Research Article

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Phenotypic and genotypic pyrethroid resistance of *Aedes albopictus*, with focus on the 2017 chikungunya outbreak in Italy

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Abstract

BACKGROUND: The highly invasive mosquito species *Aedes albopictus* has become a major health concern in temperate areas due to its role as vector of exotic arboviruses. Pyrethroid insecticides represent the main tools for limiting the circulation of such mosquito-borne viruses. The present work aim to extend previous reports on phenotypic pyrethroid-resistance in European *Ae. albopictus*, to identify its genetic basis and to monitor the geographical distribution of resistant genotypes, with a particular focus on sites experiencing the 2017 chikungunya outbreak in Italy.

RESULTS: Bioassays, performed according to World Health Organization protocols, showed full susceptibility to deltamethrin (concentration = 0.05%) and varying levels of resistance to permethrin (0.75%) and/or α-cypermethrin (0.05%) across Italy, with highest levels in the core of the 2017 chikungunya outbreak. Partial genotyping of the *VSSC* gene revealed widespread distribution of V1016G mutation and confirmed its association with pyrethroid resistance.

CONCLUSION: The results obtained show that the condition for the spread of pyrethroid resistance in *Ae. albopictus* in Europe exists under strong selective pressure due to intensive insecticide spraying to control exotic arbovirus outbreak or high levels of nuisance. The results draw attention to the need for an evidence-based implementation of mosquito nuisance control, taking insecticide resistance management into consideration.

Supporting information may be found in the online version of this article.

Keywords: insecticide resistance; mosquito; arbovirus vector; Europe

1 INTRODUCTION

Mosquitoes are the deadliest animals in the world due to their ability to transmit life-threatening diseases caused by viruses, protozoa and helminths, responsible for millions of deaths and hundreds of millions of debilitating and economically damaging illnesses every year.[1] *Anophelinae* are vectors of deadly malaria parasites, while *Culicinae* are vectors of arboviral diseases such as yellow fever, dengue, chikungunya and zika (*Aedes*) or West Nile disease and Japanese encephalitis (*Culex*). Incidence of arboviral diseases is significantly increasing due to the global movement of infected humans and the expanded distribution of vector species. The worldwide incidence of dengue, for instance, has risen 30-fold in the past three decades, and several countries are reporting their first outbreaks of the disease.[1]

Most arboviruses are not endemic in temperate regions because climatic parameters do not support the establishment of their primary vector, *Aedes aegypti*, whose distribution is mostly limited to tropical regions. However, in recent decades, *Aedes albopictus*, a secondary vector species native of Southeast Asia, has expanded its distribution worldwide thanks to the production of diapausing

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eggs capable of surviving the winter months and has stably colonized temperate urban and suburban habitats in Europe and North America.[2–6] In Mediterranean areas, *Ae. albopictus* is becoming a major public health concern[7] and has already led to several autochthonous cases of dengue in the last few years,[8,9] as well as two relevant chikungunya outbreaks. Both of these occurred in Italy, with >200 infected human cases in Emilia Romagna (North East Italy) in 2007[10,11] and ~500 cases in Lazio (Central Italy) and Calabria (South Italy) 10 years later.[12,13]

In tropical endemic regions, vector control by insecticides (especially by indoor residual spraying or the usage of impregnated bednets) coupled with larval source reduction is absolutely critical in the prevention and control of mosquito-borne diseases. Increasing levels of phenotypic and genotypic insecticide resistance (IR) in the major vector species (i.e. *Anopheles* vectors of malaria, *Ae. aegypti* and *Culex quinquefasciatus*), however, are creating a major health concern[14–16] as they risk reducing the efficacy of the main 'weapons' available today to control mosquito-borne diseases. To prevent this, the World Health Organization (WHO) has drafted guidelines to monitor IR in major vector species and to contrast its insurgence and spread.[17–19]

Pyrethroid insecticides are widely used for adulticide treatments and are the only chemicals allowed in Europe against mosquitoes,[20–22] the most commonly used being α-cypermethrin, permethrin and deltamethrin. In contrast with the extensive knowledge on IR in major tropical mosquito vector species, knowledge on pyrethroid resistance (PR) in *Ae. albopictus* is still fragmented.[14,23,24] Moreover, it is difficult to compare results from different studies, as no specific guidelines nor official *Ae. albopictus* reference colonies exist to date. So far, PR has been reported in only a few adult populations from native range in Southeast Asia,[25–28] as well as in few populations in the invasive range, in particular in the Indian subcontinent[29–31] and in Africa.[32–34] The few studies conducted on PR in *Ae. albopictus* from temperate areas have mostly reported full susceptibility,[14,24,35,36] while first evidence of resistance to one or the other compound was recently observed in Spain,[37] Italy[38] and the USA.[39,40]

Resistance mechanisms have been extensively studied in major tropical vector species, but little information is available on mechanisms underlying pyrethroid resistance in *Ae. albopictus*. Two major mechanisms causing IR in *Aedes* mosquitoes have been identified: increased levels/activity of detoxification enzyme(s) and reduced target-site sensitivity.

