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ORIGINAL PAPER

Unbiased sequence analysis of *vgsc* gene reveals circulation of novel and known knock-down resistance mutations in *Culex pipiens*, challenging vector control measures

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Abstract
Pyrethroids, targeting the voltage gated sodium channel (VGSC), are fundamental for the control of arboviral disease circulation. The spread of pyrethroid resistance among vector species represents thus a major public health concern. *Culex pipiens* is one of the most abundant European mosquito species and main vector of West Nile virus, leading cause of arboviral encephalitis worldwide. Despite this, monitoring of its resistance status and the understanding of underlying mechanisms are widely neglected. Herein, we performed an oligo-hybridization capture approach on 82 *Cx. pipiens* specimens from Italy and Greece to investigate the whole coding sequence of the *vgsc* gene for the presence of known and potential knock-down resistance (*kdr*) mutations associated with target-site resistance to pyrethroids in insects. Among the 26 non-synonymous substitutions revealed by the analysis, the super-*kdr* haplotype—i.e. the association of *kdr*-alleles 918T and 1014F, known for conferring a strongly enhanced resistance phenotype in *Musca domestica* – was revealed for the first time in mosquitoes. Three more potential *kdr* alleles were detected for the first time in *Cx. pipiens* and multiple *kdr* variants were observed for locus 1014, with allele 1014F, reaching frequencies $>80\%$. Overall, results depict a worrisome situation that could affect the ability to control West Nile virus outbreaks in southern Europe. To avoid this, resistance monitoring needs to be intensified and an enhancement of the diagnostic tool box for the easy detection of different *kdr*-variants (including in particular the super-*kdr* haplotype) and for subsequent functional studies on the resistance phenotype of detected variants, is required.

Keywords Pyrethroid resistance · *Culex pipiens* · West Nile virus · Europe · Vector control · Target-site resistance

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Key message

- Resistance to insecticides is on the rise in mosquitoes capable of transmitting diseases to humans.
- Little is known on resistance mechanisms in *Culex pipiens*, one of the most dangerous mosquitoes.
- We looked for genetic traits linked to insecticide resistance in European *Cx. pipiens* populations
- We revealed widespread presence of known and novel genetic features associated with resistance
- It is crucial to clarify possible risks of those VGSC alleles for disease vector control

Introduction

Insecticide resistance is rapidly spreading across vector populations worldwide and has been acknowledged as one of the main public health challenges to be faced in the near future (WHO 2012). Indeed, without adequate preventive actions, insecticide resistance could have a significant operational impact, limiting our ability to control arboviral disease transmission (WHO 2012). To avoid resistance becoming stable in vector populations, strategies to detect resistance and counteract its spread must be put in place as early as possible. The definition of effective insecticide resistance management strategies requires anyway a comprehensive understanding of the resistance phenotype spreading in a population and its underlying mechanisms. Despite being one of the most relevant European arbovirus vector species, information on insecticide resistance in *Culex pipiens* is severely lacking.

The northern house mosquito *Cx. pipiens* is one of the most abundant mosquito species in Europe and Italy (Harbach 2012, Mancini et al. 2017; Severini et al. 2009) where it is present with two biotypes, i.e. form *pipiens* and form *molestus* (Becker et al. 2012; Harbach 2012; Di Luca et al. 2016; Brugman et al. 2018; Bisia et al. 2020). The above biotypes exhibit important ecophysiological differences (e.g. biting preference, breeding sites and diapause) which may impact also their relative importance as vectors of arboviral diseases (Farajollahi et al. 2011; Harbach 2012; Brugman et al. 2018; Haba and McBride 2022).

Culex pipiens plays indeed a crucial role in the transmission of at least three viruses circulating at present in Europe: West Nile, Usutu, and Sindbis, with West Nile having the greatest epidemiological impact (Brugman et al. 2018). West Nile virus (WNV, Flaviviridae), the most widespread arbovirus and the leading cause of arboviral encephalitis worldwide (Reiter 2010; Ciota 2017), is transmitted in an enzoonotic transmission cycle involving mosquitoes as vectors and birds as main vertebrate hosts, but occasionally humans and equids can get infected (Brugman et al. 2018). In humans, WNV infections are often asymptomatic or mild but can lead also to a neuroinvasive form with a 10–20% fatality rate (Gyure 2009). During the last few decades, Europe has witnessed a steady increase in WNV cases, with the until now largest outbreak in 2018 with more than 1600 confirmed human cases—most of them in Italy, Greece, Romania and Hungary—and a 9% case fatality ratio, (ECDC, Surveillance atlas of infectious diseases). Large outbreaks in Europe were observed subsequently also in 2022 (> 1100 cases) and 2023 (> 700 cases). At present, no specific treatment nor vaccine against WN disease in humans is available; therefore, limiting human-vector contact and control interventions targeting *Cx. pipiens* are the only tools available to reduce disease transmission.

Mosquito control interventions, aiming at the reduction of the vector population, are considered to have one of the highest returns on investment in public health (WHO 2015, 2017). In Europe, larval control is reported as the most widely adopted method to reduce the abundance of *Cx. pipiens* and also *Aedes albopictus* (i.e. vector of arboviruses such as dengue, chikungunya and Zika) and eventually also the risk of disease outbreaks (ECDC 2012, 2020). Adulticidal treatments are recommended mainly in the case of autochthonous WNV human cases, the detection of viral circulation among mosquitoes or sentinel animals or in situations with exceptionally high risk factors (involving a higher than normal concentration of vectors and hosts) (ECDC 2012, 2020). In such scenarios, adulticidal interventions are strongly needed to reduce fast and effectively the adult mosquito population able to transmit the virus. Indeed, aerial ULV adulticiding is the only method with scientific evidence for its effectiveness in reducing the incidence of WNV human cases, but is not authorized in the EU (ECDC 2020).

