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Presence of "*kdr*" and "*s-kdr*" resistance in *Musca domestica* populations collected in Piacenza province (Northern Italy)

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Abstract

Pyrethroid insecticides combine a high efficacy against insects with a low toxicity towards warm-blooded vertebrate. For this reason they are largely used to control housefly infestations. The efficacy of these products is affected by insecticide resistance mechanisms. In particular, some mutations in the sodium channel coding sequence are responsible for target-site resistance to pyrethroid insecticides. This work presents a new molecular approach based on allele-specific PCRs to point out and to characterise this resistance mechanism in two *M. domestica* populations (PNT and TRV) collected near Piacenza (Northern Italy). The presence of different *kdr* and *s-kdr* genotypes was assessed for population PNT whilst *kdr* only was detected in population TRV. Dose-response bioassays evidenced quite high resistance factors, expecially for population PNT. This is in line with the *kdr* and *s-kdr* frequencies observed in the assayed populations.

Key words: housefly, insecticide resistance, sodium channel, PASA, Italy, haplotypes.

Introduction

The common housefly *Musca domestica* L. (Diptera Muscidae) is a well known pest for health threat as it is capable to transmit and to distribute many human and animal pathogens in the environment (Graczyk *et al.*, 2001; Forster *et al.*, 2007). This insect usually finds in farms, dairies but also urban dumps suitable environments for the development and rapid growth of its populations (Raspi and Belcari, 1989; Trentini *et al.*, 1999; Cao *et al.*, 2006). For this reason, nuisance to livestocks and people living in the surroundings cannot be neglected (Howard, 2001; Winpisinger *et al.*, 2005).

Insecticide applications are commonly used to control housefly populations. In the past, organochloride insecticides like DDT (IRAC MOA 3B, www.irac-online.org) have been widely and intensively used to limit the spread of this species. Today, pyrethroids insecticides (IRAC MOA 3A) represent an important part of the chemicals adopted for housefly control, as they combine high efficacy against this pest with a quite low toxicity towards warm-blooded vertebrates. In addition, this class of products avoid the long term environmental pollution that affected organochlorine insecticides.

Di-phenyl ethane (like DDT) and pyrethroid insecticides act as modulators of the voltage-gated sodium channels, membrane proteins that are responsible for the nerve impulses transmission. Their interactions with the channel protein result in electrical signalling alteration that culminate with the paralysis and the consequent dead of the insect.

Unfortunately, insecticide treatments could be not so efficacious because of unsatisfactory environment management strategies that do not consider the high growth rate of this pest and also because insecticide resistance mechanisms have been developed and selected in housefly populations. During the years, the insecticide abuse have exerted high selection pressure on the populations selecting different resistant mechanisms, due to an increased production of detoxifying enzymes or to structural changes in the pesticide target proteins (Liu and Pridgeon, 2002). The most important mechanism of resistance to pyrethroids is target-site insensitivity conferred by mutations which occur in the sodium channel coding sequence. They cause a reduction of the insecticide binding affinity, compromising the effectiveness of the insecticide applications and seriously affecting the integrated management strategies.

Several point mutations have been described in this target, in *M. domestica* as well as in many others insect species (Davies *et al.*, 2007; Soderlund, 2012; Rinkevich *et al.*, 2013; Dong *et al.*, 2014). The two most important non-synonymous mutations found to confer pyrethroid resistance were first described in housefly and are known as "knock-down resistance" (*kdr*) and "super-*kdr*" (*s-kdr*) (Williamson *et al.*, 1996). They are responsible for amino acid substitutions located in the same functional domain: L1014F for the former and M918T for the latter (Soderlund and Knipple, 2003).

In houseflies there is an additional mutation located in the *kdr* locus and involved in pyrethroid resistance. It is referred as "*kdr-his*" and causes a different amino acid replacement, from Leu to His (L1014H) (Liu and Pridgeon, 2002; Rinkevich *et al.*, 2012).

Insecticide bioassays do not provide enough information about the presence of these mutations and their allelic variants, while a rapid identification is possible by using a biomolecular approach. Different methods have been developed in order to detect *kdr* mutations: PCR-RFLP, allele-specific PCRs or direct sequencing (Huang *et al.*, 2004; Rinkevich *et al.*, 2006; Qiu *et al.*, 2012). On the contrary, till now *s-kdr* has been detected only

by direct sequencing (Rinkevich *et al*., 2006). In *M. domestica*, the analysis of mutation frequencies in voltage gated sodium channel can give important information about the resistance levels of the analysed populations and it could be an interesting tool to evaluate the efficacy of control strategies based on pyrethroid applications. Recent insecticide bioassays carried on an Italian population of *M. domestica* collected from a poultry farm showed the presence of high resistance levels towards several active ingredients but no evidences about the resistance mechanisms involved were provided (Pezzi *et al.*, 2011).