Cytochrome P450s are involved in the metabolism and detoxification of a wide range of compounds and members of subfamilies CYP6 and CYP9 appear to play important roles in PR in *Ae. aegypti*.[14,41,42] Also, the few studies performed on metabolic resistance in *Ae. albopictus* suggest a role for some enzymes of the CYP6 subfamily in PR,[43] although this association still needs to be validated across different geographical regions.

Reduced target-site sensitivity to pyrethroids is caused by mutations in the voltage-sensitive sodium channel (VSSC), a membrane protein fundamental in the transmission of electrical signals in the nervous system. Pyrethroids modify the VSSC gating kinetics[44] by slowing both the activation and/or inactivation of the channel and thus the correct signal transmission. Amino acid substitutions limiting the interaction of a pyrethroid with the VSSC (commonly known as *knockdown resistance* or simply *kdr*[45]) reduce susceptibility to insecticides and are widespread in many mosquito species.[46] Several such substitutions have been reported in *Ae. aegypti* with varying effects on pyrethroid susceptibility.[14,47] The most common ones are mutations F1534C and two different substitutions in position 1016 (V1016G and V1016I[14]). For *Ae. albopictus* only a few studies have been carried out[14,48]: the first *kdr* allele, F1534C, was reported in Singapore in 2011[49] and later in China,[50] Greece[51] and Brazil,[52] and two other substitutions in the same position have also been identified (F1534L[51,53] and F1534S[50,51]). A further non-synonymous mutation in position 1532 (I1532T) was observed by Xu *et al.*[51] in Italian specimens, while very recently Kasai *et al.* (in press) detected a new substitution in position 1016 of the VSSC in Vietnam and Italy, and confirmed that in *Ae. albopictus* the V1016G mutation confers much higher levels of PR than F1534C and F1534S.

The increasing risk of exotic arbovirus outbreaks vectored by *Ae. albopictus* in Europe – revealed by the 2017 chikungunya epidemics in Italy – raises concern about the PR status of local populations, since pyrethroids are the only weapons recommended in the case of autochthonous arbovirus transmission nuisance.[54,55] The need to monitor and better understand phenotypic PR as well as its genetic basis in *Ae. albopictus* has been recognized by WHO[56] as well as by the recently established Worldwide Insecticide Resistance Network.[57,58] Indeed, it is likely that PR is more widespread than presently revealed by the few pyrethroid-resistant *Ae. albopictus* populations detected so far in Europe.[37,38] In fact, populations in temperate regions have been subjected in the past to selective pressure due to the patchy and uncontrolled, yet not neglectable, use of insecticide-based space spraying by private citizens and public administrations to achieve immediate and tangible, even though short-term, effects on mosquito nuisance.[59–62]

The present study aimed to extend previous reports of phenotypic resistance to pyrethroid insecticides in *Ae. albopictus* from Europe/Mediterranean areas,[38] to identify its possible genetic basis and to monitor the geographical distribution of resistant genotypes in populations across Italy, Albania and Greece. In particular, we focused on sites experiencing the 2017 chikungunya outbreak, where extensive pyrethroid spraying was implemented.[55,63]

2 MATERIALS AND METHODS

2.1 Mosquito collections and rearing

Ovitrap collections of *Ae. albopictus* eggs were carried out from June to November 2017 by local entomology teams in 12 sites from five Italian regions (Table S1), with particular focus on sites where autochthonous chikungunya virus (CHIKV) cases occurred in the same period (i.e. RM1, RM2, LT and AZ in Lazio region and GM in Calabria region; Table S1, Fig. 1). As in Pichler *et al.*,[38] collections at each sampling site were carried out with five or more ovitraps[64] to avoid oversampling of siblings, and egg samples on germination paper were sealed in plastic bags and sent by express courier to the Department of Public Health and Infectious Diseases (DPHID) at Sapienza University in Rome.

Larvae were reared at a larval density of 100 larvae/l in the insectary of DPHID at T = 26 ± 1 °C, RH = 60 ± 5% and at 14:10 h light: dark photoperiod and fed with artificial dry cat food. Pupae were collected daily and transferred into 40 cm^3 cages. Emerged adults were identified as *Ae. albopictus* using morphological keys[65] and kept at the same temperature and humidity as larvae.

2.2 Insecticide susceptibility bioassays

Bioassays were performed, as already described in Pichler *et al.*,[38] at DPHID following test procedures using WHO test-tubes[19,56,66]