In Europe only pyrethroids—which target the voltage gated sodium channel (VGSC) and interfere with normal nervous signal transmission—are allowed for adulticidal treatments (EU Directive 2012; EU Directive 1998). However, the often excessive and erroneous usage of pyrethroids to reduce the considerable nuisance linked to mosquito bites (especially in tourist areas), together with the usage of the same active ingredients for the control of agricultural pests, has led to the selection of pyrethroid resistance (PR) in target species, putting possibly at risk our ability to control fast and effectively ongoing disease transmission (Hemingway and Ranson 2000; Soderlund and Knipple 2003; Rinkevich et al. 2013; Zhu et al. 2016).

Main mechanisms underlying pyrethroid resistance in mosquitoes are overexpression of enzymes involved in

detoxification pathways (i.e. metabolic resistance) and substitutions within the VGSC (i.e. target-site resistance). Such substitutions, alter the binding and interaction of the pyrethroid with the VGSC, increasing the dose of insecticide required for knock-down and resulting thus in reduced susceptibility of the mosquito. Therefore, such substitutions are commonly referred to as knock-down resistance (*kdr*) mutations (Davies et al. 2007; Dong et al. 2014). More than 50 such mutations in the VGSC with varying importance in resistance have been described in different insect species (Davies et al. 2007; Rinkevich et al. 2013; Dong et al. 2014), and abundant literature exists for main vector species such as *Anopheles* spp. malaria vectors (Clarkson et al. 2021), *Aedes* mosquitoes (Vontas et al. 2012; Moyes et al. 2017) or *Culex quinquefasciatus* (Komagata et al. 2009; Li et al. 2012).

Information on the resistance status and its underlying mechanisms are instead scarce for *Cx. pipiens* and few studies have specifically focused on populations from Europe. Phenotypic PR (assessed by bioassays) was reported in *Cx. pipiens* populations from countries of the Mediterranean basin including Greece, Italy, Spain and Turkey (Ben Cheikh et al. 1998; Zayed et al. 2006; Vasquez et al. 2009; Peery et al. 2012; Kioulos et al. 2013; Fotakis et al. 2017; Tmimi et al. 2018; Guntay et al. 2018; Paaijmans et al. 2019; Ser and Cetin 2019; Pichler et al. 2022; ECDC 2023), as well as in Belgium (Vereecken et al. 2022). In Italy, reduced susceptibility to permethrin was detected by WHO-tube bioassays in 9 populations out of 10 analysed, with lowest mortality rates (< 20%) observed in coastal tourist sites, suggesting a highly worrisome situation and a possible negative impact of PR on vector control measures (Pichler et al. 2022).

Similarly, information on target-site resistance mechanisms in *Cx. pipiens* is limited: so far only substitution of the leucine in position 1014 of the VGSC with phenylalanine (L1014F; Martinez-torres et al. 1999) and rarely serine or cysteine have been reported (L1014C/S; Scott et al. 2015). In Europe, substitutions L1014F/C have been detected in Greece, Italy and Turkey, reaching worrisome frequencies in some populations (Kioulos et al. 2013; Taskin et al. 2016; Fotakis et al. 2020, 2022; Guz et al. 2020; Pichler et al. 2022; ECDC 2023), while allele 1014S was detected rarely in Greece and Turkey (< 5%; Fotakis et al. 2022). In Italy, Pichler et al (2022) reported the 1014F allele in all 10 populations examined with highest frequencies in those populations from coastal tourist sites where bioassays had detected phenotypic resistance and a significant association between the presence of the 1014F allele and permethrin resistance was confirmed.

Despite these clear evidence -obtained via bioassays and genetic investigations- indicating the spread of PR across *Cx. pipiens* populations in Europe and its possible negative impact on the effectiveness of vector control measures, data are very sparse in terms of time and space (ECDC 2023) and few studies were designed for a better understanding of resistance mechanisms and phenotypes in *Cx. pipiens.*

Here we take advantage of the targeted next-generation sequencing (NGS) approach developed by Itokawa et al. (2019) which allows a high coverage sequencing of the whole *vgsc* gene. This approach allows to detect novel mutations across the whole gene, a fundamental advantage compared to other approaches limited to small stretches of the *vgsc* (investigated by partial sequencing of single exons) or to single-nucleotide polymorphisms (SNPs; investigated by PCR approaches). Thanks to this approach, it was possible to gather first information concerning the existence, distribution and frequency of known and novel mutations in the VGSC with possible or confirmed impact on PR in *Cx. pipiens* specimens from Italy and Greece.

Materials and methods

Mosquito sampling

Sampling of *Cx. pipiens* specimens was performed from June to October 2022 in 8 sites from 4 provinces from northern to southern Italy as well as one site from Attica region in Greece (Table S1).