Our study aims to point out the presence and involvement of target-site resistance to pyrethroid insecticides in two populations of *M. domestica* collected near Piacenza (Northern Italy, Po valley), analysed with a new molecular approach which allows the detection of the three known amino acidic substitutions in the voltage gated sodium channel.

Materials and methods

Insects: origin and rearing

Adult houseflies have been collected in 2010 in two livestock farms located in Piacenza province. The first one (PNT) is a modern stable which uses regular insecticide applications in order to control the housefly populations. The second one (TRV) is a small traditional stable not applying any control strategies against flies.

For each sampling area, a population has been originated from the collected insects and maintained in rearing cages under controlled conditions (21 °C ± 0.5 °C), with water and dry mixture of sucrose and milk powder (1:1) always available. A mixture of bran and milk powder (4:1) moistened with tap water was used as oviposition substrate. Eggs were collected every 2 days and transferred into a rearing substrate (bran: 25% w/w; milk powder: 5% w/w; water: 70% w/w). Puparia were collected regularly and transferred to another cage for the next generations.

A susceptible population (S-WHO), used as reference, was kindly provided by Ralf Nauen (Bayer Crop Science, Monheim, Germany) and has been maintained in the same conditions.

Genomic extraction

Genomic DNA was extracted from the heads of individual adult, following a 'salting-out' protocol already described (Panini *et al.*, 2014). Heads were homogenized using a QIAGEN TissueLyser LT for 30 s at 50 Hz in a 2 mL tube containing one stainless steel bead and 300 µL TNES buffer pH 7.5 (50 mM Tris, 400 mM NaCl, 20 mM EDTA, 0.5% SDS) with proteinase K (100 µg mL^{-1}). Homogenate was heated at 55 °C for 1 h and then proteins were precipitated with 85 µL NaCl 5 M and pelleted at 16 000 × g for 5 min. DNA was isolated from the supernatant by ethanol precipitation and resuspended in 50 µL sterile water. The DNA concentration was assessed using a Qubit Fluorimeter 2.0 instrument (Quant-iT ds DNA HS Assay kit; Invitrogen, Carlsbad, CA, USA). The amount of genomic DNA obtained was in the range 2–50 ng μL^{-1}.

PASA-PCR

The presence of *kdr* (L1014F and L1014H) and *s-kdr* (M918T) single point mutations was assessed with allele specific polymerase chain reaction amplification (PASA-PCR). A set of primers was designed (table 1) using a cDNA *M. domestica* sodium channel gene coding sequence (Gene Bank accession number: NM_001286885.1) and primer sequences from Huang *et al*. (2004). A schematic representation of the primer positions is reported in figure 1. The gene structure have been analysed by using Spidey (Wheelan *et al*., 2001), which provide tools for the alignment between mRNA and genomic DNA sequences.

PASA PCR mixtures include 0.4 µM of each primer, 1 µL of genomic DNA and 12.5 µL Dream Taq Green PCR Master Mix (Thermo Scientific, Milan, Italy) in a total reaction volume of 25 µL. For both targets amplification started with 2 min at 94 °C, followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, with a final elongation at 72 °C for 10 min.

Results of PASA-PCRs were validated by sequencing the control fragments directly amplified with the external primers, encompassing the mutations under investigation, obtained from some samples randomly selected. The obtained sequences were then aligned with the genomic reference sequence (Gene Bank accession numbers: NW_004774263.1 and NW_004765908.1) using ClustalW (Thomson *et al*., 1994).

Table 1. Primers (5'-3') used for *kdr* and *s-kdr* characterisation. Nucleotides reported in bold uppercase indicate their specificity for the susceptible or the resistant alleles. The position of the primers is reported as the nucleotide numbers of the cDNA sequence (Gene Bank accession number: X96668).

Target	Primer	Direction	Sequence 5'-3'	Position	References
kdr	K1	FW	tcgcttcaaggaccatgaat	2881-2901	
kdr	K2	RE	ttacgtttcacccagttctta	3178-3199	
kdr	K3	FW	acggtcgtgatcggcaat**C**	3035-3054	Huang *et al*., 2004
kdr	K4	FW	acggtcgtgatcggcaat**T**	3035-3054	Huang *et al*., 2004
kdr	HF_KsH_F	FW	cggtcgtgatcggcaatc**T**	3036-3055	
kdr	HF_KrH_F	FW	cggtcgtgatcggcaatc**A**	3036-3055	
s-kdr	SK1	FW	ttcgtgtattcaaattggcaaa	2709-2731	
s-kdr	SK2	RE	cgaaaagttgcattcccatc	2836-2856	
s-kdr	SK3	RE	acccattgtccggccc**A**	2767-2783	
s-kdr	SK4	RE	acccattgtccggccc**G**	2767-2783	

