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Detection and geographical distribution of the organophosphate resistance-associated Δ3Q *ace* mutation in the olive fruit fly, *Bactrocera oleae* (Rossi)

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Abstract

BACKGROUND: The olive fruit fly, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae), is the most important pest of olives. Its control is based mostly on organophosphate (OP) insecticides, a practice that has led to resistance development. OP resistance in *B. oleae* has been associated with three mutations in the acetylcholinesterase (AChE), the product of ace gene. The current study presents new diagnostic tests for the detection of the *ace* mutations and aims at monitoring the frequency of the Δ3Q mutation, which appears associated with resistance at higher OP doses in natural olive fly populations.

RESULTS: An allele-specific polymerase chain reaction (PCR), a PCR-RFLP (restriction fragment length polymorphism) and a Taq-Man test were developed for the Δ3Q mutation detection and a new duplex quantitative PCR assay was designed for the G488S and I214V mutations. Moreover, the frequency of Δ3Q mutation was examined in ten populations of eight countries around the Mediterranean basin. The highest frequencies (10%) were found in Greece and Italy, whereas a gradual decrease of Δ3Q frequency towards the western Mediterranean was noted.

CONCLUSION: Robust tests for insecticide resistance mutations at their incipient levels are essential tools to monitor the increase and geographical spread of such mutations. Three different tests were developed for AChE-Δ3Q that indicated its association with OP applications across the Mediterranean.
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Keywords: *Bactrocera oleae*; insecticide resistance; acetylcholinesterase; diagnostic test

1 INTRODUCTION

The primary approach used to control insect pests is the application of insecticides. Organophosphate (OP) insecticides have been extensively used throughout the world to control insect populations for the last 40 to 50 years. Inevitably, such extensive and continuous applications have caused the development and spread of insecticide resistance in natural insect populations. The target of OPs is acetylcholinesterase (AChE), which is responsible for the hydrolytic degradation of the neurotransmitter acetylcholine (ACh) at cholinergic synapses and consequently the termination of impulse transmission.[1,2] OPs disrupt the impulse transmission and cause insect paralysis and death by irreversible phosphorylation and deactivation of AChE.[3–5] An important mechanism of resistance to OPs is the alteration of their target in such a way that affect the binding of the OP insecticide into the active site and the efficiency of AChE phosphorylation.[6] AChE insensitivity due to point mutations in or around the active site has been described in various insect species,[7] such as *Drosophila melanogaster*,[8,9] several mosquito species,[10,11] in *Musca domestica*,[12–15] *Bactrocera dorsalis*,[16] *Ceratitis capitata*,[17] *Aphis gossypii*[18,19] and other hemipteran species.[20] Moreover, most mutations are identical in all these species,[7] suggesting that a low number of mutations can actually provide resistance. However, there are species, such as *Nephotettix cincticeps*,[21] *Boophilus microplus*,[22] and *Lucilia cuprina*,[23,24] where the OP resistant phenotype is not associated with alterations in the *ace* gene. In such cases, resistance to OPs has been attributed to elevated levels of detoxifying esterases[23,25] or glutathione-S-transferases (GSTs)[26,27] that may bind and sequester these insecticides, preventing them from reaching their target, AChE.

The olive fruit fly, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae), is the major pest of the olive fruit. The female fly leaves its eggs in the olive and the emerging larvae feed on the olive sap, thus destroying the fruit. For more than 40 years, olive fly

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populations have been mainly controlled by heavy use of OPs. Two non-synonymous point mutations and one short deletion in the *ace* gene have been reported to affect sensitivity to OP insecticides,[28,29] while metabolic resistance did not seem to have a major role.[30] The two point mutations, which are found in the active site of AChE, result in substitution of isoleucine and glycine by valine and serine in position 214 (I214V) and 488 (G488S), respectively, and cause steric hindrance of the insecticides.[28] The mutation G488S is almost always accompanied by I241V in field populations and their combination confer higher resistance over each individual mutation.[28,31–33] However, the third mutation is in striking contrast to the other two point mutations. The deletion of three glutamines (Δ3Q) is not located in the catalytic center of AChE but at the carboxyl terminal of the enzyme at position 642 and affects the glycophosphatidylinositol (GPI)-anchoring efficiency,[29] indicating a distinct OP-resistance mechanism. It has been hypothesized that the Δ3Q improves GPI anchoring, thus increasing the amount of AChE that reaches the synaptic cleft for its normal role of ACh hydrolysis, allowing the insect to survive higher insecticide dosages.[34] The only additional documented report of a resistance-associated mutation outside the catalytic region of AChE is that of the Colorado potato beetle *Leptinotarsa decemlineata*,[35] in which resistance is hypothesized to be associated with a more generalized change of the secondary structure of the protein.