In Attica region, adult mosquitoes were captured in the framework of a research project for the entomological surveillance of mosquitoes using BG Sentinel traps equipped with lure and CO_2, stored on ice for transportation to the laboratory and then preserved in ethanol until DNA extraction. Samples from Italy were collected by collaborating research groups as egg rafts or larvae from larval breeding sites such as road ditches and sewers; sampled eggs were stored on wet filter paper, and larvae were maintained in plastic containers filled with water and then sent to the Department of Public Health and Infectious Diseases (DPHID) at Sapienza University of Rome where eggs were allowed to hatch and larvae were reared at larval density of 100 larvae/l in the insectary of DPHID at T = 26 °C ± 1 °C, RH = 60 ± 5%, 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 *Cx. pipiens* using morphological keys (Severini et al., 2009), kept at the same temperature and humidity as larvae and fed with 5% sugar solution at libitum. Adult mosquitoes were subsequently freeze-killed and stored at -80 without any preservant.

Molecular analysis

DNA was extracted from whole mosquito carcasses using the DNeasy Blood and Tissue kit (QIAGEN) and subsequently quantified using a microplate reader and the Quant-iT PicoGreen kit (Thermo Fisher Scientific), according to

manufacturer's instructions. Only specimens with > 4 ng/ul DNA concentration were processed; mean DNA concentration was 13 ng/ul varying from a minimum of 4 to a maximum of 49 ng/ul. Identification of biotypes *Cx. pipiens* f. *pipiens* and f. *molestus* was performed following the protocol described by Bahnck and Fonseca (2006).

Targeted sequencing of the *vgsc* gene was conducted for 82 *Cx. pipiens* as described by Itokawa et al. 2019. Given the elevated sequence homology observed for the *vgsc* gene across dipteran species, it was possible to perform library construction and oligo hybridization capture using the biotinylated oligo probes designed for *Ae. albopictus* (Davies et al. 2007; Itokawa et al. 2019). We modified the probe set from the previous version described in Itokawa et al. (2019), resulting in Culicinae_VGSC_V2 (Table S2). In this version 35 new probes were added to cover several *vgsc* exons (Exon2 and 16.5 in *Aedes aegypti* and Exon2, 8, 14, 24, 29, and 32 for *Cx. pipiens*) which showed relatively weak capture rate due to low homology and/or short exon length (Itokawa et al. 2019). The probes were synthesized by IDT as xGen Lockdown Probes. NGS library preparation and hybridization capture enrichment were conducted with the protocol described previously (Itokawa et al. 2019). Briefly, 10–20 ng gDNA of each sample was fragmented, end-prepped, and indexed adapters ligated using NEBNext Ultra II FS DNA Library Prep Kit for Illumina (NEB) as indicated in the manufacturer's protocol. After adapter ligation reaction, ligase was heat inactivated by incubating samples at 65 °C for 20 min, and then all indexed libraries were pooled into a single tube. The pooled library was purified by 0.8 × AMpureXP (Beckman Coulter) and eluted into 20 uL H_2O. The purified pooled library was subjected to hybridization capture, washing, and final amplification (12 cycles) using xGen Hybridization and Wash Kit (IDT) and the Culicinae_VGSC_V2 probe set as indicated in the manufacturer's protocol. Paired-end sequencing (PE150) was performed on an Illumina NextSeq1000 obtaining a range of 122,156–480,752 read pairs per individual. Raw NGS reads were deposited to Sequence Read Archive (DRA) of DNA Data Bank of Japan (DDBJ) under accession number DRR528067–DRR528091 and DRR567865–DRR567921.

Bioinformatic analysis

The automated pipeline MoNaS v2.0 (https://github.com/ItokawaK/MoNaS) was employed as described by Itokawa et al. 2019 to analyse the obtained NGS read data and to filter, call and annotate functionally variants. MoNaS pipeline relies on BWA (Li and Durbin 2009), SAMTools (Li et al. 2009), BCFtools csq (Danecek and McCarthy 2017), and FreeBayes (Garrison and Marth 2012). Scaffold supercont3.182_3 (Itokawa et al. 2019) (https://github.com/ItokawaK/MoNaS), which was constructed by correcting gaps and possible consensus errors in scaffolds in the Cpip_J2 (Arensburger et al. 2010) assembly, was used as the *vgsc* gene reference. Read coverages on each exon of each sample were calculated with BEDtools (Quinlan and Hall 2010)) "mulitcov" function. Linkage disequilibrium was computed using the likelihood ratio test available in ARLEQUIN ver. 3.5. (Excoffier and Lischer 2010). Chi-square or Fisher's exact tests were performed to evaluate significant differences in allele frequencies between populations.

Results

High quality sequences of the *vgsc* gene were obtained for 82 *Cx. pipiens* specimens, 21 of which were identified as *Cx. pipiens* f. *molestus* (6 from Greece and 15 from Italy) and 61 as *Cx. pipiens* f. *pipiens* (4 from Greece and 57 from Italy). Reads were aligned to the *vgsc* gene from *Cx. quinquefasciatus* with mean coverage, (computed as number of overlapping reads) per exon per specimen varying from 38 to 5927(Figure S1). In our pipeline, variant and genotype calling were conducted by FreeBayes v1.3.6 (Garrison and Marth 2012). Filtering by quality values in VCF (QUAL > 50), identified 218 variable sites (in comparison to reference supercont3.182_3) within the *vgsc* coding sequences across all 82 mosquitoes. Total read depth per genotype varied from 14 to 478 with median 153. Of the identified variable sites, 215 were polymorphic among the analysed specimens with a distribution/exonic region as detailed in Table 1. Two hundred sites were biallelic SNPs or MNPs (Multiple Nucleotide Polymorphisms, i.e. highly linked mutations observed within few nucleotide positions). The allelic balance values (i.e. depth of the first allele in genotype/total depth) in all heterozygous genotypes ranged between 0.25 and 0.76 with median 0.51.