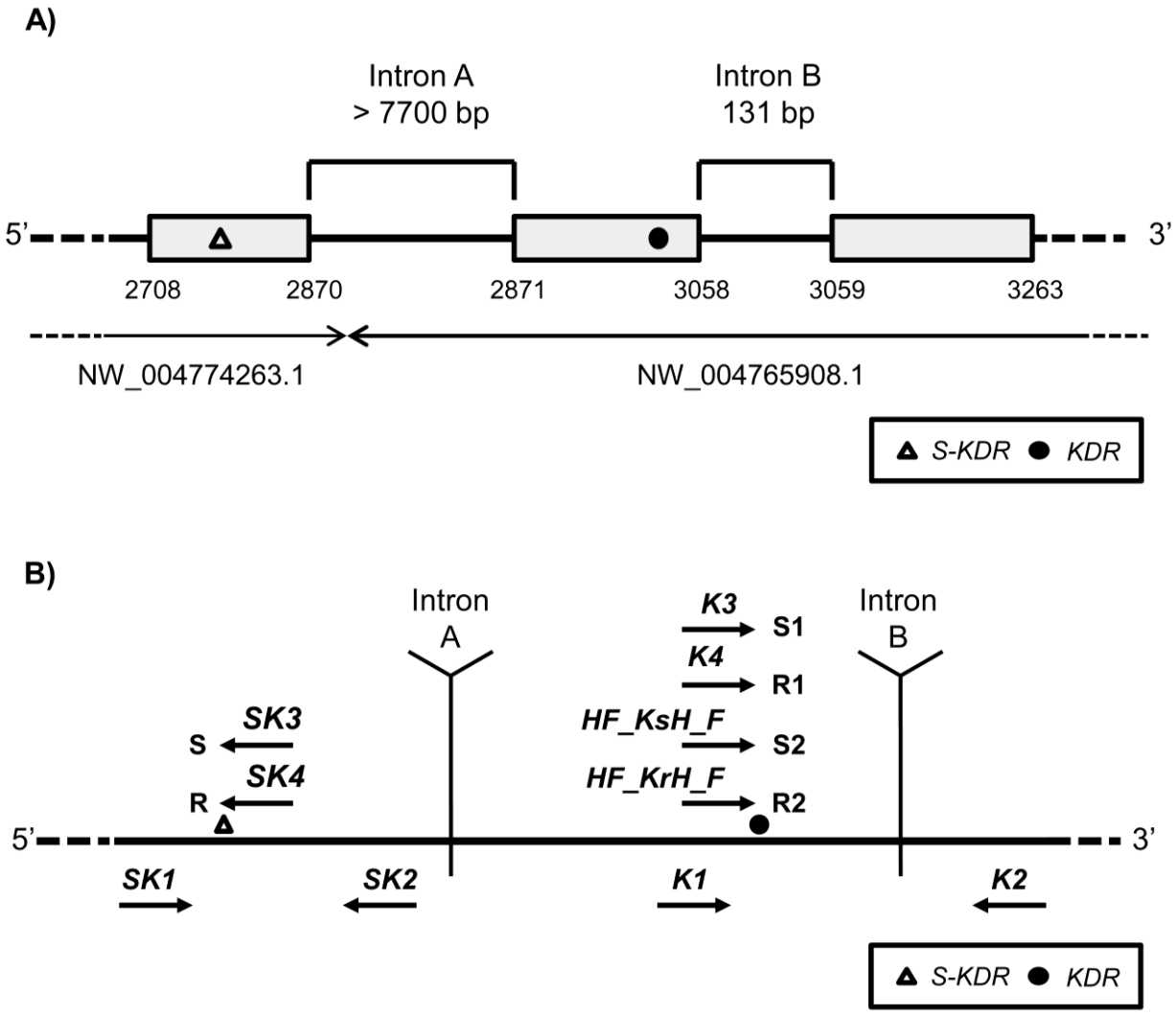

Figure 1. Scheme of *M. domestica* sodium channel gene, which focuses on the portion encompassing the mutations of interest. A) Three exons are shown as grey boxes. The first one includes the *s-kdr* locus (triangle); the second one includes the *kdr* locus (circle). B) SK1 and SK2 are the common external primers used to amplify the *s-kdr* region (145 bp); SK3 and SK4 are the internal specific primers used to amplify respectively the susceptible or the resistant alleles (72 bp). K1 and K2 are the common external primers used to amplify the *kdr* region (448 bp); K3 and K4 are the internal specific primers used to discriminate the polimorphisms of the first position of the codon (295 bp), whilst HF_KsH_F and HF_KrH_F are the internal specific primers used to discriminate the polimorphisms of the second position of the codon (295 bp).

Characterisation of *kdr* mutations

The presence of the *kdr* mutations was assessed by combining results from different specific PCR reactions. The common external primers K1 and K2 amplify a control band of 448 bp, while amplicons originated from the common primer K2 and the allele specific primers (K3, K4, HF_KsH_F and HF_KrH_F) produce a fragment of 295 bp.

