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Qualitative Sybr Green real-time detection of single nucleotide polymorphisms responsible for target-site resistance in insect pests: the example of *Myzus persicae* and *Musca domestica*

V. Puggioni, O. Chiesa, M. Panini and E. Mazzoni*

Department of Sustainable Crop Production, Section Sustainable Crop and Food Protection, Università Cattolica del Sacro Cuore, Via Emilia Parmense, 84, I-29122 Piacenza, Italy

Abstract

Chemical insecticides have been widely used to control insect pests, leading to the selection of resistant populations. To date, several single nucleotide polymorphisms (SNPs) have already been associated with insecticide resistance, causing reduced sensitivity to many classes of products. Monitoring and detection of target-site resistance is currently one of the most important factors for insect pest management strategies. Several methods are available for this purpose: automated and high-throughput techniques (i.e. TaqMan or pyrosequencing) are very costly; cheaper alternatives (i.e. RFLP or PASA–PCRs) are time-consuming and limited by the necessity of a final visualization step. This work presents a new approach (QSGG, Qualitative Sybr Green Genotyping) which combines the specificity of PASA–PCR with the rapidity of real-time PCR analysis. The specific real-time detection of *Cq* values of wild-type or mutant alleles (amplified used allele-specific primers) allows the calculation of ΔCq_{W-M} values and the consequent identification of the genotypes of unknown samples, on the basis of ranges previously defined with reference clones. The methodology is applied here to characterize mutations described in *Myzus persicae* and *Musca domestica* and we demonstrate it represents a valid, rapid and cost-effective technique that can be adopted for monitoring target-site resistance in field populations of these and other insect species.

Keywords: SNPs genotyping, qualitative real-time PCR, *M. persicae*, *M. domestica*, R81T, *kdr*, *s-kdr*, *kdr-his*, M918L, MACE

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Introduction

Insecticide resistance is a widespread phenomenon documented in a great number of insect species (Arthropod Pesticide Resistance Database; http://www.pesticideresistance.com). Most represent a serious threat for agricultural production, and others are of medical and veterinarian importance (Whalon *et al.*, 2008). Despite the large diversity of insect pests, resistance mechanisms that have so far been identified can roughly be gathered together just in a few groups: reduced penetration; enhanced production of metabolic enzymes excluding, sequestering and destroying the insecticide; target-site insensitivity (Feyereisen *et al.*, 2015).

The discovery of point mutations in target proteins associated with insecticide resistance mechanisms has increased in recent years and the coexistence of different mutations within a single specimen has been documented in several species. The establishment of different genotypes associated with resistant phenotypes depends on the life cycle, the fecundity and the reproductive mode of the insects. In particular, mating

*Address for correspondence
Fax: +39 0523 599268
Phone: +39 0523 599237
E-mail: emanuele.mazzoni@unicatt.it

is responsible for the production of different genotype combinations and can explain the presence of different resistance mechanisms within the individual, each contributing to enhance resistance factors (Fenton *et al.*, 2010; Hardstone & Scott, 2010; Feyereisen *et al.*, 2015).

Monitoring and detection of insecticide resistance is currently one of the most important aspects for insect pest management.

In recent years, we have experienced an increase of resistant cases of agricultural pests that are important for some local crop productions. The most representative example is the green peach aphid *Myzus persicae* (Sulzer) (Hemiptera: Aphididae), which is considered one of the most widely and strongly resistant species worldwide. Since 2010, we have received a considerable number of requests for single nucleotide polymorphisms (SNP) genotyping for samples collected throughout Italy (Panini *et al.*, 2014), with particular attention paid to neonicotinoid resistance. In fact, the assessment of the presence and distribution of resistant alleles is of particular importance to understand the reasons for control failures causing consistent re-infestation of this pest and to create and implement effective resistance management strategies, avoiding inefficacious insecticide applications.

Analogous considerations are true for other insect species important for urban and livestock environments, like the housefly *Musca domestica* L. (Diptera: Muscidae). As it can transmit several diseases it represent a serious pest for public health, poultry and livestock farming, and insecticide applications are commonly used for its control. In particular, previously, pyrethroids were the chemical control of choice, leading to serious resistance problems. Target-site mutations responsible for pyrethroid resistance have been documented in samples collected in different locations worldwide (Rinkevich *et al.*, 2012; Mazzoni *et al.*, 2015). Despite this, they still remain one of the main chemicals adopted for housefly control. For this reason the presence and diffusion of any target-site mechanisms, which could affect this class of product must be continuously considered and monitored to assess the resistance status of this pest.

In both the above-mentioned species, there is a consistent number of SNPs that must be checked to estimate the presence of target-site resistance mechanisms. In addition, it is possible to find different polymorphisms for a specific locus, e.g. M918T/L in the green peach aphid *s-kdr* locus (Panini *et al.*, 2015) or L1014F/H in the housefly *kdr* locus (Liu & Pridgeon, 2002). Furthermore, an important aspect to consider is the large number of samples that have to be analyzed in order to obtain frequencies of the resistant alleles, which can be representative of the real situation in the field. All of these aspects contribute to increase the number of analyses that must be performed.

A significant amount of research carried out in recent years has provided several advances in understanding the evolution of resistance mechanisms and a variety of molecular and biochemical techniques has been developed allowing the detection of resistance-associated mutations. In particular, the increasing number of insect genomic sequences available has enabled the development of several SNP genotype detection methods.

A comprehensive list of these techniques has been reviewed by Kwok (2001) and Black & Vontas (2007), while a cost, timing and performance comparison has been presented by Bass *et al.* (2007) and, more recently, by Bai *et al.* (2014). Some of the high-throughput technologies (e.g. TaqMan assay or direct sequencing reactions) are limited due to expensive equipment and/or the high costs and expiring date of the reagents. Other affordable assays (e.g. allele-specific PCRs or PCR–RFLP) require several steps (amplification, restriction analysis, PCR and visualization in gel electrophoresis), limiting the number of samples that can be analyzed in a short time.