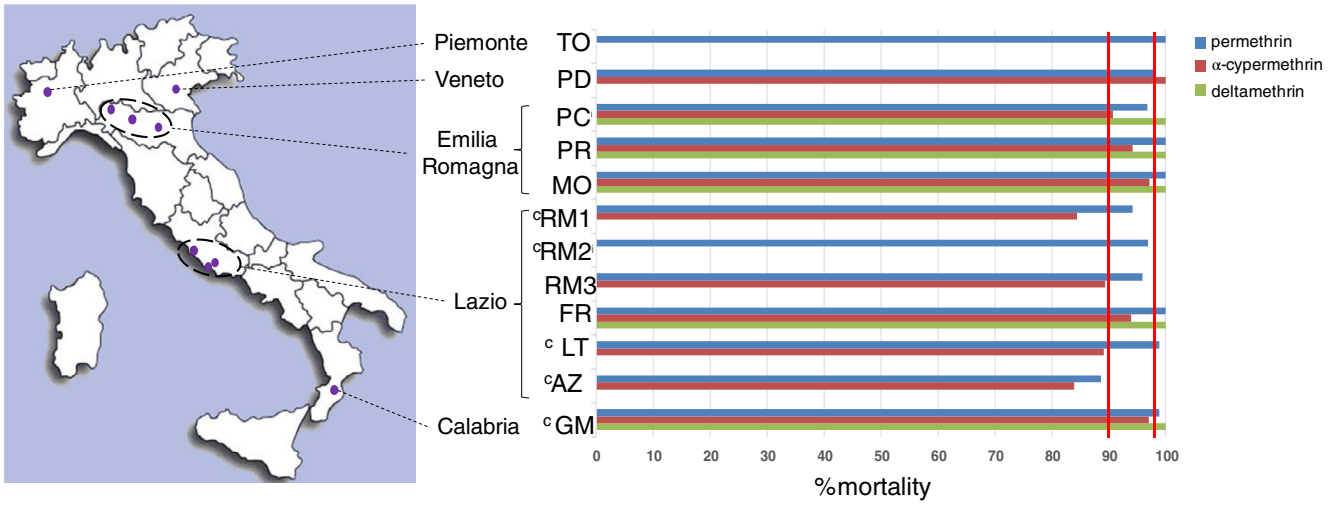

Figure 1. Distribution of *Aedes albopictus* populations and mortality (%) after 1-h exposure to pyrethroids: blue, permethrin 0.75%; red, α-cypermethrin 0.05%; green, deltamethrin 0.05%. Red vertical lines indicate 90% and 98% mortality thresholds (19, 56). [c]Sites for which CHKV cases in 2017 have been reported. Site codes are as in Table S1.

lined with filter papers impregnated with one of the following insecticides: permethrin (concentration = 0.75%), α-cypermethrin (0.05%), deltamethrin (0.05%) (Vector Control Research Unit, School of Biological Sciences, 11800 Minden, Penang, Malaysia). Insecticide concentrations were higher than the tentative concentrations proposed by WHO guidelines for *Aedes* mosquitoes (i.e. 0.03% for delthamethrin and 0.25% for permethrin[56]) and were selected based on the dosages most frequently used in the literature for *Ae. albopictus* in order to allow comparison of results with previous studies[26,29–31,33,66,67] and avoid the risk of overestimating insecticide resistance. The 0.05% concentration for deltamethrin was consistent with data available on a candidate *Ae. albopictus* susceptible reference strain.[53] Insecticide-impregnated (and control) papers were discarded after being used in six bioassays.

Bioassays were performed in the insectary at the same conditions as mosquito rearing (see above) by using 3- to 5-day old unfed *Ae. albopictus* females, either directly emerged from field-collected eggs (F0) or from their offspring (F1). Table 1 reports the total number of replicates/site/pyrethroid (in most cases three to four, as recommended by WHO[19]) and the total number of tested mosquitoes (in most cases 20–25 per replicate). The number of knocked-down mosquitoes (i.e. mosquitoes unable to stand or fly in a coordinated way[19]) was recorded every 10 min during exposure time. After 1 h of exposure, the mosquitoes were transferred into tubes with untreated papers and allowed a 24-h recovery, after which they were classified as dead or survived and the percentage mortality was computed. Control tubes (i.e. tubes lined with filter papers impregnated with only the insecticide excipient, but without the active ingredient) were set up and manipulated as the test-tubes.

Mean values of mortality were computed for each population. When mortality in control cages exceeded 5%, Abbott's correction for natural mortality was applied. According to WHO guidelines,[19,56] populations were considered 'susceptible' if mortality at 24 h 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 (Knock Down) curves for each population and to calculate 50% (KDT50) and 95% (KDT95) knock-down times. Pearson's correlation coefficient was computed to evaluate correlation between KDT values and percentage mortality. All analyses were carried out as described in Pichler *et al.*[38] using R software version 3.3.3.[68]

2.3 VSSC genotyping

After bioassays for a subset of dead or surviving mosquitoes, genomic DNA was extracted from half of the carcasses using the DNeasy®-Blood and tissue kit (Qiagen), and partial genotyping of domain II and III of the *VSSC* gene was performed. Since we could not afford to test all populations for all three pyrethroids, only mosquitoes exposed to permethrin, either in the present or in previous studies (Pichler *et al.*38; Kasai *et al* in press), were included in this analysis to be able to compare results with field data by Kasai *et al.* (in press). Genotyping was performed on samples collected from nine Italian regions, one site in Albania and one site in Greece (sampled either in the present study or in Pichler *et al.*[38]; Table 2), following the PCR protocol described by Kasai *et al.*,[49] with successive modifications (Kasai *et al.* in press). Briefly, the primers used were aegSCF20: GACAATGTGGATCGCTTCCC and aegSCR21: GCAATCTGGCTTGTTAACTTG; PCR reaction was performed in a 25 μL volume containing 10x reaction Buffer, 0.2 μM of each primer, 0.1 mM of each dNTP, 3 mM of MgCl2, 1 U of TaqPolymerase (BIOTAQ™ Bioline) and 1 μL of DNA extracted from a whole mosquito. Thermocycle conditions were 94 °C for 2 min followed by 35 cycles of 98 °C for 10 s, 55 °C for 30 s and 72 °C for 1 min, with a final elongation at 72 °C for 5 min. PCR products were purified using the SureClean Kit (Bioline) and sequenced at BMR Genomics s.r.l. (Padua, Italy).