Specifically, PCRs were organised in two separate groups in order to detect the nucleotide substitutions in the first and second position of the *kdr* codon, respectively.

The first PCR group combines the reverse common primer K2 and the forward primers K3 (PCR-S1) or K4 (PCR-R1), specifically designed to detect nucleotide C or T in the first position of the codon. It allows the determination of the Phe substitution (**T**TT) but it is not able to discriminate between wild-type susceptible Leu (**C**TT) and resistant His (**C**AT).

The second PCR group combines the reverse common primer K2 and the forward primers HF_KsH_F (PCR-S2) or HF_KrH_F (PCR-R2), specifically designed to detect nucleotide T or A in the second position of the codon. It allows the determination of the His substitution (C**A**T) but it is not able to discriminate between wild-type susceptible Leu (C**T**T) and resistant Phe (T**T**T).

Results derived from the cross-check of the two PCR groups allow the correct genotyping of the *kdr* locus. A schematic overview is presented in figure 2.

Amplicons were visualised on 2% agarose gel stained with Midori Green (NIPPON Genetics EUROPE GmbH) (figure 3).

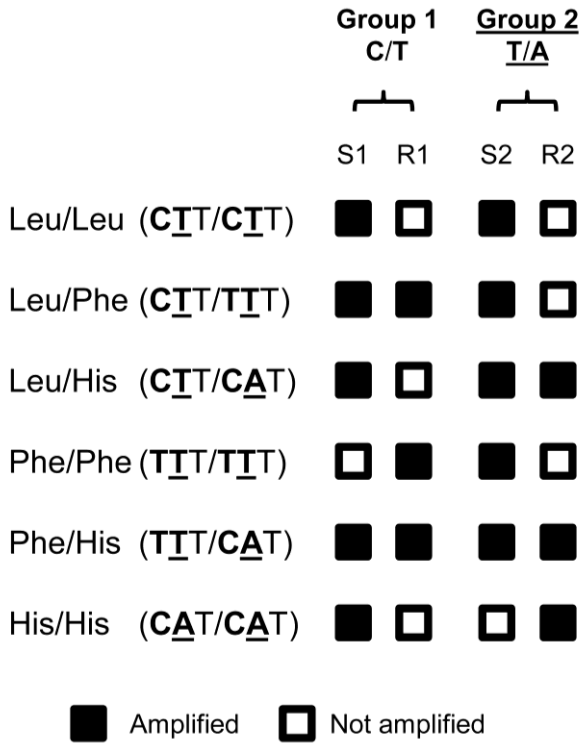

Figure 2. Schematic representation of the amplification obtained with the PCRs used in this study. Black boxes indicate positive amplification for the corresponding genotype. PCRs S1 and R1 are used to discriminate nucleotide C/T in the first position of the codon; PCRs S2 and R2 are used to discriminate nucleotide T/A in the second position of the codon.

Characterisation of *s-kdr* mutations

The presence of the *s-kdr* mutation was assessed by using 3 different PCR reactions. The combination of the common external primers SK1 and SK2 amplifies a 145 bp control band . The combination of SK1 with SK3 or SK4 amplifies respectively the susceptible or the resistant allele, both producing 72 bp bands . Amplicons were visualised on 3.5% agarose gel stained with Midori Green (figure 4).

Insecticides bioassays

Susceptibility toward technical grade methoxychlor (Sigma-Aldrich) was investigated by using topical application bioassays, according to Huang *et al*. (2004).

Insecticide solution in acetone (1 µL) was applied on the notum of three days old females with an Hamilton micro-syringe. For each experiment, 5-6 different concentrations of insecticide were prepared and used to treat pools of 20 houseflies; a control group treated with acetone only have been included in each replicates. Each group was maintained inside a vented plastic cylinder, with water and food (dry milk and sucrose, 1:1 ratio) supplied *ad libitum* and mortalities were assessed 24 hours after the treatment.

The experiment was replicated at least 4 different times for each population (PNT, TRV and S-WHO). Data obtained from the bioassays were pooled together, corrected for control mortality by using Abbott's formula (Abbott, 1925) and concentration-mortality relationships were estimated by probit analysis using POLO-Plus software (LeOra Software, Berkeley, CA) (Finney, 1971).

Results and discussion

Genotyping of *kdr* and *s-kdr* mutations

Three different alleles for the *kdr* locus are known in *M. domestica*: the wild-type codon 'CTT' (Leu) and two different resistant codons 'TTT' (Phe) and 'CAT' (His) (Rinkevich *et al.*, 2006).

