraadt CJ, van Vliet AJ, Bartumeus F, et al. Approaches to passive mosquito surveillance in the EU. Parasit Vectors. 2015;8:9.
24. Werner D, Kampen H. *Aedes albopictus* breeding in southern Germany, 2014. Parasitol Res. 2015;114:831–4.
25. Werner D, Zielke DE, Kampen H. First record of *Aedes koreicus* Diptera: Culicidae) in Germany. Parasitol Res. 2016;115:1331–4.
26. Vezzani D. Artificial container-breeding mosquitoes and cemeteries: a perfect match. Trop Med Int Health. 2007;12:299–313.
27. Schaffner F, Angel G, Geoffroy B, Hervy JP, Rhaiem A, Brunhes J. The mosquitoes of Europe (CD-Rom). Montpellier: IRD Éditions & EID Méditerrannée; 2001.
28. Fonseca DM, Widdel AK, Hutchinson M, Spichiger SE, Kramer LD. Fine-scale spatial and temporal population genetics of *Aedes j. japonicus*, a new US mosquito, reveal multiple introductions. Mol Ecol. 2010;19:1559–72.
29. Zielke DE, Werner D, Kampen H, Schaffner F, Fonseca D. Unexpected patterns of admixture in German populations of *Aedes japonicus japonicus* (Diptera: Culicidae) underscore the importance of human intervention. PLoS ONE. 2014;9:e99093.
30. Pritchard JK, Stephens M, Donnelly P. Inference of population structure using multilocus genotype data. Genetics. 2000;155:945–59.
31. Earl DA, von Holdt BM. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conserv Genet. 2012;4:359–61.
32. Peakall R, Smouse PE. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research – an update. Bioinformatics. 2012;28:2537–9.
33. Zielke DE, Ibáñez-Justicia A, Kalan K, Merdic E, Kampen H, Werner D. Recently discovered *Aedes japonicus japonicus* (Diptera: Culicidae) populations in The Netherlands and northern Germany resulted from a new introduction event and from a split from an existing population. Parasit Vectors. 2015;8:40.

34. Fonseca DM, Campbell S, Crans WJ, Mogi M, Miyagi I, Toma T, Bullians M, Andreadis TG, Berry RL, Pagac B, Sardelis MR, Wilkerson RC. *Aedes* (*Finlaya*) *japonicus* (Diptera: Culicidae), a newly recognized mosquito in the United States: analyses of genetic variation in the United States and putative source populations. J Med Entomol. 2001;38:135–46.
35. Rochlin I, Kawalkowski M, Ninivaggi DV. Comparison of Mosquito Magnet and Biogents Sentinel traps for operational surveillance of container-inhabiting *Aedes* (Diptera: Culicidae) species. J Med Entomol. 2016;53:454–9.

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Occurrence and spread of the invasive Asian bush mosquito Aedes japonicus japonicus (Diptera: Culicidae) in West and North Germany since detection in 2012 and 2013, respectively
--Manuscript Draft--

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Article Type:	Research Article
Full Title:	Occurrence and spread of the invasive Asian bush mosquito Aedes japonicus japonicus (Diptera: Culicidae) in West and North Germany since detection in 2012 and 2013, respectively
Short Title:	Ae. japonicus in West and North Germany
Corresponding Author:	Helge Kampen Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health Greifswald, GERMANY
Keywords:	Aedes japonicus japonicus; Asian bush mosquito; invasive species; West Germany; North Germany; populations; spread; vector; Mueckenatlas
Abstract:	The invasive Asian bush mosquito Aedes japonicus japonicus was first recognised as established in Germany in 2008. In addition to the first known and quickly expanding population in the southwestern part of the country, three separate populations were discovered in West, North and southeastern Germany in 2012, 2013 and 2015, respectively, by means of the 'Mueckenatlas', a German instrument of passive mosquito surveillance. Since the first findings of mosquito specimens in West and North Germany, these regions were checked annually for continuing colonisation and spread of the species. Both affected areas were covered by a virtual 10x10km2 grid pattern in the cells of which cemeteries were screened for immature stages of the mosquito. The cells were considered populated as soon as larvae or pupae were detected, whereas they were classified as negative when no mosquito stages were found in the cemeteries of at least three different towns or villages. Presence was also recorded when Ae. j. japonicus adults were submitted to the 'Mueckenatlas' from the respective cell or when there was evidence of local occurrence in localities other than cemeteries. Based on this approach, a significant expansion of the populated area was documented in West Germany since the first detection of Ae. j. japonicus in 2012 (increase in positive grid cells by more than 400%), while the North German population appears not to be expanding so far (reduction of positive grid cells by ca. 30% since 2013). As Ae. j. japonicus finds suitable climatic and ecological conditions in Germany, the differential expansion of the two populations might be attributed to the West German population being older and thus more firmly established than the closely related but younger North German population that might still be in its founder phase. However, geographic spread of all German populations in the future is anticipated. Continuous surveillance is recommended, as Ae. j. japonicus is a competent vector of several pathogens in the laboratory.
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Additional data availability information:	

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1   **Occurrence and spread of the invasive Asian bush mosquito *Aedes japonicus***

2   ***japonicus* (Diptera: Culicidae) in West and North Germany since detection in**

3   **2012 and 2013, respectively**

4

5   **Short title:** *Aedes japonicus* in West and North Germany

6

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19   **Abstract**

20   The invasive Asian bush mosquito *Aedes japonicus japonicus* was first recognised as

21   established in Germany in 2008. In addition to the first known and quickly expanding

22   population in the southwestern part of the country, three separate populations were

23   discovered in West, North and southeastern Germany in 2012, 2013 and 2015, respectively,

24   by means of the 'Mueckenatlas', a German instrument of passive mosquito surveillance.

25   Since the first findings of mosquito specimens in West and North Germany, these regions

26   were checked annually for continuing colonisation and spread of the species. Both affected

27   areas were covered by a virtual 10x10km^2 grid pattern in the cells of which cemeteries were

28   screened for immature stages of the mosquito. The cells were considered populated as soon

29   as larvae or pupae were detected, whereas they were classified as negative when no

30   mosquito stages were found in the cemeteries of at least three different towns or villages.

31   Presence was also recorded when *Ae. j. japonicus* adults were submitted to the

32   'Mueckenatlas' from the respective cell or when there was evidence of local occurrence in

33   localities other than cemeteries. Based on this approach, a significant expansion of the

34   populated area was documented in West Germany since the first detection of *Ae. j.*

35   *japonicus* in 2012 (increase in positive grid cells by more than 400%), while the North

36   German population appears not to be expanding so far (reduction of positive grid cells by ca.

37   30% since 2013). As *Ae. j. japonicus* finds suitable climatic and ecological conditions in

38   Germany, the differential expansion of the two populations might be attributed to the West

39   German population being older and thus more firmly established than the closely related but

40   younger North German population that might still be in its founder phase. However,

41   geographic spread of all German populations in the future is anticipated. Continuous

42   surveillance is recommended, as *Ae. j. japonicus* is a competent vector of several pathogens

43   in the laboratory.

44

45   **Key words:** *Aedes japonicus japonicus,* Asian bush mosquito, invasive species, West

46   Germany, North Germany, populations, spread, vector, Mueckenatlas.

47 **Introduction**

48 As a result of globalisation and the worldwide trade with used tyres, lucky bamboo and

49 water-holding machinery, *Aedes* mosquitoes are regularly transported around the world and

50 introduced into non-endemic areas [1]. The Asian bush mosquito *Aedes* (*Hulecoeteomyia*)

51 *japonicus japonicus* is one of the top-ranked invasive mosquito species of the world [2].

52 Originating from East Asia (Korea, Japan, Taiwan, southern China, southeastern Russia),

53 where winters can be extremely cold [3], *Ae. j. japonicus* is well adapted to climatic

54 conditions in certain parts of North America and in Central Europe. It showed up in the

55 United States in the 1990s where it is now widely distributed in 33 states including Hawaii

56 [4]. In 2001, it was detected farther north in Canadian Quebec and Ontario [5, 6], in 2013 in

57 Newfoundland [7] and in 2014 in Vancouver, British Columbia [8]. In Europe, *Ae. j. japonicus*

58 was first established in Belgium in 2002, followed by Switzerland, Germany, Austria,

59 Slovenia, The Netherlands, Croatia, Hungary and France between 2008 and 2013 [4]. At

60 present, there are seven apparently separate populations in Europe with the Belgian

61 population being the only one with evidence for its mode of introduction as it is restricted to

62 the premises of two used tyre-trade companies [9]. By contrast, little information exists on

63 where the other European populations came from, how and where they entered Europe and

64 how they dispersed within Europe. Molecular population analyses showed two microsatellite

65 genetic signatures in Europe suggesting that at least two independent introduction events

66 took place [10, 11].

67 In Germany, four distinct *Ae. j. japonicus* populations have been detected, one in 2008 in the

68 southwestern part of the country, one in 2012 in the central western region, one in 2013 in a

69 more northern area, and one in 2015 in the Southeast [12-15; Fig. 1], with distances of at

70 least 190km between the closest known boundaries of these in 2013. According to genetic

71 analyses by Zielke et al. [11, 15], the northern and the southeastern German populations are

72 probably offshoots of the West German and the Austrian/Slovenian populations,

73 respectively, while the Southwest German population, extending cross-border into

74 Switzerland and France [17, 18], has a different genetic makeup [10, 11, 15].

75 Although the species naturally breeds in tree holes and rock pools, it accepts a wide variety

76 of artificial water containers such as rain water casks, jars, flower vases, pots and dishes [18].

77 This marked lack of specificity in choice of breeding site and environment provides *Ae. j.*

78 *japonicus* with excellent developmental opportunities and is the reason why the species can

79 also be found in cemeteries, either in urban, suburban or near natural settings (e.g. forests).

80 Cemeteries are opportune facilities from both the mosquito's and the investigator's point of

81 view [19]: they usually offer not only large quantities of breeding sites but also appropriate

82 habitats for adult mosquitoes such as bushes and trees that provide shade and shelter.

83 Moreover, food sources for adult mosquitoes are present in the form of flowering plants and

84 blood hosts (human visitors, birds and small mammals). For the collector, cemeteries are

85 easily accessible (in contrast to private premises), and a large number of potential breeding

86 sites can be checked for larvae and pupae within a limited period of time.

87 *Aedes j. japonicus* is considered a potential vector of disease agents although its vector

88 competencies have mainly been demonstrated in the laboratory. Experimentally, it was able

89 to transmit Japanese encephalitis, West Nile, dengue, chikungunya, Rift Valley fever and

90 Getah viruses [20-24], while in the field, it was found infected with Japanese encephalitis,

91 West Nile and La Crosse viruses [25-27]. In addition to its vector potential, the species is

92 discussed to replace indigenous mosquito species [28, 29].

93 The West and North German *Ae. j. japonicus* populations dealt with in this study were

94 discovered in 2012 and 2013, respectively, by means of the 'Mueckenatlas', a citizen science

95 project addressing the spatiotemporal mapping of mosquito species in Germany [30]. The

96 purpose of the present study was to track the spread of these two populations since their

97 detection, using regular field collections of mosquito developmental stages.

98

99 **Materials and methods**

100 *Mosquito collection*

101 The findings presented are based on two surveillance approaches, a passive and an active

102 one. In the scope of the passive surveillance instrument 'Mueckenatlas', citizens are

103 requested to collect adult mosquitoes in their private surroundings, kill them by freezing

104 overnight and send them by post to the research institutions involved [30]. Upon arrival,

105 they are identified to species morphologically or, if necessary (i.e. in the case of damaged

106 and incomplete specimens or cryptic species), genetically. The 'Mueckenatlas' was launched

107   in April 2012, and to date mosquitoes from all over Germany have been submitted, including

108   numerous *Ae. j. japonicus* specimens from their four German distribution areas.

109   The active surveillance approach, initially a reaction to the submission of *Ae. j. japonicus*

110   from West and North Germany, includes on-site monitoring by visiting areas suspected of

111   being colonised and then searching for mosquito larvae and pupae. After reception of the

112   first *Ae. j. japonicus* specimens from West and North Germany, the immediate surroundings

113   of their collection sites (usually private gardens) were inspected for water containers

114   harbouring immature stages of the species, followed by the closest cemeteries. When it

115   became clear that *Ae. j. japonicus* had become established, further surveillance was based

116   on a grid pattern of 10x10km^2 cells virtually laid over the affected region. Generally, up to

117   three cemeteries per cell were checked for the presence of *Ae. j. japonicus* developmental

118   stages by scrutinizing flower vases, supply wells, bowls and other artificial water-holding

119   containers. If larvae were found, all cells surrounding the positive cell (sometimes more)

120   were subjected to the same procedure. If no larvae could be detected in three cemeteries of

121   different villages or towns in the same grid cell, the cell was considered negative. A positive

122   cell was also recorded for any case of *Ae. j. japonicus* found outside of a cemetery, i.e. by

123   'Mueckenatlas' sample submissions or arbitrary larval findings in gardens or wooded areas.

