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Genomic and functional study on the Tiger Mosquito, *Aedes albopictus*, in Italy

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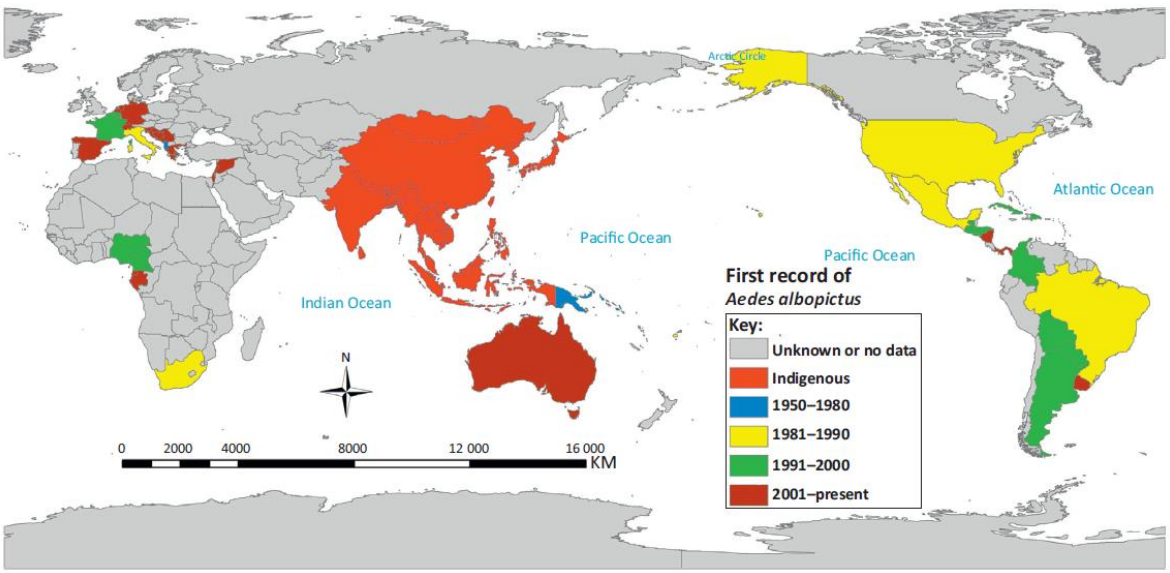
1. Introduction

1.1. *Aedes albopictus*: A Global Invader

Aedes (Stegomyia) albopictus, part of the family Culicidae, subfamiliy Culicinae, is a mosquito species commonly known as the Asian tiger mosquito or Forest day mosquito and was first described by Skuse (Skuse 1894) as a species, characterized by a black and white stripy pattern on the legs and other parts of the body, native to tropical and subtropical regions of South-East-Asia. During the last few decades this mosquito species has managed to spread all over the world (Figure 1.1) getting ranked among the 100 worst invasive species (ISSG) and being considered the most invasive mosquito species, as well as an increasing threat to public health. Similar to what already observed for the closely related species *Aedes aegypti* (Goubert et al. 2016; Powell and Tabachnick 2013; Hawley 1988), *Ae. albopictus* seems to have undergone a process of domestication with a progressive adaptation to urban and sub-urban habitats, which offered alternative blood sources and anthropogenic larval breeding sites. As already observed for *Culex pipiens* and *Ae. aegypti* (Lounibos 2002), this adaptation to humans was a fundamental factor allowing the rapid spread of *Ae. albopictus*.

Figure 1.1. *Aedes albopictus* distribution range, taken from Bonizzoni et al 2013. The map shows the first record of *Ae. albopictus* by country as reported in published literature.



Biology and Invasion success:

Reasons for the success of *Ae. albopictus* can be sought in its ability to adapt easily to a very large range of habitats thanks to its ecological and physiological flexibility.

General life history. Aedes albopictus females lay drought-resistant eggs singly in soil or above the water level; subsequently repeated inundations favor and stimulate egg-hatching. At optimal conditions larval development will be completed in 5-10 days with the formation of pupae and the subsequent hatching of adult mosquitoes which live 4 to 8 weeks (Hawley 1988). The species is multivoltine, resulting in 5-17 generations per year depending on rainfall and temperature. Mean winter temperatures of > 0°C are necessary for egg overwintering while a mean annual temperature of >11°C, as well as > 500mm of annual rainfall are necessary for adult survival and activity (Medlock et al. 2015; Straetemans 2008). Mean summer temperatures of 25-30°C are ideal for mosquito development, but the species has shown high climate adaptability (see below) and has managed to establish also in areas with lower mean temperatures and annual rainfall (Severini et al. 2008; Benedict et al. 2007).

Breeding sites and feeding habits. Historically, the species occurred in rural and forest habitats of Southeast Asia, with tree holes or bamboo stumps as typical larval habitat and a prevalently zoophilic biting behavior. Subsequently, *Ae. albopictus* has adapted extremely well to urban and suburban environments by switching from natural breeding sites (i.e. tree holes, bamboo stumps, bromeliads) to artificial, human-made containers (i.e. tires, flower pots, cemetery urns etc.). The species is not known to breed in brackish or salt water (Buhagiar 2009).

The tiger mosquito is known for being a very aggressive and opportunistic daytime biter (Hawley 1988), which prefers to feed and rest outdoors (exophilic and exophagic), although gravid females have been found indoors in Rome, Italy (Valerio et al. 2009). The species bites preferentially mammals, however, the females can feed upon most groups of vertebrates including reptiles, birds and amphibians (Kamgang et al. 2012; Helene Delatte et al. 2010) and host choice depends highly on host abundance and availability. Therefore, in urban areas where both humans and *Ae. albopictus* can reach very high densities (Toma et al. 2003), this species feeds almost exclusively on humans (Valerio et al. 2009). Obviously, such plastic feeding habits

are one of the reasons for the invasiveness of *Ae. albopictus*, allowing the species to extend its range and to occupy easily new habitats.

Adaptation to cold climate. Differently from *Ae. aegypti*, *Ae. albopictus* has been able to adapt also to temperate climates thanks to the production of photoperiodic diapausing eggs which are cold-hardy and desiccation resistant (Delatte et al. 2009; Kobayashi, Nihei, and Kurihara 2002). While populations in tropical regions with mean temperatures above 10°C are active throughout the year with no overwintering stage (Mitchell 1995), in temperate regions females exposed to shortening daylight hours in autumn as well as lower mean temperatures (as in winter time), are able to produce eggs in which the larvae enter dormancy and can survive also cold spells of -10°C (Urbanski et al. 2012; Nawrocki and Hawley 1987). The critical photoperiod threshold varies between sites according to latitudinal variations in the length of favorable growing season. Generally, production of diapausing eggs occurs below 13–14 hours of daylight, while the return of permissive climatic (mean temperatures around 10 to 11°C) and photoperiodic conditions (>11 hours of daylight per day), favors the resumption of development (Toma et al. 2003; Hanson and Craig 1994). Furthermore, some invasive populations, such as the one from Rome, Italy, have shown to be capable to overwinter also in the adult stage (Romi, Severini, & Toma, 2006), supporting that a fast cold-acclimation and, more general, climate adaptation has been a crucial element for the successful spread of the species (Urbanski et al. 2012).
Moreover, the desiccation resistant eggs appeared to be perfect for long-distance transport and in fact the passive dispersal of diapausing eggs has allowed the species to spread in few decades all across the world via the main transport routes, especially via the commerce of used tires and lucky bamboo (Scholte and Schaffner 2007; Juliano and Luonibos 2005).

Interactions with resident species. Once *Ae. albopictus* has been introduced in a new area its establishment depends not only on climatic and environmental factors but also on the interactions with existing species which can lead to the exclusion of native or invasive species or to a stable coexistence. Most studies on competitive interaction of *Ae. albopictus* involved its interaction with the main dengue vector *Ae. aegypti* due to its public health relevance. These two species compete primarily at the larval stage, and it has been hypothesized that the reduction of the local *Ae. aegypti* population observed in Brazil, as well as the range restriction of *Ae. aegypti* observed in La Rèunion since the introduction of *Ae. albopictus*, was due to

superiority of *Ae. albopictus* in larval competition (Bagny et al. 2009; Braks et al. 2004). Extensive studies in laboratory and in field conditions have been performed to evaluate outcomes of these interaction showing that interspecific competition varies strongly with eco-climatic conditions, as well as the time since invasion (Guo et al. 2016; Lounibos et al. 2016; Alto, Bettinardi, and Ortiz 2015).

In Italy, the species has colonized urban and suburban niches partially overlapping with the ones exploited by the indigenous *Cx. pipiens*; studies on survival and growth parameters carried out on the two species suggested a greater capacity to exploit food resources and thus a more rapid growth of *Ae. albopictus* compared to *Cx. pipiens* (Carrieri et al. 2003).

Global expansion

Thanks to human migrations and, later on, intercontinental trade *Ae. albopictus* colonized first several Islands in the southern Pacific and in the Indian ocean and started then spreading during the 20th century across the whole world with Antarctica being at the moment the only not colonized continent. This long distance spread of *Ae. albopictus* is mainly explained by the importation of egg- infested used tires (Hawley 1988; Reiter and Sprenger 1987) as well as the shipping of ornamental plants, mainly Lucky Bamboo (Dracaena spp.), packaged in standing water (Scholte et al. 2007; Madon et al. 2002;). Also, the transport of trucks and cars by sea from *Ae. albopictus*-infested areas is supposed to be a possible, but less important way of introduction (Medley, Jenkins, and Hoffman 2015; Scholte and Schaffner 2007). On a smaller, local scale, possible ways of dispersal are, again, the transport of used tires, but also the passive transportation of eggs or adult mosquitoes by private transport and/or trucks (Medley, Jenkins, and Hoffman 2015), while natural dispersal of *Ae. albopictus* seems to be very limited (Marini et al. 2010).

In North-America, after some sporadic detections of larvae and pupae in used tires coming from South-East-Asia in 1946 and again in 1972 (Madon et al. 2002), the first detection of a substantial population of *Ae. albopictus* dates back to 1985 in Texas, USA (Sprenger and Wuithiranyagool 1986). Currently the species is reported in more than 30 states, mainly at the east-coast (Morens and Fauci 2014).

First reports of the tiger mosquitoes in Central- and South-America were made in the ‘80s and ‘90s in Brazil (Forattini 1986) and Mexico (Ibanez-Bernal and Martinez-Campos 1994), maybe reflecting an invasion process starting from Texas and moving southwards. Since then the

species has spread across whole central- and most south-American countries (Scholte and Schaffner 2007).

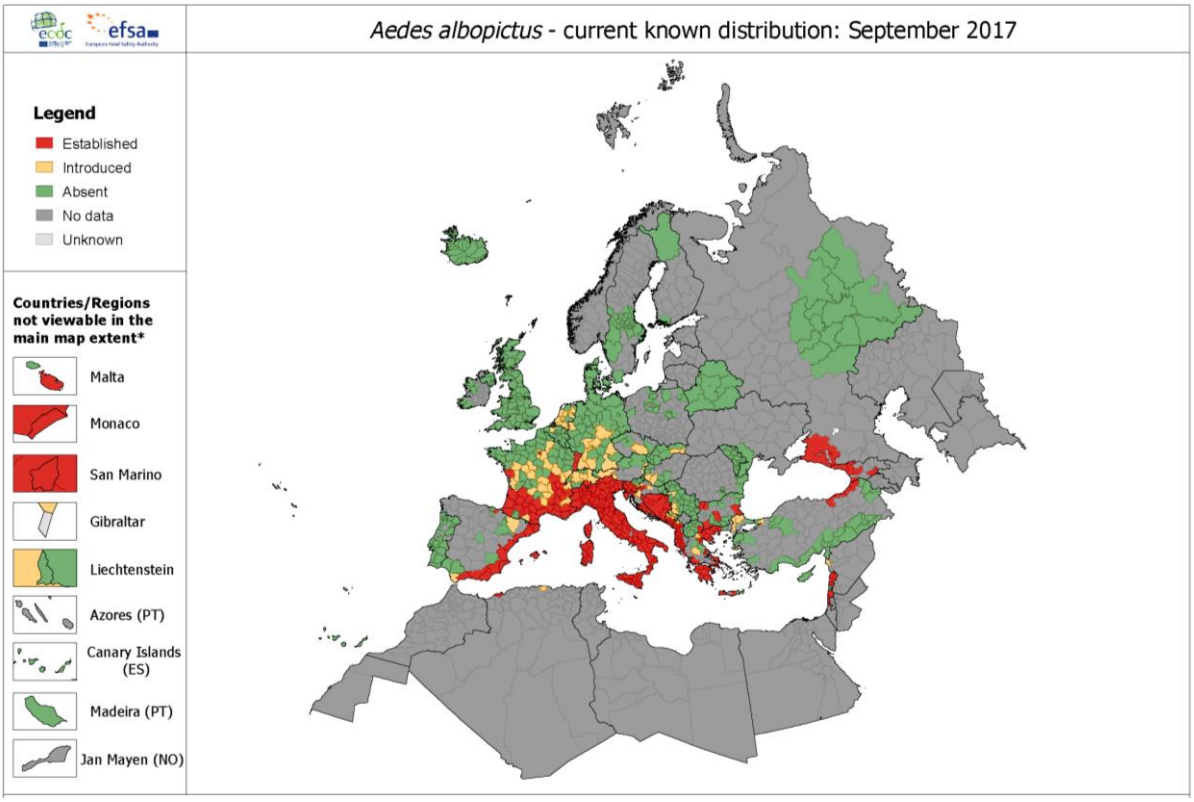
In Africa, the species was first reported in 1989 in car-tire import inspections in South Africa (Cornel and Hunt 1991), where the infestation was initially controlled. More recently the species was found in Nigeria from where it appears to have spread to Camerun (Simard et al. 2005), Equatorial Guinea (Toto et al. 2003) and Gabon (Krueger and Hagen 2007) where the species is now established.

In New Zealand and Australia, the species has been captured several times, mainly close to ports (Laird et al. 1994), but so far it has established only on some of the Torres Islands north of Queensland (Ritchie et al. 2006).

In Europe, *Ae. albopictus* was first reported in Albania in 1979 (Adhami and Reiter 1998), in Genoa (north-west Italy) in 1990 (Sabatini, Raineri, Trovato, & Coluzzi, 1990) and in Padua (north-east Italy) in 1991 (Dalla Pozza and Majori 1992). In the following years, it spread across whole Italy and has now established in most areas <600m above sea level reaching highest densities in urban areas (Valerio et al. 2009; ECDC 2009). The most abundant populations are found in the regions of Veneto and Friuli-Venezia-Giulia, large parts of Lombardia and Emilia-Romagna as well as the coastal areas of central Italy (ECDC 2009; Scholte and Schaffner 2007). Following the invasion of Italy, *Ae. albopictus* spread in all European countries around the Mediterranean basin (Scholte and Schaffner 2007; Figure 1.2) and established populations have been found also in Bulgaria, Russia, Romania as well as Switzerland where the species has probably arrived thanks to recurrent introductions from the bordering Italian regions (Medlock et al. 2015; Wymann et al. 2008). Occasional reports of *Ae. albopictus* specimens have come also from other European countries such as Austria, Czech Republic, Germany, and Slovakia, with the Northern-most reports of *Ae. albopictus* in Europe coming from the Netherlands (Medlock et al. 2015; Scholte et al. 2007), but until now these populations seem not to have established outside greenhouses.

Studies modelling the risk of future expansion, considering also global warming projections and increasing urbanization, suggest that the tiger mosquito will expand further and that also northern Europe will become more suitable for establishment due to wet and warmer conditions, while hotter and drier summers in Southern Europe could there slightly decrease the risk of stable colonization (Caminade et al. 2012; ECDC 2009)

Figure 1.2. Current known distribution of *Aedes albopictus* in Europe updated at September 2017. Data were obtained through the VectorNet project for ECDC (https://ecdc.europa.eu)



1.2. Vector status and public health concern

Aedes albopictus is not only a significant biting nuisance but it has also been demonstrated to be a field vector of Chikungunya (CHKV), Dengue (DENV) and Zika virus (ZIKV), and several laboratory studies have demonstrated its ability to serve as a vector for more than 20 arboviruses (Gratz 2004; Grard et al. 2014). Moreover, due to its opportunistic feeding behavior *Ae. albopictus* has the potential to act as a ‘‘bridge vector’’ of zoonotic pathogens (e.g. canine dirofilariosis by *Dirofilaria* spp.) to humans (Faraji et al. 2014; Paupy et al. 2009; Benedict et al. 2007).

Despite this, the public health impact of the Asian tiger mosquito was often minimized, being considered a far less efficient vector compared to the more anthropophilic *Ae. aegypti*. Anyway, this has been denied by several Dengue and Chikungunya epidemics worldwide with *Ae. albopictus* being confirmed as the only or main vector (Paupy et al. 2009; Reiter, Fontenille, and Paupy 2006; Gubler and Clark 1995). In Europe the tiger mosquito has been responsible

for several autochthonous cases of Dengue recorded in France (Succo et al. 2016) and in Croatia (Gjenero-Margan et al. 2011).

A first autochthonous Chikungunya outbreak occurred in north-east Italy in 2007 (Angelini et al. 2007; Rezza et al. 2007) with more than 200 reported cases. It has been established that this outbreak was linked to an epidemic which involved the whole Indian Ocean Islands as well as surrounding countries and has subsequently spread to Italy. Interestingly, this outbreak was caused by a new CHKV strain which carried a single adaptive mutation (the A226V mutation in the envelope protein E1) enhancing the replication and transmission of this virus in *Ae. albopictus* (Vazeille et al. 2008), highlighting thus how viral emergence can be related to changes in vectors or hosts (Chevillon et al. 2008). Indeed, competence for virus transmission has been found to be highly variable within and among vector populations and depends on specific combinations of mosquito genome and viral genetic characteristics (Azar et al. 2017; Vazeille et al. 2016; Lambrechts et al. 2009; Lambrechts, Fellous, and Koella 2006).

In 2017 a further Chikungunya outbreak has been reported in Italy with several related clusters involving autochthonous transmission of Chikungunya virus, causing almost 300 reported cases mainly in Lazio and Calabria regions. Despite the absence of the A226V mutation in this outbreak the transmissibility of the virus has been high, underlining that the absence of the mutation does not prevent the occurrence of multi-foci outbreaks, as long as the environmental conditions are suitable for virus transmission (ECDC 2017; Venturi et al. 2017).

1.3. Vector control methods

Given the absence of specific vaccines for all *Aedes*-borne virosis except yellow fever, vector control measures are the only way to prevent their transmission. New control methods, including the development of sterile insect technologies, as well as transgenic and paratransgenic approaches, are currently being studied and tested in the field (McGraw and O'Neill 2013), but presently control of *Aedes* vectors is primarily based on chemical and/or biological insecticides, as well as community engagement for habitat management (Moyes et al. 2017; Baldacchino et al. 2015).