In the TRV population (n = 30 specimens) only two *kdr* alleles have been found: 40% of the analysed individuals carried the susceptible allele L1014, whilst 60% of them showed the presence of the resistant allele F1014. In particular, the majority of the flies were heterozygous resistant (53%) and a small percentage carried the homozygous resistant genotype (7%) (table 2).

On the contrary, all the three possible *kdr* alleles have been detected in the PNT population (n = 40 specimens), with different genotype combinations. The most diffuse genotypes were L1014 + F1014 (35%) and F1014 + H1014 (32.5%). In addition, homozygous resistant flies for both amino acidic substitutions were found: 17.5% for the F1014 allele and 7% for the H1014 allele (table 2).

The same flies were analysed for the presence of the *s-kdr* mutation (M918T). It was found only in the PNT population: 50% of the individuals showing a heterozygous genotype and 2.5% a homozygous resistant genotype (table 3).

In addition, the mutation was found only in the presence of *kdr* F1014. No specimens bearing "*s-kdr*" mutation without "*kdr*" (F1014) have been found. This is in

Table 2. Percentage of specimens with different *kdr* genotype combinations in the two analysed population, TRV (n = 30) and PNT (n = 40).

kdr genotype	flies (%)	
	TRV	PNT
L/L	40	2.5
L/F	53.3	35
L/H	0	5
F/F	6.7	17.5
F/H	0	32.5
H/H	0	7.5

Table 3. Percentage of specimens with different *s-kdr* genotype combinations in the two analysed population, TRV (n = 30) and PNT (n = 40).