In this work, we combine the basics of real-time PCRs and allele-specific PCRs to develop a new affordable assay (QSGG: Qualitative Sybr Green Genotyping) to detect known target-site mutations in *M. persicae* and *M. domestica* (Nabeshima *et al.*, 2003; Bass *et al.*, 2011; Rinkevich *et al.*, 2013; Panini *et al.*, 2015). The approach links rapid detection of the Sybr Green real-time technique with the capability of PASA–PCRs to discern univocally the presence of a particular SNP of interest.

Materials and methods

Insects

M. persicae populations were collected around Italy from different hosts. During field sampling aphids were directly stored in acetone and then kept at −20°C till DNA extraction. Populations of reference clones were available in the rearing collection of the Department of Sustainable Crop Production (Università Cattolica del Sacro Cuore, Piacenza, Italy), where each strain is maintained as a colony of parthenogenetic females under controlled conditions (Mazzoni & Cravedi, 2002).

M. domestica populations were collected from various sites in Northern Italy, stored in acetone and kept at −20°C till DNA extraction. A reference susceptible strain S-WHO was kindly provided by Ralf Nauen (Bayer Crop Science, Monheim, Germany) and reared as described in Mazzoni *et al.* (2015).

Reference specimens

Specimens of *M. persicae* and *M. domestica* with well-defined genotypes were used as references (table 1). They were previously characterized by direct sequencing (Sanger method) for the presence of the following mutation of interest: L1014F/H (*kdr* and *kdr-his*) and M918T (*s-kdr*) for houseflies (Mazzoni *et al.*, 2015); L1014F (*kdr*), M918T/L (*s-kdr*), R81T for aphids (Panini *et al.*, 2014, 2015). The same procedure was here adopted to characterize the presence of MACE (S431F).

DNA extraction

Genomic DNA was extracted from single specimens of *M. persicae* or from the head of individual adults of *M. domestica* by a 'salting-out' protocol, as already described (Panini *et al.*, 2014). After some trials with different DNA dilutions without quantification, real-time PCR analyses were performed using 1:10 and 1:5 dilutions for aphid and housefly samples, respectively.

PCR protocols for SNP detection

The presence of target-site mutations was assessed by real-time PCR. Different assays were developed for different *loci*. A full list of primers used is reported in table 2. In some allele-specific primers a mismatch was incorporated in position 4, starting from 3′ end, to improve their specificity.

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Table 1. Reference specimens and known nucleotide polymorphisms associated with insecticide resistance in *M. domestica* and *M. persicae*.

Organism	Target	Population	Codons	Amino acids
M. domestica	*kdr*	SWHO	CTT/CTT	Leu/Leu
		PNT_M11	CTT/TTT	Leu/Phe
		PNT_P6	CTT/CAT	Leu/His
		PNT_P25	TTT/TTT	Phe/Phe
		PNT_M9	TTT/CAT	Phe/His
		PNT_P14	CAT/CAT	His/His
	s-kdr	SWHO	ATG/ATG	Met/Met
		PNT_M12	ATG/ACG	Met/Thr
		PNT_M4	ACG/ACG	Thr/Thr
M. persicae	R81T	1X	AGA/AGA	Arg/Arg
		97H1	AGA/ACA	Arg/Thr
		99H1	ACA/ACA	Thr/Thr
	MACE	1X	TCA/TCA	Ser/Ser
		13H	TCA/TTT	Ser/Phe
		43H	TTT/TTT	Phe/Phe
	kdr	1X	CTC/CTC	Leu/Leu
		62H2	CTC/TTC	Leu/Phe
		92H6	TTC/TTC	Phe/Phe
	s-kdr	1X	ATG/ATG	Met/Met
		62H2	ATG/ACG	Met/Thr
		92H6	ACG/ACG	Thr/Thr
		167H6	ATG/CTG	Met/Leu
		384C	ATG/TTG	Met/Leu
		114H65	CTG/CTG	Leu/Leu
		175H2	ACG/CTG	Leu/Thr
		125.06	ACG/TTG	Leu/Thr

All PCR reactions were performed using iTaq™ Universal SYBR® Green Supermix (Bio-Rad) and were run on a BIO-RAD CFX96™ Real-Time system. In general, PCR reactions (10 µl) contained 5 µl iTaq™, 0.4 µM of each primer and 3 µl of diluted genomic DNA, and were performed as follows: initial denaturation at 95°C (5 min) followed by 40 cycles of denaturation at 95°C (5 s), annealing temperature depending on the locus (30 s) and elongation at 72°C (60 s). A final melt curve step was included, ramping from 65 to 95°C by 0.5°C every 5 s.

The possible genotypes can be distinguished by calculating the difference between the Quantification Cycle (Cq for short) values obtained from PCRs to detect wild-type allele (PCR-W) and mutant allele (PCR-M), as follows:

$$\Delta Cq_{W-M} = Cq_W - Cq_M,$$

ΔCq_{W-M} data were analyzed with the IBM SPSS version 23 Statistical Package using EXAMINE, ONEWAY and NPAR TESTS procedures.

M. domestica s-kdr

To detect M918T (ATG→ACG) in para-type sodium channel gene (NaCh), the following PCR reactions ($T_{annealing}$ 62.3°C) were used: (a) PCR-W_{HfSK} combines primer HF_Sk7_Re with allele-specific primer HF_SkX5s_FW to detect codon ATG (Met); (b) PCR-M_{HfSK} combines primer HF_Sk7_Re with allele-specific primer HF_SkX6r_FW to detect codon ACG (Thr).