The amino acid positions checked for possible *kdr* mutations were S989P, I1011M/V, F1014L and V1016G/I in domain II, and I1532T and F1534C/S in domain III, according to numbering of the most abundant splice variants of the house fly VSSC (GenBank accession no. AAB47604). The Hardy–Weinberg equilibrium (HWE) was tested for each sampling site and for each detected mutation. Odds ratios were computed, and a chi-square test was performed to evaluate the possible association between the detected *kdr* genotypes and permethrin susceptibility.

Table 1. Results of WHO tube bioassays performed on *Aedes albopictus* populations from Italy

Active ingredient	Region	Site	Tested generation	rep	*N* tot tested	KDT50 (95% CI)	KDT95 (95% CI)	Mortality % (95%CI)
0.75% permethrin	Piemonte	TO	F1	3	66	19 (17.8–20.28)	34.77 (31.74–40.8)	100
	Veneto	PD	F1	4	88	21.61 (20.23–23.08)	50.98 (46.22–58.83)	97.73 (93.15–99.62)
	Emilia Romagna	PC	F0	4	94	18.79 (17.41–20.28)	51.99 (46.45–61.17)	96.81 (91.93–98.20)
		PR	F0	4	101	10.93 (9.86–12.12)	26.88 (24.14–31.58)	100*
		MO	F0	4	103	15.81 (14.87–16.81)	33.13 (30.39–37.64)	100*
	Lazio	†RM1	F1	4	87	26.69 (24.87–28.65)	71.39 (63.24–85.87)	94.25 (88.05–97.90)
		‡RM2	F1	4	96	22.41 (20.81–24.13)	55.53 (49.64–65.9)	96.87 (92.10–98.21)
		RM3	F1	4	97	23.85 (22.69–25.07)	42.38 (39.53–47.06)	95.88 (90.68–98.70)
		FR	F1	2	43	16.21 (14.78–17.78)	34.61 (30.62–43.55)	100
		†LT	F0	3	93	15.25 (14.14–16.44)	36.79 (33.27–42.61)	98.85*(95.35–99.94)
		†AZ	F1	3	97	23.68 (22.19–25.27)	61.47 (55.34–71.49)	88.66 (81.36–93.94)
	Calabria	†GM	F1	3	89	15 (14.15–15.9)	27.24 (25.03–31.36)	98.88 (95.15–99.94)
0.05% cypermethrin	Piemonte	TO	NA		NA	NA	NA	NA
	Veneto	PD	F1	2	48	25.67 (23.74–27.75)	51.06 (45.9–61.84)	100
	Emilia Romagna	PC	F0	5	119	33.81 (31.92–35.81)	86.23 (77.26–101.5)	90.76 (84.69–95.09)
		PR	F0	4	94	25.21 (23.84–26.66)	51.33 (47.33–57.8)	94.22* (88.92–98.00)
		MO	F0	4	105	23.49 (22.39–24.65)	41.98 (39.24–46.39)	97.14 (92.76–99.28)
	Lazio	†RM1	F1	4	90	29.57 (27.76–31.49)	69.29 (62.4–81.26)	84.44 (76.04–90.92)
		‡RM2	NA		NA	NA	NA	NA
		RM3	F1	3	75	30.54 (28.26–33)	86.86 (75.11–110.33)	89.33 (81.05–94.98)
		FR	F1	4	83	19.2 (17.92–20.57)	47.81 (43.24–55.23)	93.98 (87.5–97.8)
		†LT	F1	4	92	28.19 (26.2–30.33)	85.52 (74.41–105.94)	89.19*(83.06–95.17)
		†AZ	F1	4	97	30.54 (28.71–32.49)	76.05 (68.17–89.62)	83.93*(77.68–91.60)
	Calabria	†GM	F1	4	100	17.09 (15.98–18.28)	39.77 (36.18–45.61)	97 (92.41–99.25)
0.05% deltamethrin	Piemonte	TO	NA		NA	NA	NA	NA
	Veneto	PD	NA		NA	NA	NA	NA
	Emilia Romagna	PC	F0	5	114	14.68 (13.77–15.65)	32.03 (29.35–36.36)	100*
		PR	F0	4	92	16.14 (15.41–16.9)	25.02 (23.34–28.41)	100
		MO	F0	4	92	12.63 (11.86–13.45)	22.95 (21.06–26.52)	100
	Lazio	‡RM1	NA		NA	NA	NA	NA
		‡RM2	NA		NA	NA	NA	NA
		RM3	NA		NA	NA	NA	NA
		FR	F1	4	93	8.91 (7.66–10.37)	24.45 (21.72–29.13)	100
		‡LT	NA		NA	NA	NA	NA
		‡AZ	NA		NA	NA	NA	NA
	Calabria	†GM	F1	2	47	14.68 (13.29–16.22)	31.63 (27.84–40.49)	100

Generation, replicates (rep) and number of mosquito females tested for pyrethroid resistance, i.e. active ingredients permethrin 0.75%, α-cypermethrin 0.05% and deltamethrin 0.05% are reported, as well as % mortality (95% confidence intervals) at 24 h after 1 h exposure and times to knock-down (KDT) of 50% and 95% of population (95% confidence intervals).
*Abbott corrected mortality.
†Sites in Italy where chikungunya cases have been reported in 2017. Site codes are as in Table S1.