s-kdr genotype	flies (%)	
	TRV	PNT
M/M	100	47.5
M/T	0	50
T/T	0	2.5

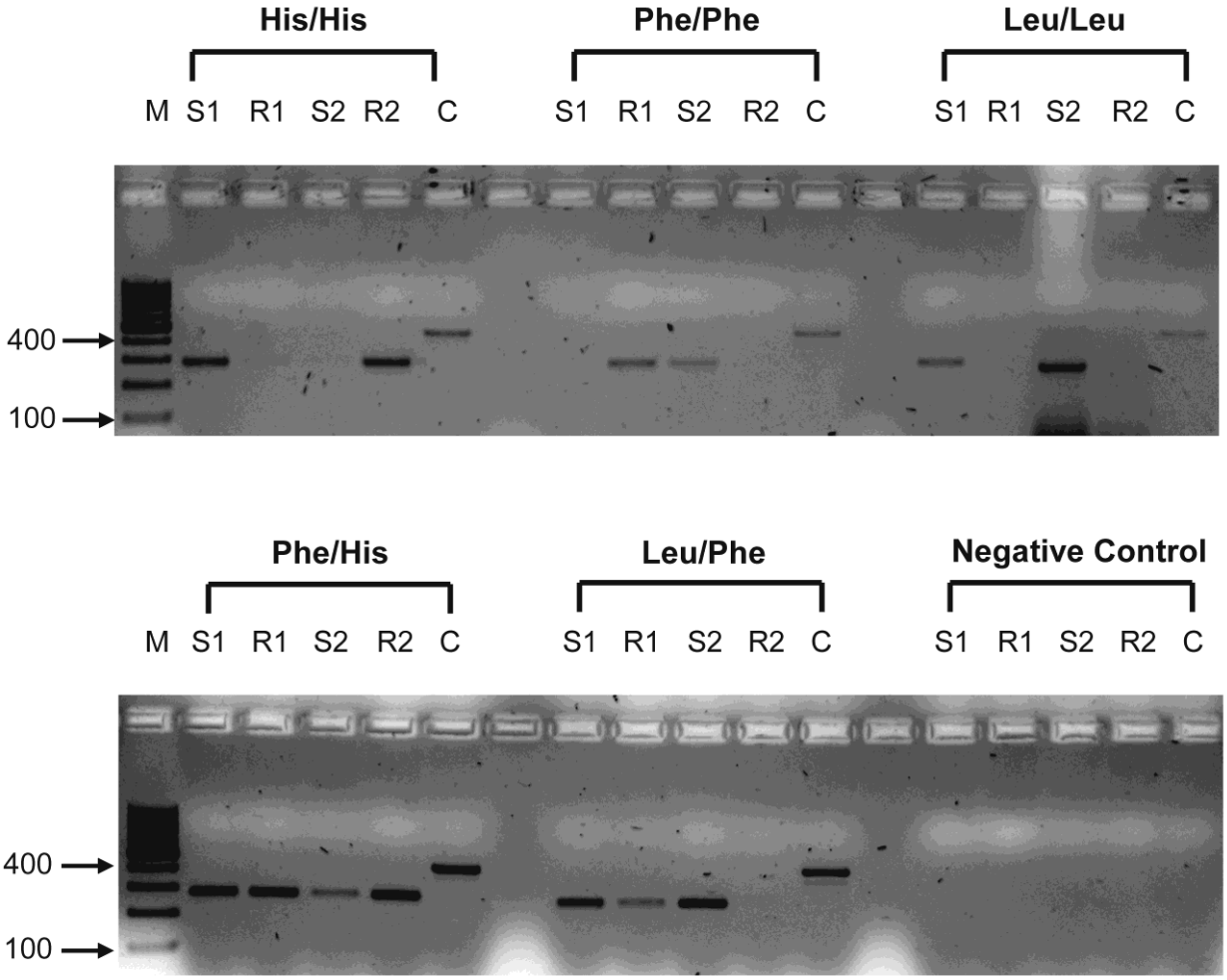

Figure 3. Amplification profile of different *kdr* genotypes: S1, R1, S2 and R2 are the allele-specific PCR reactions (295 bp); C is the control band (448 bp). DNA ladder 100 bp (Thermo Scientific) is used as marker (M).

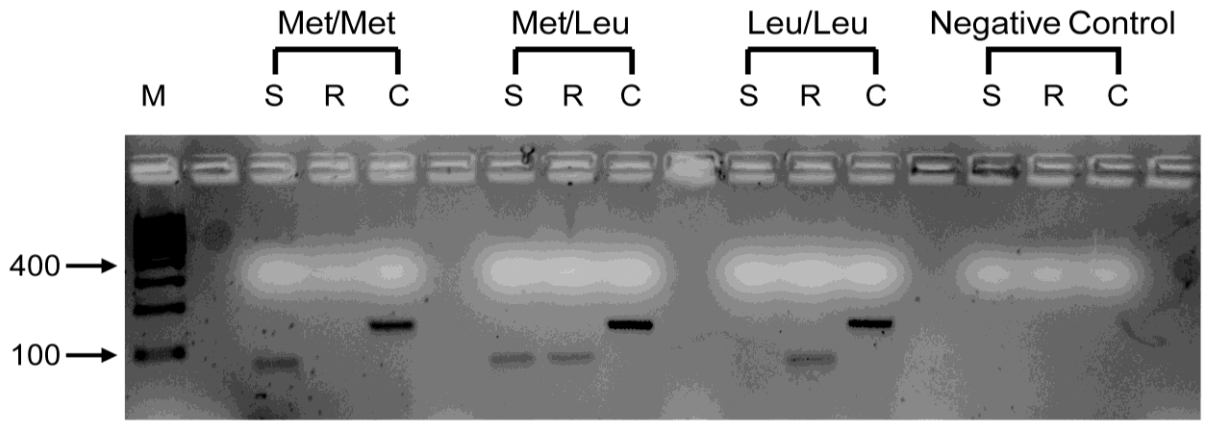

Figure 4. Amplification profile of different *s-kdr* genotypes: 1) Met/Met: homozygous susceptible (S: 72 bp); 2) Met/Leu: heterozygous (S and R: 72 bp); 3) Leu/Leu: homozygous resistant (R: 72 bp); 4) negative control of each PCR reaction. For each genotype a control band (C: 172 bp) is shown. DNA ladder 100 bp (Thermo Scientific) is used as marker (M).

agreement with literature data and support the hypothesis that "*s-kdr*" mutation is of secondary origin in respect with "*kdr*" mutation and also that the presence of "*kdr*" is compulsory to fix "*s-kdr*" mutation in the same allele (Lee *et al.*, 1999).

Haplotypes

Some of the results obtained with allele-specific PCRs have been confirmed by direct sequencing of the control band amplified with the external primers. Primers K1 and K2, encompassing the *kdr* locus, amplify a region of 448 bp and it is comprehensive of an intron about 131 bp long (intron B) (figure 1) that has been described in the literature as a very high variable region. More than 120 haplotypes, in several cases showing also insertions as well as delections, have been reported till now (Rinkevich *et al*., 2012).

Sequences derived from Italian samples carrying different *kdr* genotypes have been aligned with all the housefly sequences now available in GeneBank. The alignment of intronic region B showed that the Italian specimens with the susceptible homozigous genotype (Leu/Leu, e.g. TRV 8) present the same intronic region of a Turkish sample with same genotype (*v11*, accession number: AY850269.2). Furthemore, the Italian samples which showed the resistant homozigous (Phe/Phe, e.g. PNT 5) and heterozygous genotype (Phe/His, e.g. PNT 9) have the same intronic region of samples derived from Turkey, China and USA (*kdr2*, accession number: AY850261.2) (Rinkevich *et al*., 2012) (figure 5).

Sample carrying genotypes Leu/Leu, Phe/Phe and Phe/His showed chromatograms with single peaks; on the contrary, other samples with different genotypes (Leu/Phe and His/His) showed the presence of double peaks which suggest the presence of two different intronic region in the same specimen (data not shown).