M. persicae MACE

To detect S431F (TCA→TTT) in acetylcholinesterase 1 gene (*AChE1*), the following PCR reactions ($T_{annealing}$ 60°C) were used: (a) PCR-W_{MACE} combines primer AChE-F2 with allele-specific primer MpACEs-R to detect codon TCA (Ser); (b) PCR-M_{MACE} combines primer AChE-F2 with allele-specific primer Mace-R-Rev to detect codon TTT (Phe).

M. persicae R81T

To detect R81T (AGA→ACA) in nicotinic acetylcholine receptor β1 subunit (nAChR β1), the following PCR reactions ($T_{annealing}$ 60.5°C) were used: (a) PCR-W_{R81T} combines primer MpNACR-R514 with allele-specific primer MpNACRs-XFW-aga to detect codon AGA (Arg); (b) PCR-M_{R81T} combines primer MpNACR-F52 with allele-specific primer MpNACRr-XRE-aca to detect codon ACA (Thr).

M. persicae kdr

To detect L1014F (CTC→TTC) in para-type sodium channel gene (NaCh) the following PCR reactions ($T_{annealing}$ 58°C) were used: (a) PCR-W_{MpK} combines primer *kdr*-R4 with allele-specific primer MpKDR-XFW-ctc to detect codon CTC (Leu); (b) PCR-M_{MpK} combines primer *kdr*-F1 with allele-specific primer MpKDR-XRE-ttc to detect codon TTC (Phe). In the latter the concentration of primers was increased to 0.5 µM.

M. domestica kdr

To detect L1014F/H (CTC→TTC or CTC→CAT) in para-type sodium channel gene (NaCh) the following PCR reactions ($T_{annealing}$ 58.5°C) were used: (a) PCR-$W1_{HfK}$ combines primer K2 with allele-specific primer K3 to detect nucleotide C in position 1; (b) PCR-$M1_{HfK}$ combines primer K2 with allele-specific primer K4 to detect nucleotide T in position 1; (c) PCR-$W2_{HfK}$ combines primer K2 with allele-specific primer HF_KsH_F to detect nucleotide T in position 2; (d) PCR-$M2_{HfK}$ combines primer K2 with allele-specific primer HF_KrH_F to detect nucleotide A in position 2.

M. persicae s-kdr

To detect M918T (ATG→ACG) in para-type sodium channel gene (NaCh) the following PCR reactions ($T_{annealing}$ 68.7°C) were used: (a) PCR-$W2_{MpSK}$ combines primer MpSK-F25 with allele-specific primer MpSKs-RE to detect nucleotide T in position 2; (b) PCR-$M2_{MpSK}$ combines primer MpSK-F25 with allele-specific primer MpSKr-RE to detect nucleotide C in position 2.

To detect M918L (ATG→CTG or TTG) in the same locus the following PCR reactions ($T_{annealing}$ 63°C) were used: (a) PCR-$W1_{MpSK}$ combines primer MpSK-F25 with allele-specific primer MpSKL-XRE-atg to detect nucleotide A in position 1; (b) PCR-$M1C_{MpSK}$ combines primer MpSK-R3292 with allele-specific primer MpSKL-XFW-ctg to detect nucleotide C in position 1; (c) PCR-$M1T_{MpSK}$ combines primer MpSK-R3292 with allele-specific primer MpSKL-XFW-ttg to detect nucleotide T in position 1.

Results

For each target, PCR for the wild-type and the mutant alleles were set up. The reaction conditions were optimized using preliminary gradient PCRs to get the best thermal conditions as well as the optimal primer concentrations (data not

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Table 2. Sequences of the primers used.

Organism	Target	Primer	Sequence 5′–3′
M. domestica	*kdr*	K1[1, 5]	TCGCTTCAAGGACCATGAAT
		K2[1, 5]	TTACGTTTCACCCAGTTCTTA
		K3[5]	ACGGTCGTGATCGGCAAT**C**
		K4[5]	ACGGTCGTGATCGGCAAT**T**
		HF_KsH_F[5]	CGGTCGTGATCGGCAATC**T**
		HF_KrH_F[5]	CGGTCGTGATCGGCAATC**A**
	s-kdr	SK1[1, 5]	TTCGTGTATTCAAATTGGCAAA
		SK2[1, 5]	CGAAAAGTTGCATTCCCATC
		HF_Sk7_Re	GAGGATGATGAGGCAATCCTCC
		HF_SkX5s_FW	CACTGAATTTACTCATTTCGACTA**T**
		HF_SkX6r_FW	CACTGAATTTACTCATTTCGACTA**C**
M. persicae	MACE	AChE-R3[1, 2]	CGAGACACCACCACGTTTTCCTC
		AChE-F2[1, 2]	GAGCCAGAATACACAGAGAAGCG
		MpACEs-R	GCTCCGTCAAATAATAAAATAT**TG**
		Mace-R-Rev[3]	GCTCCGTCAAATAATAAAATAT**AA**
	R81T	MpNACR-F52[1, 4]	CCATTGTTCGTATAGTTACAGAATC
		MpNACRr-XRE-aca	TATATTAAGTAGGTTACTCACACGT**G**
		MpNACR-R514[1, 4]	GAGATAAATCGCTGAGTAGATTTC
		MpNACRs-XFW-aga	GATAATGAAATCAAACGTTTGGTTGA**G**
	kdr	*kdr*-F1[1, 2]	TCGTGGCCCACACTGAATCT
		kdr-R4[1, 2]	GTTCATGTAAGATACATGAATTC
		MpKDR-XFW-ctc	CTACTGTTGTCATTGGTCAC**C**
		MpKDR-XRE-ttc	ATAGTACTTATACATACCAGGA**A**
	s-kdr	MpSKr-RE	GCACCGATGGTTCGACCC**G**
		MpSKs-RE[4]	GCACCGATGGTTCGACCC**A**
		MpSK-F25[4]	TGAAACTGATGGCGATGAGCCCTA
		MpSK-R3292[4]	GTAGGTTCTGGATAGCAATTGTTGC
		MpSKL-XFW-ctg	CCCACACTGAATCTTTTAATATCCCTA**C**
		MpSKL-XFW-ttg	CCCACACTGAATCTTTTAATATCCCTA**T**
		MpSKL-XRE-atg	GCACCGATGGTTCGACGCA**T**