Based on the guidelines for the control and monitoring of invasive mosquito species, released by the World Health Organization (WHO) and the European Centre for Disease Prevention and Control (ECDC), interventions at the larval level should be prioritized over adult control,

because of the higher expected impact and lower environmental costs (WHO 2012b; ECDC 2012). Larval control methods include:

i) Source reduction. This consists in removing or making unavailable breeding sites, such as containers, water tanks, flower pots, etc. Such campaigns have shown to reach temporary suppression of larval stages and can be an effective long-term strategy to reduce mosquito abundance (Baldacchino et al. 2015; Fonseca et al. 2013; Abramides et al. 2011; Grantham, Anderson, and Kelley 2009), but require a strong community-involvement and a solid educational effort, which are difficult to achieve, particularly in areas at low risk of disease transmission.

ii) Application of chemical Insect Growth Regulators (IGR). IGRs such as pyriproxyfen, methoprene or diflubenzuron, inhibit the insect molting process by limiting the chitin synthesis or by inhibiting/mimicking the juvenile hormone, and exhibit also an ovicidal effect (Suman et al. 2013; Bellini et al. 2009). IGRs have shown to be highly effective, especially when targeting the most productive breeding sites (Ocampo 2014), but they may act also on non-target insect species, such as aquatic invertebrates (Pauley, Earl, and Semlitsch 2015; Walker et al. 2005) and development of resistance starts to be reported (Grigoraki, Puggioli, et al. 2017; Douris et al. 2016).

iii) Application of biological larvicides. The microbial larvicide *Bacillus thuringiensis* var. *israelensis* (Bti) (Guidi et al. 2013), sometimes in combination with another microbial larvicide *Lysinibacillus sphaericus* (Lsph), are increasingly used in Europe. The two toxins act synergistically and disrupt the cell membranes, after being activated in the larval gut (Boyce et al. 2013). This complex mechanism of action makes Bti very specific on target species and prevents selection of resistance mechanisms. The effectiveness of this biological control method has been demonstrated (Boyce et al. 2013), but formulation and application methods need to be improved to extend the duration of Bti's residual control (Marcombe et al. 2011)

Adulticide interventions are recommended only in the presence of infected human travelers coming from endemic countries, in order to prevent autochthonous disease transmission, or in the case of extremely intense nuisance (WHO-EMCA 2013). Such interventions consist mainly in the application of pyrethroids (e.g. α-cypermethrin, permethrin and deltamethrin), the only insecticides allowed in Europe for adulticide interventions (EU Directive 98/8). These

insecticides act by preventing the closure of the voltage-gated-sodium channel, have high insecticidal potency and a rapid knockdown effect (WHO 2005). Often their application is done in combination with piperonyl butoxide (PBO), a synergist which enhances the action of insecticides mainly by inhibiting the cytochrome P-450 system, which is fundamental in insecticide detoxification pathways. Pyrethroids are considered relatively safe for humans, but are known to be toxic to non-target insect species, aquatic invertebrates and fish, and thus aerial application should be avoided (Bellini, Zeller, and Van Bortel 2014). An additional major drawback is the high risk of development of resistance to these compounds, as already shown in major mosquito vector species, such as *Anopheles gambiae* and *Ae. aegypti* (Moyes et al. 2017; Scott, Hardstone, and Kasai 2015; Ranson et al. 2011). It is therefore advisable to check periodically for pyrethroid resistance in local mosquito populations and to use insecticides carefully, to slow the evolution of resistance which could seriously reduce the efficacy of these major vector control tools in the near future (Ranson et al. 2011).

1.4. Aim of the PhD project

Italy is nowadays the most widely *Ae. albopictus* infested European country and has experienced two chikungunya outbreaks, highlighting the public health relevance of this mosquito species also in temperate regions. This PhD project consisted in functional as well as genomic studies aimed to shed light on two relevant, but so far neglected, aspects related to *Ae. albopictus* in Italy.

The functional study consists in the analysis of the susceptibility of locally established populations across Italy to pyrethroid insecticides by specific bioassays. Resistance to pyrethroid insecticides has been reported in *Ae. albopictus* populations from the native range (South-East-Asia), while the few studies conducted until now on invasive populations in Italy highlighted full susceptibility. However, the use of pyrethroids by private citizens and sometimes public administrations to reduce the nuisance created by high mosquito densities in several Italian urban areas where the species has become a permanent pest, creates an actual risk of insurgence of resistance mechanisms. The results of the study here reported represent the first assessment of the susceptibility of local populations sampled across Italy to the 3 most used pyrethroids - α-cypermethrin, permethrin and deltamethrin – and is aimed to serve as a reference for future studies on this subject and to help preventing resistance spreading by implementing rationale use of insecticide products.

The second study subject consists in the analysis of the population genetic structure and invasion history of *Ae. albopictus* by a population genomic approach, with particular focus on Italy. This can provide precious information on possible ways of introduction and transportation of the species in Italy and thus help in avoiding introduction of further *Ae. albopictus* populations, as well as of other invasive mosquito species. Moreover, identification of source populations known to be characterized by a lack of complete susceptibility to insecticides could allow to prevent the spread of resistance mechanisms in Italy. Finally, information on the introduction source(s) can also be of help in assessing the public health threat related to the invasion by populations with different vector competence.

2. Resistance to pyrethroid insecticides in adult Italian *Aedes albopictus* populations

2.1 Introduction

Control of mosquito-borne-diseases (MBD) relies heavily on the control of vector populations. Unfortunately, the abundant usage of insecticides not only for vector control but also for the control of agricultural pests has led to the spread of insecticide resistance (IR), nowadays observed among all the major vector species (WHO 2012a; Hemingway and Ranson 2000; WHO 1992).

The mechanisms that enable insects to resist the action of insecticides can be:

i) Behavioral: i.e. any modification of the insect's behavior helping to avoid lethal doses of the insecticide, for example, by becoming more exophilic or a shift in biting times (Mathenge et al. 2001).

ii) Metabolic: i.e. a modification in the mosquitoes' enzymatic detoxification systems including gene amplification or transcriptional up-regulation of detoxification enzymes, mainly esterases, monooxygenases and glutathione S-transferases (Hemingway et al. 2004; Brogdon and Mcallister 1998). An enhancement in the activity of these enzymes enables insects to metabolize insecticides before their toxic effect.

iii) Target-site: i.e. a modification within the target of the insecticide, reducing or avoiding an efficient interaction with the insecticide. For example, organophosphate and carbamate insecticides act on the acetylcholinesterase (AChE) and several mutated forms of AChE causing reduced susceptibility to these insecticides exist (Russell et al. 2004). Target site mutations (known as kdr) in the amino acid sequence in the voltage gated sodium channel (VGSC) of nerve cell membranes, target of pyrethroids and chlororganic compounds (such as DDT), have been detected in several insect species (Farnham and Sawicki 1976) and have been widely studied in the malaria vector *Anopheles gambiae* (Santolamazza et al. 2008; Ranson et al. 2000; Martinez-Torres 1998).

iv) Reduced penetration: i.e. modifications in the insect cuticle or digestive tract preventing or slowing down the penetration of insecticides (Mougabure-Cueto and Picollo 2015).

Particularly worrying are the increasing levels of resistance to pyrethroid compounds which are currently the only active ingredients allowed for adulticidal interventions in Europe (WHO-EMCA 2013; EU Directive 528/2012; EU Directive 98/8) and the only recommended ones for the treatment of bed-nets, a fundamental tool for reducing for example Malaria-incidence (van den Berg et al. 2012).

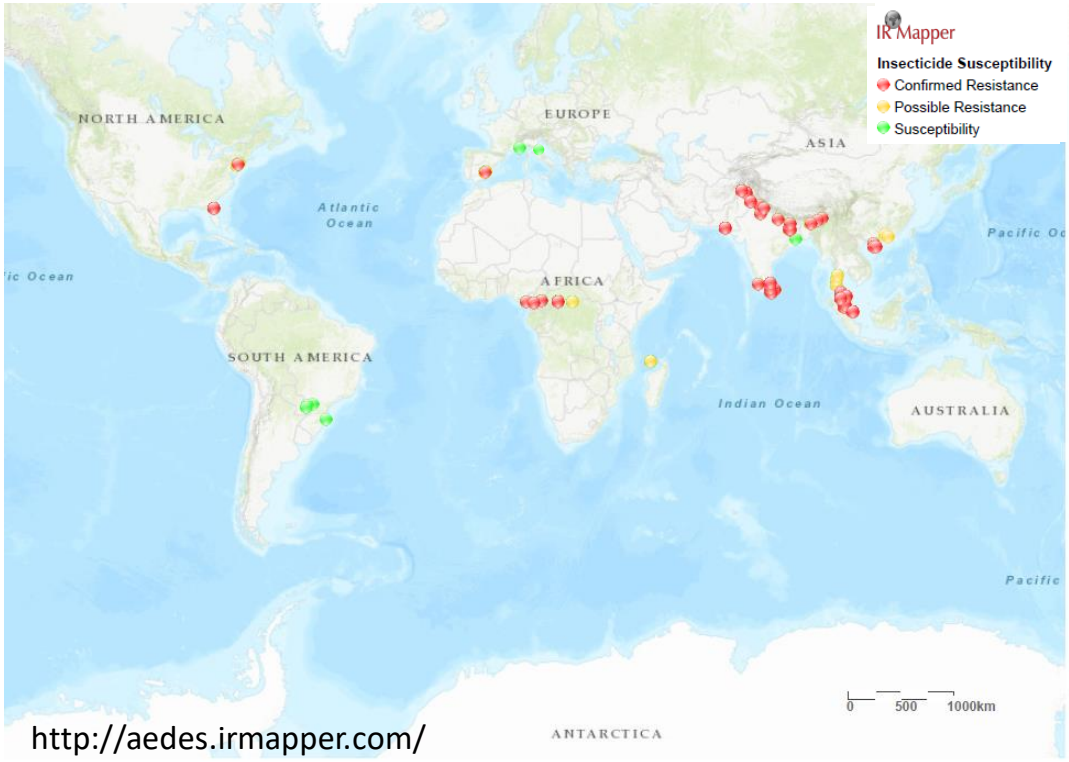
The spread of IR is further enhanced by the phenomenon of cross-resistance (i.e. one resistance mechanism allows the insect to resist also another insecticide class) for example between pyrethroids and DDT, both acting on the VGSC. This situation has been recognized by WHO which drafted several guidelines for monitoring IR and avoiding a further spread, recommending the application of integrated (thus combined) vector control strategies and a periodical evaluation of insecticide susceptibility of vector species (WHO 2014, 2013, 1998). In addition, a Worldwide Insecticide Resistance Network has been established, with the aim to track IR at a global scale and to develop coordinated strategies for early detection and management of resistance (Moyes et al. 2017; Corbel et al. 2016).

In contrast with the extensive knowledge on IR in major tropical mosquito vector species (Smith, Kasai, and Scott 2016; Ranson et al. 2011), knowledge on IR in *Ae. albopictus* is still highly fragmented and clear guidelines for the assessment of IR are missing, as pointed out by Moyes et al. (2017) and Vontas et al (2012): available data documenting IR are highly clustered, and their comparison is difficult since generated using different methods (Figure 2.1). Resistance to pyrethroids has been reported in the last years in adult populations from South-East Asia, the native range of *Ae. albopictus*, (Chuaycharoensuk et al. 2011; Ishak et al. 2015; Lee et al. 2014; Thanispong et al. 2015), as well as from the Indian subcontinent (Arslan et al. 2016; Kushwah et al. 2015; Sivan et al. 2015) and Africa (Kamgang, et al. 2011; Ngoagouni et al. 2016), while almost no reports came so far from temperate areas, except those from Richards et al. (Richards et al. 2017), who recorded reduced susceptibility to permethrin in the USA, and Bengoa et al. (2017) who revealed first signs of resistance of Spanish *Ae. albopictus* populations to cypermethrin and possible resistance to deltamethrin and permethrin.

In Italy, national vector control guidelines (Romi et al. 2011), in agreement with ECDC ones (ECDC 2012) recommend to prioritize larval over adult control interventions. However, private citizens and some public administrations may favor the usage of adulticidal control measures, since they have immediate and tangible, even though short-termed, effects on mosquito

nuisance. Moreover only rarely evaluations on the effectiveness of control measures (Farajollahi et al. 2012; Fonseca et al. 2013; Manica et al. 2017), including susceptibility of vector species to insecticides, are performed: in early 2000 Romi et al. (Romi et al. 2003) did not find any signs of resistance of adult *Ae. albopictus* populations from Rome and other sites across the country, and also Vontas et al (2012) observed full susceptibility in one population from Rome in 2009.

Figure 2.1. Insecticide susceptibility studies performed until 2017 on *Ae. albopictus*, adapted from IRmapper.com.



2.2. Materials & Methods

Mosquito collections and rearing

Ovitrap collections of *Ae. albopictus* eggs and rearing to adults were carried from May to October 2016 in 16 sites across Italy, as well as in two sites from Albania and one from Greece by entomology teams from several collaborating research institutions (see Table 2.1), Collections at each sampling site were conducted with ≥ 5 ovitraps to avoid oversampling of siblings, and, whenever possible, in a site where adulticide treatments using pyrethroids were known to have been performed during the sampling season (labelled with TR in site acronyms), as well as in a second untreated site in the same area (labelled with NT) (Table 2.1). A lab-strain from Athens, Greece, selected for resistance to temephos, was also included in the study to evaluate a possible cross-resistance between organophosphates and pyrethroids. Egg samples sealed in plastic bags were sent by express courier to the Department of Public Health and Infectious Diseases (DPHID) at Sapienza University of Rome.

Larvae were reared at larval density of 0.05 larvae/ml in the insectary of DPHID at T=26 ±1°C, RH=60 ± 5% and at 14:10h light:dark photoperiod and fed with artificial dry cat-food. Pupae were collected daily and transferred into 40 cm-cubic cages. Emerged adults were identified as *Ae. albopictus* using morphological keys (Severini et al. 2009) and kept at the same temperature and humidity as larvae until used for the bioassays. When samples from field collected eggs were not sufficient to complete the experiments, adults were blood-fed and the progeny (F1) was used for bioassays (Table 2.2, 2.3 and 2.4).

Table 2.1. Sampling sites and performed bioassays (P=0.75% permethrin; C=0.05% α-cypermethrin; D=0.05% deltamethrin) for *Aedes albopictus*.TR= insecticide treatments were performed during sampling season; NT = no insecticide treatments were reported during sampling season. * non-pyrethroid. Below the table research institutions participating in sampling are listed.

								Adulticide treatments						
Country	Region	Province	Site-code	Site	Lat	Long	Bioassays	Active ingredient	Target species	for how many years	Spraying method	Treated area (ha)	Time of spraying	Schedule
Italy	Trentino [1]	Trentino	TN-NT1	Zambana	46.150615	11.0978867	P,C,D	-	-	-	-	-	-	-
	Trentino [1]	Trentino	TN-NT2	San Michele	46.187769	11.13276	P,C,D	Buprofezin*	*Sc. titanus/ cicadellidi*	-	motorized sprayer	>1ha	day time	once per year (june)
	Veneto [2]	Padova	PD-NT	Brugine	45.295875	11.9822526	P	-	-	-	-	-	-	-
	Veneto [2]	Venezia	VE-TR	Spinea	45.493505	12.1407815	P,C,D	permethrin, tetramethrin, PBO	-	-	-	-	-	weekly
	Emilia-Romagna [3]	Ferrara	FE-TR1	Lido di Spina	44.648288	12.2324679	P,C,D	permethrin, tetramethrin	*Ae. caspius*	from 1991	Cold fogging	10-207	night	up to 20 treatments per season
	Emilia-Romagna [3]	Ferrara	FE-TR2	Lido di Volano	44.796377	12.2565185	P	permethrin, tetramethrin	*Ae. caspius*	from 1991	Cold fogging	ott-85	night	up to 20 treatments per season
	Liguria [4]	Imperia	IM-NT	Imperia	43.931274	8.060794	P,C,D	-	-	-	-	-	-	-
	Marche [5]	Ancona	AN-NT	Ancona	43.609425	13.495495	P,C,D	-	-	-	-	-	-	-
	Lazio [6]	Rome	RM-NT	Rome-Rebibbia	41.922409	12.573722	P	-	-	-	-	-	-	-
	Lazio [6]	Rome	RM-TR1	Rome-Verano	41.901660	12.5236161	P,C,D	-	*Ae. albopictus*	from 2000 to 2015	Cannon sprayer	80 h	dusk (7-8 PM)	monthly during summer
	Lazio [6]	Rome	RM-TR2	Rome-Policlinico	41.903394	12.5070702	P, C	permethrin, tetramethrin, PBO	*Cx pipiens, Ae. albopictus*	from 2012?	Cannon sprayer	40 h	night (1 AM)	every 3 weeks from June to
	Campania [7]	Naples	NA-TR	Procida	40.751975	14.015302	P	etofenprox, tetramethrin, PBO	*Cx pipiens, Ae. albopictus*	since 1990	back pack sprayer	3.7 h	night (22.00-23.00)	monthly (April to September)
	Puglia [8]	Bari	BA-NT	Valenzano	41.074018	16.8454081	P,C,D	-	-	-	-	-	-	-
	Puglia [8]	Bari	BA-TR	Bari	41.122129	16.8441071	P	cypermethrin, deltametrhin	*Cx pipiens, Ae. albopictus*	2010	Cannon sprayer	60 h	night	weekly
	Sicilia [9]	Messina	ME-NT	Messina-Site A	38.216720	15.565800	P,C	-	-	-	-	-	-	-
	Sicilia [9]	Messina	ME-TR	Messina-Site B	38.232769	15.551017	P,C	cypermethrin	*Ae. albopictus*	from 2012	Cannon sprayer	0.1	early morning	twice during mosquito season
Albania	Albania [10]	Vlorë County	AL-TR	Borsh	40.056360	19.8320601	P,C	cypermethrin	*Ae.albopictus, Cx.pipiens*	5 years (2012 to date)	back pack sprayer	2 ha	dusk	monthly
	Albania [10]	Vlorë County	AL-NT	Vlore	40.450808	19.4464868	P,C,D	alphamethrin	*Ae.albopictus, Cx.pipiens*	2 years, 2014-2015	cold fogging	10 ha	night	every 2 weeks
Greece	Greece[11]	Athens	GR-NT	Athens	38.018889	23.7275335	P	-	-	-	-	-	-	-
	Greece[11]	Athens	GR-LAB	Athens Lab-strain	Lab-strain		P,C,D	-	-	-	-	-	-	-

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Insecticide susceptibility bioassays

Bioassays were performed according to WHO protocols (WHO 1998, 2013) in test tubes lined with filter papers impregnated with one of the following insecticides: permethrin (0.75%), α-cypermethrin (0.05%) or deltamethrin (0.05%) (Vector Control Research Unit, School of Biological Sciences, 11800 Minden, Penang, Malaysia). Insecticide concentrations were selected based on the dosages most frequently used for *Ae. albopictus* in order to allow comparison of results with previous studies (Arslan et al. 2016; Ngoagouni et al. 2016; Ishak et al. 2015; Kushwah et al. 2015; Sivan et al. 2015; Pocquet et al. 2014; Wan-norafikah et al. 2013). The 0.05% concentration for deltamethrin was chosen based on data available on a candidate *Ae. albopictus* susceptible reference strain (Marcombe et al. 2014). Insecticide impregnated (and control) papers were discarded after being used in 6 bioassays.

Bioassays were performed in the insectary at the same conditions of mosquito rearing (see above) by using ~25 unfed *Ae. albopictus* females (3 to 5-day old), either directly emerged from field collected eggs/larvae (F0), or from their progenies (F1) (Table 2.2, 2.3 and 2.4). Mosquitoes were exposed to insecticides for 1 hour and the number of knocked down mosquitoes (i.e. mosquitoes unable to stand or fly in a coordinated way; WHO 2013) was recorded every 10 minutes during exposure time; mortality was recorded at 24 hours post-exposure. Depending on mosquito availability, 3-4 replicates/population/insecticide were performed and for each population/insecticide also a control tube (i.e. lined with filter papers impregnated only with the insecticide excipient but without the active ingredient) was set up and manipulated as the test tubes.

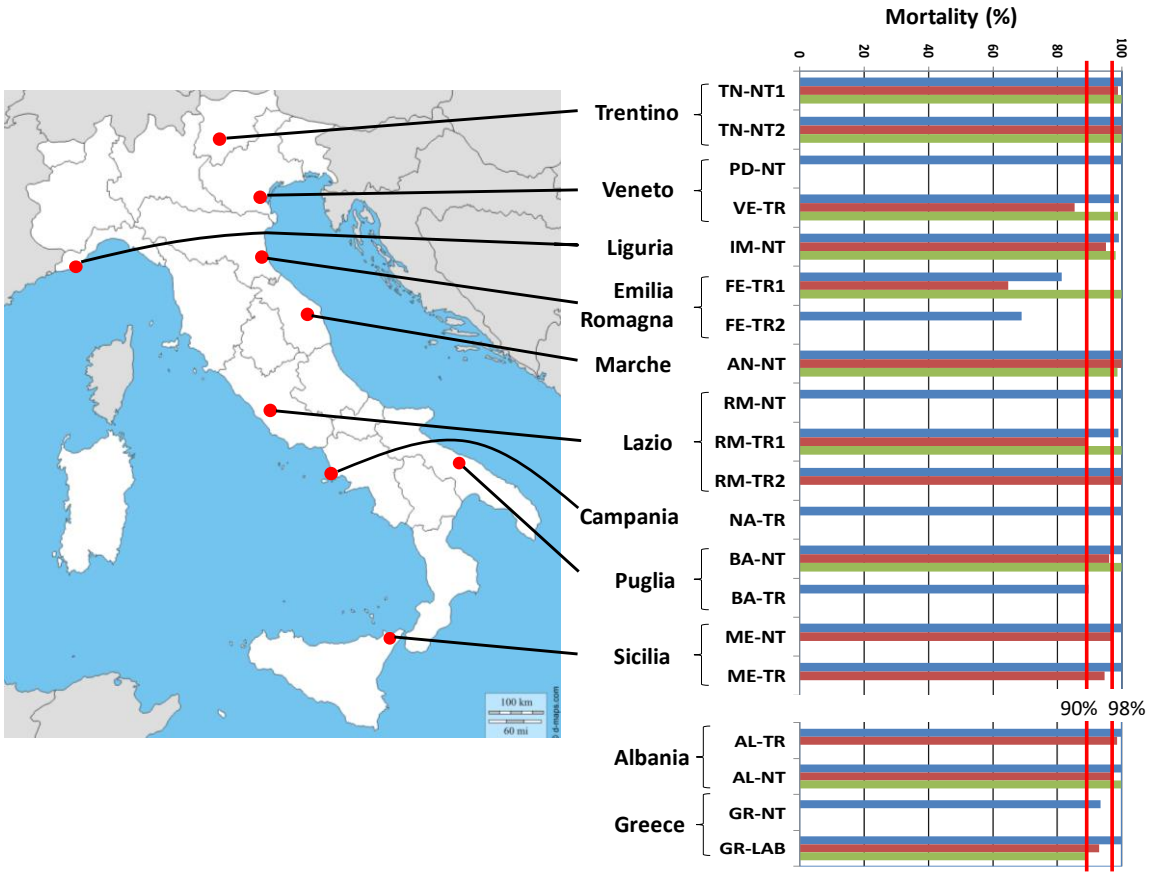
Mean values of mortality were computed for each population (R software 3.3.3). According to WHO guidelines (WHO 2013) populations were considered “susceptible” if mortality at 24 hours after exposure was ≥ 98%, “possibly resistant” if mortality ranged between 90% and 97% and “resistant” if mortality was ≤ 90%.