Among the polymorphic loci, 26 resulted in non-synonymous mutations of which 24 were biallelic while the remaining 2 loci were multiallelic (Table S3). Impact on pyrethroid susceptibility is highly suspected or confirmed for mutations causing substitutions in 5 amino acid positions of the VGSC (Table 2). Overall allelic frequencies for mutations in these 5 loci varied between 0.6% (allele 253L) and 59% (allele 1014F; Table 3 and Fig. 2).

Mutation V253L, situated in exon 7 within the linker connecting helix S4 and S5 of domain I, was detected only once in heterozygosis in one *Cx. pipiens* f. *molestus* specimen from Italy (Bari; Fig. 1, Tables 3 and S4).

Mutation M918T, situated in exon 19d forming the linker connecting helix S4 and S5 of domain II was detected only in heterozygosis in 8 Italian specimens (4 *Cx. pipiens* f. *molestus* and 4 *Cx. pipiens* f. *pipiens*) all of which carried also the *kdr* allele 1014F in homo- or heterozygosis (Fig. 1, Tables 3, S4 and S5).

Table 1 Distribution of polymorphic loci and non-synonymous mutations per exon detected in 82 *Culex pipiens* specimens from Italy and Greece

Exon	Estimated length (Davies et al. 2007)	N of polymorphic loci	N of non-synonymous mutations
3	156	1	–
4	206	3	2
7	213	5	1
8	61	5	1
11	276	6	–
14	242	16	2
15	100	1	–
17	278	5	–
18	174	11	–
20	188	7	1
21	247	9	1
22	212	6	2
23	220	2	1
24	266	20	–
25	174	11	–
27	123	2	–
28	195	12	–
29	246	4	2
30	271	12	2
31	305	17	–
32	954	47	10
19c	163	5	–
19d		4	1
26 k	123	5	–
26 l		2	–
Total		**218**	**26**

For locus 1014, situated in Exon 20 forming helix 6 of domain II, 4 different amino acid variants were detected: the wildtype (susceptible) allele 1014L and 3 *kdr* alleles, 1014F, C, S (Fig. 1, Tables 3 and 4).

The wildtype allele 1014L was observed at an overall frequency of 30%, with frequencies per region ranging from 5% (Attika-region) to 70% (Trentino) and only 12 homozygote specimens (9 *Cx. pipiens* f. *pipiens* and 3 *Cx. pipiens* f. *molestus* from Italy) across the whole sample (Table 4 and S4). The *kdr*-allele 1014F, encoded by two different triplets TTT or TTC, represented the most frequent allele with an overall allelic frequency of 59%, varying from 25% (Trentino) to 87% (Ferrara) per province; 41% of specimens overall were identified as homozygotes for the 1014F allele (Table 3). Among the two alternate triplets encoding for allele 1014F the triplet TTT (allele 1014F ^{TTT}) was more frequent in both biotypes and was detected at an overall frequency of 46%, varying from 5% (Trentino) to 77% (Ferrara). Allele 1014F ^{TTC} was observed at an overall frequency of 13% and, while absent from the Greek sample, in Italy its frequency varied from 9% (Ferrara) to 18% (Bari) (Table S6). Allele 1014C, present at an overall frequency of 10%, was most frequent in Greece (allelic frequency = 50%) where it represented the most common variant in position 1014. In Italy, allele 1014C was detected only in Bari province (in both biotypes) at a frequency of 21%, in heterozygosis with allele 1014F ($N=5$) or the wildtype allele 1014L ($N=2$). Allele 1014S was observed only in one *Cx. pipiens* f. *pipiens* specimen from Trentino region, in heterozygosis with the wildtype allele 1014L (Table 4 and S4).

Mutation F1534L, encoded by triplettes TTA or CTC, situated in Exon 29, forming helix 6 of domain III was detected at an overall frequency of 8% (Figs.1, 2, Table 3). Allele 1534L ^{TTA} was detected in all 4 Italian provinces in a total of 10 specimens (4 *Cx. pipiens* f. *molestus* and 6 *Cx. pipiens* f. *pipiens*), two of which were homozygotes. Allele

Table 2 Missense mutations detected within the VGSC for Italian and Greek *Culex pipiens* specimens with possible or confirmed impact on pyrethroid susceptibility

Amino acid position (coordinates for *M. domestica*)	Coordinates for *Cx. quinquefasciatus* (supercont3.182_3)	Reference	Alternative	mutation type	Contig Position	Exon	*Kdr*-impact
253	263	V (GTC)	L (CTC)	SNP	550,242	Exon7	Suspected
918	931	M (ATG)	T (ACG)	SNP	514,916	Exon19d	Strong
1014	1027	L (TTA)	F(TTT,TTC); C(TGT);S(TCA)	MNP	510,975	Exon20	Strong
1534	1571	F (TTC)	L (TTA, CTC)	SNP	482,164 & 482,166	Exon29	Supportive
1879	1916	P (CCG)	S (TCG)	SNP	476,646	Exon32	Supportive