The I214V and G488S mutations are the first ones to be selected under the minimum OP pressure and have risen to high frequency. A clear correlation between extensive use of OPs and high frequency of 214 V and 488S alleles in the Mediterranean basin has been shown previously.[31–33] Furthermore, the presence of both I214V and G488S mutations in homozygocity in the least resistant individuals,[33] advocates for their importance at a minimum level of resistance, but also is indicative of a low fitness cost of these two mutations. On the contrary, the Δ3Q mutation appears associated with resistance at higher OP doses but always in combination with the two point mutations. This suggests an auxiliary and/or multiplicative role of this mutation, in addition to its inability to provide by itself a basal level of resistance. Moreover, Δ3Q was always found in heterozygosis,[29] indicating a much higher fitness cost. Be that as it may, there is still little information on the geographic distribution and variability of Δ3Q alleles in the development of OP resistance in olive fly.

The management of pesticide resistance requires rapid and efficient assays for the detection of mutants in a population. Several approaches have been proposed with polymerase chain reaction followed by fragment length polymorphism (PCR-RFLP) and allele-specific PCR being the most popular. Taq-Man chemistry and melting curve analysis have been previously used as a "closed tube" alternative to overcome PCR-RFLP disadvantages.[36] In addition, multiplex PCR assays can generate multiple sets of data in a single reaction increasing the analytical capabilities. Such assays require less biological material and are less expensive than single target assays.

In the present study, we report the development of different diagnostic tests for the detection of the Δ3Q mutation and its use to genotype olive fly populations collected throughout the Mediterranean basin. In addition, we present a new duplex quantitative polymerase chain reaction (qPCR) assay for the detection of I214V and G488S mutations. As the threat of insecticide resistance continues to loom large, diagnostic assays for resistance-associated alleles in natural populations and monitoring their distribution would be useful in selection and application of appropriate resistance management strategies.

Table 1. Frequency of Δ3Q mutation in Mediterranean basin

Location	F_R [a]	R/N [b]	HW $\chi 2$
Greece (Crete)	0.125	8/32	c
Italy (Vasto)	0.1112	6/27	c
Israel (Jerusalem)	0.1	6/30	c
Israel (Sde Boker)	0.1	6/30	c
Cyprus	0.0645	4/31	c
France (Corsica)	0.0625	4/32	c
France (south coast)	0.0333	2/30	c
Spain (Cordoba)	0.0167	1/30	c
Portugal (Lisboa)	0	0/30	
Morocco	0.05	3/30	c

[a] F_R is the frequency of the resistant allele calculated as the ratio $R/2N$.
[b] N is the total number of individuals tested and R is the number of the resistant alleles found in the sample. For a diploid organism, the maximum value for R is $2N$.
[c] Indicates the Hardy–Weinberg equilibrium based on χ^2 test. All populations were in Hardy–Weinberg equilibrium, apart from the population of Portugal that was monomorphic

2 MATERIAL AND METHODS

2.1 Insect

Olive fly samples were collected from olive fruits from ten different locations in the Mediterranean basin. The number of olive flies and locations of the sample sites are shown in Table 1. Genomic DNA was extracted from single flies as described by Ashburner.[37]

2.2 Diagnostic tests

Primers and sequence-specific oligonucleotide probes used in diagnostic tests were designed from the known mRNA sequence of *Boace*.[28] The primers and the probe used in real-time PCR were designed using Oligo 6 software (TIB MOLBIOL, Germany).

2.3 Detection of Δ3Q mutation

2.3.1 PCR-RFLP

PCR for the amplification of exon 10 (where the Δ3Q mutation is located) was performed in 20 μL reaction volume and ∼10 ng genomic DNA was used as a template. The amplification reactions contained a final concentration of 1xTaq buffer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 10 pmoles of each primer (Boace10F: TGAAGTCAAACCATCATCCG and Boace10R: GACAGCGCCAACATGAACG) and 1 unit of Taq polymerease (GoTaq® Promega). The amplification reaction was performed as earlier at annealing temperature 51 °C and extension time 30 s. PCR products of exon 10 were incubated with 5 units *Mwo*I (New England Biolabs) for 2 h in a 40 μL reaction volume. Digestion products were electrophoresed in a 2% Low Range Ultra Agarose gel (New England Biolabs).

2.3.2 Allele-specific PCR

About 10 ng genomic DNA was amplified in a 20 μL reaction volume with primers Ex10wt3′F-IMP (CTTCCTCCCTGCAACAATAG) and Boace10R (to test the presence of the wild type allele of exon 10) or with primers Ex10mut3′F-IMP (CTTCCTCCCTGCAACAATAC)

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Table 2. Sequences of PCR primers and hybridization probes (HybProbe) for melting curve analysis of I214V and G488S mutations