124   The inspected cemeteries, which were selected at random by exploring villages, towns or

125   city districts in the desired grid cell, varied in size, number and type of potential breeding

126   sites available, and density of vegetation. As these fixed parameters rendered the various

127   sampled cemeteries incomparable, it was not considered appropriate to examine the same

128   number of water containers in each cemetery. Instead one hour at most was spent in a

129   cemetery, with every flower vase, dish, bowl and other container with water checked in

130   smaller cemeteries and at least 80 water containers checked in larger ones. The selection of

131   the inspected containers was also arbitrary but focused on vases under beech trees (*Fagus*

132   spec.) and in other shaded and vegetated areas if present. Also, containers were checked in

133   different sectors of large cemeteries. The search was stopped immediately when larvae of

134   *Ae. j. japonicus* were unambiguously identified by specific morphological and behavioural

135   characteristics [13]. This identification in the field was later confirmed in the laboratory

136   genetically by CO1 barcoding [31, 32] of alcohol-fixed larvae from every site considered

137   positive. In cases of uncertainty (e.g. early stage larvae), individuals suspected of being *Ae. j.*

138 *japonicus* were transferred to beakers together with some water they were developing in,

139 and kept until adult emergence. Adults were then identified to species morphologically [33].

140 As the latter procedure did not always allow a decision about presence or absence on the

141 spot, the situation was equalised with the non-finding of *Ae. j. japonicus*, and the search was

142 continued as described.

143 After the first detections of *Ae. j. japonicus* in West and North Germany, the two affected

144 areas (Fig. 1) were repeatedly surveyed, if possible twice a year, once in May and once in

145 August, based on the observation in some areas of the United States that there are two

146 larval population peaks during the breeding season [34]. Population densities were not

147 determined but efforts necessary to find *Ae. j. japonicus* larvae can be conveyed by the

148 numbers of cemeteries that had to be examined.

149

150 *Statistics*

151 With regard to a minimum population density, the sensitivity and specificity of the detection

152 of *Ae. j. japonicus* in the cemeteries can be described as follows: supposing that the larvae

153 are evenly spread over an infinite number of potential breeding sites, their detection has a

154 probability of 99% (95%) as long as at least 5.6% (3.7%) of the examined sites are populated

155 (binomial model). Given the documented expertise and experienced collecting approach of

156 the persons looking for the mosquitoes and the genetic identification techniques applied,

157 the specificity of correct species determination can be considered 100%. False positive

158 results were, therefore, excluded.

159 To determine the spatiotemporal expansion of the area colonised by *Ae. j. japonicus*, the

160 sign test was applied. As all grid cells were of equal size, only the ratio of positive and

161 negative grid cells within a defined area needed consideration.

162 To check for the difference in investigation effort between the populated areas and the

163 years of data collection, the chi-square test was applied to counts of cemetery visits per grid

164 cell necessary to find *Ae. j. japonicus* aquatic stages in relation to the maximum possible

165 number of visits (which was preset at three).

166 For both the sign test and the chi-square test, significance was set at $p < 0.05$.

167   All analyses were performed in R [35].

168

169   **Results**

170   *West German population*

171   The first *Ae. j. japonicus* specimens from West Germany were submitted to the

172   'Mueckenatlas' in July 2012. Based on a 10x10km^2 grid pattern, the area affected was

173   checked for developmental mosquito stages in August 2012, May and August 2013, May and

174   August 2014, and May and August 2015. The findings differed between the two inspections

175   per year and have been summed for each year in Fig. 2.

176   According to detections of specimens in the field, *Ae. j. japonicus* spread considerably from

177   2012 to 2015 (Fig. 2). Numbers of grid cells with larval findings increased from 21 in 2012 to

178   31 in 2013 (plus 47.6%), 52 in 2014 (plus 67.7%) and 89 in 2015 (plus 71.2%), totalling 424%

179   from 2012 to 2015 (Table 1). Although population densities were not determined, larvae

180   could generally be found with less effort in the centre of the populated area than in

181   peripheral areas (Figs. 2a-2d), where they were often detected only after extensive search

182   and in limited numbers (sometimes only two specimens per cemetery).

183   With few exceptions, 'Mueckenatlas' submissions could be verified by mosquito

184   demonstrations in the field. In both 2012 and 2013, however, two cells (M5 and I7 in 2012,

185   H4 and H8 in 2013) were positive by 'Mueckenatlas' submission whereas no *Ae. j. japonicus*

186   individuals could be found in cemeteries of the respective cells (Figs. 2a, 2b). Cell M5, from

187   where an *Ae. j. japonicus* specimen had been submitted to the 'Mueckenatlas' in 2012,

188   remained negative until 2014 (Fig. 2c), and only in 2015 were larvae demonstrated in this

189   cell (Fig. 2d), although in a cemetery of a town different from where the 'Mueckenatlas'

190   submission originated.

191   Generally, the numbers of grid cells rated positive in August were higher than those in May

192   (Table 1) although not all cells positive in May were necessarily positive in August. For

193   example in May 2014, five cells in the periphery of the populated area (G7, F10, G10, H10,

194   J11) were negative in August (Fig. 2c).

195 Similarly, most cells positive in one year were confirmed positive in the next. As an

196 exception, *Ae. j. japonicus* larvae could not be detected in 2015 in five cells that had been

197 positive in 2014 (J4, G8, F10, J11, K11; Figs. 2c, 2d). Four of these (J4, G8, J11, K11) and some

198 other negative cells (F8, M10, L13, L14) were completely or predominantly surrounded by

199 positive cells in 2015 (Fig. 2d), causing a somewhat patchy picture of colonisation.

200 Cell Q14 was only checked in early September 2015 due to three submissions of *Ae. j.*

201 *japonicus* to the 'Mueckenatlas' in August. While the species was eventually found in two

202 cemeteries in that cell, the surrounding cells could not be sampled in the expiring mosquito

203 season. The negative cells east of cells N14 and O15 therefore convey the visual impression

204 that positive cell Q14 is isolated from the West German population (Fig. 2d).

205 The only grid cell with *Ae. j. japonicus* findings beyond a cemetery during the field surveys

206 was cell I3 in August 2014, where larvae were detected in numerous tree holes in a beech

207 forest. Shortly before, several adult specimens collected in this forest had been submitted to

208 the 'Mueckenatlas'. After the breeding sites were identified, a cemetery about 2km away in

209 direct line was also shown to be colonised in August 2014 whereas another cemetery in the

210 same cell, 2.4km away, seemed to be free of *Ae. j. japonicus*.

211 The annual increase in grid cells positive for *Ae. j. japonicus*, as initially based on subjective

212 observations and interpreted as a spreading mosquito population, is supported statistically.

213 To check the hypothesis of a geographic spread of the mosquito population within a defined

214 area, the area examined in 2012 was annually evaluated (Fig. 2a). Within this area (framed in

215 bold in Figs. 2a-d), all cells were assessed regarding their observed status compared to the

216 previous year. The potential status of the two cells not sampled within the frame in 2015

217 (E10, E11), either positive or negative, had no effect on the outcome of the statistical

218 analysis. The hypothesis that the ratio of positive and negative squares remains stable from

219 one year to the following must be rejected according to the sign test (p> 0.05), whereby

220 multiple testing (year-to-year comparison) is considered. From 2012 to 2013, for example,

221 12 shifts from negative to positive were registered, but only one shift from positive to

222 negative (p=0.0017). Similarly, 18 shifts from negative to positive versus one shift from

223 positive to negative were observed for the period 2013–2014 (p<0.0001), and 16 shifts from

224 negative to positive versus 5 shifts from positive to negative for the period 2014–2015

225 (p=0.0133).

226 In the West German population, 226 cemeteries had to be visited in 82 cells of the reference
227 area in August 2012 to find *Ae. j. japonicus* larvae, according to the preset criteria, averaging
228 2.76 cemeteries per cell. Only 140 cemeteries were inspected in 80 cells in August 2015,
229 averaging 1.75 visits per cell. Thus, the examination effort was considerably less in 2015
230 compared to 2012 (chi-square test: X^2=8.2428, df=1, p=0.00409). The effort increased when
231 examining less populated peripheral areas.

232

233 *North German population*

234 A specimen of *Ae. j. japonicus* from North Germany was submitted to the 'Mueckenatlas' for
235 the first time in late summer 2012, but the collection area could be visited only in May 2013.
236 As for West Germany, the annual extent of geographic spread was assessed based on
237 cemetery inspection in a 10x10km^2 grid pattern. Survey data exist from May and August
238 2013 and May and August 2014, while only the August field study could be carried out in
239 2015. For 2013 and 2014, the data of the two annual surveys were again summed for Fig. 3.

240 The situation for the North German *Ae. j. japonicus* population was different from that in
241 West Germany in that the area colonised (Figs. 3a-3c) decreased in the three years of
242 observation. A reduction from 12 positive grid cells in 2013 to 10 in 2014 (reduction of
243 16.7%) and 8 in 2015 (reduction of 20%) was registered, giving a total reduction of one third
244 from 2013 to 2015 (Table 1). Over the years, only five cells (F2, G2, C3, H3, F4) were
245 consistently positive, and in none of the years did the cells display a spatially non-disrupted
246 population. Despite this, *Ae. j. japonicus* larvae were found in two cells in 2015 (H2, F3)
247 where no specimens had been detected previously.

248 In 2013 and 2014 (Figs. 3a, 3b), one submission each to the 'Mueckenatlas' was registered,
249 while in 2015 (Fig. 3c) two submissions were received. The submission in 2013 and one of
250 the submissions in 2015 did not correlate with positive cemetery inspections in the
251 respective cells.

252 As in West Germany, cells usually remained positive in August when they had been positive
253 in May of the same year. Cells I3 and C5 were exceptions in 2013, and 2013 was the only
254 year with more cells positive in May than in August (Table 1).

255 Except for 2012 in West Germany, the numbers of cemeteries per cell that had to be

256 checked to find *Ae. j. japonicus* were higher in North Germany than in West Germany,

257 suggesting that the population density was generally lower (Figs. 3a-3c). In North Germany,

258 a minimum of 2.69 cemeteries had to be visited per cell in August 2013 until *Ae. j. japonicus*

259 was found. In that collection period, a total of 129 cemeteries were inspected in 48 cells

260 which increased to 137 cemeteries in 49 cells in August 2015 (2.80 visits per cell). A

261 statistical difference as to the annual average examination effort per cell was not found (chi-

262 square test: X^2=0.0028, df=1, p=0.9577). Both measurements (i.e., August 2013 and August

263 2015) can therefore be considered jointly and result in an average collection effort of 2.74

264 cemetery visits per cell. This value corresponds to the efforts in the West German study area

265 in August 2012 (chi-square test: X^2=0.0199, df=2, p=0.099).

266 Despite the decline of two positive grid cells per year in the North German *Ae. j. japonicus*

267 distribution area, the hypothesis that the ratio of negative and positive squares are

268 comparable over time, corresponding to a more or less stable population area, cannot be

269 rejected (p>0.1).

270

271 **Discussion**

272 The emergence and spread of the invasive Asian bush mosquito *Ae. j. japonicus* in North

273 America and Europe are attributed to the broad ecological tolerance and adaptability of this

274 mosquito species [36]. Particularly, the resistance of its eggs to low temperatures, an

275 extended season of activity from early spring to late autumn and the low grade of

276 specialisation in the choice of breeding sites, with the immature stages tolerating high

277 organic concentrations, support the survival and establishment of the species in non-native

278 areas [9].

279 Not only are the eggs of *Ae. j. japonicus* dispersed by continental and intercontinental

280 transport and subsequently succeed in establishing new populations at some of their

281 destinations, but populations may also quickly expand once firmly established. The factors

282 determining an increase in population densities and a subsequent geographic spread of the

283 population are not clear, and there is evidence that populations remain more or less static in

284 terms of area coverage over many years, e.g. in Belgium [9]. Observations from the United

285   States suggest that *Ae. j. japonicus* might need one to three years for breeding site numbers

286   and population densities, and thus detection frequencies, to significantly increase [37].

287   We tried to assess the continuing presence and the rate of geographic spread of *Ae. j.*

288   *japonicus* populations by checking cemeteries in a grid cell pattern. Although this approach

289   lacks standardisation, and thus comparability, it has successfully been applied in various

290   studies targeting *Aedes* species and enables a good overview of their spatial distribution [e.g.

291   16, 38]. Accordingly, *Ae. j. japonicus* showed a significant spread of a population in West

292   Germany but a more or less static population in North Germany between 2012 and 2015,

293   and 2013 and 2015, respectively.

294   Although no specific data on population densities were collected during the years of

295   observation, generally both more cemeteries needed to be checked to find *Ae. j. japonicus*

296   specimens and the numbers of specimens encountered were much lower in peripheral grid

297   cells than in central grid cells in West Germany. The same situation applies to the

298   comparison of the North and the West German populations in that efforts to find larvae

299   were comparable between most grid cells in the North German population and cells in the

300   periphery of the West German population. Thus, based on the finding that the genetic

301   makeup of the North German population suggests a close relationship with the West

302   German population, Zielke et al. [11] concluded that the North German population is an

303   offshoot of the West German one, probably as a consequence of passive vehicle transport of

304   founder individuals along a connecting motorway. Therefore, the North German population

305   is likely to be younger than the West German one and probably not as firmly established.