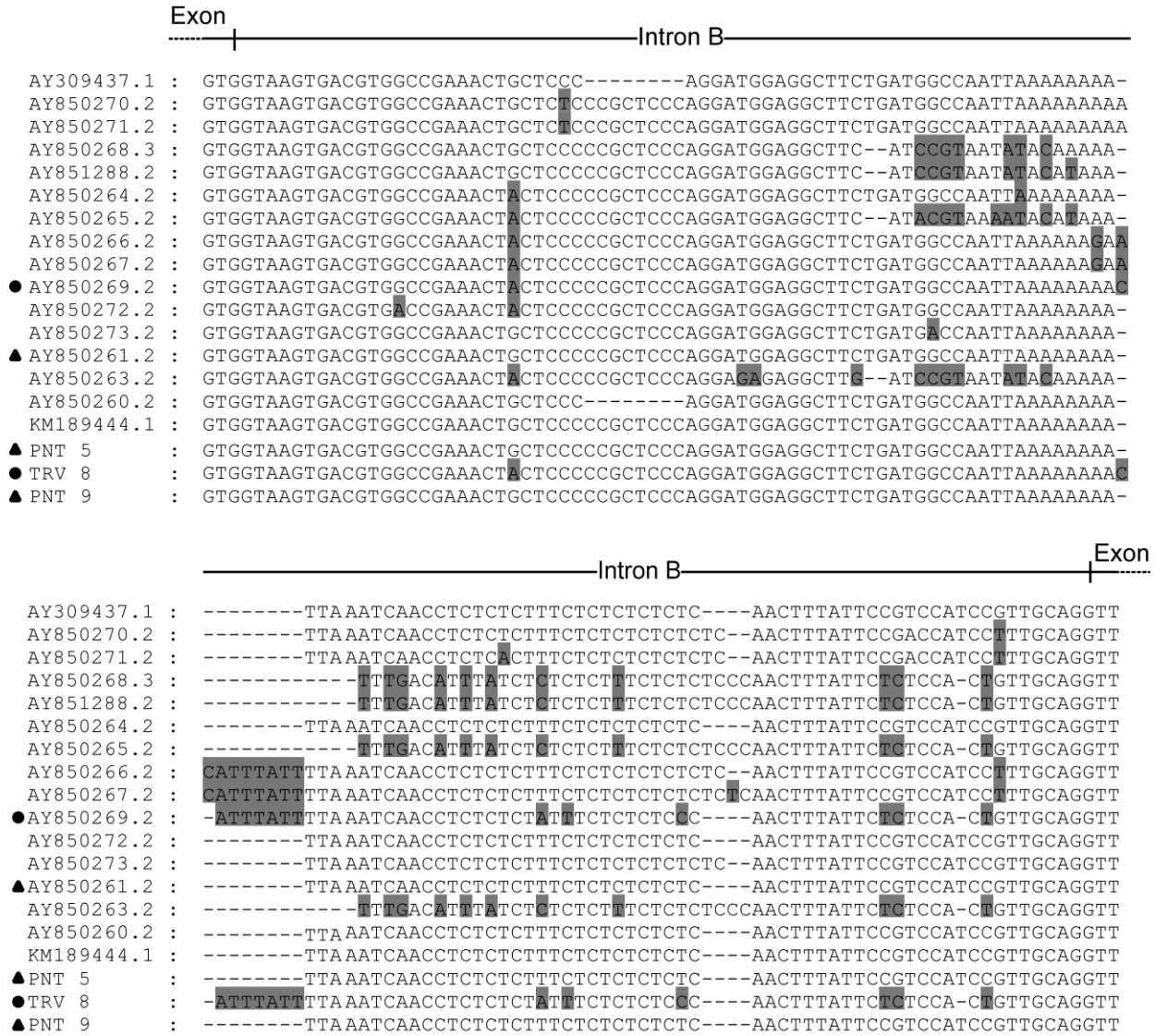

Figure 5. Alignment of intronic region B of all the sequences available in GeneBank. Intronic region variations are in gray boxes. The symbols identify two different haplotypes: the samples PNT 5 (Phe/Phe) and PNT 9 (Phe/His) have the same intronic sequence of AY805261.2 (*kdr2*, triangle) while TRV 8 (Leu/Leu) has 100% identity with AY850269.2 (*v11*, circle).

Table 4. Log-dose probit-mortality data derived from methoxychlor topical application bioassays against adult flies. LC_{50}: lethal concentration that is expected to cause 50% of mortality; CI 95%: confidence interval limits at 95%; R.F.: resistance factor calculated as the ratio between LC_{50} of resistant strain and LC_{50} of susceptible strain S-WHO.