For knock-down assessment, a log time-probit statistical model was applied to compute KD-curves for each population and to calculate 50% (KDT50) and 95% (KDT95) knock-down times (WHO, 2013). A binomial Generalized Linear Model (GLM) was carried out to test the effect of insecticide control activities on mosquitoes and to evaluate if there is any significant difference between KD-curves of populations from treated and untreated sites.

2.3. Results

Susceptibility to permethrin, α-cypermethrin and deltamethrin as well as KDTs were assessed in 20, 14 and 10 *Ae. albopictus* populations respectively (Figure 2.2). Mortality in control tubes was always <5%, except for the permethrin bioassay of the Greek field-population from Athens (mortality = 8%), for which Abbott-corrected values are reported (Abbott 1987). No knockdown was observed in control tubes during the one-hour exposure to insecticides.

Figure 2.2. Distribution of *Aedes albopictus* tested populations and mortality (%) after 1h exposure to pyrethroids. Permethrin 0.75%: blu; α-cypermethrin 0.05%: red; deltamethrin 0.05%: green. Red vertical lines indicate 90% and 98% mortality thresholds (WHO 2013; 2016). Sites for which adulticide treatments have been reported during the sampling season are labelled with –TR. Sites in which adulticide treatments were not carried out during the sampling season are labelled with –NT. GR-LAB= laboratory-selected temephos resistant colony.



2.1. Permethrin. Bioassays suggested resistance to permethrin only in the treated populations from Ferrara province in Emilia Romagna (mortality: FE-TR1 =81.3%, FE-TR2 =68.9%) and from Bari province in Puglia (BA-TR mortality=89.6%), while the field-population from Athens (Greece) appeared to be possibly resistant (GR-NT, mortality= 93.5). Consistently, these populations showed the highest KDT50 and KDT95 values. A large variability of KDT50 and KDT95 values was observed across Italy (KDT50: 13'-43'; KDT95: 23'-154'; see Figure 2.3 and Table 2.2), with significantly higher values in populations from treated sites in Veneto and Puglia, when compared to populations from neighboring untreated sites ($p<0.05$; Figure 2.4).

Figure 2.3. Knock down time and 95% confidence interval of 50% (KDT50, blu) and 95% (KDT95, yellow) of *Aedes albopictus* exposed to permethrin 0.75%. Sites for which adulticide treatments have been reported during the sampling season are labelled with –TR. Sites in which adulticide treatments were not carried out during the sampling season are labelled with –NT. GR-LAB= laboratory-selected temephos resistant colony

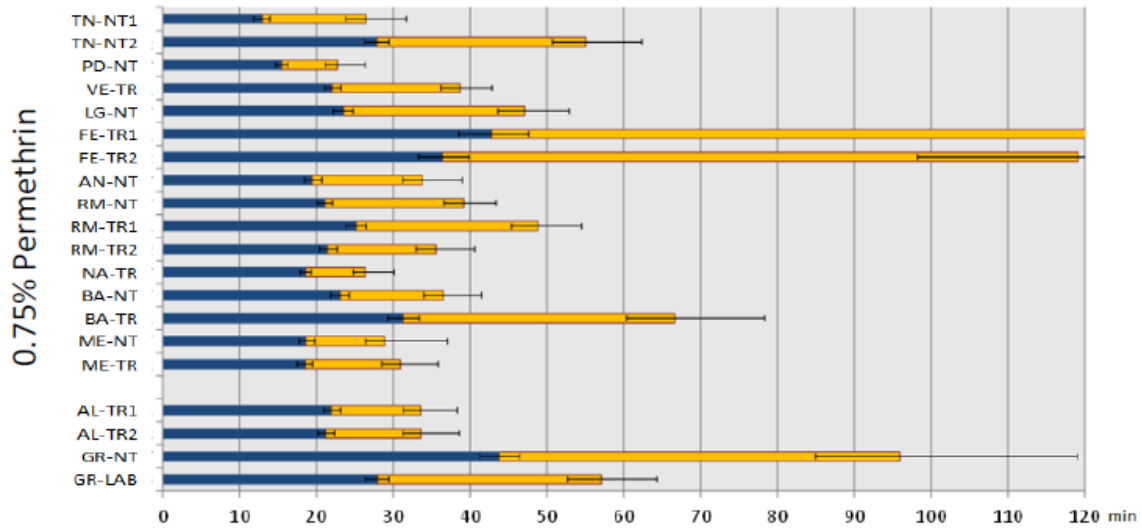
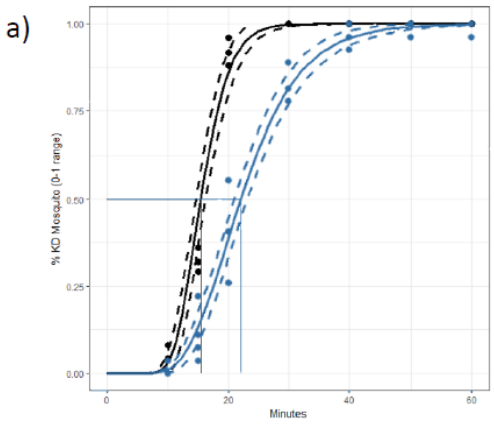


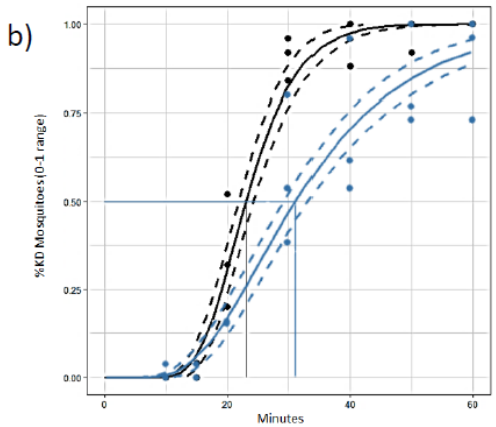
Table 2.2. Results of WHO tube bioassays performed on *Aedes albopictus* populations from Italy, Albania and Greece. Generation and number of mosquito females tested for resistance to permethrin 0.75% are reported, as well as mortality (%) at 24 hours after 1h exposure and times to knock-down (KDT) of 50% and 95% of population (95% confidence intervals). Sites in which adulticide treatment were not carried out during the sampling season are labelled with –NT. GR-LAB= laboratory-selected temephos resistant colony. Results indicating resistance or possible resistance according to WHO (2013, 2016) are highlighted in bold.

	region/country	site-code	treatment	tested generation	N tested	Mortality % (95% CI)	KDT50 (95% CI)	KDT95 (95% CI)
0.75% Permethrin	Trentino	TN-NT1	N	F0	74	100	12.9 (11.9- 14.0)	26.5 (23.9 - 31.8)
		TN-NT2	Y	F0	82	100	27.9 (26.3 - 29.5)	55.1 (50.7 - 62.4)
	Veneto	PD-NT	N	F0	74	100	15.5 (14.7- 16.3)	22.7 (21.2 – 26.4)
		VE-TR	Y	F0	108	99.0	22.1 (21.1- 23.2)	38.7 (36.1 - 42.8)
	Emilia Romagna	FE-TR1	Y	F0	74	**81.3**	42.8 (38.4- 47.6)	154.2 (121.8 – 260.9)
		FE-TR2	Y	F0	75	**68.9**	36.4 (33.2 - 39.9)	119.2 (98.2 – 172.1)
	Liguria	IM-NT	N	F1	100	99.0	23.5 (22.2- 24.8)	47.1 (43.5 - 52.9)
	Marche	AN-NT	N	F0	75	100	19.5 (18.4- 20.7)	33.8 (31.2 - 39)
	Lazio	RM-NT	N	F0	122	100	21.1 (20.1 - 22.1)	39.3 (36.7 - 43.3)
		RM-TR1	Y	F0	96	99.0	25.2 (23.9- 26.6)	48.9 (45.4 – 54.6)
		RM-TR2	Y	F1	77	100	21.5 (20.4-22.7)	35.5 (33 -40.6)
	Campania	NA-TR	N	F0	99	100	18.6 (17.9- 19.4)	26.4 (24.9 – 30.1)
	Puglia	BA-NT	N	F0	75	100	23.1 (21.9- 24.3)	36.6 (34.1– 41.6)
		BA-TR	Y	F1	77	**89.6**	31.3 (29.4- 33.3)	66.7 (60.4 - 78.3)
	Sicilia	ME-NT	N	F0	50	100	18.6 (17.5- 19.8)	29 (26.5 – 37.1)
		ME-TR	Y	F0	75	100	18.5 (17.5- 19.6)	30.9 (28.5 – 35.8)
	ALBANIA	AL-TR	Y	F0	77	100	22 (20.9- 23.1)	33.5 (31.3– 38.3)
		AL-NT	Y	F1	74	100	21.3 (20.2- 22.4)	33.6 (31.2– 38.5)
	GREECE	GR-NT	N	F1	100	**93.5**	43.8 (41.2 - 46.5)	95.9 (85 -119)
		GR-LAB	lab	F1	100	100	27.9 (26.5 - 29.5)	57.1 (52.7 - 64.4)

Figure 2.4. Comparison of knock-down curves obtained for *Aedes albopictus* exposed to permethrin 0.75%. Dotted lines show 95% confidence intervals, dots observed data. a) Populations from Veneto; Blue= VE-TR; Black= PD-NT; b) Populations from Puglia; Blue= BA-TR; Black= BA-NT; Below the graphs the summary statistics of the binomial Generalized Linear Model (GLM) carried out to test the effect of insecticide control activities on mosquitoes. The interaction term (LogTime*treated) allows to test if the relationship between the proportion of dead mosquitoes and logtime is statistically different between treatment statuses.



	Estimate	Std.Error	z-value	Pr(>|z|)
Intercept	-11,2057	1,4139	-7,926	2,27E-15
LogTime	4,1071	0,5114	8,03	9,71E-16
treated	2,1198	1,5257	1,389	0,165
LogTime:treated	-1,1711	0,5428	-2,157	3,10E-02



	Estimate	Std.Error	z-value	Pr(>|z|)
Intercept	-11,2194	0,9048	-12,4	2,00E-16
LogTime	3,5741	0,2857	12,512	2,00E-16
treated	3,7424	1,0547	3,548	0,000388
LogTime:treated	-1,4024	0,3249	-4,316	1,59E-05

2.2. α-**cypermethrin**. resistance to α-cypermethrin was suggested for the treated populations from Ferrara province (FE-TR1, mortality = 64.8%) Venezia province (VE-TR, mortality = 85.3%) and Rome (RM-TR1, mortality = 89.2%). Consistently, these populations showed the longest KDT50 and KDT95 values.

Results, suggestive of possible resistance were obtained for several other tested populations, (see Figure 2.5) while full susceptibility was observed only for 4 Italian populations (mortality: TN-NT1=98.7%; TN-NT2 =100%; AN-NT=100%; RM-TR2=100%), and one population from Vlore-county in Albania (AL-TR, mortality =98.6%). Large variability for KDT50 and KDT95 was observed across Italy (KDT50: 22'-62'; KDT95: 40'-186') but no significant differences were detected among populations in neighboring treated vs untreated sites (Figure 2.5 and Table 2.3).

Figure 2.5. Knock down time and 95% confidence interval of 50% (KDT50, blu) and 95% (KDT95, yellow) of *Aedes albopictus* exposed to α-cypermethrin 0.05%. Sites for which adulticide treatments have been reported during the sampling season are labelled with –TR. Sites in which adulticide treatments were not carried out during the sampling season are labelled with –NT. GR-LAB= laboratory-selected temephos resistant colony

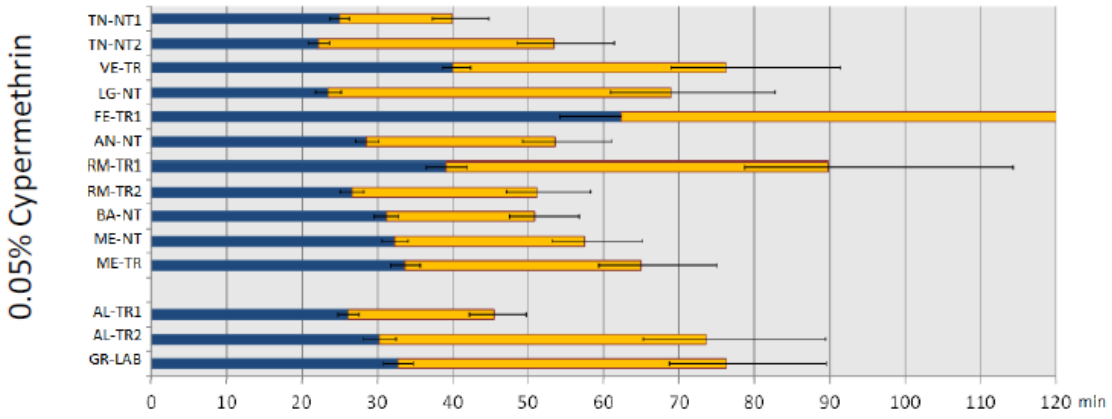


Table 2.3 Results of WHO tube bioassays performed on *Aedes albopictus* populations from Italy, Albania and Greece. Generation and number of mosquito females tested for resistance to α-cypermethrin 0.05% are reported, as well as mortality (%) at 24 hours after 1h exposure and times to knock-down (KDT) of 50% and 95% of population (95% confidence intervals). Sites in which adulticide treatment were not carried out during the sampling season are labelled with –NT. GR-LAB= laboratory-selected temephos resistant colony. Results indicating resistance or possible resistance according to WHO (2013, 2016) are highlighted in bold

	region/country	site-code	treatment	tested generation	N tested	Mortality % (95% CI)	KDT50 (95% CI)	KDT95 (95% CI)
0.05% α - Cypermethrin	Trentino	TN-NT1	N	F1	78	98.7	25 (23.7-26.3)	39.9 (37.3-44.8)
		TN-NT2	Y	F1	90	100	22.2 (20.9 - 23.7)	53.5 (48.5– 61.4)
	Veneto	PD-NT	N	NA	NA	NA	NA	NA
		VE-TR	Y	F0	75	**85.3**	40 (37.7- 42.3)	76.2 (68.9 – 91.5)
	Emilia Romagna	FE-TR1	Y	F1	73	**64.8**	62.3 (54.2-71.6)	186.3 (142.7 - NA)
		FE-TR2	Y	NA	NA	NA	NA	NA
	Liguria	IM-NT	N	F1	100	**95.0**	23.4 (21.8-25.2)	69 (60.9 - 82.7)
	Marche	AN-NT	N	F0	75	100	28.5 (26.9- 30.2)	53.6 (49.3 - 61)
	Lazio	RM-NT	N	NA	NA	NA	NA	NA
		RM-TR1	Y	F1	74	**89.2**	39.1 (36.5 - 41.9)	89.8 (78.7 – 114.3)
		RM-TR2	Y	F1	78	100	26.6 (25.1-28.2)	51.2 (47.1 - 58.3)
	Campania	NA-TR	N	NA	NA	NA	NA	NA
	Puglia	BA-NT	N	F0	76	**96.1**	31.2(29.6- 32.8)	50.9 (47.5 – 56.8)
		BA-TR	Y	NA	NA	NA	NA	NA
	Sicilia	ME-NT	N	F1	76	**96.7**	32.3 (30.6-34.1)	57.5 (53.2 - 65.1)
		ME-TR	Y	F1	75	**94.7**	33.7 (31.8-35.7)	64.9 (59.4 - 75)
	ALBANIA	AL-TR	Y	F0	72	98.6	26.1 (24.7 - 27.6)	45.5 (42.2 – 51.3)
		AL-NT	Y	F1	75	**97.3**	30.3 (28.2 - 32.5)	73.6 (65.3 – 89.4)
	GREECE	GR-NT	N	NA	NA	NA	NA	NA
		GR-LAB	lab	F1	100	**93.0**	32.7 (30.8 - 34.7)	76.3 (68.8 - 89.5)

2.3. Deltamethrin. All the 8 Italian populations tested, as well as the Albanian one, were fully susceptible to deltamethrin while resistance was observed only in the Greek laboratory colony (mortality = 89.0%). KDT50 and KDT95 were highest in RM-TR1, but no significant differences were observed among treated and untreated sites (Figure 2.6 and Table 2.4).

Figure 2.6. Knock down time and 95% confidence interval of 50% (KDT50, blu) and 95% (KDT95, yellow) of *Aedes albopictus* exposed to deltamethrin 0.05%. Sites for which adulticide treatments have been reported during the sampling season are labelled with –TR. Sites in which adulticide treatments were not carried out during the sampling season are labelled with –NT. GR-LAB= laboratory-selected temephos resistant colony

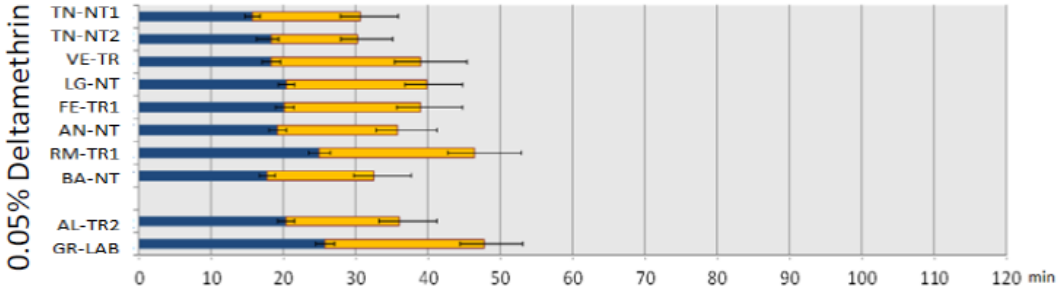


Table 2.4 Results of WHO tube bioassays performed on *Aedes albopictus* populations from Italy, Albania and Greece. Generation and number of mosquito females tested for resistance to deltamethrin 0.05% are reported, as well as mortality (%) at 24 hours after 1h exposure and times to knock-down (KDT) of 50% and 95% of population (95% confidence intervals). Sites in which adulticide treatment were not carried out during the sampling season are labelled with –NT. GR-LAB= laboratory-selected temephos resistant colony. Results indicating resistance or possible resistance according to WHO (2013, 2016) are highlighted in bold

	region/country	site-code	treatment	tested generation	N tested	Mortality % (95% CI)	KDT50 (95% CI)	KDT95 (95% CI)
0.05% Deltamethrin	Trentino	TN-NT1	N	F1	78	100	15.7 (14.7 - 16.8)	30.7 (27.9 - 35.9)
		TN-NT2	Y	F1	75	100	18.3 (17.3 - 19.4)	30.3 (27.9 – 35.1)
	Veneto	PD-NT	N	NA	NA	NA	NA	NA
		VE-TR	Y	F1	77	98.7	18.3 (17.1 -19.6)	39 (35.4 - 45.4)
	Emilia Romagna	FE-TR1	Y	F1	78	100	20.2 (19 - 21.5)	39 (35.7 - 44.8)
		FE-TR2	Y	NA	NA	NA	NA	NA
	Liguria	IM-NT	N	F1	100	98.0	20.4 (19.3 - 21.6)	39.9 (36.8 - 44.8)
	Marche	AN-NT	N	F1	77	98.7	19.2 (18.1-20.4)	35.8 (32.9 - 41.3)
	Lazio	RM-NT	N	NA	NA	NA	NA	NA
		RM-TR1	Y	F1	74	100	25 (23.5 - 26.5)	46.4 (42.8 - 52.9)
		RM-TR2	Y	NA	NA	NA	NA	NA
	Campania	NA-TR	N	NA	NA	NA	NA	NA
	Puglia	BA-NT	N	F0	77	100	17.8 (16.7 - 18.9)	32.5 (29.8 - 37.7)
		BA-TR	Y	NA	NA	NA	NA	NA
	Sicilia	ME-NT	N	NA	NA	NA	NA	NA
		ME-TR	Y	NA	NA	NA	NA	NA
	ALBANIA	AL-TR	Y	NA	NA	NA	NA	NA
		AL-NT	Y	F1	78	100	20.4 (19.2 - 21.6)	36.1 (33.3 - 41.3)
	GREECE	GR-NT	N	NA	NA	NA	NA	NA
		GR-LAB	lab	F1	100	**89 .0**	25.8 (24.5 - 27.1)	47.8 (44.5 - 53.1)

2.4. Discussion

We here report the first evidence of resistance to permethrin and α-cypermethrin in adult *Ae. albopictus* populations from Italy. The lowest mortality rates (<70%) were detected in populations from two sites along the Adriatic coast in Comacchio area (Emilia-Romagna region, North-East-Italy). No detailed data on adulticide usage in Italy are available, but it is relevant to note that the two sites are highly touristic and insecticide spraying is extensively conducted since 1991 during the summer seasons to reduce nuisance mostly due to *Aedes caspius* and *Cx. pipiens* (Bellini and Veronesi 1994). In fact, preliminary results on sympatric *Cx. pipiens* showed mortality rates <20% after exposure to 0.75% permethrin (FE-TR2, data not shown), confirming that mosquito populations in the area are likely to be exposed to high adulticide pressure. It would be interesting to test susceptibility to pyrethroids of *Ae. albopictus* populations collected in neighbouring localities where no or scattered adulticide treatments are conducted.