3 RESULTS

3.1 Insecticide susceptibility bioassays

Susceptibility, as well as knock-down times (KDTs)s, to permethrin, α-cypermethrin and deltamethrin were assessed for 12, 10 and 5 *Ae. albopictus* populations, respectively (Fig. 1, Table 1). No knock-down was observed in control tubes. The results for each compound, along with 95% confidence intervals for mortality, as well as knock-down times, are shown in Table 1.

3.1.1 *Permethrin*

Resistance to permethrin was detected only in the population from Anzio (AZ; mortality = 88.7%), while possible resistance was observed in three other populations collected in Lazio (RM1, RM2, RM3), as well as in the PC population from Emilia Romagna and the PD population from the Veneto region. All these populations, with the exception of RM3, showed the highest (>46′22″) and overlapping KDT95 values. Overall KDT50 and KDT95 values

Table 2. Genotype and allele frequency for mutated and wildtype alleles for loci V1016G, I1532T and F1534C, and total number of specimens/sampling region (N) genotyped

	V1016G						I1532T						F1534C					
	Genotype			Allele			Genotype			Allele			Genotype			Allele		
Region	G/G	V/G	V/V	Freq (G)	Freq (V)	*N*	T/T	I/T	I/I	Freq (T)	Freq (I)	*N*	C/C	F/C	F/F	Freq (C)	Freq (F)	N
Italy, Trentino	0	0	13	0	1	13	–	–	–	–	–	–	–	–	–	–	–	–
Italy, Piemonte	0	0	10	0	1	10	–	–	–	–	–	–	–	–	–	–	–	–
Italy, Veneto	0	5	30	0.07	0.93	35	1	6	18	0.16	0.84	25	–	–	25	0	1	25
Italy, Emilia Romagna	15	32	38	0.36	0.64	85	5	25	24	0.32	0.68	54	–	–	54	0	1	54
Italy, Marche	0	0	24	0	1	24	–	8	15	0.17	0.83	23	–	–	23	0	1	23
Italy, Lazio	9	20	25	0.35	0.65	54	–	–	–	–	–	–	–	–	–	–	–	–
Italy, Puglia	3	9	20	0.23	0.77	32	–	1	19	0.03	0.97	20	–	–	20	0	1	20
Italy, Calabria	0	1	8	0.06	0.94	9	–	–	–	–	–	–	–	–	–	–	–	–
Italy, Sicilia	0	0	30	0	1	30	–	3	25	0.05	0.95	28	–	–	28	0	1	28
Albania, Vlore	0	0	21	0	1	21	–	5	17	0.11	0.89	22	–	–	22	0	1	22
Greece, Athens	0	0	20	0	1	20	–	–	19	0	1	19	9	7	3	0.66	0.34	19
Total	**27**	**67**	**239**	**0.18**	**0.82**	**333**	**6**	**48**	**137**	**0.16**	**0.84**	**191**	**9**	**7**	**175**	**0.07**	**0.93**	**191**

No mutation was found in positions 989, 1011 and 1014.

showed large variability (KDT50 11′–27′, KDT95 27′–71′; Fig. 2) with a good correlation between KDT50, KDT95 and mortality ($r_{KDT50/mortality} = -0.72$, df = 10, $P < 0.01$; $r_{KDT95/mortality} = -0.78$, df = 10, $P < 0.01$).

3.1.2 *α-cypermethrin*

Resistance to α-cypermethrin was detected in four out of five populations collected in Lazio in 2017 (RM1, RM3, AZ, LT), while a possible resistance was observed for all the other tested populations except the PD population from Veneto, which showed 100% mortality. The highest KDT values were observed for resistant populations, as well as for the PC population from Emilia Romagna, and a good correlation was detected between KDT50, KDT95 and mortality ($r_{KDT50/mortality} = -0.65$, df = 8, $P < 0.05$; $r_{KDT95/mortality} = -0.73$, df = 8, $P < 0.05$; Fig. 2). Variability for knock-down times was large across sampling sites: KDT50 17′–34′ and KDT95 40′–87′.

3.1.3 *Deltamethrin*

All tested populations were fully susceptible to deltamethrin with 100% mortality. The highest KDT95 values were observed for the PC population from Emilia Romagna (KDT95 32′; Fig. 2). Limited variability was observed among populations and confidence intervals were largely overlapping.

3.2 VSSC genotyping

Genotyping of *VSSC* gene was performed on *N* = 333 (for domain II) and *N* = 191 (for domain III) specimens from Italy, Albania and Greece, either dead or survived after 0.75% permethrin bioassays. Major attention was paid to mutations in domain II, as V1016G mutation had already been observed in Asia and Italy and found to be significantly associated with pyrethroid resistance (Kasai *et al.* in press). Overall, mutations were detected in positions 1016 in domain II and 1532 and 1534 in domain III; for all the other investigated loci only wild-type alleles were observed. Genotyping results were deposited to VectorBase[69] with the exception of those related to the 22 specimens from Emilia Romagna genotyped by Kasai *et al.* (in press). Genotypic and allelic frequencies are grouped based on geographic origin in Table 2, and on permethrin test results in Table 3. No significant deviations from HWE were detected in any sampling site for any mutation.