306   The assumption that the West and North German populations are of different ages is also

307   supported by the area coverage, as expressed by the number of positive grid cells. This

308   number was generally lower in May than in August, probably due to low population

309   densities at the beginning of the season. Probably, low abundance and patchy occurrence,

310   rather than absence, were the reasons for cells without *Ae. j. japonicus* surrounded by cells

311   with findings.

312   *Aedes j. japonicus* could not be found in the cemetery of the town of the 2012

313   'Mueckenatlas' submission from grid cell M5 in West Germany. Specimens were only found

314   in a cemetery of another town of that cell in 2015. It is possible that *Ae. j. japonicus* has

315   occurred in that cell since 2012 but in a population density below the detection limit.

316   Despite the expansion of the West German populated area, grid cell M5 remained in its

317   periphery, where abundances must be assumed to have been very low even in late August,

318   until 2015. Alternatively, *Ae. j. japonicus* might not have been discovered in that cell because

319   it did not colonise cemeteries. In grid cell I3 in West Germany, for example, *Ae. j. japonicus*

320   was first detected as adults in a beech forest in 2014 and sent for identification. An on-site

321   inspection showed the species to be widely distributed in tree holes in that forest. In the

322   closest cemetery, however, it could not be found, and only a few specimens were detected

323   in the second closest cemetery.

324   *Aedes j. japonicus* has the competitive advantage over several indigenous mosquito species

325   with similar ecological niches to be active from very early until very late in the season [36]. It

326   can therefore quickly develop high abundances in the centre of established populations and

327   might outcompete indigenous mosquito species [34]. In central grid cells of the West

328   German population, masses of larvae were observed in water basins of several cemeteries in

329   early April while few or no specimens of other species could be found. The water

330   temperatures at that time, which were generally below 10°C and as low as 4°C (unpublished

331   data), agree with the onset of larval development at 4.5-5°C as measured in southern New

332   Hampshire, USA, by Burger & Davis [34].

333   In summary, *Ae. j. japonicus* as an intruder does not appear to have competitive

334   disadvantages as opposed to indigenous mosquito species. It is highly adaptable to the

335   German climate and tends to expand as soon as certain population densities are reached.

336   This is the case in the West German population, probably as well as in the southwestern

337   German population, but not yet in the North German population. The results indicate that

338   the speed of active spread is rapid once a population is firmly established. The West German

339   population is, therefore, predicted to breach the border to Belgium in the west and to merge

340   with the Southeast German population in the near future, possibly already in 2016. This may

341   lead to a highly mixed 'superpopulation' with broad genetic diversity and, thus, an even

342   greater adaptability. Although *Ae. j. japonicus* can no longer be eradicated from Germany

343   and must now be considered a permanent component of the country's mosquito fauna,

344   further monitoring might produce valuable information on the establishment and

345   spatiotemporal expansion of an invasive mosquito species as well as a potential vector of

346   disease agents.

347

Supporting Information

349 **Table S1:** Location, grid cell assignment and monitoring results of the sites checked for *Ae. j.*
350 *japonicus* in West Germany.

351 **Table S2:** Location, grid cell assignment and monitoring results of the sites checked for *Ae. j.*
352 *japonicus* in North Germany.

353

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360

361 **Literature**
362

363 1.   Medlock J, Hansford KM, Versteirt V, Cull B, Kampen H, Fontenille D, et al. (2015) An
364   entomological review of invasive mosquitoes in Europe. Bull Entomol Res 105: 637–663.

365

366 2.   ISSG (Invasive Species Specialist Group) (2009) Global Invasive Species Database –
367   *Ochlerotatus japonicus japonicus*. Available online:
368   http://www.issg.org/database/species/ecology.asp?si=1396&fr=1&sts=sss&lang=EN
369   (accessed 30 April 2016).

370

371 3.   Kamimura K (1976) On the Japanese species of the family Culicidae. In: Science of
372   Mosquitoes. Edited by Sasa M, Kurihara T, Kamimura K. Tokyo, Japan: Hokuryukan: 150–
373   188 [In Japanese].
374

375   4. Kampen H, Werner D (2014) Out of the bush: The Asian bush mosquito *Aedes japonicus*
376   *japonicus* (Theobald, 1901) (Diptera, Culicidae) becomes invasive. Parasit Vectors 7: 59.
377

378   5. Savignac R, Back C, Bourassa J (2002) Biological Notes on *Ochlerotatus japonicus* and
379   other mosquito species new to Quebec. Joint Meeting 68th Annual Meeting of the
380   American Mosquito Control Association and the West Central Mosquito & Vector
381   Control Association; Denver, CO, 16-21 February 2002. Abstracts: 21–22.
382

383   6. Thielman A, Hunter FF (2006) Establishment of *Ochlerotatus japonicus* (Diptera:
384   Culicidae) in Ontario, Canada. J Med Entomol 43: 138–142.
385

386   7. Fielden MA, Chaulk AC, Bassetta K, Wiersma, YF, Erbland, M, Whitney, H, Chapman TW
387   (2015) *Aedes japonicus japonicus* (Diptera, Culicidae) arrives at the most easterly point
388   of North America. Canadian Entomologist 147: 737–740.
389

390   8. Jackson M, Belton P, McMahon S, Hart M, McCann S, Azevedo D, Hurteau L (2016) The
391   first record of *Aedes* (*Hulecoeteomyia*) *japonicus* (Diptera: Culicidae) and its
392   establishment in western Canada. J Med Entomol 53: 241–244.
393

394   9. Versteirt V, Schaffner F, Garros C, Dekoninck W, Coosemans M, van Bortel W (2009)
395   Introduction and establishment of the exotic mosquito species *Aedes japonicus*
396   *japonicus* in Belgium. J Med Entomol 46: 1464–1467.
397

398   10. Zielke DE, Werner D, Kampen H, Schaffner F, Fonseca DM (2014) Unexpected patterns of
399   admixture in German populations of *Aedes japonicus japonicus* (Diptera, Culicidae)
400   underscore the importance of human intervention in the dispersal of this invasive
401   mosquito species. PLoS One 9: e99093.
402

403   11. Zielke DE, Ibañez-Justicia A, Kalan K, Merdič E, Kampen H, Werner D (2015) Recently
404   discovered *Aedes japonicus japonicus* (Diptera: Culicidae) populations in The Nether-
405   lands and northern Germany resulted from a new introduction event and from a split
406   from an existing population. Parasit Vectors 8: 40.

74

407

408   12. Becker N, Huber K, Pluskota B, Kaiser A (2011) *Ochlerotatus japonicus japonicus* – a

409   neozoon in Germany and a revised list of the German mosquito fauna. Eur Mosq Bull 9:

410   88–102.

411

412   13. Kampen H, Zielke D, Werner D (2012) A new focus of *Aedes japonicus japonicus* (Diptera,

413   Culicidae) distribution in western Germany: rapid spread or a further introduction

414   event? Parasit Vectors 5: 284.

415

416   14. Werner D, Kampen H (2013) The further spread of *Aedes japonicus japonicus* (Diptera,

417   Culicidae) towards northern Germany. Parasitol Res 112: 3665–3668.

418

419   15. Zielke DE, Walther D, Kampen H (2016) Newly discovered population of *Aedes japonicus*

420   *japonicus* (Diptera: Culicidae) in Upper Bavaria, Germany, and Salzburg, Austria, is

421   closely related to the Austrian/Slovenian bush mosquito population. Parasit Vectors 9:

422   163.

423

424   16. Schaffner F, Kaufmann C, Hegglin D, Mathis A (2009) The invasive mosquito *Aedes*

425   *japonicus* in Central Europe. Med Vet Entomol 23: 448–451.

426

427   17. Krebs T, Bindler P, L'Ambert G, Toti C, Perrin Y, Jourdain F (2014) First establishment of

428   *Aedes japonicus japonicus* (Theobald, 1901) (Diptera: Culicidae) in France in 2013 and its

429   impact on public health. J Vector Ecol 39: 437–440.

430

431   18. Andreadis TG, Anderson JF, Munstermann LE, Wolfe RJ, Florin DA (2001) Discovery,

432   distribution, and abundance of the newly introduced mosquito *Ochlerotatus japonicus*

433   (Diptera: Culicidae) in Connecticut, USA. J Med Entomol 38: 774–779.

434

435   19. Vezzani D (2007) Artificial container-breeding mosquitoes and cemeteries: a perfect

436   match. Trop Med Int Health 12: 299–313.

437

438   20. Takashima I, Rosen L (1989) Horizontal and vertical transmission of Japanese
439   encephalitis virus by *Aedes japonicus* (Diptera: Culicidae). J Med Entomol 26: 454–458.
440

441   21. Sardelis MR, Turell MJ (2001) *Ochlerotatus j. japonicus* in Frederick County, Maryland:
442   discovery, distribution, and vector competence for West Nile virus. J Am Mosq Control
443   Assoc 17: 137–141.
444

445   22. Schaffner F, Vazeille M, Kaufmann C, Failloux A-B, Mathis A (2011) Vector competence
446   of *Aedes japonicus* for chikungunya and dengue viruses. Eur Mosq Bull 29: 141–142.
447

448   23. Turell MJ, Byrd BD, Harrison BA (2013) Potential for populations of *Aedes j. japonicus* to
449   transmit Rift Valley fever virus in the USA. J Am Mosq Control Assoc 29: 133–137.
450

451   24. Takashima I, Hashimoto N (1985) Getah virus in several species of mosquitoes. Trans R
452   Soc Trop Med Hyg 79: 546–550.
453

454   25. Chagin KP, Kondratiev PI (1943) Vectors of autumnal (Japanese) encephalitis in
455   Primor'ye region and measures for controlling them. Med Parazitol Parazit Bolezni 12:
456   34–44 [in Russian].
457

458   26. CDC (Centers for Disease Control and Prevention) (2012) Mosquito species in which
459   West Nile virus has been detected, United States, 1999–2012.
460   http://www.cdc.gov/westnile/resources/pdfs/mosquitospecies1999-2012.pdf (accessed
461   30 April 2016).
462

463   27. Harris MC, Dotseth EJ, Jackson BT, Zink SD, Marek PE, Kramer LD, et al. (2015) La Crosse
464   virus in *Aedes japonicus japonicus* mosquitoes in the Appalachian Region, United States.
465   Emerg Infect Dis 21: 646–649.
466

467   28. Andreadis TG, Wolfe RJ (2010) Evidence for reduction of native mosquitoes with
468   increased expansion of invasive *Ochlerotatus japonicus japonicus* (Diptera: Culicidae) in
469   the northeastern United States. J Med Entomol 47: 43–52.

470

471   29. Rochlin I, Gaugler R, Williges E, Farajollahi A (2013) The rise of the invasives and decline

472   of the natives: insights revealed from adult populations of container-inhabiting *Aedes*

473   mosquitoes (Diptera: Culicidae) in temperate North America. Biol Invasions 15: 991–

474   1003.

475

476   30. Kampen H, Medlock JM, Vaux AG, Koenraadt CJ, van Vliet AJ, Bartumeus F, et al. (2015)

477   Approaches to passive mosquito surveillance in the EU. Parasit Vectors 8: 9.

478

479   31. Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification

480   of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates.

481   Mol Mar Biol Biotechnol 3: 294–299.

482

483   32. Hébert PD, Ratnasingham S, de Waard JR (2003) Barcoding animal life: cytochrome c

484   oxidase subunit 1 divergences among closely related species. Proc Biol Sci 270 (Suppl 1):

485   S96–S99.

486

487   33. Schaffner F, Angel G, Geoffroy B, Hervy JP, Rhaiem A, Brunhes J (2001) The mosquitoes

488   of Europe (CD-Rom). Montpellier, France: IRD Éditions & EID Méditerrannée.

489

490   34. Burger JF, Davis H (2009) Discovery of *Ochlerotatus japonicus japonicus* (Theobald)

491   (Diptera: Culicidae) in southern New Hampshire, U.S.A. and its subsequent increase in

492   abundance in used tire casings. Entomol News 119: 439–444.

493

494   35. R Core Team (2015). R: A language and environment for statistical computing. R

495   Foundation for Statistical Computing, Vienna, Austria. https://www.r-project.org/

496   (accessed 30 April 2016).

497

498   36. Kaufman MG, Fonseca DM (2014) Invasion biology of *Aedes japonicus japonicus*

499   (Diptera: Culicidae). Annu Rev Entomol 59: 31–49.

500

501   37. Morris JA, Lampman RL, Ballmes G, Funes J, Halvorsen J, Novak RJ (2007) First record of

502   *Aedes japonicus japonicus* in Illinois: defining its spatial distribution and associated

503   mosquito species. J Am Mosq Control Assoc 23: 243–251.

504

505   38. O'Meara GF, Gettman AD, Evans LF Jr, Scheel FD (1992) Invasion of cemeteries in Florida

506   by *Aedes albopictus*. J Am Mosq Control Assoc 8: 1–10.