Mortality rates suggestive for resistance (<90%) were obtained also for populations from Puglia (BA-TR) when exposed to permethrin, and Veneto (VE-TR) and Lazio (RM-TR1) when exposed to α-cypermethrin. Four additional populations from Italy (from treated as well as untreated sites) showed mortality rates indicative of possible resistance to α-cypermethrin (mortality <98%). Further tests on larger sample sizes are needed to confirm these preliminary results.

Evidence of lower susceptibility to both pyrethroids is also provided from the significant increase in the time to knockdown observed in some populations. In fact, large variability for KDT50 and KDT95 was observed across Italy, likely reflecting differential adulticide usage. Significant differences between treated and untreated sites were found in the case of permethrin: populations collected in treated sites in Veneto and Puglia showed higher KDT50 and KDT95 values than populations collected in the same region in neighboring but untreated sites, suggesting that adulticide spraying carried out at high frequency during the whole season in these sites lowered the species' susceptibility. This appeared not to be the case in Lazio and Sicilia possibly due to less effective or more recent adulticide treatments.

Differently from what observed for permethrin and α-cypermethrin, all Italian populations were susceptible to deltamethrin. Similar results were obtained in Greece (Vontas et al. 2012), Spain (Bengoa et al. 2017) and the US (Marcombe et al. 2014). This result is consistent with the

hypothesis of a lower usage of this insecticide in Italy but could also indicate that the deltamethrin dosage used was inappropriate for *Ae. albopictus*.

Mechanisms producing the permethrin/α-cypermethrin resistance phenotype in Italian populations will be evaluated in future studies. While target-site-resistance mechanisms, which typically induce cross-resistance (Smith, Kasai, and Scott 2016; Flores et al. 2013; Brengues et al. 2003; Chandre et al. 1999), are widespread and well-known in *Anophelines* (Ranson et al. 2011), far less information is available for *Ae. albopictus*. Several target site mutations have been identified in this species but their association with IR is still unclear (Moyes et al. 2017; Smith, Kasai, and Scott 2016) and appears to be less strong compared to other mosquito species. Also, the lack of cross-resistance to different pyrethroids in the Comacchio population suggests that multiple resistance mechanisms, possibly including detoxification pathways (Ishak et al. 2016; Kasai et al. 2007) may be involved.

Aedes albopictus populations from Albania were found fully susceptible to all pyrethroids tested, with relatively low KDTs, despite being sampled in insecticide treated sites. On the other hand, the field population from Athens (which was shown to be susceptible to deltamethrin in 2009; Vontas et al. 2012) did not show full susceptibility to permethrin and exhibited KDT95 values higher than all other tested populations, except those from Comacchio. Surprisingly, however, no public pyrethroid space-spraying has been carried out in Athens since 2007, although a selective pressure by intensive treatments performed by private citizens cannot be excluded. The lower susceptibility of the field-collected population from Greece to permethrin could be explained by a different origin of the Greek population compared to the Italian and Albanian ones, as suggested by the genomic study discussed in chapter 3 of the present PhD thesis as well as by previous studies (Manni et al. 2017), but also by cross-resistance between organophosphates and pyrethroids, as already reported for other mosquito species (Rodríguez et al. 2002; Wirth and Georghiou 1999). In fact the same amplified CCEs responsible for the Temephos-resistance of the laboratory colony have been observed also in Greek field-populations (Grigoraki, Pipini, et al. 2017) and could be associated with a reduced susceptibility to permethrin which can be hydrolysed by CCEs as shown in other insect species (Usmani and Knowles 2001).

Data herein presented need to be interpreted with caution considering some limitations inherent to the study design and sampling efforts. First, WHO provides specific diagnostic dosages based

on data available only for *Ae. aegypti*, *Culex quinquefasciatus* and *Anopheline* mosquitoes. The dosages used in this study tested were higher than those recently recommended as tentative for *Aedes* mosquitoes (WHO 2016), and this choice was made in order to obtain comparable results with previous studies (see Materials and & Methods). This implies that our results certainly do not overestimate resistance levels, but may underestimate them. Further studies on a susceptible reference colony are needed to more precisely estimate diagnostic dosages, the lack of which strongly limits the possibility to compare and interpret results across studies (Vontas et al. 2012). Second, we chose to preform bioassays with F0 females or, when not possible, F1 progenies, in order to avoid loss of selective pressure and inbreeding under laboratory conditions. This choice, however, implied that in some cases we did not have the possibility to have 4 replicates, as required by WHO (WHO 2013) to confirm resistance. Third, the classification of "treated site" in the study is heterogeneous as it reflects different mosquito control activities carried out in Italy, Albania and Greece, including different pyrethroid compounds sprayed at different doses, different spraying methods, protocols and time-schedules. Nevertheless, it is notable that only the populations from Trentino subjected to occasional adulticide spraying (Rizzoli A.P., personal communication), together with populations from Marche (Ancona province; AN-NT), showed complete susceptibility to all the tested insecticides, while highest resistance was observed in Comacchio sites, where very intensive control activities following a well-defined monitoring plan has been implemented even before the *Ae. albopictus* invasion to reduce nuisance due to *Ae. caspius* (a very aggressive autochthonous species). In most other sites adulticide treatments were introduced only after the colonization of the areas by invasive *Ae. albopictus*.

Overall, our report of first evidence of resistance to permethrin and α-cypermethrin in adult *Ae. albopictus* Italian populations represents a first step to fill a gap of knowledge on resistance to pyrethroids in invasive populations now fully established in Europe, where the species is becoming an increasing health threat. The results show that resistance to the most commonly used pyrethroids (i.e. permethrin and α-cypermethrin in Italy) is arising in areas where the species has been well established for several years, reaches high densities and creates high nuisance. Coupled with possible resistance observed recently in Spain (Bengoa et al. 2017) and the high levels of resistance found in the only west European *Ae. aegypti* population from Madeira island (Seixas et al. 2017), the results should serve as a warning for all Europe and

encourage further efforts in monitoring this phenomenon and in standardizing protocols for IR detection and guidelines for IR management in *Aedes* vector species in temperate areas. Studies of this kind are in fact highly needed to support local public health authorities in managing and planning effective control measures and to maintain insecticide-based vector control options effective. The large Chikungunya outbreak (ECDC 2017) that occurred in central Italy in summer 2017 clearly highlights the urgency of more extensive studies to better understand and monitor the spread of resistance phenotypes with a higher spatial and temporal coverage particularly in areas where the risk of arbovirus autochthonous transmission is predicted to be not negligible (Moyes et al. 2017; Schaffner, Medlock, and Van Bortel 2013; Tilston, Skelly, and Weinstein 2009; Liu-Helmersson et al. 2016), as well as the implementation of synergic and coordinated actions aimed at controlling the mosquito population abundance at the larval stage.

3. Population genomics and invasion history of *Aedes albopictus* in Italy

3.1. Introduction

Despite the impressive worldwide spread of *Aedes albopictus* during the last few decades and its epidemiological importance (see above), few detailed studies on its population genetic structure and invasion history have been performed. These studies highlighted high genetic variability within sampling sites and lack of genetic structure according to geography, independently of whether the considered populations were native or invasive and often contradictory results among studies on the species' invasion history were obtained (e.g., the case of Greece or Brazil; Kamgang, Brengues, et al. 2011; Manni et al. 2017; Birungi and Munstermann 2002; Kambhampati, Black, and Rai 1991a).

In fact, phylogeographic studies performed until now on *Ae. albopictus* suffered from two major limitations: low numbers of field-sampled populations included in the study and/or limited effectiveness of genetic markers utilized.

Effective genetic markers should be selectively neutral and sufficiently variable to allow investigations on genetic differentiation and genetic clustering of individuals. Moreover, they should be easily scored, allow comparisons among specimens and, ideally, of datasets from different studies (Goubert et al. 2016). Population genetic studies on *Ae. albopictus* were first performed using polymorphic enzymes (e.g. Chareonviriyaphap et al. 2004; Urbanelli et al. 2000; Black et al. 1988; Kambhampati, Black, and Rai 1991), and, later on, mitochondrial DNA (mtDNA; e.g. Battaglia et al. 2016; Ismail et al. 2015; Beebe et al. 2013; Zhong et al. 2013; Porretta et al. 2012; Kamgang et al. 2011; Delatte et al. 2011;) and microsatellites (e.g. Manni et al. 2017, 2015; Porretta et al. 2006; Delatte et al. 2013; Porretta et al. 2006).

While polymorphic enzymes showed a remarkable resolution, and allowed to investigate genetic relationship between individuals and populations, they are known to have some important drawbacks such as a reduced number of informative markers available or the possible non-neutral evolution of some of the protein variants examined (Schlötterer 2004).

Studies on mtDNA instead, suffered, besides rare exceptions (Battaglia et al. 2016; Beebe et al. 2013), from low comparability, due to differences in the amplified fragment, and low levels of genetic variation among samples. In addition, mtDNA markers reflect the demography only in terms of the maternal line and may thus not be well-suited for unravelling complicated demographic histories as the *Ae. albopictus* one.

Microsatellite studies instead, despite being highly variable, showed low reproducibility and comparability given that most authors developed new sets of markers. Anyway, recent studies (Manni et al. 2017, 2015; Maynard et al. 2017; Medley, Jenkins, and Hoffman 2015) have defined new sets of microsatellites allowing fine-scale genetic analysis. Also, the usage of insertion polymorphisms created by transposable elements (TEs) has been evaluated as it has been demonstrated that some TE families have several thousand well-conserved copies in the tiger mosquito (Goubert et al. 2017).

In this present study we took advantage of a collaboration with the Department of Ecology and Evolutionary Biology (EEB) at Yale University to investigate the population structure and invasion history focusing on Italian populations by genotyping Single Nucleotide Polymorphisms (SNPs) across the whole genome. SNPs are powerful genetic markers, densely distributed across eukaryotic genomes and provide a basis for high-resolution analysis of historical biogeography and population structure (Wray 2013). To obtain a densely distributed, genome-wide set of markers, we used a double-digest Restriction site-associated DNA sequencing approach (ddRADseq; Peterson et al. 2012) which is currently one of the most popular methods for genotyping SNPs across the whole genome in non-model species. Moreover, this approach improves, compared to other RADseq methods, the recovery of the same genomic regions across all specimens, therefore increasing also comparability between samples included in different studies (Peterson et al. 2012).
The collaboration with EEB allowed us to merge SNP-data on European populations with a SNP-dataset produced at EEB in order to obtain a worldwide dataset including 29 populations from both, the native and the invasive range.

3.2. Materials and Methods

Mosquito collection and preparing for downstream processing

For European samples, collection of *Ae. albopictus* eggs, larval rearing and morphological identification were carried out as described in paragraph 2.2, while samples from the worldwide range were already available at EEB. All the specimens were preserved for downstream analysis dry at -80°C (Figure 3.1; Table 3.1). DNA extraction and sequencing library preparation were performed at the Department of Public Health and Infectious Diseases at Sapienza University for the European specimens and at EEB for all the other populations following the same protocol (see below). Also, sequencing of libraries including the European and the remaining specimens was performed in different runs and only afterwards results obtained for the two datasets were merged for data analysis.

Figure 3.1. Approximate position of sampling sites worldwide (left) and in Italy (right). In the worldwide map colors correspond to different macro-areas, numbers can be looked up in table 3.1;
red= North-America, light-blue = South-America, purple =Africa, grey = Japan; black = S-E-Asia, yellow = Europe.

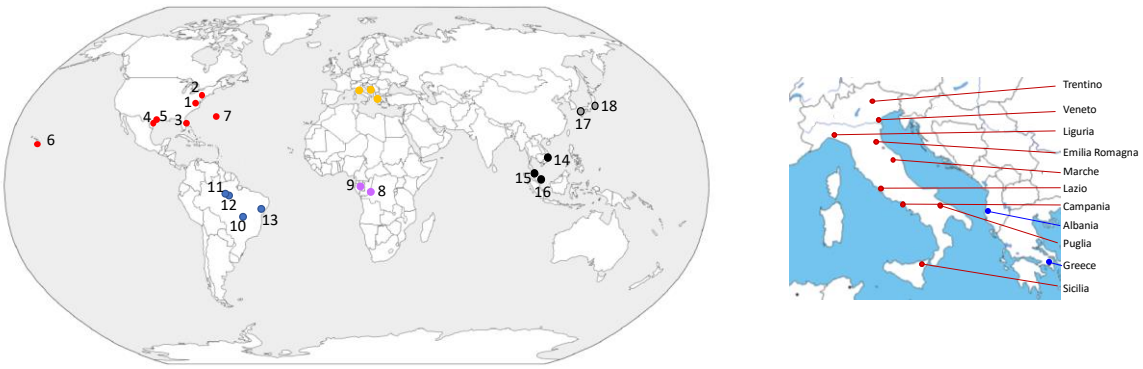


Table 3.1. Population information for the *Aedes albopictus* samples used in this study including the sampling region-site, sampling year, number of individuals included in the study and the codes used as abbreviation for sampling sites. For populations outside Europe see also Kotsakiozi et al. 2017.

	country	sampling region	Range	Number of specimens	sampling year	code
Europe	Italy	Trentino - San Michele	invasive	7	2016	TN
		Veneto - Spinea	invasive	7	2016	VE
		Liguria - Imperia	invasive	7	2016	LG
		Emilia Romagna - Lido di Volano	invasive	7	2016	ER
		Marche - Ancona	invasive	7	2016	MA
		Lazio - Roma	invasive	7	2016	LZ
		Campania - Procida	invasive	7	2016	CA
		Puglia - Bari	invasive	7	2016	PG
		Sicilia - Messina	invasive	7	2016	SI
	Albania	Vlore	invasive	7	2016	AL
	Greece	Athens	invasive	7	2016	GR
	USA	1. USA - Manassas, Virginia	invasive	4	2010	MAN
		2. USA-Newark, New Jersey	invasive	4	2008	NEW
		3. USA- Florida	invasive	6	2006	FLO
		4. Texas1-Brownsville	invasive	4	2010	BRO
		5. Texas2-Corpus Christi	invasive	4	2001	CORP
		6. Hawai	invasive	4	2006	HAW
	UK	7. Bermuda	invasive	4	2015	BER
	DRC	8. Kinshasa	invasive	4	2011	DRC
	Gabon	9. Franceville	invasive	4	2015	GAB
	Brazil	10. Brasilia	invasive	4	2015	BRA
		11. Itacoatiara, Amazon State	invasive	4	2015	COAT
		12. Presidente Figueiredo, Amazon State	invasive	4	2015	PRES
		13. Salvador	invasive	6	2001	SALV
	Vietnam	14. Phu Hoa	native	4	2015	VTN
	Malaysia	15. Kuala Lampur	native	5	2006	KLP
	Singapore	16. Sentosa Island	native	4	2014	SIN
	Japan	17. Kagoshima	native	4	2008	KAG
		18. Tokyo	native	6	2008	TOK

DNA extraction and Quantification

DNA extraction was performed on whole mosquitoes using the DNeasy Blood and Tissue kit (Qiagen), according to the manufacturer's instructions but with the addition of RNAse A; DNA was eluted afterwards in double-distilled water (ddH2O). Extracted DNA (5ul) was run on a 1.5% agarose gel stained with Midori Green Advance DNA Stain (Nippon Genetics Europe GmbH) in order to include in downstream analysis only specimens with high-quality, unfragmented DNA, and quantified using the Quant-iT™ PicoGreen kit (Thermo Fisher Scientific ™) according to manufacturer's instructions using a microplate reader. For the following steps approximately 650 ng of DNA/specimen diluted in 50ul of ddH2O were used.

Barcoding

For a subset of specimens (N=16) morphological species identification was confirmed by DNA barcoding. Fragments (of approximately 600bp) of the mitochondrial Cytochrome c oxidase subunit I (COI) gene were amplified, using polymerase chain reaction (PCR) primers and protocol described in Folmer et al. 1994, sequenced (BMR s.r.l., Padua, Italy) and compared with sequences already available at the Barcode of Life Data System (Ratnasingham and Hebert 2007). Barcoding confirmed morphological species identification for all the 16 specimens sequenced (sequence data are available in paragraph 7.1).

Double-digest Restriction site-Associated DNA sequencing library preparation

Double-digest Restriction-site-Associated DNA sequencing is a reduced representation sequencing approach which permits genotyping of multiple individuals with substantially reduced sequencing investment compared to whole genome sequencing. Differently to previously developed RAD-seq approaches (Andrews et al. 2016; Peterson et al. 2012) this method is not based on random shearing of the genome but instead uses two restriction enzymes (RE) and fine-tuned size selection to recover genomic regions randomly distributed across the genome. Only the subset of genomic restriction fragments generated by cuts of both REs (i.e. having one end from each cut) and which fall within the size-selection window will be included in the final sequencing library, favoring thus the recovery of the same genomic region from different individuals and increasing the number of comparable sites genotyped across all specimens.

Sequencing libraries were prepared according to Peterson et al (2012) and Gloria-Soria et al. (2016). Approximately 650 ng of DNA were doubled-digested using NlaIII and MluCI (NEB) restriction enzymes in incubation at 37°C for 3h. To confirm that digestion has been successful 4 ul of digested DNA were run on a 1.5% agarose gel stained as above. Prior to the successive step digested DNA was purified using Agencourt AMPure XP magnetic beads (Beckman Coulter Genomics) and quantified (using Quant-iT™ PicoGreen kit) in order to obtain 200ng of DNA for the ligation step during which P1 and P2 barcoded adaptors (Table 3.2) were added to the DNA fragments using T4 ligase (NEB).