Amino acid positions are given for the *Musca domestica* (AAB47604) and the *Cx. quinquefasciatus* reference genomes (supercont3.182_3). Between parenthesis triplettes encoding for the reference and alternative amino acids. Contig position refers to Cpip_supercont3.182_3 (Itokawa et al. 2019)

Table 3 Allelic frequency for loci involved in pyrethroid resistance split per sampling area

		Amino acid position											
		253		918		1534		1879		1014			
	N	V	**L**	M	**T**	F	**L**	P	**S**	L	**F**	**C**	**S**
Italy-Trentino	10	1	**0**	1	**0**	0.8	**0.2**	1	**0**	0.7	**0.25**	**0**	**0.05**
Italy-Ferrara	26	1	**0**	0.94	**0.06**	0.98	**0.02**	1	**0**	0.13	**0.87**	**0**	**0**
Italy-Rome	19	1	**0**	0.97	**0.03**	0.92	**0.08**	1	**0**	0.58	**0.42**	**0**	**0**
Italy-Bari	17	0.97	**0.03**	0.88	**0.12**	0.85	**0.15**	1	**0**	0.15	**0.65**	**0.21**	**0**
Greece-Attica region	10	1	**0**	1	**0**	1	**0**	0.9	**0.1**	0.05	**0.45**	**0.5**	**0**
Total	82	0.994	**0.006**	0.95	**0.05**	0.92	**0.08**	0.99	**0.01**	0.3	**0.59**	**0.1**	**0.01**

In bold alleles with known or supposed impact on pyrethroid susceptibility. *N* = number of *Culex pipiens* specimens genotyped

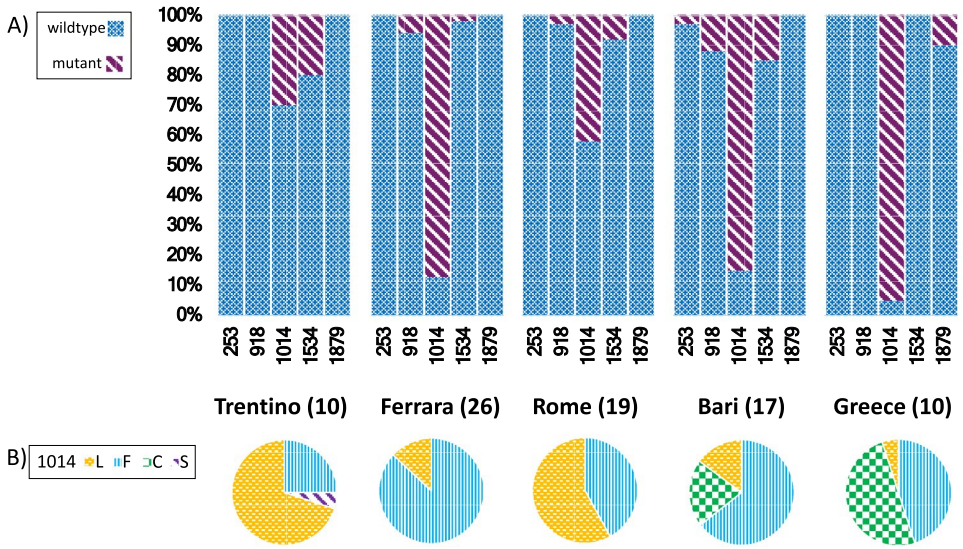

Fig. 1 Results obtained for 82 *Culex pipiens* specimens from Italy and Greece (split per sampling area) for the genotyping of 5 amino acid positions within the VGSC with potential impact on PR. Number of specimens analysed is shown between parentheses. Panel A) Frequency of wildtype or mutant alleles observed for 5 amino acid positions within the VGSC (253, 918, 1014, 1534, 1879). Panel B) Frequency of different amino acid variants observed for position 1014 of the VGSC. L = wildtype allele, F, C, S = mutant (*kdr*)alleles

Table 4 Genotype frequency for amino acid position 1014 of the VGSC split per sampling area

		L1014F/C/S genotype						
Sampling province	N	L/L	L/F	L/C	L/S	F/C	C/C	F/F
Italy-Trentino	10	5	3	–	1	0	–	1
Italy-Ferrara	26	3	1	–	–	0	–	22
Italy-Rome	19	4	14	–	–	0	–	1
Italy-Bari	17	–	3	2	–	5	–	7
Greece-Attica region	10	–	0	1	–	3	3	3
Total	82	12	21	3	1	8	3	34

L = wildtype (susceptible allele) F/C/S = *kdr* alleles *N* = number of *Culex pipiens* specimens genotyped

1534L ^{CTC} was detected only once in heterozygosis in a *Cx. pipiens* f. *pipiens* specimen from Bari province (Table S4).

Mutation P1879S, situated in exon 32 forming the carboxyl terminal domain, was detected only in heterozygosis in 2 Greek *Cx. pipiens* f. *pipiens* specimens with both specimens carrying also mutations in position 1014: one was a homozygote for allele 1014F while the other was a heterozygote 1014F/1014C (Fig. 1, Tables 3, S4 and S5).

Differences between biotypes were investigated by performing a Fisher exact test considering only mutations detected at an overall frequency > 5% (i.e. mutations in position M918T, F1534L and L1014F/C) and specimens from Italian provinces where both biotypes were detected. For none of these loci significant differences between biotypes were detected (Tables 5 and S7).