507

508   **Legends to figures:**

509

510   **Figure 1:** Map showing the geographic locations of the four known *Ae. j. japonicus*

511   populations in Germany (population encircled in green was neither detected nor studied by

512   the authors; for population encircled in red see [15]). Insets mark the two populations/areas

513   surveyed.

514

515   **Figure 2:** Area of West Germany in the federal states of North Rhine-Westphalia, Rhineland-

516   Palatinate and Hesse checked for *Ae. j. japonicus* in 2012 (a), 2013 (b), 2014 (c) and 2015 (d).

517   Red squares: grid cells positive, green squares: grid cells negative by cemetery inspection,

518   circles: grid cells with *Ae. j. japonicus* submissions to the 'Mueckenatlas'; figures in the grid

519   cells denote findings in May/August of the respective year with '1', '2', '3' representing the

520   number of cemeteries inspected until *Ae. j. japonicus* was found, 'X' representing three

521   negative cemeteries in case only one of the two collection seasons was positive, and '-'

522   meaning not sampled. The area framed in bold marks the reference area used for statistical

523   analysis.

524

525   **Figure 3:** Area of North Germany in the federal states of North Rhine-Westphalia and Lower

526   Saxony checked for *Ae. j. japonicus* in 2013 (a), 2014 (b) and 2015 (c). Red squares: grid cells

527   positive, green squares: grid cells negative by cemetery inspection, circles: grid cells with *Ae.*

528   *j. japonicus* submissions to the 'Mueckenatlas'; figures in the grid cells denote findings in

529   May/August with '1', '2', '3' representing the number of cemeteries inspected until *Ae. j.*

530   *japonicus* was found, 'X' representing three negative cemeteries in case only one of the two

531   collection seasons was positive, and '-' meaning not sampled. The area framed in bold marks

532   the reference area used for statistical analysis.

533

534   **Table 1:** Number of grids cells positive for *Ae. j. japonicus* (number of 'Mueckenatlas'

535   collection sites:number of submitted specimens are given in parentheses).

Table 1

	2012		2013			2014			2015		
	August	Total*	May	August	Total*	May	August	Total*	May	August	Total*
West Germany											
'Mueckenatlas'	7 (10:14)	21	---	8 (11:27)	31	---	9 (14:56)	52	---	8 (13:16)	89
field sampling	19		13	29		33	48		32	89	
North Germany											
'Mueckenatlas'	---	---	---	1	12	---	1	10	---	2	8
field sampling	---	---	11	9		6	10		---	7	

*As the same grid cells may have been positive in both collection periods per year and/or by both collection approaches, totals are less than sums.

Figure 1
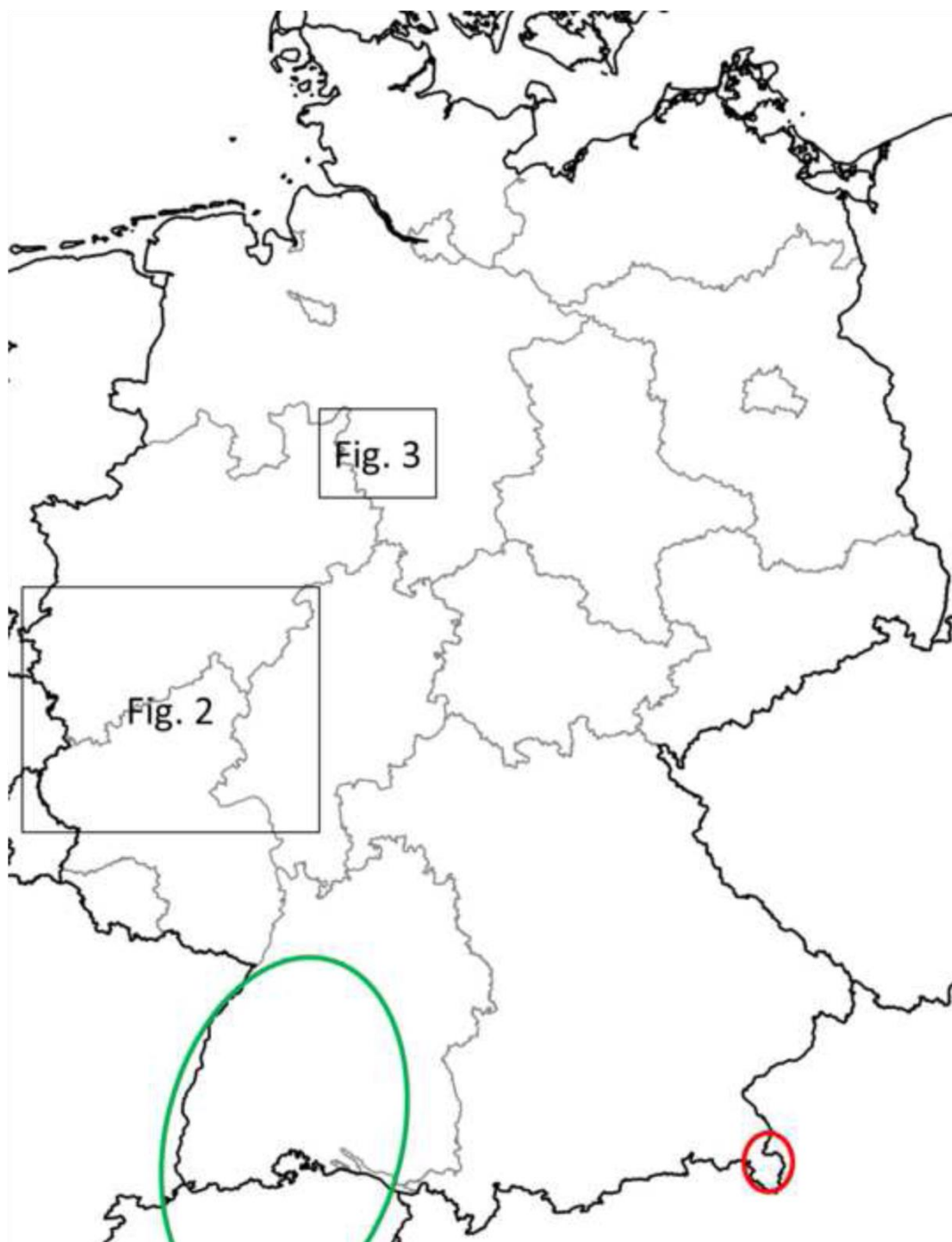
Figure 2a

Figure 2b

Figure 2b

Figure 2c

84
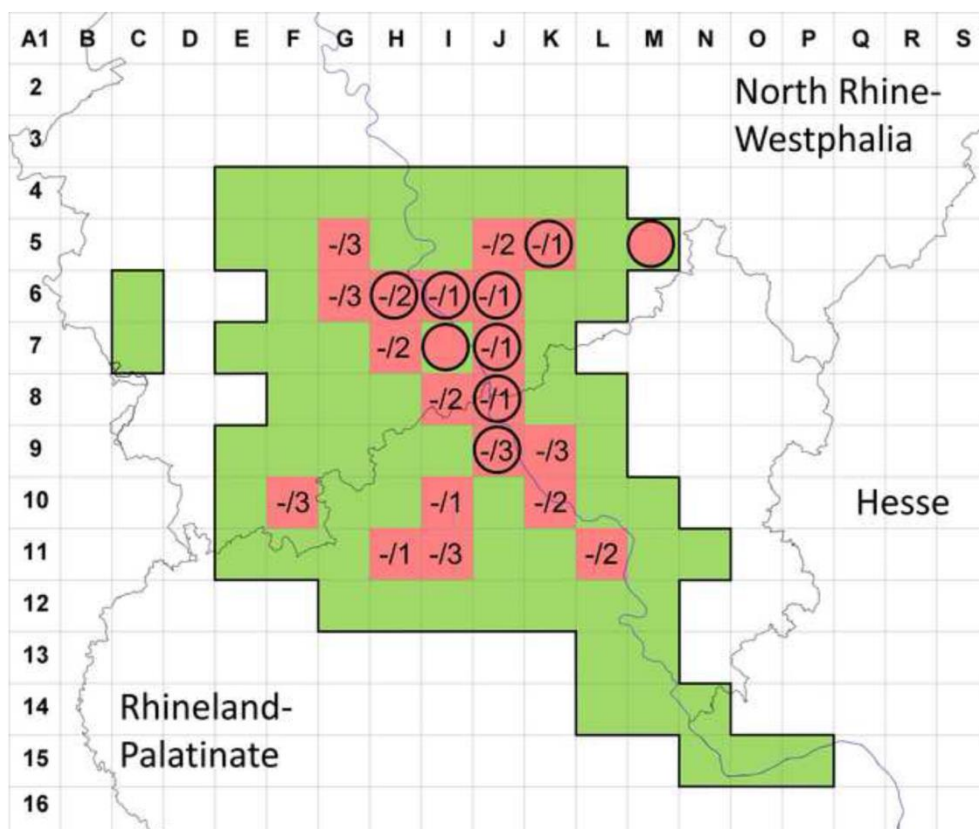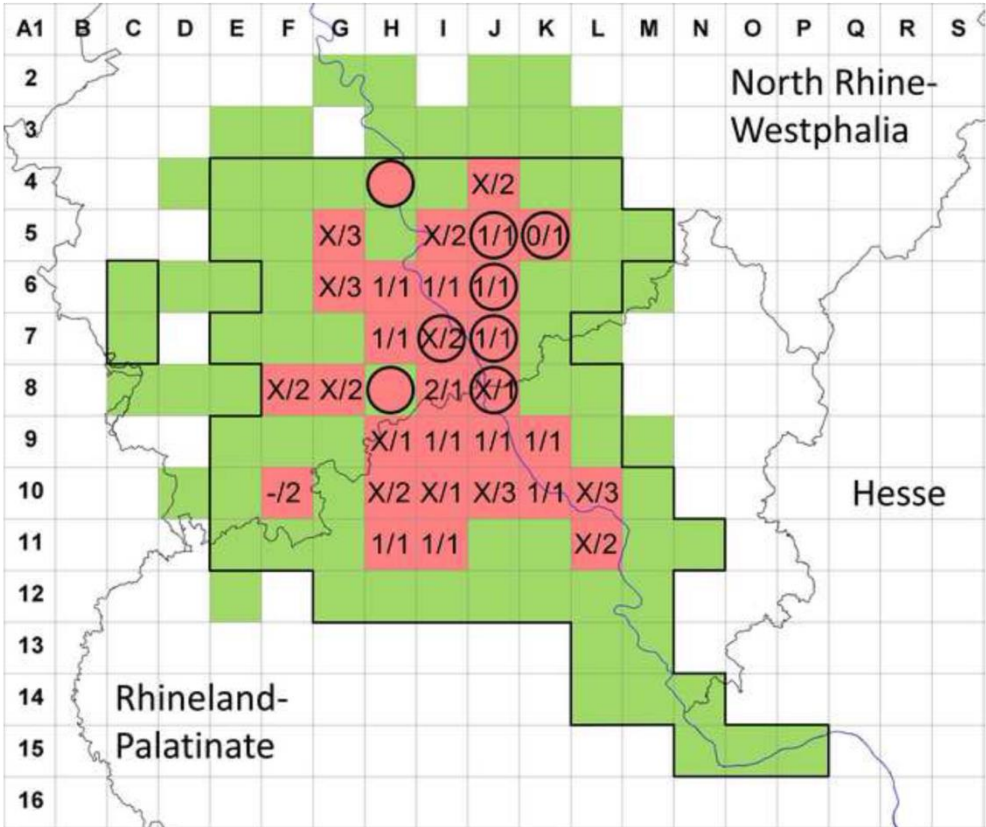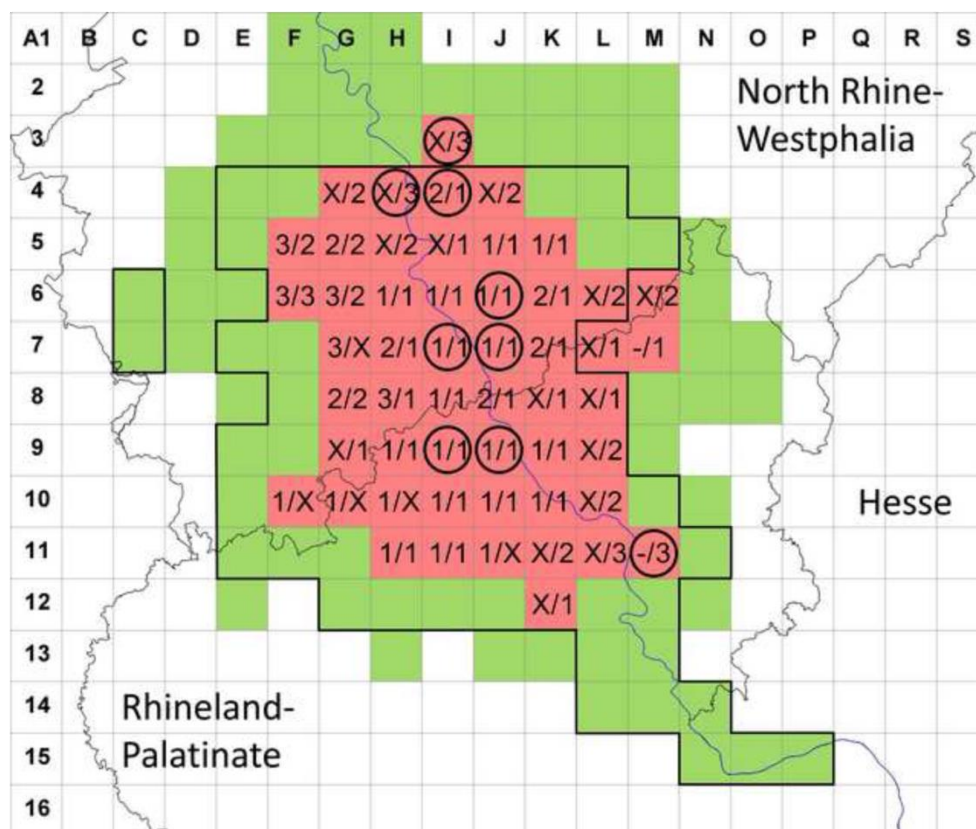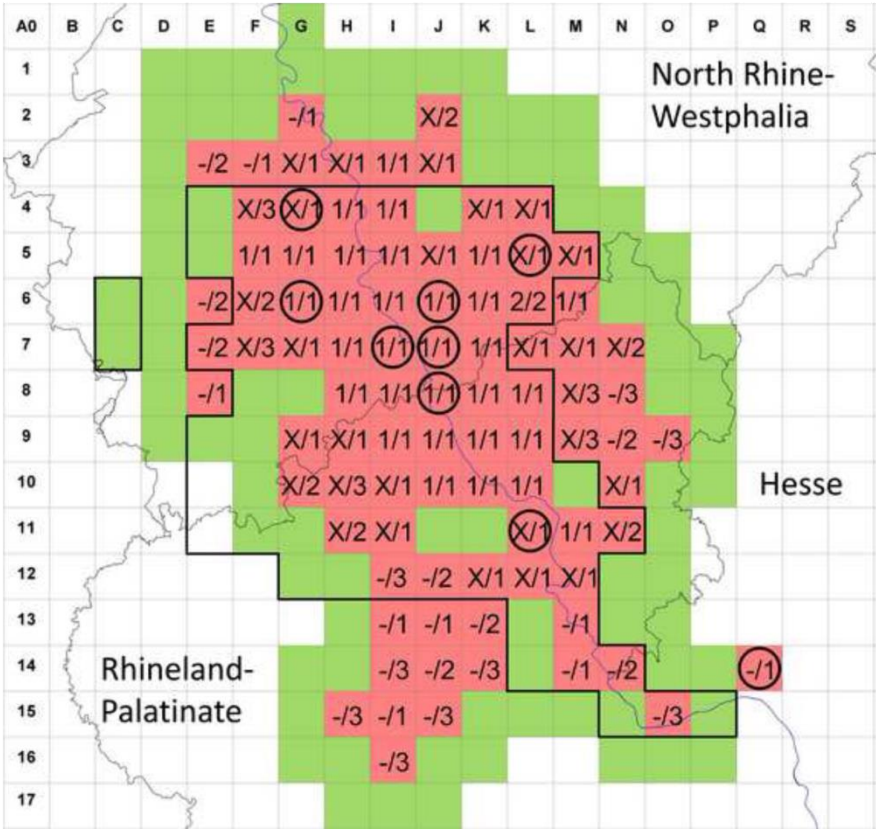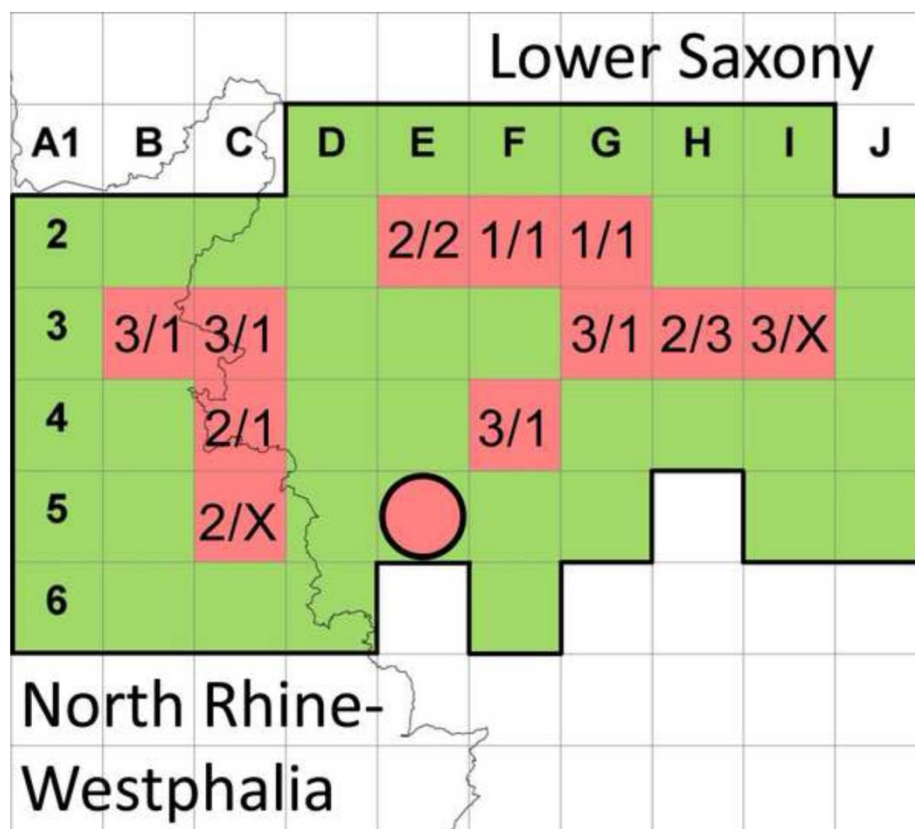
Figure 2d