The presence of 'X' letter in the primer names indicates a mismatch corresponding to the underlined nucleotide. Nucleotides in bold at 3′ end indicate the specificity for the wild-type or the mutant allele.
[1]primer used for sequencing.
[2]Cassanelli *et al.* (2005).
[3]Fontaine *et al.* (2011).
[4]Panini *et al.* (2014).
[5]Mazzoni *et al.* (2015).

shown). After this, PCRs on a certain number of referent specimens where run for 40 cycles and their Cqs were evaluated using threshold automatically calculated by the Bio-Rad CFX Manager™ software.

When both PCRs (wild-type and mutant) gave Cqs higher than 30 they were scored as negative amplification and not considered in our statistics. When only one reaction gave positive amplification, the sample was definitively scored as homozygous, wild-type (W) or mutant (M), according to the primer set used (fig. 1a). In all the remaining cases, the difference between Cq wild-type and Cq mutant values (ΔCq_{W-M}) was calculated (fig. 1b) even if one of the Cqs was higher than 30 (fig. 1c) and used to score the correct genotype. Final melt curves always confirmed the absence of non-specific amplification products (data not shown).

ΔCq_{W-M} values are expected to be negative when both alleles are wild-type, near 0 for heterozygous genotype or positive when both alleles are mutant.

A range of 20–40 reactions were performed for each target, and data were validated as follow. The Shapiro–Wilk test ($P > 0.05$) showed that ΔCq_{W-M} values for reference samples were approximately normally distributed for each target and genotype (Supplementary table 1). The same sets of ΔCq_{W-M} values were analyzed with one way analysis of variance (ANOVA) comparing different genotypes within each target. *F* statistics revealed extremely significant differences of ΔCq_{W-M} means (table 3). The comparison using the Student–Newman–Keuls (SNK) test confirmed that ΔCq_{W-M} means of homozygous wild-type, heterozygous and homozygous mutated genotype were statistically different ($P < 0.05$).

Discrimination of one polymorphism

Target-site resistance to neonicotinoids (R81T), dimethylcarbamates (MACE: S431F) and pyrethroids (*kdr*: L1014F in *M. persicae*; *s-kdr*: M918T in *M. domestica*) are caused by one amino acidic substitution, giving three possible genotypes: homozygous wild-type (W/W), heterozygous (W/M), mutant homozygous (M/M). These targets were characterized as above described and, as expected, homozygous wild-type genotypes produced negative ΔCq_{W-M}; heterozygous samples produced ΔCq_{W-M} mean values near 0; mutant homozygous genotypes gave positive ΔCq_{W-M} mean values depending on the considered target (table 4; Supplementary fig. 1).

Discrimination of two or three alternative polymorphisms

Other assays were developed to characterize target-site mutations, which are due to multiple possible nucleotide

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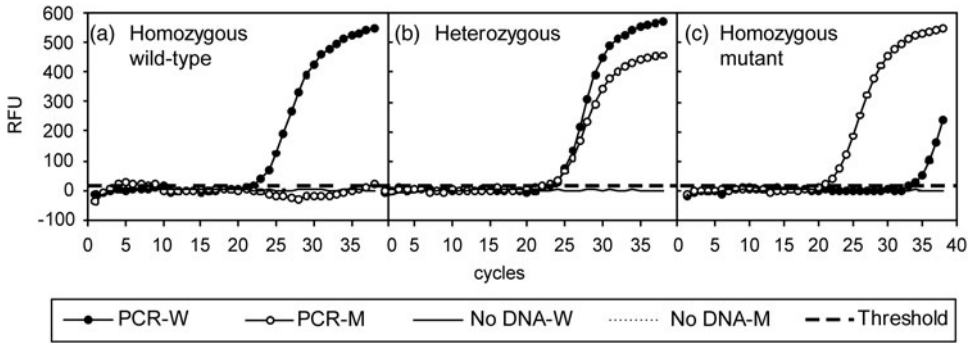

Fig. 1. Examples of real-time fluorescence curves (i.e. *M. persicae* MACE) obtained in presence of different genotypes: (a) homozygous wild-type; (b) heterozygous; (c) mutant homozygous (PCR-W, PCR with specific primer for wild-type allele; PCR-M, PCR with specific primer for mutant allele).

Table 3. Analysis of variance (ANOVA) performed on ΔCq_{W-M} values obtained from reference samples.

Species	Target	*F*	df	*P*
M. domestica	*kdr* (L1014F)	206.2	2/27	<0.001
	kdr-his (L1014H)	341.8	2/27	<0.001
	s-kdr (M918T)	2004.5	2/27	<0.001
M. persicae	MACE (S431F)	912.2	2/18	<0.001
	nAChR (R81T)	1607.1	2/27	<0.001
	kdr (L1014F)	1106.1	2/24	<0.001
	s-kdr (M918T)	860.8	2/30	<0.001
	s-kdr (M918L-ctg)	665.4	3/34	<0.001
	s-kdr (M918L-ttg)	1114.8	2/18	<0.001

substitutions involving different positions within the same codon, like *kdr* in *M. domestica* (L1014F and L1014H) or *s-kdr* in *M. persicae* (M918T and M918L).