Table 3.2. Sequence detail for barcoded adaptors P1 and P2 and Illumina indexed primers used in dd-RADseq library preparation.

	barcode number	P1. adaptor	Seq1	Seq2
barocoded adaptors	2	AAGGA_flex_P1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAAGGACATG	/5Phos/TCCTTAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
	4	ACACA_flex_P1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACACACATG	/5Phos/TGTGTAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
	8	AGCTA_flex_P1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGCTACATG	/5Phos/TAGCTAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
	9	ATACG_flex_P1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTATACGCATG	/5Phos/CGTATAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
	12	CAACC_flex_P1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCAACCCATG	/5Phos/GGTTGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
	20	CTGAT_flex_P1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTGATCATG	/5Phos/ATCAGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
	23	CTTGG_flex_P1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTTGGCATG	/5Phos/CCAAGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
	24	GACAC_flex_P1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGACACCATG	/5Phos/GTGTCAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
		flex_P2.1	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	/5Phos/AATTAGATCGGAAGAGCGAGA
Illumina indexed primers	Index1_ATCACG	PCR2	CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACGTGTGC	
	Index3_TTAGGC	PCR3	CAAGCAGAAGACGGCATACGAGATGCCTAAGTGACTGGAGTTCAGACGTGTGC	
	Index5_ACAGTG	PCR4	CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTCAGACGTGTGC	
	Index8_ACTTGA	PCR5	CAAGCAGAAGACGGCATACGAGATTCAAGTGTGACTGGAGTTCAGACGTGTGC	
		PCR1 primer	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACG	

Illumina indexes (Table 3.2) were added in the following PCR reaction using Phusion® High-Fidelity DNA Polymerase (NEB) at the following conditions: Initial denaturation of 1 min at 98°C; 8 cycles of 5 sec at 98°C for denaturation, 15 sec at 68°C for annealing and 15 sec at 72°C for extension, followed by a final extension step of 5 min at 72°C.

Following the Illumina indexing PCR products were again purified using Agencourt AMPure XP magnetic beads (Beckman Coulter Genomics) and quantified (using Quant-iT™ PicoGreen kit) in order to pool 16 barcoded and indexed specimens into each library. Selection of fragments of 215 bp was performed for all the final libraries using the Blue Pippin electrophoresis platform (Sage Science) and a final quality check was performed using a Bioanalyzer (AGILENT). Libraries were finally sequenced (75bp paired-read sequencing), using the Illumina Hi-Seq 2000 platform at the Yale Center for Genome Analysis.

Raw sequence processing and SNP filtering

Variant calling and filtering was performed twice, once for samples from Italy, Albania and Greece (the "European dataset"), and once for the world-wide dataset, including the European dataset as well as specimens form 18 populations sampled at a worldwide scale (hereafter "world-wide dataset"; Table 3.1).

Sequence data (reads) were de-multiplexed and mapped against the *Ae. albopictus* reference genome (Chen et al. 2015) using Bowtie2 v.2.1.0 (Langmead and Salzberg 2012) and Samtools v. 1.3 (Li et al. 2009) using the paired option. Unmapped reads and reads with mapping quality below Q10 were removed from the database. Also, specimens with extremely low sequencing depth (10.000-20.000 reads per specimen) were excluded from further analysis. Variant calling was performed using bcftools and variant filtering using vcftools v. 0.1.14.10 (Danecek et al.

2011) with the following parameters: biallelic SNPs with genotype depth (minDP) >7.0X, coverage of 70% and minor allele frequency (MAF) of 0.05. Linkage disequilibrium (LD) filtering was performed using PLINK 1.9 (Purcell et al. 2007) using the --indep option with a window size of 1000 variant counts, 50 as step and 1 as variance inflation factor (VIF).

Evaluation of genetic diversity and differentiation

On a worldwide scale genetic diversity per sample was estimated by computing individual observed heterozygosity (Ho) using vcftools and estimating then mean Ho per sampling locality. Due to the small sample-size, a non-parametric Kruskal-Wallis (KW) test was used to compare the mean Ho between populations.

For European specimens also expected heterozygosity (H_exp), Number of private alleles and inbreeding coefficient F*IS* were assessed. Expected heterozygosity per population was computed using the R-package adegenet 2.0.1 (Jombart 2012) and obtained heterozygosity values were compared among populations using the permutation test available in the same R-package (N simulations = 500). The number of private alleles was computed using Arlequin v.3.5.2 (Laurent Excoffier and Lischer 2010), while F*IS*-values were computed and averaged over loci using the R-package hierfstat (Goudet 2005); F*IS*-95% confidence intervals were obtained performing 100 bootstraps using the same R-package.

The partitioning of genetic variation among and within populations of *Ae. albopictus* at a European level was evaluated by a hierarchical analysis of molecular variance using locus by locus AMOVA (Excoffier, Smouse, and Quattro 1992) implemented in Arlequin v.3.5.2.2 (AMOVA; with 1,000 permutations to test the significance of fixation indices. Groups were defined in the following way: group1=Albania (AL); group2=Greece (GR); group3= northern Italy (TN, VE, ER, LG), group4=central Italy (MA, LZ, CA), group5= southern Italy (PG, SI) Italy.

Levels of genetic differentiation were evaluated for both, the worldwide and the European dataset. F*ST* values were computed between all populations pairs using Arlequin v.3.5 on the LD filtered datasets with 1,000 permutations and a significance level of 0.05.

Isolation by distance (IBD) was evaluated performing a Mantel test between a matrix of geographic and genetic distances (classical Euclidean Distance) on the European as well as the

worldwide dataset using the R-package adegenet 2.0.1. For each Mantel-test 999 replicates were performed and, to distinguish continuous clines of genetic differentiation from a patchy distribution of genetically different populations (e.g. distant and differentiated populations) the genetic distances were plotted against geographic distances.

Evaluation of evolutionary relationships among populations

To ascertain how many groups of genetically distinct populations occurred in the European dataset and to evaluate their relationship with the populations present in the worldwide dataset different clustering and phylogenetic approaches were performed:

Maximum Likelihood approach

The Software ADMIXTURE (Alexander, Novembre, and Lange 2009) was used to implement an individual clustering approach on the LD filtered datasets on a worldwide and on an European scale. The best number of genetic clusters (K) was chosen based on the cross-validation procedure available in ADMIXTURE.

Least-squares estimation approach

This individual based clustering approach, implemented in the LEA-package (Frichot and François 2015) in R 3.3.3 was applied only to the European LD filtered dataset as an alternative to the maximum likelihood approach used by ADMIXTURE (Alexander et al. 2009) which did not reveal any population structuring at the European level. Similar to Bayesian clustering programs, this method estimates individual admixture coefficients from the genotypic matrix (Pritchard, 2000) and computes an entropy criterion that evaluates the quality of fit of the statistical model to the data using a cross-validation technique (similar to the one implemented in ADMIXTURE).

Scenarios with K = 1–12 were explored performing 100 runs for each K. The cross-entropy criterion was used to choose the number of clusters which best explained the genotypic data as well as the best of the 100 runs (Frichot et al. 2014; Alexander & Lange 2011), as recommended by the software's manual.

Discriminant Analysis of Principal Components (DAPC)

DAPC was implemented using the R-package adegenet (Jombart et al. 2010). This method performs first a PCA con the raw data in order to perform afterwards a Discriminant Analysis on the retained principal components to provide an efficient description of the genetic clusters using a few synthetic variables (discriminant functions), which maximize the between-group variance and minimize the within-group variance (Jombart et al. 2010). DAPC was not performed on an individual-based but on a population-based level. To define the best number of discriminat functions to retain the a-score function of the adegenet package was used as recommended by the software's manual.

Principal Component Analysis (PCA)

PCA was performed using the R package LEA (Frichot & Francois 2015) on individuals in both datasets (worldwide and European). Tracy-Widom tests were performed to determine the number of significative eigenvalues and to calculate the percentage of variance explained by each principal component (Patterson, Price, and Reich 2006; Tracy and Widom 1994;).

Maximum Likelihood tree reconstruction

Evolutionary relationships among populations (on a worldwide and a european scale) were evaluated using the Maximum Likelihood (ML) approach implemented in RAxML (Stamatakis 2014) using 1,000 botstraps and the General Time Reversible (GTR) model of evolution along with the CAT approximation of rate heterogeneity. An ascertainment bias correction to the likelihood calculations, and the standard correction by Paul Lewis (Lewis 2001), when only variant sites are included in the data set, were applied. For this analysis the LD-filtered datasets were used and the R-package pcadapt (Luu, Bazin, and Blum 2017) was used for a multivariate analysis aiming at the identification of SNPs possibly under selection considering qvalues lower than 0.05 for detection of outlier SNPs which were excluded in order to not bias the analysis.

Inference on invasion history

A preliminary analysis on invasion history using an Approximate Bayesian Computation approach has been performed using the software DIYABC (Cornuet et al. 2015) for simulation of competing invasion scenarios and the R-package abcrf (Pudlo et al. 2016) for selecting the best suited model. Details on this analysis are given in paragraph 7.2 but are not discussed in the present thesis since results were inconclusive.

3.3. Results

Variant calling

After alignment with minimum mapping quality Q10, 3-10 million reads with a mean number of 2,000,000 SNPs per mosquito were obtained for further filtering.

The worldwide dataset consisted after SNP calling and filtering in 156 specimens (Table 3.1; 42,900 SNPs (32,197 in the LD filtered one) with a mean number of 38,283 SNPs per specimen, an average depth of 31.9X (SD=13.1) and an amount of missing data per specimen of 16.6 % (s.d.= ± 7.9) and 16.6 % (s.d.= ± 8.2) per locus.

When considering only the European dataset 103,289 SNPs (47,475 in the LD filtered one) were retained after SNP calling and filtering for 77 specimens (Table 3.1; 7 for each of the 11 populations included), with a mean number of 86,000 SNPs per specimen and an average depth of 25.5X (SD=7.7). The amount of missing data per specimen was 16.4 % (s.d.= ± 5.9) and 16.4 % (s.d.= ± 8.2) per locus.

Genetic diversity and differentiation

Observed heterozygosity computed for the worldwide dataset are reported in Table 3.3 for the worldwide dataset, and in Table 3.4 for the European dataset. Values range between 0.15 (VTN) and 0.23 (FLO) and highest values are observed among the American and Italian populations. Interestingly, populations from the native range in South-East-Asia (VTN, KLP, SIN) show comparatively lower values, similar to the ones observed in Africa and Brazil. Results obtained for Kruskal-Wallis (KW) tests performed to evaluate statistical differences between populations are reported in Figure 3.2 and confirm significant differences among populations.

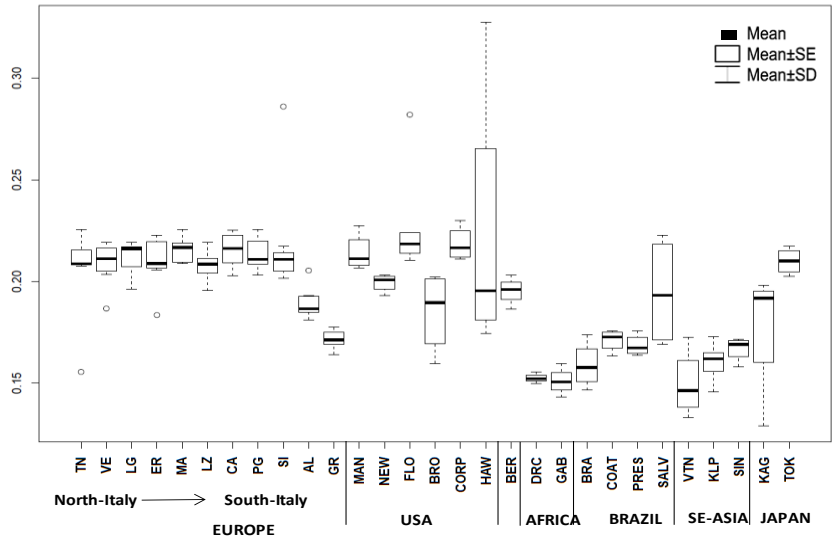
Table 3.3. Observed Heterozygosity (H_obs) computed for the worldwide dataset on 40,900 SNPs.

	Italy											USA						UK	Africa		Brazil				South-East-Asia			Japan	
sample-code	TN	VE	LG	ER	MA	LZ	CA	PG	SI	AL	GR	MAN	NEW	FLO	BRO	CORP	HAW	BER	DRC	GAB	BRA	COAT	PRES	SALV	VTN	KLP	SIN	KAG	TOK
H_obs	0.205	0.208	0.211	0.209	0.215	0.207	0.215	0.213	0.219	0.189	0.171	0.214	0.199	0.227	0.185	0.218	0.223	0.195	0.152	0.150	0.158	0.171	0.168	0.194	0.149	0.160	0.166	0.177	0.210

Data on genetic diversity obtained for the European dataset on 103,289 SNPs, are reported in Table 3.4. Observed heterozygosity and results obtained for KW-test, performed on an European as well as an Italian scale are given in Figure 3.2 and show significant differences among populations when considering all the Italian, Greek and Albanian specimens, while no significant differences are observed when focusing only on Italian populations.

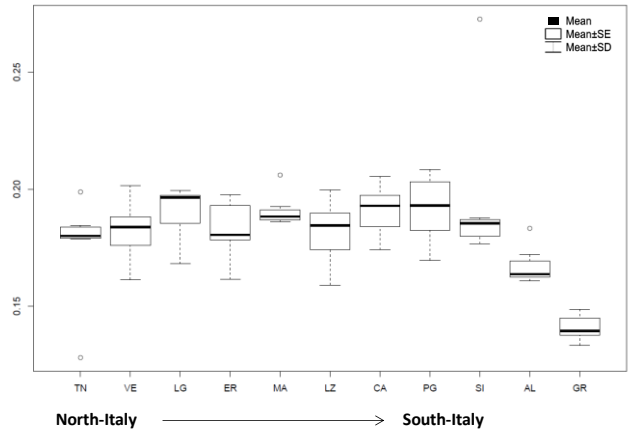
Figure 3.2. Mean Individual observed heterozygosity per population as estimated using vcftools for the a) worldwide SNPs datasets (SNPs=42,900; specimens= 156) and b) the European dataset (SNPs= 103,289; specimens=77). The mean, standard deviation (SD) and the standard error (SE) are presented. Results for the non-parametric Kruskal-Wallis test, implemented to test for differences between populations ($p<0.05$) at a worldwide, European and Italian scale, are shown right to each graph.

a)



	World (N=156)
adjusted H	111.2
d.f.	28
P value:	7,22E-09

b)



	Europe (N=77)	Italy (N=63)
adjusted H	33.477	7.70
d.f.	10	8
P value:	0.0002264	0.464

Expected heterozygosity varied between 0.207 (PG) and 0.228 (MA) for Italian populations, while lower values were observed for populations from Albania (0.195) and Greece (0.182).
Results for pairwise comparisons of expected heterozygosity among European samples are shown in Table 3.5 and demonstrate significant differences mainly when comparing populations from different countries.
Also, the number of private alleles detected suggests important differences between Italian, Albanian and Greece populations, with specimens from Greece and Albania showing highest presence of private substitutions (138 and 80 respectively). Within Italy the highest number of private alleles is observed for populations from Liguria and Puglia, with 23 and 17 private alleles respectively.
F*IS* for Italian populations varies between 0.193 (PG) and 0.293 (TN). Populations from Puglia and Liguria show the lowest values and the 95% CI intervals are not overlapping with any other Italian population. The Albanian population shows an F*IS* value similar to the one of Italian populations (0.256) while the F*IS* value observed for the Greek population (0.349) is the highest among the European populations and 95% CI interval is not overlapping with any other European population.
Hierarchical locus per locus AMOVA results and average F-statistics are reported in Table 3.6 and show that most of the genetic variance is explained at the individual level (68.07%) while only 5.70% of variance is observed among populations.

Table 3.4. Basic diversity statistic computed for the European dataset on 103,289 SNPs. H_exp= expected heterozygosity, H_obs= observed heterozygosity, F*IS*= inbreeding coefficient, CI 0 95% F*IS* confidence interval.

	sample-code	private subst. sites	H_exp	H_obs	F*IS*	F*IS* CI	F*IS* CI
Italy	TN	7	0.218	0.176	0.293	0.286	0.295
	VE	1	0.216	0.182	0.262	0.258	0.266
	LG	23	0.209	0.189	0.199	0.193	0.203
	ER	6	0.217	0.183	0.259	0.254	0.262
	MA	0	0.228	0.190	0.266	0.263	0.269
	LZ	0	0.222	0.181	0.286	0.281	0.289
	CA	7	0.215	0.190	0.230	0.223	0.232
	PG	17	0.207	0.191	0.193	0.187	0.197
	SI	2	0.223	0.195	0.229	0.224	0.233
	AL	80	0.195	0.167	0.256	0.249	0.257
	GR	138	0.182	0.140	0.349	0.336	0.346

Table 3.5. Simulated p-value after 500 simulations for comparison of expected Heterozygosity values among European populations. Grey-shaded cells identify pairwise comparisons for populations within Italy. Bold values identify non-significant comparison.

	TN	VE	PG	ER	MA	LZ	CA	PG	SI	Albania	Greece
TN	-										
VE	**0.778**	-									
PG	**0.072**	**0.088**	-								
ER	**0.974**	**0.420**	**0.136**	-							
MA	0.006	0.012	0.006	0.02 0	-						
LZ	**0.292**	**0.058**	0.038	**0.334**	**0.116**	-					
CA	**0.310**	**0.680**	**0.318**	**0.460**	0.026	**0.112**	-				
PG	**0.192**	**0.144**	**0.696**	0.090	0.040	0.090	**0.376**	-			
SI	**0.710**	**0.312**	0.016	**0.444**	**0.240**	**0.736**	**0.138**	0.042	-		
Albania	0.002	0.002	0.018	0.006	0.002	0.004	0.002	**0.110**	0.002	-	
Greece	0.002	0.006	0.008	0.002	0.002	0.002	0.002	0.006	0.002	0.01	-

Genetic differentiation computed as F*ST* -values are shown in Table 3.7 and Table 3.8 for the worldwide and the European dataset. Almost all the pairwise comparisons are significant except few, most of them including the population from Marche-Italy (MA). For Italian populations lowest values are observed in comparisons with the native range (especially Japanese populations) and the populations from the USA (except BRO), while highest differentiation is observed in comparison with populations from Africa and Brazil. Within Europe highest F*ST*-values are observed between countries, while within Italy the least differentiated populations appear to be the ones from Marche and Lazio and the most differentiated ones the one from Veneto and Puglia.

Table 3.6. Analysis of molecular variance (AMOVA) performed in Arlequin v.3.5.2.2

Source of variation	Percentage variation	Average F-Statistics
among groups	3.55	F*CT* = 0.036**
among populations within groups	5.70	F*SC* = 0.059**
among individuals within populations	22.68	F*IT* = 0.319**
within individuals	68.07	F*IS* = 0.250**
**= highly significant(p<0.001) after 1023 permutation		

Table 3.7. Population pairwise F*ST* computed for European populations on the LD filtered dataset (N SNPs = 47,475) in Arlequin v.3.5.2.2. Bold values show highest differentiated populations within a region and italic values show non-significant differentiation

	TN	VE	EG	ER	MA	LZ	CA	PG	SI	AL	GR
TN	0										
VE	0.033	0									
EG	0.066	0.068	0								
ER	0.040	0.042	0.077	0							
MA	0.031	0.045	0.051	0.043	0						
LZ	0.041	0.078	0.071	0.050	*0.011*	0					
CA	0.058	0.079	0.074	0.063	0.022	0.032	0				
PG	0.078	**0.096**	0.094	0.079	0.040	0.040	0.061	0			
SI	0.041	0.042	0.056	0.044	*0.023*	0.040	0.037	0.065	0		
AL	0.096	0.120	0.120	0.105	0.070	0.073	0.090	0.110	0.094	0	
GR	0.109	0.128	**0.132**	0.112	0.080	0.086	0.101	0.124	0.099	0.116	0

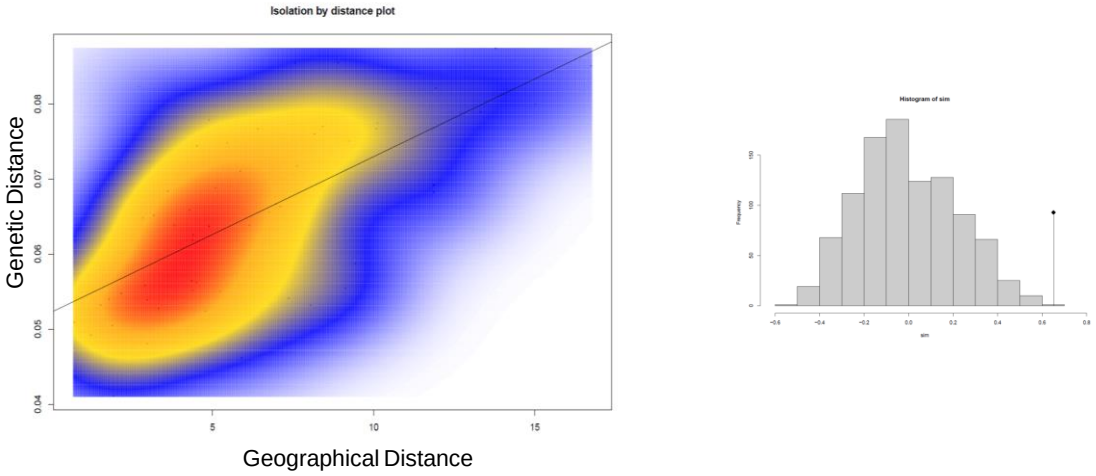
Table 3.8. Population pairwise F*ST* computed for worldwide LD filtered dataset (N SNPs= on 32,197) in Arlequin v.3.5.2.2. Bold values show highest differentiated populations within a region and italic values show non-significant differentiation. Grey-shaded cells identify pairwise comparisons for populations from the same geographic region.