V1016G mutation was observed (in homo- or heterozygosis) in 18% of Italian genotyped specimens (in five of the nine analyzed regions), with the highest frequencies in Emilia Romagna, Lazio and Puglia (Table 2, Fig. 3). In 17 specimens the V1016G mutation was observed along with I1532T mutation, both in heterozygosis, while no mosquito with both V1016G and F1534C mutations was found. The frequency of the V1016G allele was clearly higher in specimens that survived a 1-h permethrin exposure, compared to completely susceptible specimens (Fig. 4; Table 3). A significant association between the allele G1016 and resistance to permethrin was observed (chi-square = 154.02, df = 1, $P < 0.0001$; Table 3).

I1532T mutation was observed (in homo- or heterozygosis) in 16% of all the genotyped specimens sampled in Italy (in five of the seven analyzed regions) and in Albania. It was never observed in combination with the mutant allele in position 1534. No association of this mutation with the resistant phenotype was observed (chi-square = 1.25, df = 1, $P = 0.26$; Table 3).

F1534C mutation was observed (in homo- or heterozygosis) in 7% of genotyped specimens, all from Greece (Table 2). The mutation never appeared in association with the other genotyped mutations. Chi-square test confirmed a significant

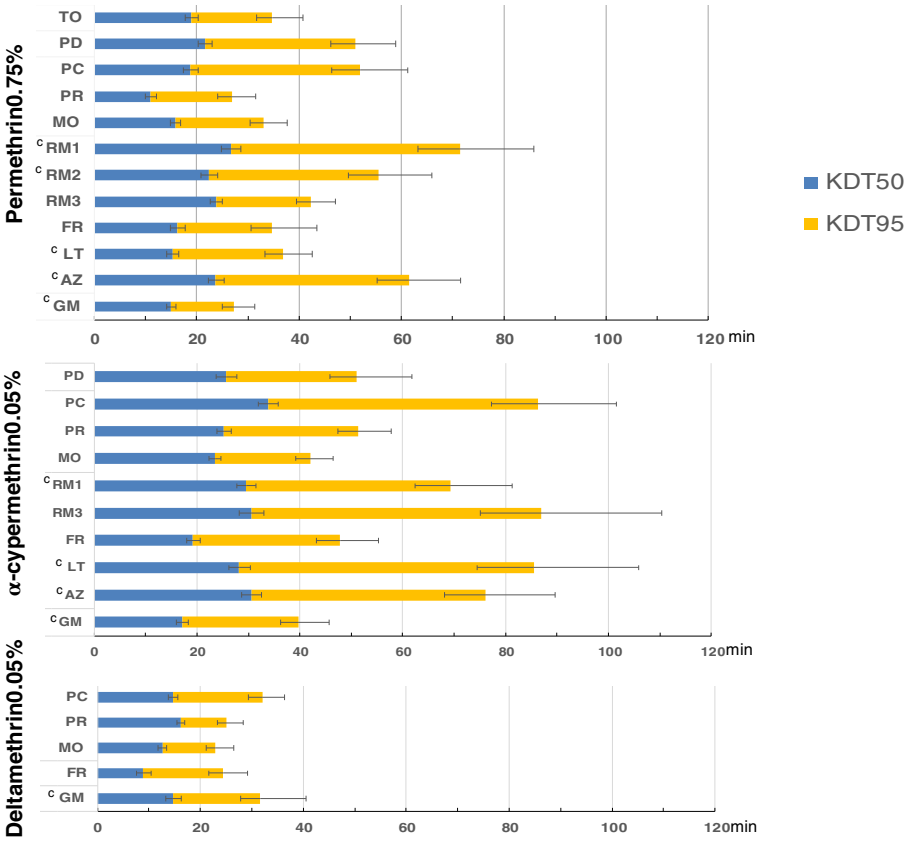

Figure 2. Knock-down time and 95% confidence interval for 50% (blue, KDT50) and 95% (yellow, KDT95) of *Aedes albopictus* populations exposed to pyrethroids (permethrin 0.75%, α-cypermethrin 0.05% and deltamethrin 0.05%). C sites for which CHKV cases in 2017 have been reported. Site codes as in Table S1.

association between the allele C1534 with resistance to permethrin (chi-square = 15.11, df = 1, $P < 0.0001$; Table 3).

4 DISCUSSION

The present study represents the first investigation in Europe on phenotypic and genotypic resistance of *Ae. albopictus* to pyrethroid insecticides, following the recent evidence of resistance to permethrin and α-cypermethrin in Italy[38] as well as the recent report of a PR-associated substitution in position 1016 of the VSSC in field *Ae. albopictus* specimens from Italy (Kasai *et al.* in press).