In *M. domestica*, the presence of L1014F/H (*kdr*/*kdr-his*) was assessed combining results from four different allele-specific real-time PCR reactions, organized in two separate groups to detect nucleotide substitutions in the first and the second position of the codon, respectively.

The first PCR group combines the reverse common primer K2 and the forward primers K3 (PCR-$W1_{HfK}$) or K4 (PCR-$M1_{HfK}$), specifically designed to detect nucleotide C or T in the first position of the codon. This reaction detects the Phe substitution (TTT) but it is not able to discriminate between wild-type susceptible Leu (CTT) and resistant His (CAT).

Smaller ΔCq_{W1-M1} values were obtained in comparison to those observed for the other considered targets. They ranged from 3.35 in the presence of 'thymine' to −5.16 in the presence of 'cytosine' (table 5).

The second PCR group combines the reverse common primer K2 and the forward primers HF_KsH_F (PCR-$W2_{HfK}$) or HF_KrH_F (PCR-$M2_{HfK}$), specifically designed to detect nucleotide T or A in the second position of the codon. This reaction detects the His substitution (CAT) but it is not able to discriminate between wild-type susceptible Leu (CTT) and resistant Phe (TTT). A wider range of values was obtained and ΔCq_{W2-M2} mean values were more clearly separated, ranging from −13.6 in the presence of 'thymine' to 9.18 in the presence of 'adenine' (table 5).

The combination of the results allows the identification of the correct genotype (table 6; Supplementary fig. 2).

In *M. persicae*, the presence of M918T/L (*s-kdr*) is further complicated by two possible polymorphisms (Italian and French) for the leucine. For this reason, five different specific real-time PCRs are needed, organized in two separate groups to detect the nucleotide substitutions in the first and second position of the codon.

PCR group checking the 'classic' *s-kdr* (M918T) includes two different reactions to determine the second position of the codon. It combines the forward common primer MpSK-F25 with the reverse primers MpSKs-RE (PCR-$W2_{MpSK}$) or MpSKr-RE (PCR-$M2_{MpSK}$), specifically designed to detect nucleotide T or C. It allows the determination of the Thr substitution (ACG) (fig. 2a), but it is not able to discriminate between wild-type susceptible Met (ATG) and resistant Leu (CTG or TTG).

The other PCR group, which evaluates the presence of the 'new' *s-kdr* (M918L), includes three different reactions to determine the first position of the codon. The first PCR combines the forward primer MpSK-F25 with the reverse primer MpSKL-XRE-atg ($W1_{MpSK}$) specifically designed to detect nucleotide A. The second combines the reverse primer MpSK-R3292 with the forward primer MpSKL-XFW-ctg ($M1C_{MpSK}$) specifically designed to detect nucleotide C. The third combines the reverse primer MpSK-R3292 with the forward primer MpSKL-XFW-ttg ($M1T_{MpSK}$) specifically designed to detect nucleotide T (fig. 2b). These reactions identify Leu substitution (CTG or TTG) and the presence of wild-type susceptible Met (ATG) but it is not able to discriminate between mutant homozygous (CTG/CTG and TTG/TTG) and mutant heterozygous (ACG/CTG and ACG/TTG) because the primer MpSKL-XRE-atg cannot amplify codon ACG (fig. 3). The combination of the results allows the identification of the correct genotype (table 7; Supplementary figs 3 and 4).

PCRs to detect M918T, gave ΔCq_{W2-M2} values widely separated, ranging from −10.2 in the presence of 'thymine' to 10.9 in the presence of 'cytosine'.

To discriminate ΔCq_{W-M} means measured in presence of codons CTG and TTG two separate ANOVA were applied.

Considering the A/C substitution, ΔCq_{W1-M1C} mean values showed good differentiation between different genotypes: wild-type homozygous (ATG/ATG; $\Delta Cq_{W1-M1C} = -12.2$), heterozygous (ATG/CTG; $\Delta Cq_{W1-M1C} = -1.7$) and mutant homozygous genotype (CTG/CTG; $\Delta Cq_{W1-M1C} = 6.1$). Finally, the heterozygous genotype generated by the presence of both mutant codons (ACG/CTG) produced ΔCq_{W1-M1C} mean value

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Table 4. ΔCq_{W-M} mean values measured for targets with one polymorphism in the corresponding codons.

Target Genotype	*M. domestica* *s-kdr* Mean ± SD	*n*	*M. persicae* MACE Mean ± SD	*N*	R81T Mean ± SD	*n*	*kdr* Mean ± SD	*n*
M/M	7.73 ± 0.49 a	10	12.71 ± 1.41 a	7	7.99 ± 0.37 a	10	5.93 ± 1.07 a	9
W/M	−0.53 ± 0.58 b	10	−0.04 ± 0.50 b	7	0.02 ± 0.59 b	10	−1.16 ± 0.58 b	9
W/W	−6.62 ± 0.45 c	10	−18.67 ± 1.87 c	7	−12.19 ± 1.20 c	10	−12.78 ± 0.83 c	9

SD, standard deviation; *n*, number of reference samples analyzed.
Means with different letters are statistically different (SNK test).

Table 5. *M. domestica kdr* locus.

First position Nucleotide detected	ΔCq_{W1-M1} Mean ± SD	*n*	Second position Nucleotide detected	ΔCq_{W2-M2} Mean ± SD	*n*
T	3.35 ± 0.63 a	5	A	9.18 ± 0.76 a	5
T/C	−0.25 ± 0.59 b	10	A/T	0.39 ± 0.77 b	10
C	−5.16 ± 1.08 c	15	T	−13.59 ± 2.50 c	15

Mean values of ΔCq_{W-M} recorded for the two PCR groups to detect SNPs in positions 1 and 2. Means with different letters are statistically different (SNK test) (SD, standard deviation; *n*, number of reference samples considered for each genotype).