		Italy											USA							Africa		Brazil				S-E-ASIA			Japan	
		TN	VE	LG	ER	MA	LZ	CA	PG	SI	AL	GR	MAN	NEW	FLO	BRO	CORP	HAW	BER	DRC	GAB	BRA	COAT	PRES	SALV	VTN	KLP	SIN	KAG	TOK
Italy	TN	0																												
	VE	0.025	0																											
	LG	0.049	0.054	0																										
	ER	0.030	0.028	0.059	0																									
	MA	0.030	0.038	0.042	0.034	0																								
	LZ	0.031	0.062	0.054	0.037	*0.015*	0																							
	CA	0.041	0.058	0.056	0.046	0.026	0.022	0																						
	PG	0.062	**0.078**	0.074	0.068	0.038	0.030	0.047	0																					
	SI	0.027	0.032	0.049	0.034	0.024	0.028	0.028	0.052	0																				
	AL	0.076	0.100	0.095	0.082	0.056	0.061	0.076	0.091	0.075	0																			
	GR	0.085	0.105	0.104	0.089	0.062	0.070	0.080	0.100	0.082	0.098	0																		
USA	MAN	0.027	0.031	*0.043*	0.042	*0.021*	0.047	0.050	0.068	0.036	0.081	0.082	0																	
	NEW	0.040	0.034	0.058	0.053	*0.032*	0.064	0.067	0.086	0.048	0.096	0.097	0.002	0																
	FLO	0.079	0.092	0.099	0.086	0.050	0.080	0.083	0.107	0.075	0.115	0.104	0.048	0.078	0															
	BRO	0.169	0.170	0.183	0.170	0.124	0.175	0.17	0.200	0.170	0.204	0.189	0.145	0.156	0.177	0														
	CORP	0.035	0.040	0.058	0.044	*0.016*	0.042	0.050	0.075	0.036	0.081	0.073	*0.008*	0.024	0.038	0.137	0													
	HAW	0.068	0.085	0.093	0.071	*0.036*	0.057	0.074	0.093	0.068	0.098	0.091	0.052	0.075	0.082	**0.185**	*0.034*	0												
	BER	0.080	0.088	0.100	0.081	0.054	0.082	0.082	0.109	0.071	0.120	0.113	0.046	0.073	0.082	0.181	0.049	0.095	0											
Africa	DRC	0.142	0.165	0.165	0.146	0.095	0.132	0.141	0.163	0.138	0.170	0.117	0.144	0.156	0.167	0.258	0.138	0.148	0.179	0										
	GAB	0.108	0.130	0.124	0.109	0.065	0.090	0.102	0.122	0.102	0.125	0.075	0.100	0.117	0.125	0.221	0.093	0.103	0.134	0.111	0									
Brazil	BRA	0.105	0.119	0.118	0.108	0.063	0.095	0.108	0.117	0.102	0.130	0.089	0.095	0.108	0.121	0.220	0.090	0.104	0.136	0.139	0.097	0								
	COAT	0.119	0.130	0.135	0.123	0.080	0.119	0.127	0.144	0.117	0.154	0.131	0.107	0.113	0.134	0.237	0.107	0.134	0.148	0.194	0.143	0.077	0							
	PRES	0.183	0.190	0.196	0.182	0.129	0.175	0.183	0.203	0.177	0.219	0.195	0.172	0.175	0.203	0.305	0.161	0.192	0.222	**0.259**	0.210	**0.139**	0.109	0						
	SALV	0.102	0.123	0.121	0.108	0.051	0.098	0.108	0.124	0.105	0.137	0.106	0.107	0.115	0.125	0.237	0.099	0.119	0.150	0.178	0.124	0.028	0.073	0.135	0					
S-E-ASIA	VTN	0.105	0.124	0.122	0.105	*0.062*	0.085	0.099	0.120	0.098	0.126	0.083	0.100	0.109	0.122	0.218	0.088	0.090	0.125	0.129	0.081	0.091	0.136	0.202	0.115	0				
	KLP	0.086	0.111	0.108	0.093	*0.036*	0.075	0.083	0.109	0.085	0.111	0.058	0.090	0.106	0.112	0.205	0.077	0.088	0.122	0.110	0.061	0.082	0.133	0.201	0.107	0.062	0			
	SIN	0.079	0.096	0.095	0.080	*0.041*	0.061	0.076	0.095	0.073	0.102	0.040	0.067	0.085	0.093	0.188	0.061	0.072	0.102	0.100	0.063	0.073	0.120	0.182	0.095	**0.065**	0.035	0		
Japan	KAG	0.050	0.057	0.061	0.053	*0.029*	0.050	0.056	0.077	0.052	0.088	0.094	0.030	0.046	0.085	0.176	0.038	0.070	0.082	0.152	0.108	0.111	0.120	0.193	0.113	0.105	0.100	0.074	0	
	TOK	0.043	0.040	0.061	0.052	0.035	0.059	0.062	0.081	0.047	0.092	0.101	0.023	0.032	0.085	0.173	0.037	0.074	0.080	0.161	0.119	0.113	0.122	0.187	0.102	0.114	0.103	0.087	0.040	0

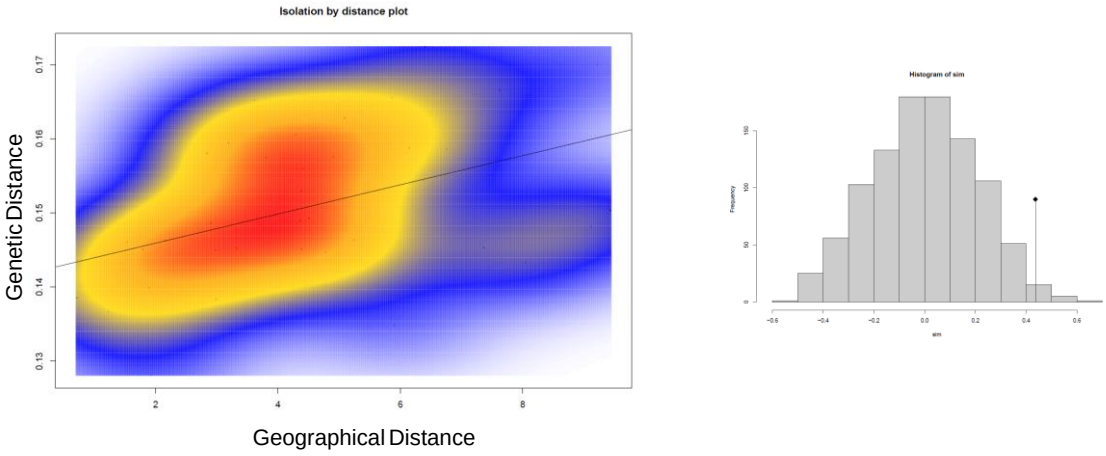
No indications for IBD were observed, as expected, on a worldwide scale (Simulated p-value = 0.753) while results for European and only Italian populations (in Figure 3.3.) indicate some genetic differentiation along with geographic distance (Europe: p-value = 0.001; Italy: p-value=0.012).

Figure 3.3. Isolation by distance represented as scatterplot of genetic (classical Euclidean distance) vs. geographic distances for a) European specimens (N= 77) and b) Italian specimens (N=63); the estimated local density of points is plotted in a kernel-smoothed colour scale with blue indicating low and red high density of points. At the right side: histograms represent permuted values (i.e., under the absence of spatial structure), the dot represents the original value of the correlation between the distance matrices.

a)



b)



Evaluation of evolutionary relationships among populations

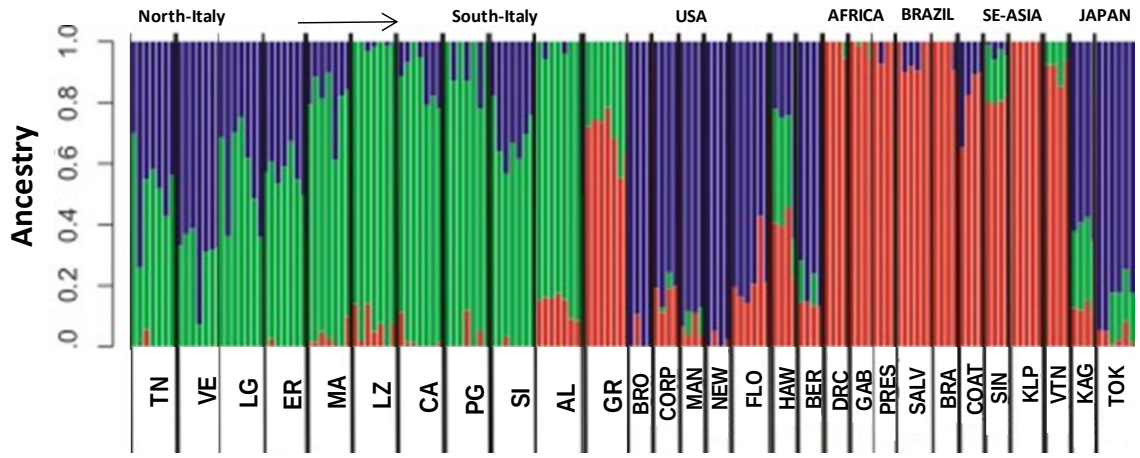
Maximum Likelihood approach

The Ancestry proportion graph for the worldwide dataset is shown in Fig 3.4. The results using the ML-approach are not shown for the European dataset since K=1 was supported as the best run. The best number of clusters for the worldwide dataset was 3 with the second-best value being K=4 (shown in Figure 3.4.). Admixture analysis for the global dataset supported the existence of three genetically distinct clusters, with native populations from Japan clustering in a different group compared to native populations from South-East-Asia (S-E-Asia). Greek and African specimens appear to be more closely related to S-E-Asia (red cluster in Figure 3.4.a), while north-American and partially North-Italian populations cluster mainly with Japanese populations (blue cluster in Figure 3.4a). Brazilian populations instead are grouped together with populations from S-E-Asia and Africa when using K=3 while they cluster independently when using K=4 (Figure 3.4b). Interestingly, Albanian specimens form a cluster almost missing in the native range and the same cluster is observed also among Italian populations (green cluster in Figure 3.4a).

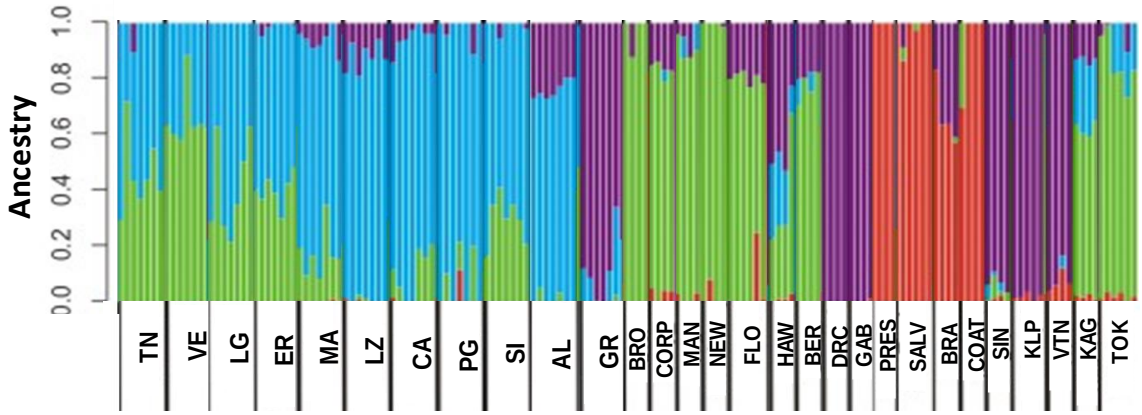
Several European specimens, as well as the population from Hawai (HAW), Florida (FLO) and Japan (KAG) show sign of genetic admixture. Especially, all the specimens from Trentino (TN), Veneto (VE), Liguria (LG), Emilia Romagna (ER) and Sicilia (SI) show Q values<0.75. The same is observed for the population from Hawai (HAW) and Japan (KAG). Admixture is observed also for some specimens of the populations from Marche (MA), Greece (GR) and Florida (FLO).

Figure 3.4. Admixture graph obtained for 156 specimens included in the worldwide dataset (N SNPs=32,197after LD filtering) a) clustering observed for best k=3; b) clustering observed for second best k=4.

a)



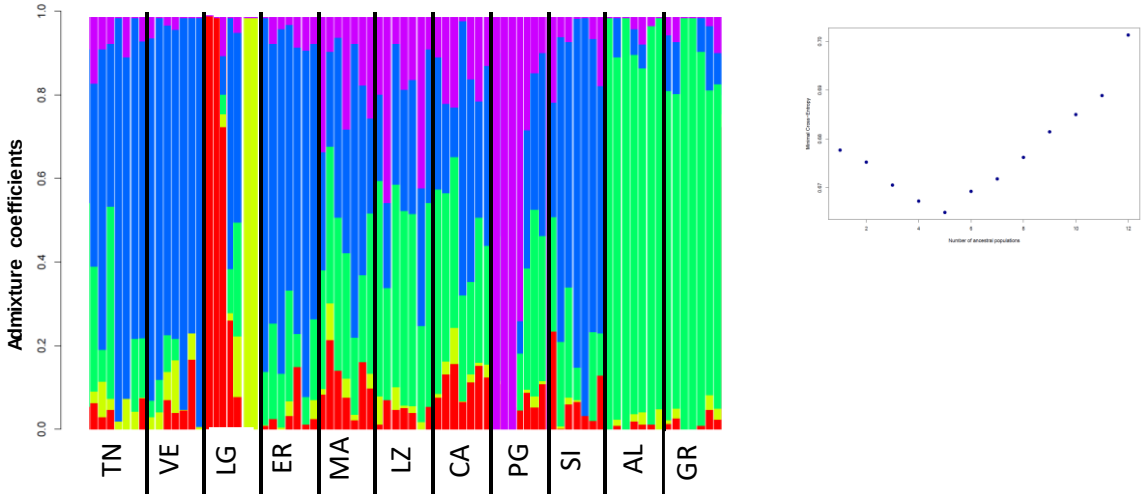
b)



Least-squares estimation approach

Results for the least-squares estimation approach implemented for the European dataset are shown in Figure 3.5. The best number of clusters identified was 5, as shown by the cross-entropy plot in Figure 3.5b. Even using this approach high levels of admixture can be observed; only populations from Greece (GR), Albania (AL) and Veneto (VE) show Q-values >0.75 for all the specimens; all other populations have varying levels of admixture with highest levels in Central- Italy (MA, LZ and CA).

Figure 3.5. Admixture graph obtained using a least-square-estimation approach for 77 specimens included in the European dataset (N SNPs=47,475 after LD filtering) at the left the clustering observed for best k=5; at the right side the cross-entropy criterion plot based on which the best number of clusters was chosen.



Discriminant Analysis of Principal Components (DAPC) and Principal Component Analysis (PCA)

DAPC results are shown in Figure 3.6 and 3.7 for the worldwide and the European dataset respectively. For the worldwide dataset (principal components retained according to a-score = 13) a situation similar to the one observed in ADMIXTURE can be observed when plotting the first two Discriminant Analysis (DA) axis. Japanese populations cluster closely together with the north-American ones while the South-East Asian populations cluster together with population from Africa and Brazil. Greek specimens cluster distinctly but appear to be more closely related to the S-E-Asian cluster. Albanian and Italian populations cluster closely together and distinct from all the other populations, although they appear to be more closely related with the USA-Japan cluster.

DAPC only on European populations (principal components retained according to a-score = 14) highlights a clear differentiation between specimens from the three countries (Figure 3.7). Within Italy a slight North-South differentiation, already observed in the ADMIXTURE and the LEA analysis can be observed, with the exception of the population from Sicilia which clusters closely with populations from northern Italy.

Results of DAPC analysis are confirmed widely by the PCA analysis shown in Figures 3.8 and 3.9 on the worldwide as well as the European dataset. Tracy – Widom test indicates significant signs of population structure ($p<0.05$) until PC 16 for the worldwide dataset and PC9 for the European one. For the European dataset also the PCA-plot for axes 2 and 3 is shown, highlighting the differentiation of specimens from Puglia and Liguria, already observed in clustering analysis performed using the least-square-estimation approach.

Figure 3.6. Discriminant Analysis of Principal Components (DAPC) for 29 *Ae. albopictus* populations. The graph represents the individuals as dots, Italian specimens are shown using a yellow gradient (light-yellow= North-Italy, dark-yellow= South-Italy)

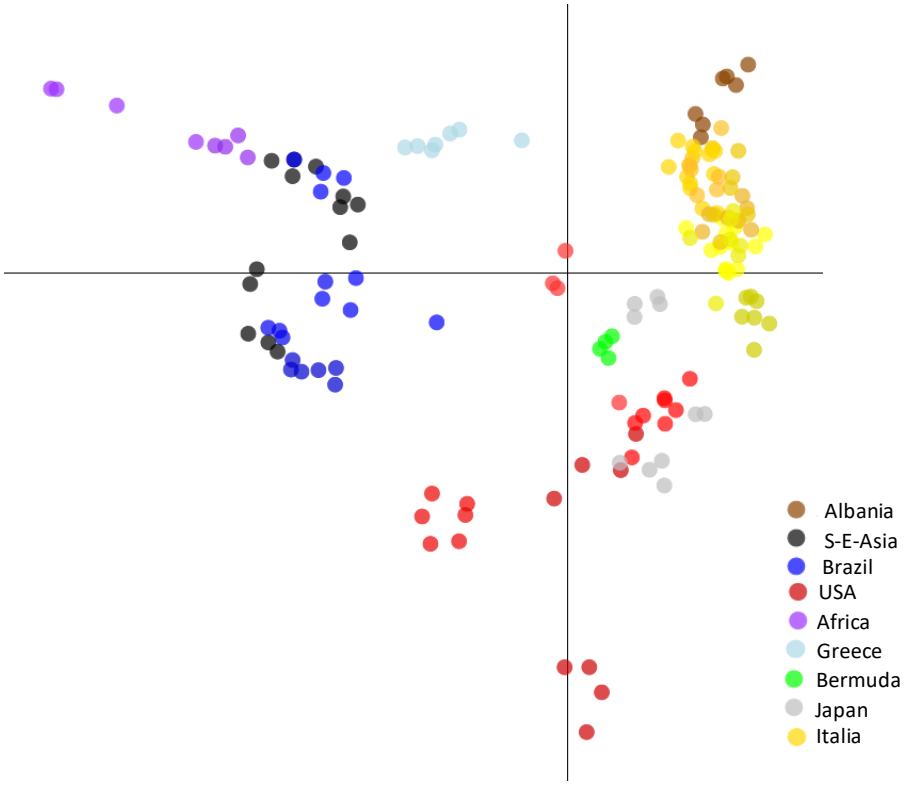


Figure 3.7. Discriminant Analysis of Principal Components (DAPC) for European *Ae. albopictus* populations (specimens = 77) on 103,289 SNPs. The graph represents the individuals as dots and the populations as inertia ellipses. A barplot of eigenvalues for the discriminant analysis (DA eigenvalues) is displayed in the inset.