All but one population showed reduced susceptibility to α-cypermethrin and 5 out of 12 populations showed reduced susceptibility to permethrin, confirming higher levels of resistance to α-cypermethrin than to other pyrethroids, as already reported from Italy in 2016[38] and from Spain in 2013.[37] The presence of at least one population showing complete susceptibility for each insecticide tested ensures the quality of the used filter papers, despite the lack of a susceptible reference colony. Full susceptibility to deltamethrin in Europe was confirmed[24,35,38] for all populations tested, although in the present study only a few of the populations showing resistance to the former two chemicals were exposed also to deltamethrin due to the limited number of available specimens. It is interesting to note that the mortality values observed (88.6–100% for permethrin and 83.93–100% for α-cypermethrin), in agreement with Pichler *et al.*,[38] may underestimate actual resistance as, in the absence of specific WHO diagnostic pyrethroid dosages for *Ae. albopictus*, dosages higher than those recently recommended for *Aedes* mosquitoes in general[56,66] were used in the bioassays.

Data on phenotypic susceptibility to permethrin and α-cypermethrin in the two most extensively studied regions (Emilia Romagna and Lazio), coupled with data from Pichler *et al.*,[38] suggest important differences at small geographical/temporal scales, consistent with the patchy distribution of resistant phenotypes already observed worldwide.[51] In Emilia Romagna, in particular, no clear signs of resistance were detected in the three inland populations analyzed in the present study, while 69% and 81% mortalities were observed in 2016 against permethrin in two coastal sites (Lido di Spina and Lido di Volano) and 65% mortality against α-cypermethrin was observed in one of them (Lido di Spina).[38] This is consistent with preliminary data on sympatric *Culex pipiens* (data not shown) and may be explained by a more extensive insecticide spraying to reduce mosquito nuisance in touristic/coastal areas compared to inland ones.[69]

Genotyping of domain II of the *VSSC* gene across Italy revealed a widespread distribution of V1016G mutation (from north to south Italy). This mutation was first detected in *Ae. albopictus* populations from Vietnam as well as in a population from Rome (Italy) by Kasai *et al.* (in press), who confirmed its involvement in resistance by exposing a V1016G homozygous strain to pyrethroids and clarified that the G1016 allele confers a much higher level of resistance to pyrethroids than C1534 or S1534 alleles, which have been reported previously in *Ae. albopictus*. Interestingly, mutations in position 1016 (V1016G and V1016I) are known for causing pyrethroid resistance in *Ae. aegypti,* alone or in combination with other mutations.[14,71,72] Our results provide further evidence

Table 3. Genotypic and allelic frequencies for the mutated and wild-type alleles for loci V1016G, I1532T and F1534C, grouped per insecticide resistance test results

Permethrin resistance test outcome	V1016G					I1532 T					F1534C					
	G/G	V/G	V/V	Freq (G)	Freq (V)	*N*	T/T	I/T	I/I	Freq (T)	Freq (I)	*N*	C/C	F/C	F/F	Freq (C)	Freq (F)	*N*
Dead	0.01	0.14	0.08	0.85	0.92	261	0.03	0.24	0.74	0.14	0.86	152	0.02	0.04	0.94	0.04	0.96	152
Survivor	0.32	0.43	0.25	0.56	0.47	72	0.05	0.29	0.66	0.20	0.80	41	0.15	0.02	0.83	0.16	084	41
Total	0.08	0.20	0.72	0.18	0.82	333	0.03	0.25	0.72	0.16	0.84	193	0.05	0.04	0.92	0.07	0.93	193
OR (95%CI)	13.13(8.40–20.54)						0.7(0.37–13.14)						4.58(2–10.49					
P-value	<0.0001						0.264						<0.0001					

No mutation was found in positions 989, 1011 and 1014. Odds ratio (OR) and *P* value to test for a significant association between the presence of the mutant allele and the resistance phenotypes are reported. The total number of specimens (*N*) genotyped is reported for each category. G = glycine, V = valine, T = Tthreonine; I = isoleucine, C = cysteine, F = phenylalanine.

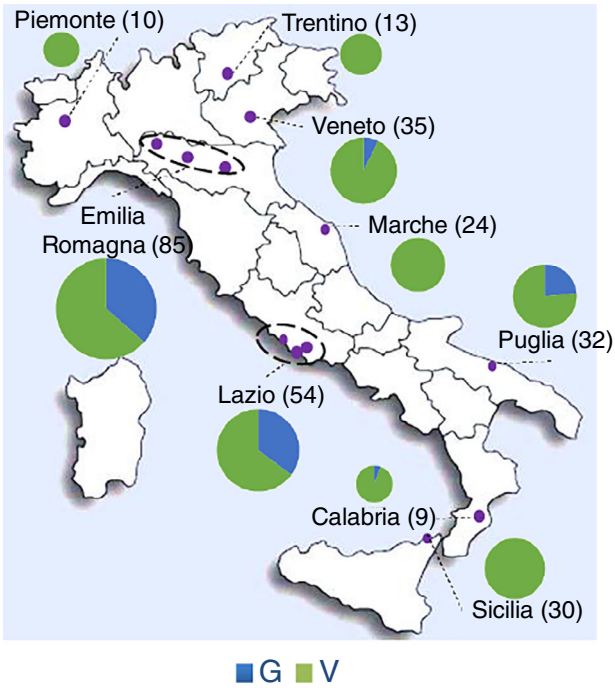

Figure 3. Locus V1016G allelic frequency in nine regions across Italy. Pie dimension is relative to the number of specimens genotyped, shown in brackets next to region names. G = blue; V = green.