Table 6. Schematic representation of PCRs used to detect known mutations in *M. domestica kdr* locus.

Codified residues		First position (C/T)		Second position (T/A)	
Amino acids	Triplets	$W1_{HfK}$	$M1_{HfK}$	$W2_{HfK}$	$M2_{HfK}$
Leu/Leu	CTT/CTT	√		√	
Leu/Phe	CTT/TTT	√	√	√	
Leu/His	CTT/CAT	√		√	√
Phe/Phe	TTT/TTT		√	√	
Phe/His	TTT/CAT	√	√	√	√
His/His	CAT/CAT	√			√

Ticks indicate positive amplification for the corresponding genotype.

equal to 8.1. Similar results were obtained analyzing the presence of A/T nucleotides in position 1 even if slightly lower ΔCqs were observed in samples with codons ACG/TTG ($\Delta Cq_{W1-M1T} = 5.9$) (table 8).

Application of QSGG method to unknown genotype samples

After the assessment of ΔCqs ranges calculated from the reference samples, a series of threshold values were defined using the limits of data distributions rounded to the nearest integer (table 9). It was decided to adopt this approach instead of using confidence intervals to limit the number of doubtful cases and because theoretical distribution limits were well separated.

Several specimens of *M. domestica* and *M. persicae* were then analyzed with the QSGG method to verify its capability to characterize samples with unknown genotype. Sets of PCRs were performed as above described and ΔCq values were scored on the basis of the established threshold limits (table 9).

The distributions of unknown samples well fitted those estimated with reference populations (fig. 4). The statistical significance of this comparison was evaluated for each target and genotype with the non-parametric Mann–Whitney *U* test and in almost every case no statistically significant differences were found (Supplementary table 1). The only statistically significant difference was observed in MACE homozygous mutated distribution (M/M), due to the small number of samples analyze likely because of the rareness of this genotype in field populations. Nonetheless all the ΔCq_{W-M} observed were without any doubt higher than the threshold limit (fig. 4d).

When no statistically significant differences were detected, examples of small deviations from the predicted ranges were observed. Examples were represented by MACE homozygous wild-type (W/W) and R81T mutant homozygous (M/M) where some ΔCqs were higher than expected but in both case this did not affect genotype assignment.

Discussion

Recent advances in molecular biology allowed the development of several techniques for SNPs detection. The opportunity and feasibility to detect variation of individual nucleotides is well established and represents a key tool for multiple application, including genetic population studies and specific point mutation characterization (Tsuchihashi & Dracopoli, 2002; Black & Vontas, 2007). Many efforts have been finalized for the identification of target-site resistance in a wide number of insect pests and there is now an increasing consciousness of the importance of early detect resistance in field populations to prevent the misuse and abuse of insecticide products that are not efficacious anymore.

A great variety of techniques have been developed, which are based on different chemistries and signal detection methods. As consequence, some of them are efficient buy very costly, whilst others represent cheaper but time-consuming solutions.

Briefly, if we compare the most common methodologies today available for SNP genotyping (Bai *et al.*, 2014), the two most specific and accurate are those which rely on the sequencing of DNA traits of interest: Sanger or pyro-sequencing. In addition to the obvious reliability of those techniques, it is

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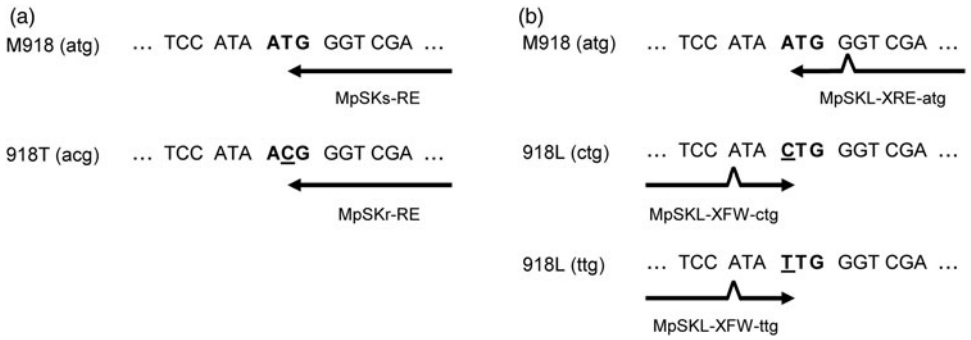

Fig. 2. Schematic representations of allele-specific primers for *s-kdr* locus in *M. persicae*. (a) 'classic' *s-kdr* (M918T). (b) 'new' *s-kdr* (M918L, codons CTG and TTG). Mismatch in position 4 from 3′ end is indicated.

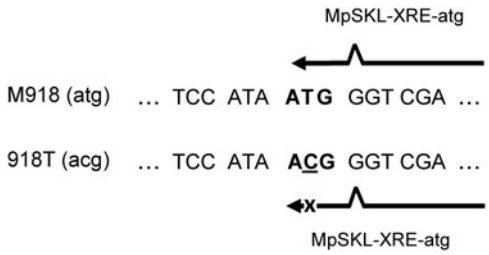

Fig. 3. Possible pairing of primer MpSKL-XRE-atg. The cross indicates the natural mismatch which prevents amplification despite the presence of nucleotide A in position 1. Mismatch in position 4 from 3′ end is indicated.

worth to consider the high costs required per sample, together with the inconvenience of a certain waiting time for the results, especially if the sequences are produced by external sequencing services. Another important technique largely used to detect point mutations is the fluorescence assay based on TaqMan probes. Despite the previous ones, real-time TaqMan assay do not require any pre-amplification steps for templates preparation (like the linkage with streptavidin-coated magnetic beads as for the pyrosequencing), and results can be obtain in only one single step; also, using different dyes it is possible to combine different allele-specific probes in a single reaction. Despite those certainly advantages, it remains quite expensive because of the need of fluorescent probes and, although their costs become a minor problem if a high number of samples are analyzed in a reduced period of time, their performance is strictly dependent on their expiring date. Furthermore, if more than two alleles can be present, single tube multiplex TaqMan analysis with more than two probes could not work in some cases, as reported by Fontaine *et al.* (2013) which reported unsatisfactory results with this technique for simultaneously detection of three codons of *s-kdr* locus in *M. persicae*.