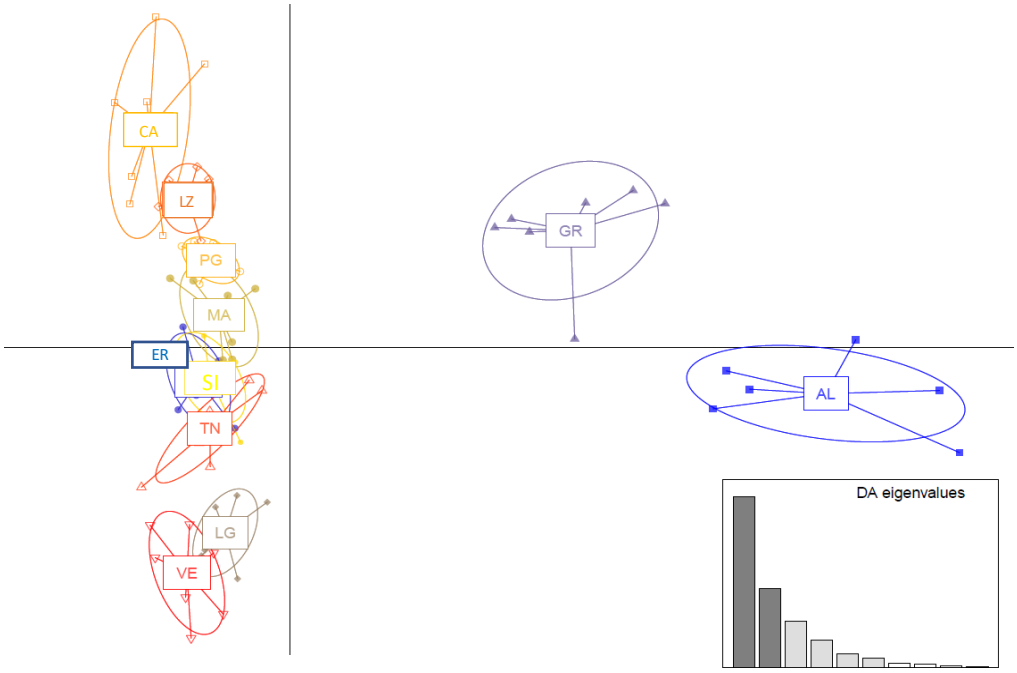


Figure 3.8. Principal Components Analysis (PCA) presenting the projection of all 156 *Ae. albopictus* specimens included in the worldwide dataset, (N=156) on the first two PCs.

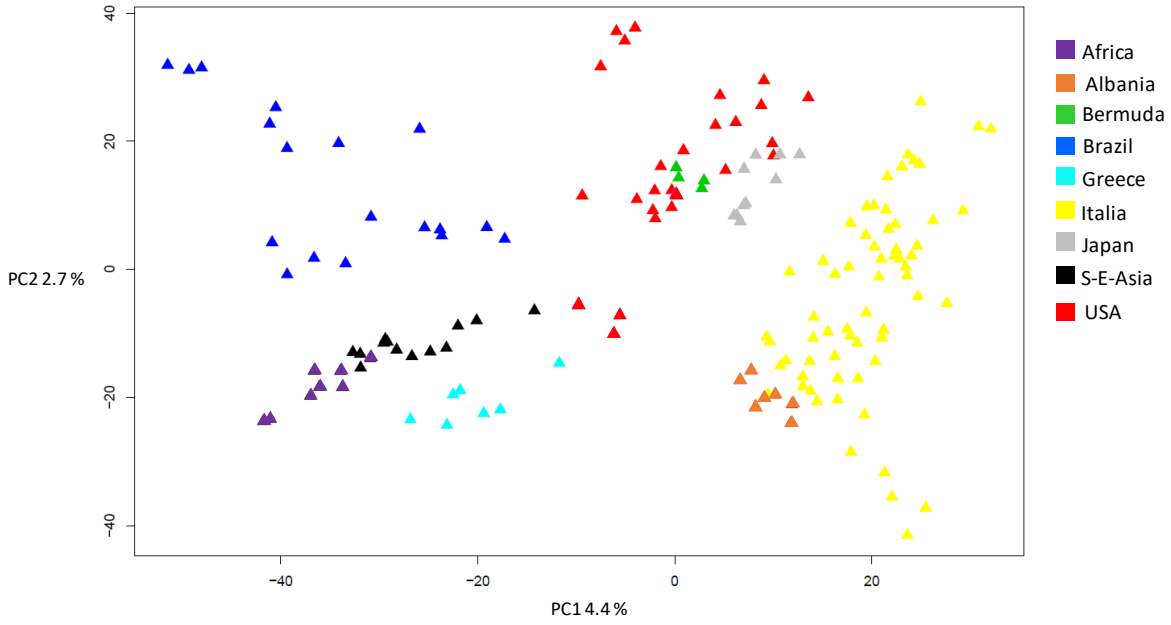
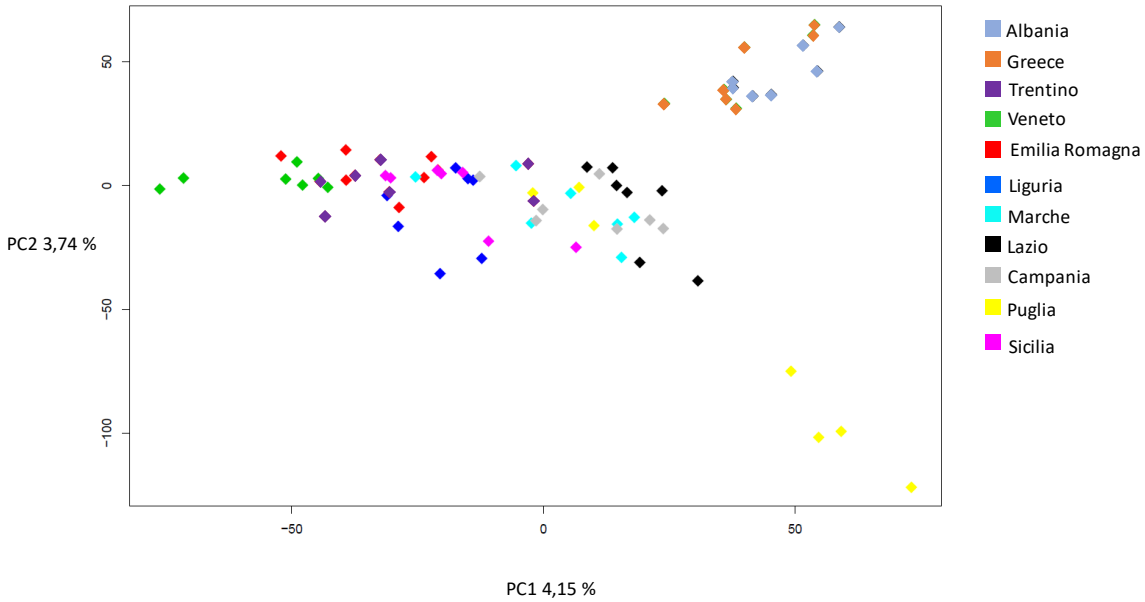
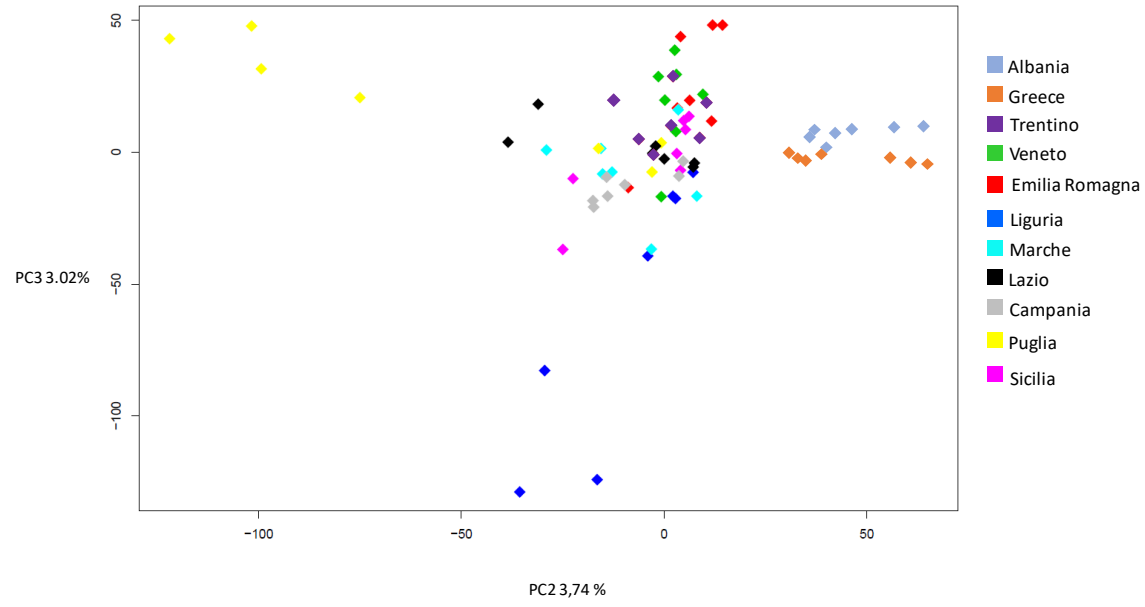


Figure 3.9. Principal Components Analysis (PCA) presenting the projection of all European *Ae. albopictus* specimens (N=77) on the first two PCs (a) and axes 2 and 3 in b).

a)



b)

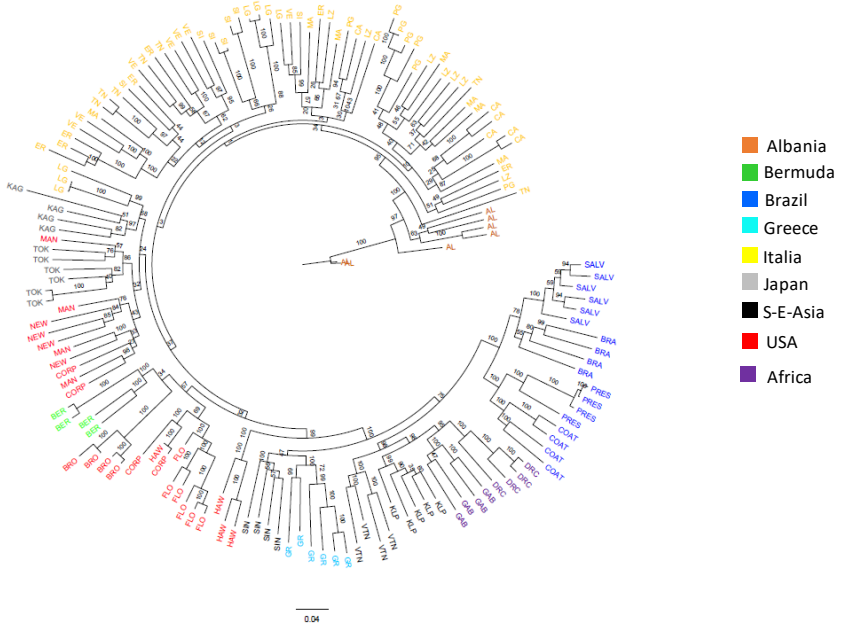


Maximum Likelihood tree reconstruction

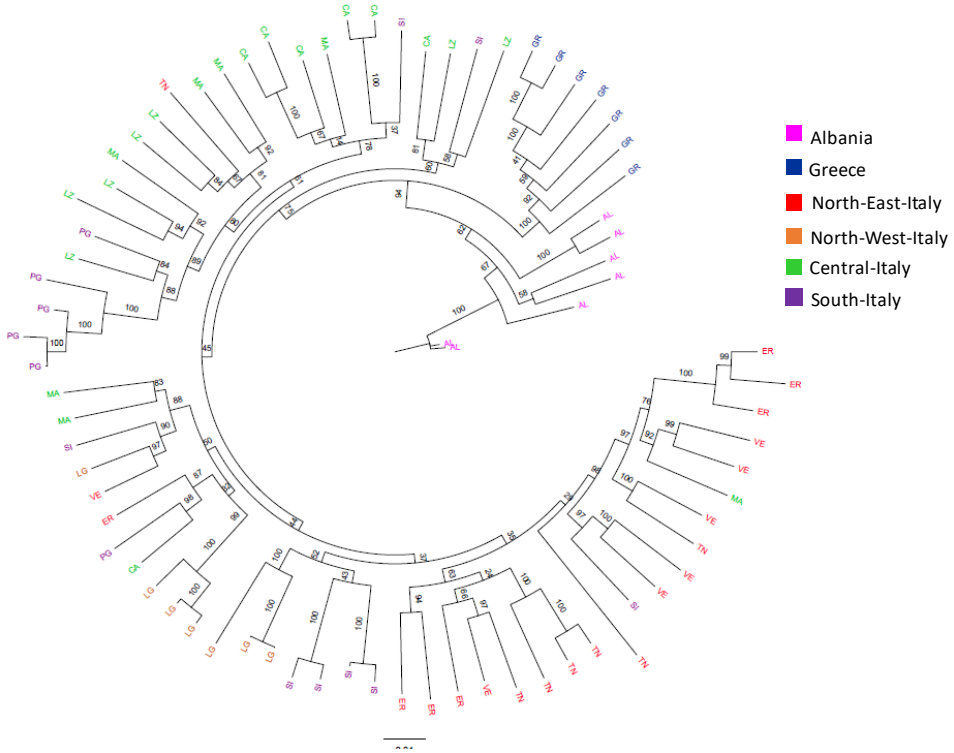
PCADAPT analysis identified 2568 outlier loci for the worldwide dataset and 5988 outliers for the European dataset which were excluded from the already LD filtered datset to construct an unrooted ML tree using RAxML, shown in Figure 3.10. The results confirm what already observed with previous analysis: on a worldwide scale Albanian specimens cluster with Italian populations and are more closely related to Japanese and North-American populations compared to populations from S-E-Asia or South-America, while Greek specimens cluster with the S-E-Asian populations. Within Europe clustering according to country is detected and missing bootstrap support for differentiation among Italian populations can be observed. As already in previous analysis a general trend of North-Center/South differentiation can be observed within Italy, with the exception of Sicilian specimens which appear to be closely related to specimens from North-Italy.

Figure 3.10. Maximum Likelihood unrooted phylogenetic trees re-constructed using a) 37,076 SNPs for the Worldwide dataset (N specimens= 156) and b) 41,487 SNPs for the European dataset (N specimens= 77). Bootstraps percentages are indicated on the nodes.

a)



b)



3.4. Discussion

Aedes albopictus has spread during the last few decades all across the world and has managed to colonize all continents except Antarctica. In the present work we tried to shed light on the spread of this mosquito focusing on Italy, the currently most heavily infested European country. The usage of a ddRADseq protocol allowed us to join our data on European samples with a worldwide dataset and to investigate relationships among populations on a global scale. A paper on results obtained for the worldwide dataset, presented partially herein, was accepted for publication by the journal "Ecology and Evolution" with the title "Population genomics of the Asian tiger mosquito, *Aedes albopictus*: insights into the recent worldwide invasion" by Kotsakiozi et al. (2017).

Marker - identification

The ddRAD approach we used was able to recover more than 40,000 SNPs from across the whole genome for the global dataset, with high consistency in marker recovery across specimens, also when library preparation was performed in different laboratories and libraries were sequenced in different moments, as in the present case. In fact, the use of two restriction enzymes during the ddRAD library preparation increases, as explained in paragraph 3.2, the possibility of retrieving the same fragments to be sequenced across all individuals and reduces the amount of missing data compared with other RADseq methods (Andrews et al. 2016; Peterson et al. 2012). This demonstrates also that the present approach will allow to combine data from different studies using the same protocol more easily compared to other markers (see paragraph 3.1). Moreover, the identified markers can be a baseline for the set-up of genotyping approaches which can be more readily scored (e.g. SNP-arrays).

Differentiation and invasion history

Worldwide

The successful genotyping of thousands of SNPs across the whole genome made it possible to detect significant population structure at a worldwide level, in the native as well as in the

invasive range, demonstrating the relative power of these genetic markers compared to microsatellites or mtDNA, used in recent studies (Manni et al. 2017, 2015; Porretta et al. 2012), which failed to detect genetic differentiation within the native range, thus limiting the possibility to assign the origin of the invasive samples.

Clustering analysis (Figure 3.4) performed on a worldwide scale revealed the existence of at least 3 different genetic groups worldwide (two of which in the native range). This result, supported also by PCA based analysis (Figure 3.6, 3.8) and the ML tree reconstruction (Figure 3.10), confirms what was suggested by allozyme studies (Urbanelli et al. 2000; Kambhampati, Black, and Rai 1991a), which have shown signs of differentiation between the southern insular populations and northern ones, as well as between western (India, Sri Lanka) and eastern populations of the native area, reflecting thus a differentiation due to ecophysiological traits, such as photoperiodic diapause and the cold-tolerance of eggs.

Aedes albopictus populations from North-America appear in our study closely related to Japanese populations, corroborating the hypothesis that Japan hosted the source population of the north-American invasion (Birungi and Munstermann 2002; Urbanelli et al. 2000). In addition, our analysis detects some signs of possible introgression: the population from Hawai, where *Ae. albopictus* has been reported already in the late 19th century (Kuno 2012; Rai 1991), shows important signs of genetic admixture which may be explained by a more complex invasion process with multiple introduction of different *Ae. albopictus* populations. More feeble signs of admixture can be observed also in the population from Florida, suggesting a possible second invasion from S-E-Asia with subsequent mating between the different source populations, although the retention of shared ancestral polymorphisms cannot be excluded.

South-American and African populations show instead closer genetic relationship with S-E-Asian populations, in agreement with previous studies which suggested separate invasion events for North- and South-America (Battaglia et al. 2016; Birungi and Munstermann 2002). Anyway, the origins of the South-American populations appear to be unclear. In fact, the second best result for ADMIXTURE clustering analysis (K=4, Figure 3.4b), as well as the phylogenetic analysis (Figure 3.10) support the existence of a separate, well defined, cluster including only specimens from South-America. This suggests that these populations derived from a single invasion event from a native population which probably was not sampled for the present study. The S-E-Asian origin of African populations instead confirms what was suggested by Kamgang et al. using COI and microsatellites (Kamgang, Brengues, et al. 2011), even if more recent

studies on specimens from Central African Republic (Kamgang et al. 2013) highlighted the relatedness of samples with both, tropical and temperate populations, thus suggesting multiple sources of introductions. Our data do not support this latter scenario, anyway this might be due to the low number of African specimens included.

F*ST* values between each pair of populations indicate that the degree of differentiation within the invasive or native range is not very different from what observed when comparing populations from the invasive with those from the native range (Table 3.8), suggesting the absence of great differentiation between ancestral and derived populations.

Europe

Our results suggest that Europe has experienced at least three different invasions; in fact, all the three clusters identified at a global scale were detected also within Europe.

Albania, the country where *Ae. albopictus* was detected first in Europe (Adhami and Reiter 1998), is assigned in the ADMIXTURE analysis to a cluster completely missing in the native range, and this result was supported also by the ML tree reconstruction. This corroborates results of Manni et al. (2017) which suggested that Albania was invaded by Chinese populations which were unfortunately not sampled for the present study.

For Italy a much more complicated colonization process with at least two different source populations (Figure 3.4) can be hypothesized based on our data. In fact, Italian populations appear to be related not only to the USA-Japan cluster, as already observed by Urbanelli et al. (1990) and Birungi & Munstermann (2002), but also to the Albanian cluster. Moreover, a large part of the Italian specimens shows signs of admixture, with Q-values >0.75, suggesting a complex invasion history (see below).

Greece, where first reports of *Ae. albopictus* have been made only in 2003 (Samanidou-Voyadjoglou et al. 2005), appears to have undergone a completely distinct invasion history and shows a clear genetic relationship with populations from S-E-Asia in all the analysis we performed, as already hypothesized by Manni et al (2017). In addition, we detect also some signs of admixture which may be explained by some introgression between populations from the two bordering countries, Albania and Greece, although other scenarios, including ancestral polymorphisms and invasion of Greece by the same source population as Albania cannot be excluded.

Generally, genetic differentiation (F*ST*-values in Table 3.7) among European populations is low but appears to be coherent with results obtained using ML- and PCA-based approaches, and, together with differences in heterozygosity and the number of private alleles (Table 3.4), reflects the different invasion histories of the three countries examined. Also, the significant signs of IBD (Figure 3.3) within Europe may be explained by differences in source populations between Albania, Greece and Italy, rather than a progressive differentiation along with geographic distance. Most previous studies in fact, revealed a lack of genetic structure according to geography (Goubert et al. 2016), possibly due to *Ae. albopictus'* low natural dispersion capabilities (Marini et al. 2010).

Italy

Focusing on Italy, low genetic differentiation is observed as shown not only by F*ST* values, but also by the missing bootstrap support in the ML tree reconstruction (Figure 3.10) and the identification of a single cluster in the ADMIXTURE approach (not shown). Despite this, some signs of differentiation between sampling sites where identified and our data confirm that Italy has experienced a complex invasion history, as suggested previously (Manni et al. 2017; Zhong et al. 2013; Urbanelli et al. 2000). This is supported by signs of genetic admixture in several Italian samples (Figure 3.4 and 3.5), which may be explained by crosses between different source populations, as well as the presence of two populations (LG, PG) with specimens which appear to be highly differentiated from other Italian samples (Figure 3.5 and 3.9). This local differentiation could be a consequence of local inbreeding (see below) or of the existence of further source populations which we are missing in the present dataset. Indeed, Manni et al (2017) suggests a close relationship between some specimens from northern Italy with populations from La Réunion, which was invaded during the 18th century, probably by populations from S-E-Asia. The presence of several different invading populations in Italy can thus have increased genetic diversity and adaptive potential of the established populations (Kolbe et al. 2004).