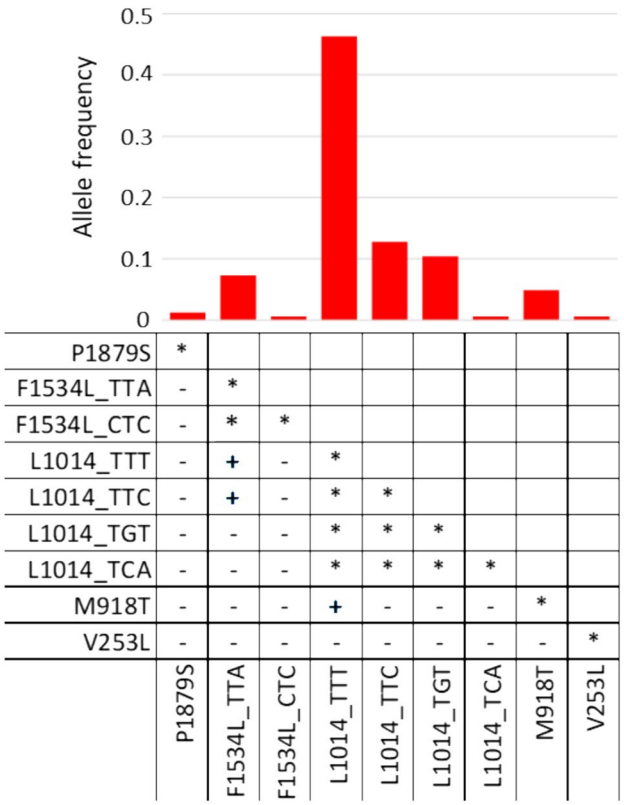

Fig. 2 Overall frequencies (bars) of identified *kdr* mutations along with significant linkage disequilibrium (LD) plot between pairs of mutations; + = significant LD detected; − = no significant LD; * = no LD computed

A linkage disequilibrium test performed on all specimens using the likelihood ratio test available in ARLEQUIN ver. 3.5 revealed significant linkage between allele 1534L (encoded by the triplet TTA) and the two triplets encoding 1014F (TTT and TTC), as well as between the mutation M918T and the allele 1014F (encoded by the triplet TTT; see Fig. 2).

Discussion

The novel targeted sequencing approach developed by Itokawa et al. (2019) allowed to: 1) detect for the first time the *super-kdr* haplotype (918 T + 1014F) in mosquitoes 2) find three substitutions possibly associated with pyrethroid resistance never described before in *Cx. pipiens* (i.e. variants in position 253, 1534 and 1879) and 3) evaluate the spread of different *kdr* variants in position 1014 of the VGSC, already known for its importance in conferring resistance in *Cx. pipiens*.

Mutation M918T, situated in pyrethroid receptor site I (PyR1), is detected herein for the first time in mosquitoes, and in clear linkage disequilibrium with allele 1014F: all specimens carrying the allele 918T (detected only in heterozygosis) were also heterozygotes ($N=4$) or homozygotes ($N=4$) for allele 1014F (encoded by the triplet TTT). In the latter specimens thus the two alleles 918 T and 1014F are for sure present as a haplotype on the same chromosome; this haplotype, 918T + 1014F, was reported for the first time in *Musca domestica* and has since been observed in several other insect species (Dong et al. 2014). Electrophysiological studies on insect sodium channels expressed in *Xenopus* oocytes revealed that the combination of the two alleles is associated with an up to 1000 fold reduction in susceptibility to pyrethroids, compared to a ~ 100-fold reduction observed when only allele 1014F is present (Vais et al. 2000), leading thus to define it as *super-kdr* haplotype (Lee et al. 1999). The increased resistance phenotype of the *super-kdr* haplotype was confirmed conducting bioassays on *M. domestica* with resistance ratios being 5 to 10 times higher for strains containing the *super-kdr* haplotype compared to strains carrying only resistance alleles in position 1014 (Scott et al. 2013; Sun et al. 2016). Given the significant role of this haplotype in enhancing the PR phenotype its first discovery in *Cx. pipiens* and mosquitoes in general is of highest relevance. It will be crucial to confirm the role of this haplotype on the resistance phenotype also in *Cx. pipiens* and to monitor its spread across Italy and Europe.

Table 5 Allelic frequency for loci involved in pyrethroid resistance split per *Culex pipiens* biotype and country

			Amino acid position											
			253		918		1534		1879		1014			
biotype	Country	N	V	**L**	M	**T**	F	**L**	P	**S**	L	**F**	**C**	**S**
molestus	Greece	6	1	**0**	1	**0**	1	**0**	1	**0**	0	**0.42**	**0.58**	**0**
	Italy	15	0.97	**0.03**	0.87	**0.13**	0.83	**0.17**	1	**0**	0.30	**0.53**	**0.17**	**0.00**
pipiens	Greece	4	1	**0**	1	**0**	1	**0**	0.75	**0.25**	0.13	**0.5**	**0.38**	**0**
	Italy	57	1	**0**	0.96	**0.04**	0.95	**0.05**	1	**0**	0.34	**0.63**	**0.02**	**0.01**

In bold alleles with known or supposed impact on pyrethroid susceptibility. N = number of specimens genotyped