Cheaper methods are available and routinely used for the same purposes, like the classic restriction length polymorphism (PCR–RFLP) and the allele-specific PCR-based assays (PASA–PCR). Excluding the former method, which is strictly dependent on the creation/destruction of restriction sites in case of nucleotide mutations, the latter has been widely used during the years for SNPs genotyping, as it represents one of the most feasible and economic technique, which do not require any specific laboratory equipment or costly kit reagents, as it is confirmed by Bai *et al.* (2014) through the comparison of different genotyping assays. Nevertheless, limitations are impose by the timing of the experiment, which necessarily depends on a visualization step in agarose gel after the amplification. Recently, comparisons of costs and advantages of using allele-specific real-time PCR (similar to QSGG method), with restriction fragment length polymorphism (RFLP) and TaqMan techniques was discussed by Dhas *et al.* (2015). All the above mentioned techniques have been largely adopted for the characterization of point mutations related to insecticide resistance in several pests, including *M. persicae* and *M. domestica*. Specifically, most of the studies carried out in housefly populations relied on Sanger sequencing (Rinkevich *et al.*, 2012), while pyrosequencing was applied to detect M918T/L and L1014F mutations in aphid samples (Panini *et al.*, 2015). TaqMan assays were set up to detect other important point mutations found in *M. persicae* (Anstead et al., 2004, 2008; Puinean et al., 2013), as well as PCR–RFLP and PASA–PCRs were applied to both species (Huang *et al.*, 2004; Cassanelli *et al.*, 2005; Qiu *et al.*, 2012; Panini *et al.*, 2014; Voudouris *et al.*, 2016).

To improve the diagnosis of target-site resistance in insect pests, in the present study we described an alternative approach for SNPs genotyping, which combines the specificity of PASA–PCR with the rapidity of the Sybr Green real-time detection. The increasing fluorescence is monitored, as in a classic real-time PCR, but it is considered just for the qualitative discrimination and not for quantitative purposes.

In this approach, the low-throughput of the classic allele-specific PCR is overcame, and 96-well microtiter plates can be used in order to process a high number of samples with a consistent reduction of the protocol run time. Furthermore, as the analyses are based on differences of Cq values (ΔCq), gDNA quantification is not compulsory. Initial efforts for the protocol optimization are needed (primer design – if they are not already available in the literature – and temperature annealing), but not more than other techniques. On the contrary, in the proposed QSGG analysis, the amplicon length is not a critical parameter and primers can be improved with the addition of mismatches to increase their allele specificity, whilst for example TaqMan probes must follow specific constraints. Finally, after the statistic validation of standard samples, the analyses can be easily performed when are needed, without any dependence with fluorescent probes availability.

Similar approaches were already been considered by other authors witch performed Sybr Green analysis combining allele-specific PCRs with quantitative real-time PCR with

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Table 7. Schematic representation of the PCRs used to detect known mutations in *M. persicae s-kdr* locus.

Codified residues Amino acids	Triplets	First position (A/C/T) $W1_{MpSK}$	$M1C_{MpSK}$	$M1T_{MpSK}$	Second position (T/C) $W2_{MpSK}$	$M2_{MpSK}$
Met/Met	ATG/ATG	√			√	
Met/Thr	ATG/ACG	√			√	√
Thr/Thr	ACG/ACG					√
Met/Leu	ATG/CTG	√	√		√	
Met/Leu	ATG/TTG	√		√	√	
Leu/Leu	CTG/CTG		√		√	
Thr/Leu	ACG/CTG		√		√	√
Thr/Leu	ACG/TTG			√	√	√
Leu/Leu	CTG/TTG (*)		√	√	√	
Leu/Leu	TTG/TTG (*)			√	√	

Ticks indicate positive amplification for the corresponding genotype (*: never detected).

Table 8. *M. persicae s-kdr* locus.

First position Genotype	ΔCq_{W1-M1C} Mean ± SD	*n*	First position Genotype	ΔCq_{W1-M1T} Mean ± SD	*n*	Second position Genotype	ΔCq_{W2-M2} Mean ± SD	*n*
ACG/CTG	8.13 ± 0.65 a	12	ACG/TTG	5.87 ± 0.71 a	7	ACG/ACG	10.90 ± 1.24 a	11
CTG/CTG	6.14 ± 1.00 b	10	TTG/TTG	Never detected		ACG/ATG	0.32 ± 0.50 b	11
ATG/CTG	−1.65 ± 1.41 c	9	ATG/TTG	−1.73 ± 1.01 b	7	ATG/ATG	−10.22 ± 1.58 c	11
ATG/ATG	−12.19 ± 1.07 d	7	ATG/ATG	−13.62 ± 0.55 c	7			

SD, standard deviation; *n*, number of reference samples considered for each genotype.
Mean values of ΔCqs recorded for the two PCR groups to detect SNPs in position 1 and 2. Means with different letters are statistically different (SNK test). Genotype TTG/TTG was never detected.

Table 9. ΔCq_{W-M} values used as thresholds for genotype assignment.