As for the European dataset, slight signs for IBD (Figure 3.3) were detected within Italy, probably due to some north-south differentiation which can be observed also when considering the ADMIXTURE graph (Figure 3.4) which shows an increasing presence of the Albanian-like cluster in central/south-Italy while in northern Italy the USA/Japan-like cluster increases. A similar pattern was detected also using the least-square-estimation clustering approach, as well

as PCA based methods and can be explained by human-aided spread, maybe by cars, which play an important role in dispersal on a reduced geographic scale, as proved by Medley et al. (2015), while natural dispersal may contribute to a lesser extent to the mosquitoes' spreading. The observed north-south pattern could also have alternative, mutually not exclusive, explanations such as differences in source populations between northern and southern Italy, and/or differences in diapausing tendencies between populations, as already observed elsewhere (Hanson and Craig 1994). Anyway, this last hypothesis seems to be contradicted by the southern-most Italian population included in the study (SI), which appeared to be more similar to northern populations compared to other central-south populations (see Figure 3.4, 3.5, 3.10).

Genetic diversity

It is commonly supposed that invasion processes go along with bottlenecks and a clear reduction in genetic diversity, however, influence on genetic diversity is modulated and can be counterbalanced by several factors such as the number of invaders and the frequency of introduction (that is, the propagule pressure) (Bock et al. 2015; Lawson Handley et al. 2011; Dlugosch and Parker 2008). In *Ae. albopictus* almost no studies, except the one of Kambhampati et al. (1991) on Brazilian populations did find clear support for strong founder effects. The present study confirms this: differences among observed heterozygosities appear to be significant at a worldwide scale (Figure 3.2) but don't seem to be related to bottlenecks during invasion events but rather to differences in source populations, and also focusing on European samples, heterozygosity appears to be similar (or even higher) to values observed in the possible source populations (Table 3.3). Medley et al. (2015), who obtained similar results for North-American populations, proposed that repeated and possibly massive introductions may have helped to maintain such high genetic variability.

Several studies on *Ae. albopictus* population structure (Manni et al. 2015; Zhong et al. 2013; Kambhampati, Black, and Rai 1991b; Black et al. 1988) in the native as well as the invasive range and using different types of markers, observed that the largest part of genetic variation is observed at the lowest hierarchical level, often defined as the variation between individuals within a population, while differentiation among groups or populations in groups is comparatively low. More recent studies revealed anyway that this so-called high local

differentiation may be due to a lack of variation at the intra-individual level (i.e. F*IS;* Manni et al. 2015; Delatte et al. 2013) that corresponds in a hierarchical AMOVA to the covariance of alleles of a given locus within individuals of the same population. The same pattern is observed also in the present study (see Table 3.5) where the individual level represents almost 70% of the genetic variance, suggesting, along with the high F*IS* values observed (see Table 3.4), a high rate of inbreeding at a local scale.

We cannot exclude that the F*IS*-values we observed were also inflated by the possible presence of siblings in the genotyped samples (although we tried to avoid this by using several ovitraps per sampling site, see M&M), anyway the repeated observation of high intra-individual genetic covariance or significant F*IS* values across different studies, suggests that *Ae. albopictus* populations share this pattern globally, with high genetic drift accompanying the establishment of very local populations (i.e. individuals found in a given sampling site), which exhibit low dispersion rate and restricted gene flow. A typical population of *Ae. albopictus* would thus be a network of interconnected breeding sites, each having a high level of inbreeding (Goubert et al. 2016).

Conclusions

The usage of genome-wide SNP-markers allowed us to reveal the existence of genetic structure in the Asian tiger mosquito at a global as well as at a more local, European scale. This was possible despite the availability of a limited number of samples from the native range; adding further populations from the native range might allow in future studies the detection of further differentiation within this range and thus a more detailed reconstruction of invasion histories.

The evolutionary scenario emerging from the present study can be defined as highly complex, with several independent introductions and the absence of strong founder effects, resulting in large and highly variable populations in the invasive range. In Italy, the presence of an extremely long coastline and thus several major ports, together with a possible lack in control efforts, has probably facilitated these repeated and possibly massive introductions of *Ae. albopictus*, with local differentiation and admixture between different source populations increasing genetic variability and evolutionary potential of the established populations.

The present findings can also have relevant implications on control and monitoring efforts of *Ae. albopictus*. Indeed, the suggested origin of the Greek population from S-E-Asia, where

insecticide resistant *Ae. albopictus* populations have been reported several times, could offer a possible explanation for the differences in susceptibility to pyrethroid insecticides between populations from Greece and Albania/Italy, described in chapter 2. A deeper knowledge on the origin of invasive populations could thus provide valuable help for planning effective control measures.

4.Overall Conclusions

Aedes albopictus represents a striking example of a fast and extremely successful invasive species and gives the opportunity to study in real-time the mechanisms which allowed adaptation to new ecological contexts, the possible links between genetic diversity and invasion success and its impact on resident mosquito species, as well as on human wellbeing. Moreover, the recent outbreak of tropical Chikungunya virus in Central Italy has further highlighted the public health importance of the species also in temperate regions and the need of an increased research effort to understand the dynamics of the invasion process, to anticipate public health risks and to optimize control strategies.

The present PhD thesis tried to give a concrete contribution to a better knowledge of the species focusing on Italy, the most heavily infested country in Europe. The usage of genome-wide SNP markers allowed to investigate the population structure and the worldwide spread of *Ae. albopictus* and showed the existence of a complex invasion history, suggesting that high propagule pressure sustained the introduction of the species in Europe and that subsequent admixture events may have helped to maintain a high genetic diversity in Italian populations. The typical breeding structure of the species, confirmed by high inbreeding coefficients within samples, suggests that we are observing a species with high differentiation at a very small geographical scale. This population structure too, might allow the species to maintain a high adaptive potential at a local level.

In addition, our functional study on insecticide resistance allowed us to detect for the first time signs of resistance to commonly used pyrethroids in some Italian *Ae. albopictus* populations exposed to prolonged strong selective pressure by the implementation of pyrethroids to reduce the nuisance. The genetic relationships detected among European samples and populations from the native range offer a possible explanation for some of the observed differences in insecticide susceptibility. The results also highlight the need of a continuous monitoring to avoid the spread and further insurgence of resistance phenotypes which could significantly lower the effectiveness of insecticide based control interventions, which represent the only available tool to control the spread of *Ae. albopictus*-borne arbovirus autochthonous transmission after introduction from endemic countries.

Overall, this PhD thesis highlights the importance of combining genetic and functional studies to obtain a more complete picture of the invasion process and of its possible consequences on public health and on the effectiveness of vector control measures.

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7. Appendix

7.1. Sequence alignment

Sequence alignment in the mega-format of approximate 600 bp of the COI region of 16 Italian *Ae. albopictus* specimens included in the present study as well as 2 specimens from North-America and from Japan available at the Barcode of Life Database (Ratnasingham and Hebert 2007). After trimming of low quality bases at the extremes of the sequences, alignment has been performed using the ClustalW algorithm (Thompson, Higgins, and Gibson 1994) in MEGA 7 (Kumar, Tamura, and Nei 1994).

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TT----------

7.2. Reconstruction of population invasion history

To infer the possible invasion history of Italian *Ae. albopictus* populations, competing scenarios regarding population divergence at a global scale were defined and simulated using an Approximate Bayesian Computation (ABC) method. Simulations as well as computation of summary statistics for the competing scenarios were obtained using the software package DIYABC v2.0 (Cornuet et al. 2015), and the best scenario was chosen based on the random forest (RF) criterion implemented in the R-package abcrf (Pudlo et al. 2016), which, compared to traditional ABC approaches reduces the model classification error (prior error rate) and is robust to the number and choice of summary statistics, even if superfluous or strongly correlated statistics are included. Moreover, according to Pudlo et al (2016) and Fraimout et al. (2017) the RF approach provides a more efficient discrimination among models and a better estimation of posterior probability, while the computing effort is strongly reduced.

Analysis were performed on the LD filtered worldwide dataset. Due to computational limits, it was necessary to subset the whole SNP-dataset into 5 random sets of 4000 SNPs. Simulation were repeated for at least 3 of these subsets and results compared among them. Populations were grouped together according to geographical criteria and previous knowledge on the European invasion history. We defined thus two groups for the native range (Japan and South-East-Asia) as well as 5 groups outside Italy (Africa, Brazil, USA, Albania Greece). As in Fraimout et al. (2017) ABC modelling was carried out including the most differentiated populations per group (based on F*ST* estimates) when more than 3 populations were available (Table 7.1).

Table 7.1. Populations used for each group in ABC analysis. Only population for which at least two populations were grouped together are listed. For sample codes see Table 2.1.

group	samples
S-E-ASIA	VTN + KLP+ SIN
USA	BRO+NEW+FLO
Japan	KAG+TOK
Brazil	BRA+COAT+PRES

A preliminary analysis on the worldwide dataset was performed to establish the best number of trees to construct in RF approach. Prior error plots for different number of trees (N=500, 1000, 2000) have been drawn and number of 1000 trees appeared to be sufficient for a robust scenario

choice (Fraimout et al. 2017). According to Pudlo et al. (2016) and Fraimout et al. (2017) a number of 10,000 simulations per scenario should be enough for scenario choice, but to be more conservative between 20,000 and 30,000 simulations were made per competing scenario and datasets were summarized using the whole set of summary statistics proposed by DIYABC.
A step by step approach was implemented starting from the putative ancestral area to more recently invaded regions. Scenarios were defined considering historical knowledge of the first record in invaded countries and the data obtained from population genetic analyses (i.e., population structure and ancestry). When more than 6 scenarios had to be tested, two different runs, one without admixture and one with only admixture -based scenarios were performed and the best-suited scenarios of each of the two runs were then compared. In a first set of analysis source populations from the same continent were not taken into account: these populations were included afterwards to verify if population in the invasive range were acting as bridge-heads. The competing scenarios were set up using prior definitions and distribution of demographic parameters, as described in Table 7.2 with parameters kept broad when no prior information was available. This is the case for the effective population sizes, for which broad priors (500–100,000) were maintained. The time ranges (expressed as numbers of generations and considering around 5 - 17 generations/year, Medlock et al. 2015) were defined based on historical data, if available.

Table 7.2. Priors used for historical parameters in scenario simulations

historical parameters	min	max
effective population size	500	100,000
duration(gen)of bottleneck	10	100
bottlenecked population size	10	1000
admixture rate	0.001	0.999

Unfortunately for all the different populations examined (defined as target population in Table 7.3) according to this approach the best scenario for all the different target populations was an admixture event of the two native samples (Japan and S-E-Asia) and thus no further information was obtained. This result can be explained by a generally low genetic differentiation of populations (as shown also by low F*ST* values), the lack of further samples from the native range as well as the complex scenario which might have led to highly admixed invasive populations (as shown in paragraph 3.3). Also, as described in paragraph 3.3, populations may undergo strong differentiation processes at a very local scale and thus their provenience might

be more difficult to track. In addition, computational restrictions did not allow us to use the whole SNP-dataset all together and thus we might have lost part of the discriminative power of our marker-set.

Table 7.3. **Description of ABC models and results**. SNP-sets indicates the subset of SNPs used for the analysis, target population the invasive population investigated and source populations the invasion scenarios with '+' indicating admixture between populations. The number of compared scenarios, simulations (sim) per scenario as well as the posterior probability (post.prob.) and the prior error rate are reported. The last column summarizes the overall result when comparing results obtained for one target populations using different SNP-sets and runs.

SNP-set	target populations	source populations	N of scenarios	sim/scenario	best model	post. prob.	prior error rate (%)	overall result
1	Hawai	sea, jap, sea+jap	3	20000	sea+jap	0.527	8.0	
2	Hawai	sea, jap, sea+jap	3	20000	sea+jap	0.515	8.2	
3	Hawai	sea, jap, sea+jap	3	20000	sea+jap	0.581	7.7	
4	Hawai	sea, jap, sea+jap	3	20000	sea+jap	0.492	7.8	
5	Hawai	sea, jap, sea+jap	3	20000	sea+jap	0.543	8.1	SEA+JAP
1	USA	hawai, sea, jap, sea+jap, hawai+sea, hawai+jap	6	20000	admixture jap+sea	0.661	2.4	
2	USA	hawai, sea, jap, sea+jap, hawai+sea, hawai+jap	6	20000	admixture jap+sea	0.715	2.4	
3	USA	hawai, sea, jap, sea+jap, hawai+sea, hawai+jap	6	20000	admixture jap+sea	0.653	2.3	
4	USA	hawai, sea, jap, sea+jap, hawai+sea, hawai+jap	6	20000	admixture jap+sea	0.772	1.9	
5	USA	hawai, sea, jap, sea+jap, hawai+sea, hawai+jap	6	20000	admixture jap+sea	0.875	9.2	SEA+JAP
1	Albania	usa, sea, jap, jap+sea, sea+usa, jap+usa	6	20000	admixture jap+sea	0.633	2.3	
2	Albania	usa, sea, jap, jap+sea, sea+usa, jap+usa	6	20000	admixture jap+sea	0.666	0.7	
3	Albania	usa, sea, jap, jap+sea, sea+usa, jap+usa	6	20000	admixture jap+sea	0.626	0.9	
4	Albania	usa, sea, jap, jap+sea, sea+usa, jap+usa	6	20000	admixture jap+sea	0.554	0.7	
5	Albania	usa, sea, jap, jap+sea, sea+usa, jap+usa	6	20000	admixture jap+sea	0.598	0.8	SEA+JAP
1	Brazile	usa, sea, jap, jap+sea, sea+usa, jap+usa	6	20000	admixture jap+sea	0.506	9	
2	Brazile	usa, sea, jap, jap+sea, sea+usa, jap+usa	6	20000	admixture jap+sea	0.613	7.4	
3	Brazile	usa, sea, jap, jap+sea, sea+usa, jap+usa	6	20000	admixture jap+sea	0.574	5.6	
4	Brazile	usa, sea, jap, jap+sea, sea+usa, jap+usa	6	20000	admixture jap+sea	0.551	10.3	
5	Brazile	usa, sea, jap, jap+sea, sea+usa, jap+usa	6	20000	admixture jap+sea	0.692	4.8	SEA+JAP
5	Veneto	usa, sea, jap, bra	4	20000	usa	0.603	8	
5	Veneto	jap+sea, sea+usa, jap+usa	3	20000	admixture jap+sea	0.53	7	
5	Veneto	usa, jap+sea	2	30000	admixture jap+sea	0.737	1	
5	Veneto	alb, jap+sea	2	30000	admixture jap+sea	0.702	2	
4	Veneto	usa, sea, jap, bra	4	20000	usa	0.631	19.7	
4	Veneto	jap+sea, sea+usa, jap+usa	3	20000	admixture jap+sea	0.511	22.1	
4	Veneto	usa, jap+sea	2	30000	admixture jap+sea	0.788	4.35	
4	Veneto	alb, jap+sea	2	30000	admixture jap+sea	0.771	6.6	
1	Veneto	usa, sea, jap, bra	4	20000	usa	0.565	20.3	
1	Veneto	jap+sea, sea+usa, jap+usa	3	20000	admixture jap+sea	0.543	21.1	
1	Veneto	usa, jap+sea	2	30000	admixture jap+sea	0.774	3.1	
1	Veneto	alb, jap+sea	2	30000	admixture jap+sea	0.746	5.3	SEA+JAP
5	Puglia	usa, sea, jap, bra	4	20000	usa	0.632	18.5	
5	Puglia	jap+sea, sea+usa, jap+usa	3	20000	admixture jap+sea	0.535	20.3	
5	Puglia	usa, jap+sea	2	30000	admixture jap+sea	0.726	5.2	
5	Puglia	alb, jap+sea	2	30000	admixture jap+sea	0.747	5.0	
4	Puglia	usa, sea, jap, bra	4	20000	usa	0.582	14.98	
4	Puglia	jap+sea, sea+usa, jap+usa	3	20000	admixture jap+sea	0.518	20.6	
4	Puglia	usa, jap+sea	2	30000	admixture jap+sea	0.795	3.4	
4	Puglia	alb, jap+sea	2	30000	admixture jap+sea	0.806	5.1	
1	Puglia	usa, sea, jap, bra	4	20000	usa	0.594	20.8	
1	Puglia	jap+sea, sea+usa, jap+usa	3	20000	admixture sea+usa	0.529	27.6	
1	Puglia	usa, usa+sea	2	30000	admixture sea+usa	0.74	4.9%	
1	Puglia	alb, usa+sea	2	30000	admixture sea+usa	0.791	2.6	SEA+JAP
5	Liguria	usa, sea, jap, bra	4	20000	usa	55.9	17.3	
5	Liguria	jap+sea, sea+usa, jap+usa	3	20000	jap+sea	0.5383333	20.4	
5	Liguria	usa, jap+sea	2	30000	admixture jap+sea	0.81	4.4	
5	Liguria	alb, jap+sea	2	30000	admixture jap+sea	0.7458	4.9	
4	Liguria	usa, sea, jap, bra	4	20000	usa	61.24	23.6	
4	Liguria	jap+sea, sea+usa, jap+usa	3	20000	jap+sea	0.5503333	20.4	
4	Liguria	usa, jap+sea	2	30000	admixture jap+sea	71.51	3.8	
4	Liguria	alb, jap+sea	2	30000	admixture jap+sea	0.7338333	4.9	
1	Liguria	usa, sea, jap, bra	4	20000	usa	56.2	19.9	
1	Liguria	jap+sea, sea+usa, jap+usa	3	20000	jap+sea	0.5046167	20.2	
1	Liguria	usa, jap+sea	2	30000	admixture jap+sea	0.7108	6.3	
1	Liguria	alb, jap+sea	2	30000	admixture jap+sea	0.7458	4.9	SEA+JAP
5	Lazio	usa, sea, jap, bra	4	20000	usa	0.51	21.0	
5	Lazio	jap+sea, sea+usa, jap+usa	3	20000	jap+sea	0.5505	20.2	
5	Lazio	usa, jap+sea	2	30000	admixture jap+sea	0.74	4.5	
5	Lazio	alb, jap+sea	2	30000	admixture jap+sea	0.73525	5.0	
4	Lazio	usa, sea, jap, bra	4	20000	usa	0.58	19.2	
4	Lazio	jap+sea, sea+usa, jap+usa	3	20000	jap+sea	0.5412667	19.4	
4	Lazio	usa, jap+sea	2	30000	admixture jap+sea	0.77	5.5	
4	Lazio	alb, jap+sea	2	30000	admixture jap+sea	0.70055	5.8	
1	Lazio	usa, sea, jap, bra	4	20000	usa	0.58	23.2	
1	Lazio	jap+sea, sea+usa, jap+usa	3	20000	jap+sea	0.5194333	26.9	
1	Lazio	usa, jap+sea	2	30000	admixture jap+sea	0.72	2.1	
1	Lazio	alb, jap+sea	2	30000	admixture jap+sea	0.7346833	4.9	SEA+JAP
5	Greece	usa, sea, jap, bra	4	20000	usa	0.61	25.7	
5	Greece	jap+sea, sea+usa, jap+usa	3	20000	jap+sea	0.5202667	20.4	
5	Greece	usa, jap+sea	2	30000	admixture jap+sea	0.73	5.0	
5	Greece	alb, jap+sea	2	30000	admixture jap+sea	0.7691667	4.8	
4	Greece	usa, sea, jap, bra	4	20000	usa	0.59	21.6	
4	Greece	jap+sea, sea+usa, jap+usa	3	20000	jap+sea	0.53295	20.4	
4	Greece	usa, jap+sea	2	30000	admixture jap+sea	0.80	1.8	
4	Greece	alb, jap+sea	2	30000	admixture jap+sea	0.78265	4.9	
1	Greece	usa, sea, jap, bra	4	20000	usa	0.58	22..6	
1	Greece	jap+sea, sea+usa, jap+usa	3	20000	jap+sea	0.5645333	20.3	
1	Greece	usa, jap+sea	2	30000	admixture jap+sea	0.72	5.3	
1	Greece	alb, jap+sea	2	30000	admixture jap+sea	0.74495	4.9	SEA+JAP