Figure 3a
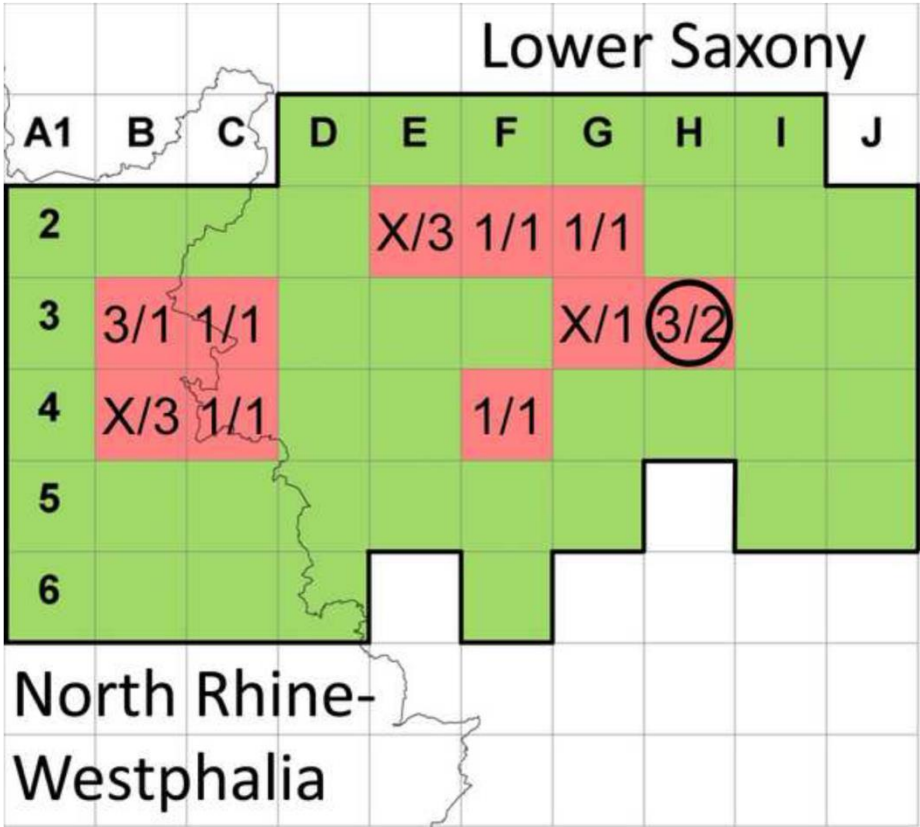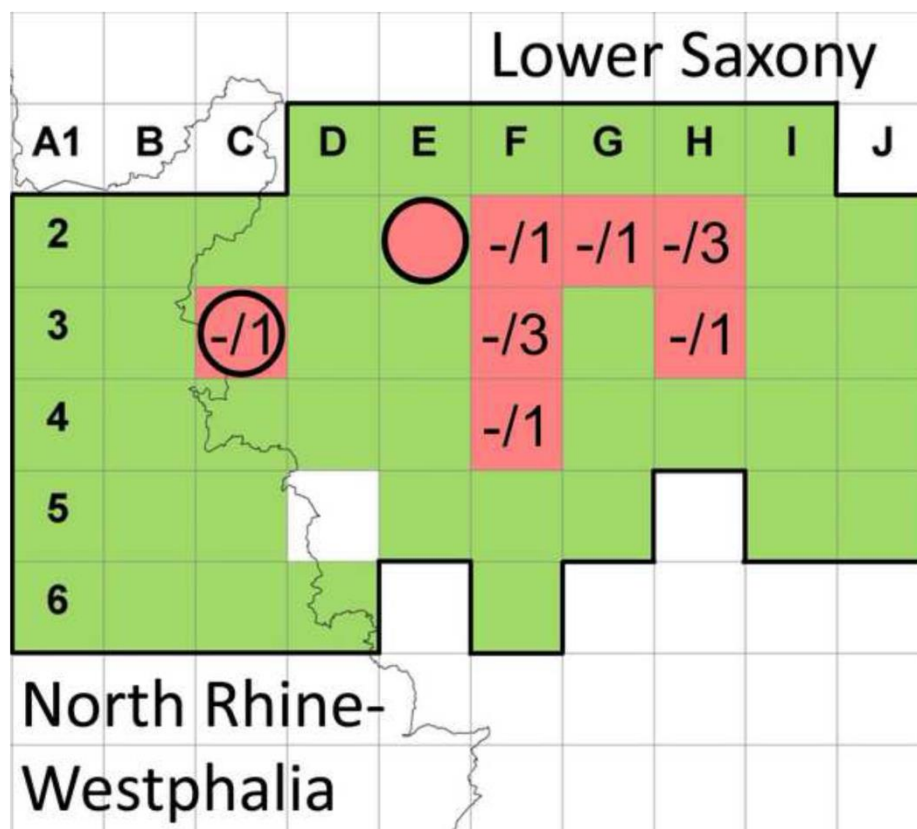
Figure 3a

Figure 3b

Figure 3c

3. SUMMARY

The present work deals with the distribution and population genetics of the invasive Asian bush mosquito *Ae. j. japonicus* which has been spreading in Germany since 2008 by human-mediated transportation and dispersal. *Aedes j. japonicus* is a vector of several arboviruses under laboratory conditions, e.g. dengue virus and chikungunya virus. It has also been found infected in the field with Japanese encephalitis virus, West Nile virus and La Crosse virus.

Following several independent submissions of *Ae. j. japonicus* specimens in the framework of the German citizen science project "Mückenatlas", a population was discovered in West Germany in 2012. The distribution area of this population was far more north than the assumed northern distribution limit of the species in South Germany. The extent of this population was defined by a short-time monitoring to an area of about 2,000 km^2. Developmental stages of five locations in West Germany were subjected to population genetic studies in order to study the relatedness of individuals within the population and with other European populations. For this purpose, seven microsatellite loci were examined and compared. In addition, part of the mitochondrial *nad*4 gene region was sequenced and screened for nucleotide polymorphisms. The results were compared to data previously collected from Swiss, Austrian/Slovenian and Belgian populations. The microsatellite signature of the West German population was quite different from the ones of the other European populations. Further, *nad*4 haplotypes never described before from Europe were found. Thus, this population must be assumed to derive from an independent introduction event of mosquitoes from overseas. The exact origin – USA or East Asia – could not be identified.

In 2013, two more populations of *Ae. j. japonicus* were detected in Europe: one in North Germany and the other in the Netherlands. The genetic makeup of individuals of these newly detected populations were analysed as described. Additionally, the genetic makeup of a broader range of individuals from Slovenia was examined, as well as that of individuals from Croatia and southern Germany. Results were compared with previous findings and showed a similar microsatellite signature and identical *nad*4 haplotypes, indicating that the North German population is a subpopulation of the West German one. The low population density and the comparably small distribution area of the North German population also suggest that

the split-off took place not long ago. By contrast, the Dutch population appears to originate from a further introduction event of mosquitoes from overseas.

In late summer 2015, the most recent German population of *Ae. j. japonicus* was detected in Upper Bavaria and adjacent Austria. Population genetics showed this population to be closely related to the formerly described Austrian/Slovenian population while being different from all other German populations suggesting it to be a split-off from the Austrian/Slovenian population.