Organism	Target	W/W	W/M	M/M
M. domestica	*kdr* (L1014F)	<−3	−2<−>1	>2
	kdr (L1014H)	<−6	−1<−>2	>8
	s-kdr	<−6	−2<−>1	>6
M. persicae	MACE	<−14	−1<−>1	>11
	R81T	<−11	−1<−>1	>7
	kdr	<−11	−2<−>0	>4
	s-kdr (*M918T*)	<−7	−1<−>2	>8
	s-kdr (*M918L: ctg*)	<−11	−4<−>0	>4
	s-kdr (*M918L: ttg*)	<−12	−4<−>0	>5

melt curve analysis for genotyping (Dall'Ozzo *et al.*, 2003; Papp *et al.*, 2003). Further improvements were obtained with high resolution melting (HRM) assays although it required more expensive equipment (Bass *et al.*, 2007).

Other studies for different purposes were performed using similar strategies for example by Fraaije *et al.* (2002) that obtained high accuracy in allele frequencies quantification and by Yu *et al.* (2005) that discriminated different *Bactrocera* species amplifying a specific region of their mitochondrial cytochrome oxidase I gene (COI).

Here we developed similar protocols looking for the discrimination of different polymorphisms that are feasible for specific nucleotide positions. We presented different solutions for genotyping point mutations already described in *M. persicae* and *M. domestica*, starting from those with just one polymorphism to reach more complex situations with multiple possible variations in the same locus. Despite a large number of PCR are required for the latter condition, strategies can be adopted to optimize the workflow and reduce the analyses. For example, the detection of *kdr* and *s-kdr* mutations can be optimized taking into account that M918T was always found only in the presence of L1014F (Soderlund & Knipple, 2003; Eleftherianos *et al.*, 2008). Therefore, in *M. persicae*, checking first the presence of the classic *s-kdr* mutation (M918T), in case of homozygous mutated samples no further investigations are necessary, reducing the number of PCRs from 7 to just 2. Similar considerations could be done on the basis of the current knowledge of specific mutation and depending on the sampling areas. An example is the new *s-kdr* mutation in green peach aphids, which is known to be caused by an A/C substitution in the majority of the Italian populations while the most common French polymorphism is A/T substitution (Roy *et al.*, 2013).

To summarize, the QSGG approach represents a valid SNP genotyping method which is high-throughput, rapid and very cost-effective. It can be easily adopted in monitoring surveys related to evaluation of target-site resistance spread and persistence, helping pest management strategies for the control of the insects here considered, as well as other possible targets or biological situations.

Supplementary material

The supplementary material for this article can be found at http://dx.doi.org/10.1017/S0007485316000675

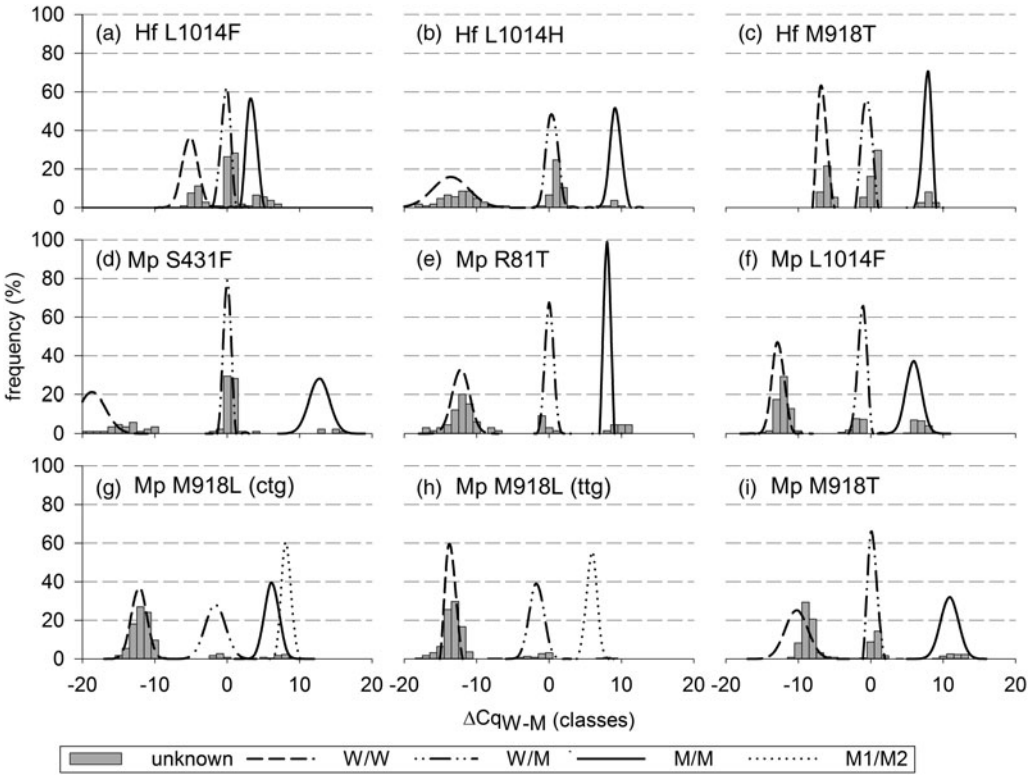

Fig. 4. Frequency distributions of ΔCq_{W-M} measured in unknown samples compared with ΔCq_{W-M} distribution from known genotypes for the investigated targets in *M. domestica* (a: *kdr*, L1014F, $n = 107$; b: *kdr-his*, L1014H, $n = 107$; c: *s-kdr*, M918T, $n = 37$) and *M. persicae* (d: MACE, $n = 85$; e: R81T, $n = 65$; f: *kdr*, L1014F, $n = 270$; g: *s-kdr*, M918L (ctg), $n = 215$; h: *s-kdr*, M918L (ttg), $n = 215$; i: *s-kdr*, M918T, $n = 340$).

Author contributions

All authors conceived and designed the experiments. V.P., O.C. and M.P. performed the experiments. E.M. and O.C. analyzed the data. All authors wrote, read and approved the manuscript.

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