Mutation V253L, found only once in heterozygosis in a specimen also carrying allele 1014F in heterozygosis, is situated in the second pyrethroid receptor site (PyR2), symmetrically to the residue in position 918 in PyR1 (Du et al. 2013; Sun et al. 2022). While nothing is known on the phenotype produced by this exact mutation, recently a substitution in the same position (V253F) was found in Brazilian *Ae. aegypti* populations where it was detected in linkage with mutation F1534C (situated in PyR1; Itokawa et al. 2021). Electrophysiological studies confirmed the role of allele 253F in reducing the channel sensitivity to both, type I and type II pyrethroids, but also in altering the channel gating, suggesting a potential negative impact on mosquito fitness. Notably, when the V253F mutation was expressed together with allele 1534C, this negative alteration of the channel activity was completely abolished, while the sensitivity to pyrethroids was further reduced compared to the presence of only one of the two mutations (Sun et al. 2022). The impact of this mutation on pyrethroid susceptibility in *Cx. pipiens* and the synergy between alleles 253L and 1014F should be further addressed in future studies.

Mutation F1534L is situated in PyR1; while absent from the Greek sample, the allele 1534L was detected in all Italian provinces (overall A.F in Italy = 9%) at frequencies ranging from 2 to 20%, representing thus the most frequent allele with possible impact on PR after 1014F in Italy. In the present study we detected two codons producing the 1534L allele, both being one mutational step away from the wildtype allele, suggesting their independent insurgence and thus strong selective pressure on the locus. Different substitutions have been described for this amino acid position in other mosquito species, with mutation F1534L being detected in *Ae. albopictus* (Chen et al. 2016; Su et al. 2019) and *Ae. aegypti* (Kushwah et al. 2020). Electrophysiological studies of these substitutions have confirmed their ability to reduce susceptibility to pyrethroids, especially—but not exclusively—of type I, while no significant modifications in channel properties were detected (Zhaonong et al. 2011; Du et al. 2013; Yan et al. 2020; Sun et al. 2022) suggesting low impact on the functionality of the channel and thus mosquito fitness. Substitutions in this position are often found in association with other potential *kdr*-alleles (e.g. in position 253, see above), but never in linkage disequilibrium with allele 1014F, as instead suggested by the present results (Fig. 2). The phenotype of possible double mutants (1014F + 1534L) needs to be evaluated: being the two mutations situated in the two pyrethroid receptor sites this may produce an increased resistance phenotype similar to other haplotypes combining mutations in both receptor sites (e.g. 253F + 1534C or 918 T + 1014F; Vais et al. 2000; Sun et al. 2022)).

Mutation P1879S is situated in the carboxyl terminal domain known for its role in the fast inactivation and closure of the VGSC (Dong et al. 2014); allele 1879S was detected in the present study only in two Greek specimens in heterozygosis, in linkage with mutations in position 1014. Mutation P1879S was detected for the first time in *Plutella xylostella* (Sonoda et al. 2008, Sonoda et al. 2010)—always in association with allele 1014F and/or other potential *kdr* mutations within the VGSC—and with strong support for its implication in PR (Rinkevich 2013; Sonoda et al. 2008). In mosquitoes this substitution has been detected by Clarkson et al (2021) in *Anopheles coluzzii* and another substitution in the same position (P1879L) has been detected in both, *An. coluzzii* and *An. gambiae*. Remarkably, also in these populations the mutation did not appear alone but in strong linkage disequilibrium with mutation L1014F, suggesting possible synergism between the two mutations.

Mutations in position 1014, situated in PyR2, are among the most well-known and widespread mutations not only in mosquitoes but also in other insect species (Dong et al. 2014). Mutation L1014F was the first *kdr*-mutation to be detected (in *M. domestica*; Williamson et al. 1993, 1996) and since then several other allelic variants were described for this position (L1014F/H/S/C/W, Rinkevich et al. 2013; Dong et al. 2014; Liu 2015; Scott et al. 2015; Du et al. 2016). Electrophysiological evidence for the impact of these mutations on pyrethroid susceptibility has been gathered since 1997 (Smith et al. 1997; Burton et al. 2011; Wang et al. 2015).

Among the different variants in position 1014, allele 1014F appears to be by far the most reported one in *Cx. pipiens* including reports from several European countries (Belgium, Greece, Italy, Romania, Turkey; Scott et al. 2015; ECDC 2023).

In the present study we observed in Italy three amino acid -variants for this position (1014F/C/S). In agreement with previous studies, allele 1014F appeared to be the most widespread (overall A.F. = 59%), with highest frequencies observed in Ferrara province (87%) where bioassays on samples from the same study site highlighted strongly reduced susceptibility to pyrethroids (mortality < 20%) in significant association with the presence of allele 1014F (Pichler et al. 2022).

The presence/distribution of the different *kdr* alleles in position 1014 across our sample may be explained by different insecticidal selective pressure or geographical connection between sites or both. For example, different selective pressure is supposed to promote the insurgence and spread of the two alleles 1014F and 1014S (first reported in *Cx. pipiens* in 1999; Martinez-torres et al. 1999; Scott et al. 2015): electrophysiological and bioassay data suggest indeed a different resistance phenotype of the two alleles with allele 1014F conferring resistance to both, pyrethroids and DDT and 1014S conferring resistance mainly to DDT (Martinez-torres et al. 1999; Burton et al. 2011).