Population	LC_{50} (g L^{-1})	CI 95%	Slope	d.f.	χ^2	R.F.
S-WHO	0.43	0.30 - 0.61	1.36 ± 0.15	21	14.1	=
TRV	86.4	39.3 - 286	0.71 ± 0.12	14	18.6	201
PNT	1240	346 - 50600	0.67 ± 0.16	14	14.2	2876

This data suggest that at least two different alleles associated with mutation L1014H are present in population PNT. Possibly, one of the allele is the same found in the intronic region of *kdr2* (see above), which correspond to the Italian specimen carrying Phe/His mutations.

These results are in agreement with data that have been already published: the intronic sequence of *kdr2* haplotype was found to be the same for different *kdr* alleles (Leu, Phe, His), whilst the intronic region of *v11* haplotype was found to be associated only with Leu (Rinkevich *et al*., 2012).

Bioassays

Preliminary bioassays were performed to estimate a valid insecticide dose range. In the case of the susceptible population (S-WHO) the optimal range of technical active ingredient was from 0.001 g L^{-1} to 10 g $L^{-1,}$ while for populations PNT and TRV the optimal range was from 1 g L^{-1} to 200 g L^{-1}. Parameters of the estimated "log-probit" mortality curves are reported in table 4.

The LC_{50} of population TRV, for which no control strategies have been adopted, was lower than the LC_{50} of population PNT, derived from a farm that regularly uses insecticide applications. Both of them were significantly higher than the LC_{50} of the susceptible strain S-WHO. Estimated resistance factors (RFs) were about 200 for population TRV and more than 2800 for population PNT (table 4).

Conclusions

Pyrethroids are commonly used insecticides to control houseflies. Target-site mutations responsible for pyrethroid resistance have been documented in samples collected in different locations worldwide (Ahmed and Wilkins, 2002; Shono *et al*., 2002; Rinkevich *et al*., 2007; 2012) but just a few information are available regarding the Italian situation (Pezzi, 2011) and no data confirm the presence of the most common mutations which are known to confer pyrethroid insensitivity.

This work evaluated the presence of *kdr*, *kdr-his* and *s-kdr* mutations in Italian samples collected in livestock farms of the Po valley. Even if only two populations have been considered, the presence of target-site mutations was reported, demonstrating for the first time their involvement in pyrethroid resistance also in Italy. Interestingly, one of those population was collected in a farm where no insecticide treatments have been used, suggesting the spread of target-site resistance around the territory.

The molecular approach here reported represents a possible alternative to the classic technologies till now adopted to investigate the above reported mutations. In fact, the allele-specific PCRs allow the rapid genotyping of the *kdr* and *s-kdr* alleles with the reduction of the costs required for the direct sequencing or for a PCR-RFLP analysis. Moreover, our set of primers can detect not only the classic *kdr* mutation (L1014F) but also the other described amino acid replacement (L1014H) in the same locus, thus avoiding the overestimation of the susceptible allele and overcoming PCR-RFLP limitation (Rinkevich *et al*., 2006).

The allele frequency of *kdr* and *s-kdr* reported in these two populations support the level of resistance that have been found in dose-response insecticide bioassays with methoxychlor, a chlorinated insecticide affecting the same target of pyrethroids.

Significant resistant factors were estimated for both the assayed populations but the highest resistance factor was detected in population PNT, collected from a farm which usually uses insecticide to control housefly populations. These results are in agreement with the calculated *kdr* and *s-kdr* frequencies: in population TRV only *kdr* mutation was detected, with the resistant allele present in 60% of the analysed specimens, whilst population PNT showed a consistent presence of *kdr* and *s-kdr* mutations, with more than 90% of the individuals carrying different *kdr* resistant genotypes and more than 50% the *s-kdr* mutation.

The study demonstrated the association of the resistance-attributable mutations in the sodium channel gene with insensitivity to methoxychlor insecticide. As it shares the same mode of action of pyrethroids (IRAC 3 - sodium channel modulators), our results suggest the possible involvement of the same mutations also in pyrethroid resistance. We cannot exclude the involvement of different resistance mechanisms like detoxification systems based on P450 enzymes. Further investigations with different insecticide products would be necessary, including pre-treatment with PBO in order to better explore the influence of metabolic resistance.

Even if this study is based on a little number of populations, it has confirmed the presence of different genotype combinations of target-site mutations that can influence the efficacy of pyrethroids applications. Furthermore, the intronic analysis of the region closed to the *kdr* locus confirms that the haplotype variability, already detected worldwide, is also present in our samples. Those results support the multiple origin of target-site resistance already discussed by other authors (Rinkevich *et al*., 2012). Moreover, the *s-kdr* mutation was not found to be associated with a specific intronic region, supporting the theory that it evolved from different *kdr* alleles.

The establishment of different combinations of resistant alleles could be due to crosses among housefly populations that are capable to spread around different geographical area and enhanced by their high reproductive potential.

Additional analysis of a higher number of housefly populations collected around Italy are then required to better explain these aspects to provide more details about the current insecticide resistance situation in this pest and to adopt and implement more efficacious insecticide resistance management strategies.

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