Figure 4. Locus V1016G genotypic and allelic frequencies for all genotyped specimens (overall) and grouped per permethrin test outcome (dead or survivor) genotypic frequencies: G/G = blue, V/G = yellow, V/V = green allelic frequencies: G = blue; V = green.

of a strong association between G1016 allele and permethrin resistance.

Mutation I1532T, already reported by Xu *et al.*[51] in Italy, was observed in the present study only in two northern Italian regions (Veneto and Emilia Romagna) and in Albania. Data confirmed the lack of association with permethrin resistance suggested by Kasai *et al.* (in press).

Mutation F1534C – reported previously in *Ae. albopictus* from Singapore,[49] China,[50] Greece[51] and Brazil[52] – was only found in the population from Athens, Greece (where it had already been detected by Xu *et al.*[51]) and not in the other populations analysed. Notably, allele C1534 is the most widespread mutation conferring pyrethroid resistance in *Ae. aegypti*[14] and several mutations in this position (F1534S, F1534L, F1534C[53–55]) are also present in *Ae. albopictus*, corroborating the hypothesis of a possible role of this amino acid in interactions with insecticides also in this species (Kasai *et al.* in press).

Mutations G1016 and C1534 were observed in populations from Italy and Greece, respectively. This creates the precondition of their co-occurrence in a single, possibly more resistant, haplotype. In

fact, although the two mutations have not so far been observed in linkage in *Ae. albopictus,*[14,48] their association was shown to lead to enhanced resistance in *Ae. aegypti*.[72,73]

Further studies on laboratory colonies and larger field samples are needed to better clarify the role of the reported mutations (and possibly of other associated mutations) in pyrethroid resistance.

5 CONCLUSION

The results obtained show the widespread presence of genetic variants associated with permethrin resistance in several *Ae. albopictus* populations across Italy, as well as in Greece, suggesting that the condition for the spread of resistance to pyrethroids in this species in Europe may exist under strong selective pressure due to intensive insecticide spraying, as in the case of the management of an exotic arbovirus outbreak.

The results are suggestive of a causality between the intensive insecticide treatments in the municipality of Anzio (AZ) during the 2017 CHIKV outbreak (http://portale.comune.anzio.roma.it/archivio10_notizie-e-comunicati_0_1133_12_1.html) and increased pyrethroid resistance in the site. Unfortunately, this cannot be conclusively established due to lack of data on the susceptibility of the local *Ae. albopictus* population before the outbreak. Anzio is a renowned tourist coastal municipality 52 km south of Rome (~44 km^2, ~55 000 inhabitants) that experienced the highest number of CHIKV cases in 2017 (~300[13,74]). Following the first case report in early September, four spraying treatments with permethrin-based insecticides were carried out once a week within a range of 200 m around each infected case following the Italian Health Minister's guidelines.[55,76] Our hypothesis is that the resulting high concentration of pyrethroids within the municipality selected the permethrin and α-cypermethrin resistance we detected. The lack of a similar result in populations from other sites in the Lazio region with reported CHIKV cases in 2017 (RM1, RM2 and LT) does not contradict this hypothesis. In these cases, the distribution of fewer infected cases across much wider areas (~80 cases in Rome and 10 cases in Latina[74]) resulted in only a few focal and scattered insecticide treatments, likely not leading to the same selective pressure as in Anzio. On the other hand, lack of resistance to permethrin in Latina (LT, 23 km from Anzio) may support the hypothesis of a lack of phenotypic resistance in the Anzio *Ae. albopictus* population before the 2017 CHIKV outbreak. Absence of observed resistance in the *Ae. albopictus* population from Guardavalle Marina (GM; Calabria region, ~120 infected cases) is likely to be associated to very limited insecticide treatments due to late detection of the outbreak at the end of the mosquito breeding season. It is also possible that resistant phenotypes already circulated in Anzio (possibly at lower frequencies) since insecticide treatments have been carried out regularly to reduce nuisance during the summer tourist seasons. In fact, in Emilia Romagna higher levels of resistance in touristic/coastal populations compared to inland ones have been observed, consistent with preliminary data on *Cx pipiens* from the same sampling sites.

Overall, the results obtained on the phenotypic resistance of several *Ae. albopictus* populations in Italy (and beyond) and on the circulation of resistant genotypes also in susceptible populations raise a public health alarm in Europe. These results draw attention to the need to study the genotypic and phenotypic resistance of *Ae. albopictus* further in Europe and beyond, as well as to regulate the use of pyrethroids for the reduction of mosquito nuisance in both public and private areas, taking insecticide resistance management into consideration. This is critical in order to limit spreading of resistance and maintain the effectiveness of current control tools in the case of arbovirus outbreaks. Synergic and coordinated actions at national and European level are required to carefully monitor the resistance status of *Ae. albopictus* (as recommended by WHO and the Italian Ministry of Health[55,66,75]). The identification of resistant-alleles opens the way to the development of an easy-to-use genotyping assay for V1016G mutations in *Ae. albopictus*, similar to that already available for *Ae. aegypti*.[76]

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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