The distribution and spread of *Ae. j. japonicus* in West and North Germany were observed from the time of detection in 2012/2013 until 2015. In this period, the West German population considerably increased its distribution area, while the North German population did not seem to expand at all. The reasons for this are seen in the North German population being younger than the West German one, the separation incident not dating back very far and the population still being in its founder phase.

Passive worldwide displacement of mosquitoes will probably increase in the future and establishment and spread of invasive species, including the Asian bush mosquito and other potential vectors of disease agents, will continue to pose challenging problems in Europe and Germany. Monitoring the dispersal of populations and performing population genetic studies to find out geographic origins as well as migration and transportation routes will help reconstructing and preventing further introduction and spread and are therefore essential tools of mosquito vector management.

4. ZUSAMMENFASSUNG

Die vorliegende Arbeit befasst sich mit der Verbreitung und Populationsgenetik der invasiven asiatischen Buschmücke *Ae. j. japonicus*, die sich seit 2008 durch Menschen-vermittelten Transport in Deutschland ausbreitet. *Aedes j. japonicus* ist unter Laborbedingungen Vektor für verschiedene Viren, unter anderem für das Dengue-Virus und das Chikungunya-Virus, und wurde im Feld mit dem Japanische Enzephalitis-Virus, dem West Nil-Virus und dem La Crosse-Virus infiziert gefunden.

2012 wurde aufgrund mehrerer unabhängiger Mücken-Einsendungen im Rahmen des Citizen-Science-Projekts "Mückenatlas" eine Population der Asiatischen Buschmücke in Westdeutschland entdeckt. Das Verbreitungsgebiet dieser Population befand sich weit nördlich der bisher angenommenen nördlichen Verbreitungsgrenze der Art in Süddeutschland. Das Ausmaß der Population wurde nach einem zeitlich begrenzten Monitoring auf eine Fläche von ca. 2000 km^2 bestimmt. Aus dieser Population wurden Individuen von fünf Orten populationsgenetischen Analysen unterzogen, um verwandtschaftliche Beziehungen innerhalb der Population und im Vergleich zu anderen europäischen Populationen aufzudecken. Hierzu wurden sieben Mikrosatelliten-Loci untersucht. Zusätzlich wurde ein Teil der mitochondrialen *nad*4-Genregion der Individuen auf Nukleotid-Polymorphismen untersucht. Die Ergebnisse wurden mit bereits zuvor erhobenen Daten von Populationen aus der Schweiz, aus Österreich/Slowenien und Belgien verglichen. Die Mikrosatellitensignatur der westdeutschen Population unterschied sich deutlich von der der anderen europäischen Populationen. Weiterhin wurden verschiedene *nad*4-Haplotypen gefunden, die zuvor nirgendwo sonst in Europa aufgetreten waren. Demnach ist zu vermuten, dass diese Population auf eine unabhängige Einschleppung von Individuen aus Übersee zurückgeht. Der genaue Ursprung – USA oder Ostasien – konnte nicht bestimmt werden.

2013 wurden zwei weitere *Ae. j. japonicus*-Populationen in Europa entdeckt: eine in Norddeutschland und eine weitere in den Niederlanden. Die genetischen Signaturen von Individuen dieser Populationen wurden wie beschrieben analysiert. Zusätzlich wurde das genetische Material einer größeren Menge von Individuen aus Slowenien sowie von Individuen aus Kroatien und Süddeutschland untersucht. Die Ergebnisse wurden mit denen aus der vorigen Studie verglichen und zeigten aufgrund einer ähnlichen Mikrosatellitensignatur und gleicher *nad*4-Haplotypen klar, dass die

norddeutsche Population eine Subpopulation der westdeutschen ist. Die geringe Populationsdichte und die vergleichsweise kleine Ausdehnung der norddeutschen Population deuten außerdem darauf hin, dass die Abspaltung nicht lange zurückliegt. Die niederländische Population scheint hingegen auf einer weiteren Einschleppung von Individuen aus Übersee zu basieren.

Im Spätsommer 2015 wurde die bisher letzte deutsche *Ae. j. japonicus*-Population in Oberbayern und dem angrenzenden Österreich entdeckt. Populationsgenetischen Analysen zufolge ist diese Population eng mit der früher beschriebenen österreichisch-slowenischen Population verwandt und unterscheidet sich von allen anderen deutschen Populationen, was darauf schließen lässt, dass es sich bei ihr um eine Abspaltung von der österreichisch-slowenischen Population handelt.

Die Ver- und Ausbreitung von *Ae. j. japonicus* in West- und Norddeutschland wurde vom Zeitpunkt der Entdeckung in 2012/2013 bis 2015 beobachtet. In dieser Periode erweiterte die westdeutsche Population ihr Verbreitungsgebiet beträchtlich, während die norddeutsche überhaupt nicht zu expandieren schien. Dies ist möglicherweise darauf zurückzuführen, dass die norddeutsche Population jünger als die westdeutsche ist, das Verschleppungsereignis noch nicht so weit zurückliegt und die Population sich noch in der Gründerphase befindet.

Die passive weltweite Verschleppung von Stechmücken wird in der Zukunft vermutlich zunehmen, und die Etablierung und Ausbreitung invasiver Spezies, inklusive der Asiatischen Buschmücke und anderer potenzieller Überträger von Krankheitserregern, werden Europa und Deutschland weiterhin vor herausfordernde Probleme stellen. Das Monitoring der Ausbreitung von Populationen und die Durchführung populationsgenetischer Analysen zur Ermittlung von geographischen Ursprüngen sowie von Wanderungs- und Transportrouten werden helfen, weitere Einschleppungs- und Ausbreitungsereignisse nachzuvollziehen und zu unterbinden und sind daher essenzielle Instrumente des Managements von Mückenvektoren.

5. REFERENCES

ALMEIDA APG, GONCALVES YM, NOVO MT, SOUSA CA, MELIM M, GRACIO AJS (2007). Vector monitoring of *Aedes aegypti* in the Autonomous Region of Madeira, Portugal. Eurosurveillance 12: pii=3311.

ANDREADIS TG, WOLFE RJ (2010). Evidence for reduction of native mosquitoes with increased expansion of invasive *Ochlerotatus japonicus japonicus* (Diptera: Culicidae) in the northeastern United States. Journal of Medical Entomology 47: 43-52.

ARMENGAUD A, LEGROS F, QUATRESOUS I, BARRE H, VALAYER P, FANTON Y, D'ORTENZIO E, SCHAFFNER F (2006). A case of autochthonous *Plasmodium vivax* malaria, Corsica, August 2006. Eurosurveillance 11: pii=3081.

BALDARI M, TAMBURRO A, SABATINELLI G, ROMI R, SEVERINI C, CUCCAGNA P, FIORILLI G, ALLEGRI MP, BURIANI C, TOTI M (1998). Malaria in Maremma, Italy. The Lancet 351: 1246-1247.

BECKER N (1997) Microbial control of mosquitoes: management of the Upper Rhine mosquito population as a model programme. Parasitology Today 13: 485-487.

BECKER N, PETRIĆ D, ZGOMBA M, BOASE C, MADON M, DAHL C, KAISER A (2010). Mosquitoes and their Control. 2nd Edition, 577 pp., Springer, Heidelberg, Germany.

BECKER N, JÖST H, ZIEGLER U, EIDEN M, HÖPER D, EMMERICH P, FICHET-CALVET E, EHICHIOYA DU, CZAJKA C, GABRIEL M, HOFFMANN B, BEER M, TENNER-RACZ K, RACZ P, GÜNTHER S, WINK M, BOSCH S, KONRAD A, PFEFFER M, GROSCHUP MH, SCHMIDT-CHANASIT J (2012). Epizootic emergence of Usutu virus in wild and captive birds in Germany. PloS One 7: e32604.

BEVINS SN (2007). Establishment and abundance of a recently introduced mosquito species *Ochlerotatus japonicus* (Diptera: Culicidae) in the Southern Appalachians, USA. Journal of Medical Entomology 44: 945-952.

BOSCH S, SCHMIDT-CHANASIT J, FIEDLER W (2012). Das Usutu-Virus als Ursache von Massensterben bei Amseln *Turdus merula* und anderen Vogelarten in Europa: Erfahrungen aus fünf Ausbrüchen zwischen 2001 und 2011. Vogelwarte 50: 109-122.

CALISTRI P, GIOVANNINI A, SAVINI G, MONACO F, BONFANTI L, CEOLIN C, TERREGINO C, TAMBA M, CORDIOLI P, LELLI R (2010). West Nile virus transmission in 2008 in north-eastern Italy. Zoonoses and Public Health 57: 211-219.

CAMERON EC, WILKERSON RC, MOGI M, MIYAGI I, TOMA T, KIM HC, FONSECA DM (2010). Molecular phylogenetics of *Aedes japonicus*, a disease vector that recently invaded Western Europe, North America and the Hawaiian islands. Journal of Medical Entomology 47: 527-535.

CASOLARI S, BRIGANTI E, ZANOTTI M, ZAULI T, NICOLETTI L, MAGURANO F, FORTUNA C, FIORENTINI C, GRAZIA CIUFOLINI M, REZZA G (2008). A fatal case of encephalitis associated with chikungunya virus infection. Scandinavian Journal of Infectious Diseases 40: 995-996.

CASTELLI F, CALIGARIS S, MATTEELLI A, CHIODERA A, CAROSI G, FAUSTI G (1993). 'Baggage malaria' in Italy: cryptic malaria explained? Transactions of the Royal Society of Tropical Medicine and Hygiene 87: 394.

CHAGIN KP, KONDRATIEV PI (1943). Vectors of autumnal (Japanese) encephalitis in Primor'ye region and mesures for controlling them. Meditsinskaia Parazitologiia i Parazitarnye Bolezni 12: 34-44 [In Russian].

CLEMENTS AN (2012). The Biology of Mosquitoes. Transmission of Viruses and Interaction with Bacteria, Volume 3. 572 pp., CABI, Cambridge, USA.

CONRATHS FJ, KÄMER D, TESKE K, HOFFMANN B, METTENLEITER TC, BEER M (2013). Reemerging Schmallenberg virus infections, Germany, 2012. Emerging Infectious Diseases 19: 513.

COX-SINGH J, DAVIS TM, LEE KS, SHAMSUL SS, MATUSOP A, RATNAM S, RAHMAN HA, CONWAY DJ, SINGH B (2008). *Plasmodium knowlesi* malaria in humans is widely distributed and potentially life threatening. Clinical Infectious Diseases 46: 165-171.

CUADROS J, CALVENTE MJ, BENITO A, AREVALO J, CALERO MA, SEGURA J, RUBIO JM (2002). *Plasmodium ovale* malaria acquired in central Spain. Emerging Infectious Diseases 8: 1506-1508.

CZAJKA C, BECKER N, POPPERT S, JÖST H, SCHMIDT-CHANASIT J, KRÜGER A (2012). Molecular detection of *Setaria tundra* (Nematoda: Filarioidea) and an unidentified filarial species in mosquitoes in Germany. Parasites & Vectors 5: 14.

CZAJKA C, BECKER N, JÖST H, POPPERT S, SCHMIDT-CHANASIT J, KRÜGER A, TANNICH E (2014). Stable transmission of *Dirofilaria repens* nematodes, northern Germany. Emerging Infectious Diseases 20: 329-331.

DAMIENS D, AYRINHAC A, VAN BORTEL W, VERSTEIRT V, DEKONINCK W, HANCE T (2014). Invasive process and repeated cross-sectional surveys of the mosquito *Aedes japonicus japonicus* establishment in Belgium. PloS One 9: e89358.

DANIS K, BAKA A, LENGLET A, VAN BORTEL W, TERZAKI I, TSERONI M, DETSIS M, PAPANIKOLAOU E, BALASKA A, GEWEHR S, DOUGAS G, SIDEROGLOU T, ECONOMOPOULOU A, VAKALIS N, TSIODRAS S, BONOVAS S, KREMASTINOU J (2011a). Autochthonous *Plasmodium vivax* malaria in Greece, 2011. Eurosurveillance 16: pii=19993.

DANIS K, PAPA A, THEOCHAROPOULOS G, DOUGAS G, ATHANASIOU M, DETSIS M, BAKA A, LYTRAS T, MELLOU K, BONOVAS S, PANAGIOTOPOULOS T (2011b). Outbreak of West Nile virus infection in Greece, 2010. Emerging Infectious Diseases 17: 1868-1872.

DELISLE E, ROUSSEAU C, BROCHE B, LEPARC-GOFFART I, L'AMBERT G, COCHET A, PRAT C, FOULONGNE V, FERRE JB, CATELINOIS O, FLUSIN O, TCHERNONOG E, MOUSSION IE, WIEGANDT A, SEPTFONS A, MENDY A, MOYANO MB, LAPORTE L, MAUREL J, JOURDAIN F, REYNES J, PATY MC, GOLLIOT F (2015). Chikungunya outbreak in Montpellier, France, September to October 2014. Eurosurveillance 20: pii=21108.