For allele 1014C possibly both, geographical connections and differential selective pressure/fitness cost impact the observed distribution: in our study 1014C is the most frequent variant in the Greek sample (AF = 50%), in agreement with what observed by Fotakis et al. (2017, 2022) who detected this variant at high frequencies (> 70%) in several Greek populations. The exclusive presence of this allele in Italy in Bari province could thus be explained by the tight maritime connections with Greece. No clear data on the resistance phenotype and fitness cost of allele 1014C (first detected in *Cx. pipiens* in 2012; Wang et al. 2012) are available; anyway the allele is suggested to have similar relevance as the 1014S allele but with a higher contribution to pyrethroid resistance (Kim et al. 2007; Zhong et al. 2013; Taskin et al. 2016). It is also noteworthy that the insurgence of the 1014C allele (encoded by TGT) requires two mutational steps from the wildtype allele 1014L(TTA) to the *kdr* allele 1014F (TTT) and then 1014C (TGT), suggesting that at least in some circumstances allele 1014C has some selective advantage in terms of fitness cost or resistance phenotype compared to the 1014F allele (Taskin et al. 2016).

Further genetic variability is introduced by the 1014F allele being encoded by two different codons, i.e. TTT and TTC. This was described previously for *Cx. pipiens* by Taskin et al. (2016) who observed allele $1014F^{TTC}$ at low frequencies. In the present study the $1014F^{TTC}$ variant reached an overall frequency of 13% and was present in all Italian provinces at frequencies ranging from 10 to 20%.

Together with the presence of alleles 1014C and 1014S this represents an important diagnostic challenge: indeed, most studies apply the allele-specific-PCR approach by Martinez-torres et al. (1999) which is able to identify allele $1014F^{TTT}$ but does not detect the variant $1014F^{TTC}$ and fails to identify correctly alleles 1014C or 1014S, leading thus to the possible underreporting of these *kdr* variants. This can hamper the evaluation of the impact of different alleles on pyrethroid susceptibility and thus mosquito control activities.

Concerning the distribution of the described variants between the two *Cx. pipiens* biotypes the present study did not produce any support for significant differences, although this estimation is strongly limited by the small number of *Cx. pipiens* f. *molestus* included. There were instead some suggestions for differences in the circulation of alleles between Italy and Greece, with specimens from Greece showing a much higher frequency of allele 1014C compared to Italy (50% vs 5%) and the exclusive presence of allele 1879S. Anyway, due to the low number of specimens analysed and all of them coming from one single sampling site, no general conclusions about the Greek sample can be made.

Overall, this study provides a clearer picture of PR in *Cx. pipiens* by highlighting the circulation of multiple previously unknown potential *kdr* alleles as well as the circulation of the *kdr* allele 1014F in association with allele 918 T, which is analogous to the *super-kdr* haplotype (described in *M. domestica*). As emphasized by Lehane et al. (2024), it will be crucial to bridge gaps between knowledge about insecticide resistance and its actual impact on operational control measures. Only for allele 1014F data on its impact on pyrethroid susceptibility exist, including studies on populations from the same sampling sites examined in the present study (Pichler et al 2022). Further work is instead still required to confirm phenotypic impacts of the newly discovered alleles including toxicological and electrophysiological studies. Additionally, it will be necessary to clarify whether the observed *kdr* allele frequencies indicate an ongoing selective sweep or some balance between selective pressures on insecticide resistance and fitness cost (Kliot and Ghanim 2012) is essential for predicting future outcomes. While in presence of selective pressure the strength of the conferred resistance may lead to an increase in frequency of *kdr* alleles, associated fitness costs may counterbalance this (Kliot and Ghanim 2012). Information on both these aspects are thus necessary to understand the spread and rise of *kdr* alleles, as well as the possibility of a population to reverse to susceptibility in the absence of selective pressure. Altogether such information will provide valuable information to decision makers for the planning of mosquito control activities and the deployment of integrated vector management plans aiming at maintaining effective the currently only chemical tool available for the control of adult mosquito populations and arboviral disease outbreaks in Europe.

Author contribution

Verena Pichler, Kentaro Itokawa, Shinji Kasai, Alessandra della Torre contributed to conceptualization, Verena Pichler, Kentaro Itokawa, Noboru Minakawa, Paola Serini contributed to methodology; Beniamino Caputo, Romeo Bellini, Rodolfo Veronesi, Claudio De Liberato, Federico Romiti, Daniele Arnoldi, Annapaola Rizzoli, Riccardo Paolo Lia, Domenico Otranto, Antonios Michaelakis, Marina Bisia contributed to sampling; Kentaro Itokawa, Verena Pichler, Carlo Maria de Marco contributed to data analysis; Verena Pichler, Kentaro Itokawa, Shinji Kasai, Alessandra della Torre contributed to writing—original draft preparation; Verena Pichler, Noboru Minakawa, Shinji Kasai, Alessandra della Torre, Beniamino Caputo, Antonios Michaelakis contributed to funding acquisition. All authors commented on previous versions of the manuscript and read and approved the final manuscript.

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Data availability The datasets generated and analysed during the current study are available in the Sequence Read Archive (DRA) of DNA Data Bank of Japan (DDBJ) under accession number DRR528067–DRR528091 and DRR567865–DRR567921.

Declarations

Conflict of interest The authors have no competing interests to declare that are relevant to the content of this article.

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