DERRAIK JG, STANLEY D, WEINSTEIN P, LESTER P, PURDIE G (2003). Presence of adult *Ochlerotatus* (*Finlaya*) *notoscriptus* (Skuse) and *Culex* (*Culex*) *pervigilans* Bergroth (Diptera: Culicidae) in tree canopy in Wellington, New Zealand. New Zealand Entomologist 26: 105-107.

DE ZULUETA J (1994). Malaria and ecosystems: from prehistory to posteradication. Parassitologia 36: 7-15.

DOBSON MJ (1994). Malaria in England: A geographical and historical perspective. Parassitologia 36: 35-60.

DOUDIER B, BOGREAU H, DEVRIES A, PONÇON N, STAUFFER WM, FONTENILLE D, ROGIER C, PAROLA P (2007). Possible autochthonous malaria from Marseille to Minneapolis. Emerging Infectious Diseases 13: 1236-1238.

EDMAN JD (2005). Journal policy on names of aedine mosquito genera and subgenera. Journal of Medical Entomology 42: 511.

EGIZI A, MORIN PJ, FONSECA DM (2014). Unraveling microbe-mediated interactions between mosquito larvae in a laboratory microcosm. Aquatic Ecology 48: 179-189.

EICHENLAUB D (1979). Malaria in Deutschland. Bundesgesundheitsblatt 22: 8-13.

FIELDEN MA, CHAULK AC, BASSETT K, WIERSMA YF, ERBLAND M, WHITNEY H, CHAPMAN TW (2015). *Aedes japonicus japonicus* (Diptera: Culicidae) arrives at the most easterly point in North America. The Canadian Entomologist 147: 737-740.

FONSECA DM, CAMPBELL S, CRANS WJ, MOGI M, MIYAGI I, TOMA T, BULLIANS M, ANDREADIS TG, BERRY RL, PAGAC B, SARDELIS MR, WILKERSON RC (2001). *Aedes* (*Finlaya*) *japonicus* (Diptera: Culicidae), a newly recognized mosquito in the United States: analyses of genetic variation in the United States and putative source populations. Journal of Medical Entomology 38: 135-146.

FONSECA DM, WIDDEL AK, HUTCHINSON M, SPICHIGER SE, KRAMER LD (2010). Fine-scale spatial and temporal population genetics of *Aedes japonicus*, a new US mosquito, reveal multiple introductions. Molecular Ecology 19: 1559-1572.

GETHMANN J, HOFFMANN B, PROBST C, BEER M, CONRATHS FJ, METTENLEITER TC (2010). Drei Jahre Blauzungenkrankheit Serotyp 8 in Deutschland – ein Überblick. Tierärztliche Umschau 65: 4-12.

GJENERO-MARGAN I, ALERAJ B, KRAJCAR D, LESNIKAR V, KLOBUČAR A, PEM-NOVOSEL I, KUREČIĆ-FILIPOVIĆ S, KOMPARAK S, MARTIĆ R, ĐURIČIĆ S, BETICA-RADIĆ L, OKMADŽIĆ J, VILIBIĆ-ČAVLEK T, BABIĆ-ERCEG A, TURKOVIĆ B, AVŠIĆ-ŽUPANC T, RADIĆ I, LJUBIĆ M, ŠARAC K, BENIĆ N, MLINARIĆ-GALINOVIĆ G (2011). Autochthonous dengue fever in Croatia, August–September 2010. Eurosurveillance 16: pii=19805.

GRANDADAM M, CARO V, PLUMET S, THIBERGE JM, SOUARÈS Y, FAILLOUX AB, TOLOU HJ, BUDELOT M, COSSERAT D, LEPARC-GOFFART I, DESPRÈS P (2011). Chikungunya virus, southeastern France. Emerging Infectious Diseases 17: 910.

GRATZ NG (1999). Emerging and resurging vector-borne diseases. Annual Review of Entomology 44: 51-75.

GUBLER DJ (2012). The economic burden of dengue. The American Journal of Tropical Medicine and Hygiene 86: 743-744.

HARRIS MC, DOTSETH EJ, JACKSON BT, ZINK SD, MAREK PE, KRAMER LD, PAULSON SL, HAWLEY DM (2015). La Crosse Virus in *Aedes japonicus japonicus* mosquitoes in the Appalachian Region, United States. Emerging Infectious Diseases 21: 646.

HAWLEY WA (1988). Biology of *Aedes albopictus.* Journal of the American Mosquito Control Association, Supplement 1: 2-39.

HAYES CG (2001). West Nile virus: Uganda, 1937, to New York City, 1999. Annals of the New York Academy of Sciences 951: 25-37.

HUBÁLEK Z, HALOUZKA J (1999). West Nile fever − a reemerging mosquito-borne viral disease in Europe. Emerging Infectious Diseases 5: 643-650.

IBÁÑEZ-JUSTICIA A, KAMPEN H, BRAKS M, SCHAFFNER F, STEEGHS M, WERNER D, ZIELKE D, DEN HARTOG W, BROOKS M, DIK M, VAN DE VOSSENBERG B, SCHOLTE E-J (2014). First report of *Aedes japonicus japonicus* (Theobald, 1901) (Diptera, Culicidae) population in The Netherlands. Journal of the European Mosquito Control Association 31: 9-13.

JÖST H, BIALONSKI A, STORCH V, GÜNTHER S, BECKER N, SCHMIDT-CHANASIT J (2010). Isolation and phylogenetic analysis of Sindbis viruses from mosquitoes in Germany. Journal of Clinical Microbiology 48: 1900-1903.

JÖST H, BIALONSKI A, SCHMETZ C, GÜNTHER S, BECKER N, SCHMIDT-CHANASIT J (2011). Short report: Isolation and phylogenetic analysis of Batai virus, Germany. The American Journal of Tropical Medicine and Hygiene 84: 241-243.

KALAN K, BUZAN VE, IVOVIĆ V (2014). Distribution of two invasive mosquito species in Slovenia. Parasites & Vectors 7 Supplement 1: 9.

KAMPEN H, SCHAFFNER F (2008). Culicidae. In: BONNEFOY X, KAMPEN H, SWEENEY K (eds), Public Health Significance of Urban Pests. World Health Organisation, Copenhagen: 347-386.

KAMPEN H, WERNER D (2015). Die wiederkehrende Notwendigkeit von Stechmücken-Surveillance und -Forschung. Bundesgesundheitsblatt Gesundheitsforschung Gesundheitsschutz 58: 1101-1109.

KAMPEN H, WERNER D (2014). Out of the bush: the Asian bush mosquito *Aedes japonicus japonicus* (Theobald, 1901) (Diptera, Culicidae) becomes invasive. Parasites & Vectors 7: 59.

KAMPEN H, ZIELKE DE, WERNER D (2012). A new focus of *Aedes japonicus japonicus* (Theobald, 1901) (Diptera, Culicidae) distribution in western Germany: rapid spread or a further introduction event? Parasites & Vectors 5: 284-290.

KREBS T, BINDLER P, L'AMBERT G, TOTY C, PERRIN Y, JOURDAIN F (2014). First establishment of *Aedes japonicus japonicus* (Theobald, 1901) (Diptera: Culicidae) in France in 2013 and its impact on public health. Journal of Vector Ecology 39: 437-440.

KRONEFELD M, KAMPEN H, SASSNAU R, WERNER D (2014). Molecular detection of *Dirofilaria immitis*, *Dirofilaria repens* and *Setaria tundra* in mosquitoes from Germany. Parasites & Vectors 7: 30.

KRÜGER A, RECH A, SU XZ, TANNICH E (2001). Two cases of autochthonous *Plasmodium falciparum* malaria in Germany with evidence for local transmission by indigenous *Anopheles plumbeus*. Tropical Medicine and International Health 6: 983-985.

KVIST L, MARTENS J, NAZARENKO AA, ORELL M (2003). Paternal leakage of mitochondrial DNA in the great tit (*Parus major*). Molecular Biology and Evolution 20: 243-247.

LAAKSONEN S, KUUSELA J, NIKANDER S, NYLUND M, OKSANEN A (2007). Outbreak of parasitic peritonitis in reindeer in Finland. The Veterinary Record 160: 835-841.

LAIRD M, CALDER L, THORNTON RC, SYME R, HOLDER PW, MOGI M (1994). Japanese *Aedes albopictus* among four mosquito species reaching New Zealand in used tires. Journal of the American Mosquito Control Association 10: 14-23.

LARISH LB, SAVAGE HM (2005). Introduction and establishment of *Aedes* (*Finlaya*) *japonicus japonicus* (Theobald) on the island of Hawaii: implications for arbovirus transmission. Journal of the American Mosquito Control Association 21: 318-321.

LA RUCHE G, SOUARÈS Y, ARMENGAUD A, PELOUX-PETIOT F, DELAUNAY P, DESPRÈS P, LENGLET A, JOURDAIN F, LEPARC-GOFFART I, CHARLET F, OLLIER L, MANTEY K, MOLLET T, FOURNIER JP, TORRENTS R, LEITMEYER K, HILAIRET P, ZELLER H, VAN BORTEL W, DEJOUR-SALAMANCA D, GRANDADAM M, GASTELLU-ETCHEGORRY M (2010). First two autochthonous dengue virus infections in metropolitan France, September 2010. Eurosurveillance 15: pii=19676.

LOUNIBOS LP (2007). Competitive displacement and reduction. Journal of the American Mosquito Control Association 23: 276-282.

LUNDSTRÖM JO (1999). Mosquito-borne viruses in western Europe: a review. Journal of Vector Ecology 24: 1-39.

MAGNACCA KN, BROWN MJ (2010). Mitochondrial heteroplasmy and DNA barcoding in Hawaiian *Hylaeus* (*Nesoprosopis*) bees (Hymenoptera: Colletidae). BMC Evolutionary Biology 10: 174.

MARCHAND E, PRAT C, JEANNIN C, LAFONT E, BERGMANN T, FLUSIN O, RIZZI J, ROUX N, BUSSO V, DENIAU J, NOEL H, VAILLANT V, LEPARC-GOFFART I, SIX C, PATY MC

(2013). Autochthonous case of dengue in France, October 2013. Eurosurveillance 18: pii=20661.

McCALL JW, GENCHI C, KRAMER LH, GUERRERO J, VENCO L (2008). Heartworm disease in animals and humans. Advances in Parasitology 66: 193-285.

MEDLOCK JM, HANSFORD KM, SCHAFFNER F, VERSTEIRT V, HENDRICKX G, ZELLER H, BORTEL WV (2012). A review of the invasive mosquitoes in Europe: ecology, public health risks and control options. Vector Borne Zoonotic Diseases 12: 435-447.

MEHLHORN H, WALLDORF V, KLIMPEL S, JAHN B, JAEGER F, ESCHWEILER J, HOFFMANN B, BEER M (2007). First occurrence of *Culicoides obsoletus*-transmitted bluetongue virus epidemic in Central Europe. Parasitology Research 101: 219-228.

MIYAGI I (1971). Notes on the *Aedes* (*Finlaya*) *chrysolineatus* subgroup in Japan and Korea (Diptera: Culicidae). Tropical Medicine 13: 141-151.

MOLAEI G, FARAJOLLAHI A, SCOTT JJ, GAUGLER R, ANDREADIS TG (2009). Human bloodfeeding by the recently introduced mosquito, *Aedes japonicus japonicus*, and public health implications. Journal of the American Mosquito Control Association 25: 210-214.

MUNSTERMANN LE, ANDREADIS TG (1999). *Aedes japonicus* in Connecticut. Vector Ecology Newsletter 30: 7-8.

MURGUE B, MURRI S, ZIENTARA S, DURAND B, DURAND JP, ZELLER H (2001). West Nile outbreak in southern France, 2000: the return after 35 years. Emerging Infectious Diseases 7: 692-696.

MYERS JH, SAVOIE A, VAN RANDEN E (1998). Eradication and pest management. Annual Review of Entomology 43: 471-491.

PAUPY C, DELATTE H, BAGNY L, CORBEL V, FONTENILLE D (2009). *Aedes albopictus*, an arbovirus vector: from the darkness to the light. Microbes and Infection 11: 1177-1185.

PEYTON EL, CAMPBELL SR, CANDELETTI TM, ROMANOWSKI M, CRANS WJ (1999). *Aedes* (*Finlaya*) *japonicus japonicus* (Theobald), a new introduction into the United States. Journal of the American Mosquito Control Association 15: 238-241.

PILASKI J, MACKENSTEIN H (1985). Nachweis des Tahyna-Virus in zwei verschiedenen europäischen Naturherden. Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene, I. Abteilung, Originale B 180: 394-420.

POPPERT S, HODAPP M, KRUEGER A, HEGASY G, NIESEN WD, KERN WV, TANNICH E (2009). *Dirofilaria repens* infection and concomitant meningoencephalitis. Emerging Infectious Diseases 15: 1844-1846.

PRAETORIUS F, ALTROCK G, BLEES N, SCHUH N, FAULDE M (1999). Importierte *Anopheles*: im Gepäck oder aus dem Flugzeug? Ein Fall von schwerer autochthoner Malaria tropica in Flughafennähe. Deutsche Medizinische Wochenschrift 124: 998-1002.

REINERT JF (2000). New classification for the composite genus *Aedes* (Diptera: Culicidae: Aedini), elevation of subgenus *Ochlerotatus* to generic rank, reclassification of the other subgenera, and notes on certain subgenera and species. Journal of the American Mosquito Control Association 16: 175-188.

REINERT JF, HARBACH RE, KITCHING IJ (2006). Phylogeny and classification of *Finlaya* and allied taxa (Diptera: Culicidae: Aedini) based on morphological data from all life stages. Zoological Journal of the Linnean Society 148: 1-101.

REZZA G, NICOLETTI L, ANGELINI R, ROMI R, FINARELLI AC, PANNING M, CORDIOLI P, FORTUNA C, BOROS S, MAGURANO F, SILVI G, ANGELINI P, DOTTORI M, CIUFOLINI MG, MAJORI GC, CASSONE A (2007). Infection with chikungunya virus in Italy: an outbreak in a temperate region. The Lancet 370: 1840-1846.

Rocconi F, Di Tommaso M, Traversa D, Palmieri C, Pampurini F, Boari A (2012). Allergic dermatitis by *Dirofilaria repens* in a dog: clinical picture and treatment. Parasitology Research 111: 493-496.

Rochlin I, Gaugler R, Williges E, Farajollahi A (2013). The rise of the invasives and decline of the natives: insights revealed from adult populations of container-inhabiting *Aedes* mosquitoes (Diptera: Culicidae) in temperate North America. Biological Invasions 15: 991-1003.

Roehrig JT (2013). West Nile virus in the United States − a historical perspective. Viruses 5: 3088-3108.

Saegerman C, Berkvens D, Mellor PS (2008). Bluetongue epidemiology in the European Union. Emerging Infectious Diseases 14: 539.

Santa-Olalla Peralta P, Vazquez-Torres MC, Latorre-Fandós E, Mairal-Claver P, Cortina-Solano P, Puy-Azón A, Adiego Sancho B, Leitmeyer K, Lucientes-Curdi J, Sierra-Moros MJ (2010). First autochthonous malaria case due to *Plasmodium vivax* since eradication, Spain, October 2010. Eurosurveillance 15: pii=19684.

Santini M, Vilibic-Cavlek T, Barsic B, Barbic L, Savic V, Stevanovic V, Listes E, di Gennaro A, Savini G (2015). First cases of human Usutu virus neuroinvasive infection in Croatia, August-September 2013: clinical and laboratory features. Journal of Neurovirology 121: 92-97.

Sardelis MR, Dohm DJ, Pagac B, Andre RG, Turell MJ (2002a). Experimental transmission of eastern equine encephalitis virus by *Ochlerotatus j. japonicus* (Diptera: Culicidae). Journal of Medical Entomology 39: 480-484.

Sardelis MR, Turell MJ, Andre RG (2002b). Laboratory transmission of La Crosse virus by *Ochlerotatus j. japonicus* (Diptera: Culicidae). Journal of Medical Entomology 39: 635-639.

SARDELIS MR, TURELL MJ, ANDRE RG (2003). Experimental transmission of St. Louis encephalitis virus by *Ochlerotatus j. japonicus*. Journal of the American Mosquito Control Association 19: 159-162.

SARTORI M, LA TERRA G, POZZOLI G, FRANZINI C (1989). A case of autochthonous malaria in Italy. Scandinavian Journal of Infectious Diseases 21: 357-358.

SCHAFFNER F, CHOUIN S, GUILLOTEAU J (2003). First record of *Ochlerotatus* (*Finlaya*) *japonicus japonicus* (Theobald, 1901) in metropolitan France. Journal of the American Mosquito Control Association 19: 1-5.

SCHAFFNER F, KAUFMANN C, HEGGLIN D, MATHIS A (2009). The invasive mosquito *Aedes japonicus* in Central Europe. Medical and Veterinary Entomology 23: 448-451.

SCHAFFNER F, VAZEILLE M, KAUFMANN C, FAILLOUX AB, MATHIS A (2011). Vector competence of *Aedes japonicus* for chikungunya and dengue viruses. European Mosquito Bulletin 29: 141-142.

SCHAFFNER F, MEDLOCK JM, VAN BORTEL W (2013). Public health significance of invasive mosquitoes in Europe. Clinical Microbiology and Infection 19: 685-692.

SCHAFFNER F, FONTENILLE D, MATHIS A (2014). Autochthonous dengue emphasizes the threat of arbovirosis in Europe. The Lancet Infectious Diseases 14: 1044.

SCHAFFNER F, MATHIS A (2014). Dengue and dengue vectors in the WHO European region: past, present, and scenarios for the future. The Lancet Infectious Diseases 14: 1271-80.

SCOTT JJ (2003). The ecology of the exotic mosquito *Ochlerotatus* (*Finlaya*) *japonicus japonicus* (Theobald 1901) (Diptera: Culicidae) and an examination

of its role in the West Nile virus cycle in New Jersey. Ph.D. thesis, Rutgers University, New Brunswick, NJ, USA.

SEIDEL B, DUH D, NOVOTNY N, ALLERBERGER F (2012). Erstnachweis der Stechmücken *Aedes* (*Ochlerotatus*) *japonicus* (Theobald, 1901) in Österreich und Slowenien in 2001 und für *Aedes* (*Stegomyia*) *albopictus* (Skuse, 1895) in Österreich 2012 (Diptera: Culicidae). Entomologische Zeitschrift 122: 223-226.

SERVICE MW (2001). The Encyclopedia of Arthropod-transmitted Infections of Man and Domesticated Animals. 1st Edition, 608 pp., CABI, London, UK.

SIGNORELLI C, MESSINEO A (1990). Airport malaria. The Lancet 335: 164.

SIMÓN F, SILES-LUCAS M, MORCHÓN R, GONZÁLEZ-MIGUEL J, MELLADO I, CARRETÓN E, MONTOYA-ALONSO JA (2012). Human and animal dirofilariasis: the emergence of a zoonotic mosaic. Clinical Microbiology Reviews 25: 507-544.

SOUSA CA, CLAIROUIN M, SEIXAS G, VIVEIROS B, NOVO MT, SILVA AC, ESCOVAL MT, ECONOMOPOULOU A (2012). Ongoing outbreak of dengue type 1 in the Autonomous Region of Madeira, Portugal: preliminary report. Eurosurveillance 17: pii=20333.

SUCHARIT S, SURATHIN K, SHRESTHA SR (1989). Vectors of Japanese encephalitis virus (JEV): species complexes of the vectors. Southeast Asian Journal of Tropical Medicine and Public Health 20: 611-621.

TAKASHIMA I, HASHIMOTO N (1985). Getah virus in several species of mosquitoes. Transactions of the Royal Society of Tropical Medicine and Hygiene 79: 546-550.

TAKASHIMA I, ROSEN L (1989). Horizontal and vertical transmission of Japanese encephalitis virus by *Aedes japonicus* (Diptera: Culicidae). Journal of Medical Entomology 26: 454-458.

Tanaka K, Mizusawa K, Saugstad S (1979). A revision of the adult and larval mosquitoes of Japan (including the Ryukyu Archipelago and the Ogasawara Islands) and Korea (Diptera: Culicidae). 987 pp., U.S. Army Medical Laboratory, Department of Entomology, Pacific: Kamitsuruma 4054, APO, AP, 96343.

Tappe D, Plauth M, Bauer T, Muntau B, Dießel L, Tannich E, Herrmann-Trost P (2014). A case of autochthonous human *Dirofilaria* infection, Germany, March 2014. Eurosurveillance 19: pii=20790.

Thielman A, Hunter FF (2006). Establishment of *Ochlerotatus japonicus* (Diptera: Culicidae) in Ontario, Canada. Journal of Medical Entomology 43: 138-142.

Tsai TF, Popovici F, Cernescu C, Campbell GL, Nedelcu NI (1998). West Nile encephalitis epidemic in southeastern Romania. The Lancet 352: 767-771.

Turell MJ, Dohm DJ, Sardelis MR, O'guinn ML, Andreadis TG, Blow JA (2005). An update on the potential of North American mosquitoes (Diptera: Culicidae) to transmit West Nile virus. Journal of Medical Entomology 42: 57-62.

Turell MJ, Dohm DJ, Mores CN, Terracina L, Wallette Jr DL, Hribar LJ, Pecor JE, Blow JA (2008). Potential for North American mosquitoes to transmit Rift Valley fever virus. Journal of the American Mosquito Control Association 24: 502-507.

Versteirt V, Schaffner F, Garros C, Dekoninck W, Coosemans M, Van Bortel W (2009). Introduction and establishment of the exotic mosquito species *Aedes japonicus japonicus* (Diptera: Culicidae) in Belgium. Journal of Medical Entomology 46: 1464-1467.

Warrell DA, Gilles HM (2002). Essential Malariology. 4th Edition, 348 pp., Arnold Publishers, London, UK.

WEISSENBÖCK H, HUBÁLEK Z, BAKONYI T, NOWOTNY N (2010). Zoonotic mosquito-borne flaviviruses: worldwide presence of agents with proven pathogenicity and potential candidates of future emerging diseases. Veterinary Microbiology 140: 271-280.

WERNER D, KAMPEN H (2013). The further spread of *Aedes japonicus japonicus* (Diptera, Culicidae) towards northern Germany. Parasitology Research 112: 3665-3668.

WERNER D, KAMPEN H (2015). *Aedes albopictus* breeding in southern Germany, 2014. Parasitology Research 114: 831-4.

WESTBY K, FRITZEN C, HUANG J, JASKE E, PAULSEN D, JONES C, MONCAYO AC (2011). La Crosse encephalitis in Eastern Tennessee: evidence of invasive mosquito (*Aedes albopictus* and *Ochlerotatus japonicus*) involvement in the transmission of an indigenous disease. The American Journal of Tropical Medicine and Hygiene 85: 374.

WHO (2009). Dengue: Guidelines for Diagnosis, Treatment, Prevention and Control. 147 pp., World Health Organization, Geneva, Switzerland.

WHO (2012). Global Strategy for Dengue Prevention and Control 2012 – 2020. 35 pp., World Health Organization Geneva, Switzerland.

WHO (2014). World Malaria Report 2014. 227 pp., World Health Organization, Geneva, Switzerland.

WIDDEL AK, MCCUISTON LJ, CRANS WJ, KRAMER LD, FONSECA DM (2005). Finding needles in the haystack: single copy microsatellite loci for *Aedes japonicus* (Diptera: Culicidae). The American Journal of Tropical Medicine and Hygiene 73: 744-748.

YUNICHEVA YU, RYABOVA TE, MARKOVICH NY, BEZZHONOVA OV, GANUSHKINA LA, SEMENOV VB, TARKHOV GA, VASILENKO LE, GUZEEVA TM, SHEVEREVA TV,

Sᴇʀɢɪᴇᴠ VP (2008). First evidence for breeding *Aedes aegypti* L. mosquito in the area of Greater Sochi and in some towns of Abkhazia. Meditsinskaia Parazitologiia i Parazitarnye Bolezni Jul-Sep: 40-43.

Zᴇʟʟᴇʀ H, Mᴀʀʀᴀᴍᴀ L, Sᴜᴅʀᴇ B, Bᴏʀᴛᴇʟ W, Wᴀʀɴs-Pᴇᴛɪᴛ E (2013). Mosquito-borne disease surveillance by the European Centre for Disease Prevention and Control. Clinical Microbiology and Infection 19: 693-698.

Zɪᴇʟᴋᴇ DE, Iʙáñᴇᴢ-Jᴜsᴛɪᴄɪᴀ A, Kᴀʟᴀɴ K, Mᴇʀᴅɪć E, Kᴀᴍᴘᴇɴ H, Wᴇʀɴᴇʀ D (2015). Recently discovered *Aedes japonicus japonicus* (Diptera: Culicidae) populations in The Netherlands and northern Germany resulted from a new introduction event and from a split from an existing population. Parasites & Vectors 8: 40.

Zɪᴇʟᴋᴇ DE, Wᴇʀɴᴇʀ D, Kᴀᴍᴘᴇɴ H (2016). Newly discovered population of *Aedes japonicus japonicus* (Diptera: Culicidae) in Upper Bavaria, Germany, and Salzburg, Austria, is closely related to the Austrian/Slovenian bush mosquito population. Parasites & Vectors 9: 163.

Zᴏʟʟᴇʀ T, Nᴀᴜᴄᴋᴇ TJ, Mᴀʏ J, Hᴏꜰꜰᴍᴇɪsᴛᴇʀ B, Fʟɪᴄᴋ H, Wɪʟʟɪᴀᴍs CJ, Fʀᴀɴᴋ C, Bᴇʀɢᴍᴀɴɴ F, Sᴜᴛᴛᴏʀᴘ N, Mᴏᴄᴋᴇɴʜᴀᴜᴘᴛ FP (2009). Malaria transmission in non-endemic areas: case report, review of the literature and implications for public health management. Malaria Journal 8: 71.