Name		5′-3 sequence
I214V	Forward	CTGATCATTTGATACACAACGGAA
	Reverse	CCCACACGATATTGAAACGAA
	Sensor HybProbe	CTGCCACACTGGATATATACAATGC-Fluorescein
	Anchor HybProbe	LC640[a]-GACATTATGTCGGCTGTGG GTAATGTCATT-Phosphate
G488S	Forward	GGATGAGGCGACTTCATTGC
	Reverse	GTGCACTACTACTACTTTACACACCG
	Sensor HybProbe	GCGCAGTGAGCGATCACT-Fluorescein
	Anchor HybProbe	LC640-CTTCACCTGCCCGACTA ATGAGTATGCCC-Phosphate

[a] LC640, Light Cycler Red 640.

and Boace10R (to test the presence of the mutant Δ3Q allele of exon 10). The amplification reaction contained a final concentration of 1xTaq buffer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 10 pmoles of each primer and 1 unit of Taq polymerease (GoTaq® Promega) and it was performed under the following conditions: initial denaturation at 94 °C for 4 min, followed by 30 cycles consisting of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. This was followed by 7 min of final extension at 72 °C. PCR products were electrophoresed in a 1.5 % agarose gel (Biorad).

2.3.3 Taq-Man assay

Real-time PCR was performed on a LightCycler 2 (Roche Applied Science) amplifying exon 10 in 20 μL reaction volume. The amplification reactions contained 50 ng of genomic DNA as a template and a final concentration of 1x of Hot Start reaction mix for PCR on the LightCycler, 10 μM of each primer (Del10-f: GAAGTCAAACCATCATCCGCTT and Del10-r:CAGTGACAGCGCCAACAT) and 4 μM of TM-ins probe (FAM-TGCAACAACAGCAACAACACT-BBQ). The LightCycler program had an initial pre-incubation step for 10 min at 95 °C followed by 45 cycles that included a denaturation step at 95 °C for 10 s, an annealing step at 60 °C for 60 s and an extension step at 72 °C for 1 s. Finally, a cooling step at 40 °C for 30 s was included.

2.4 Detection of I214V and G488S mutations

2.4.1 Duplex qPCR analysis and melting curve analysis

A multicolor procedure was used for the detection of G488S and I214V mutations in a single PCR amplification reaction. The final volume of the duplex qPCR reaction was performed in a 20 μL reaction and 50 ng of genomic DNA was used as a template. Each reaction contained 1x Master Mix HyProbe Plus for PCR on the LightCycler, 5 μM of primers I214V-F, I214V-R and G488S-R, 2.5 μM of primer G488S-F and 5 μM of each probe (Table 2). Thermal cycling for the LightCycler 2.0 was performed under the following conditions: initial denaturation (pre-incubation) at 95 °C for 10 min, followed by 35 cycles of amplification at 95 °C for 15 s, 51 °C for 20 s and 72 °C for 30 s. A final melting curve cycle was performed by raising the temperature to 95 °C for 10 s followed by an annealing temperature at 45 °C for 20 s. Finally the temperature was raised to 85 °C with continuous fluorescent acquisition, followed by a cool down to 40 °C for 30 s.

The detection of G488S mutation was performed at 640 nm fluorescence channel whereas I214V mutation was monitored at 705 nm channel. The fluorescence signal (*F*) in real-time was plotted against temperature (*T*) to produce melting curves for each sample. Melting curve analysis was performed using the Tm calling feature of the Data Analysis software v.4.05 (Roche Applied Science).

2.5 Statistical analysis

Δ3Q allele frequencies and Hardy–Weinberg equilibrium tests in ten natural olive fly populations were calculated using PopGene v. 1.31.[38]

3 RESULTS

A small OP resistance-associated deletion in *B. oleae* AChE (Δ3Q) was previously identified and a direct PCR diagnostic test was presented for monitoring the mutant allele.[29] In this study, we set out to develop three additional diagnostic tests based on the sequence data of resistant allele in order to generate more convenient and robust assays to detect the presence of the Δ3Q mutation in natural populations. In that respect, we also developed a new duplex qPCR assay for the detection of the I214V and G488S mutations. Cloned cDNAs containing either the wild type or the altered sequence of the Δ3Q mutation from a previous work were used as control templates for the development of the assays in order to provide allele specific sequences.[29]

3.1 Allele-specific PCR

The Δ3Q mutation is located at the last exon of *Bactrocera oleae ace* (exon 10). To test for the presence or absence of the Δ3Q mutation in individual flies two primers were designed: primer Ex10wt3′F-IMP, specific for the wild type allele and primer Ex10mut3′F-IMP, specific for the mutant allele. The two primers were identical except from the last 3′ base that provides the specificity for one or the other allele. In addition, both primers contained an arbitrary mutation that was introduced in the third before the last nucleotide in order to increase the specificity of the primers (Intentional Mismatch).[39,40] The nucleotide sequences of resulting diagnostic primers demonstrating allele-specific amplification are presented in Figure 1. These primers were used in conjunction with the downstream primer Boace10R. As shown in Figure 2, primer Boace10R with Ex10mut3′F-IMP amplify a 67 bp fragment only in mutated individuals. However, primers Boace10R and Ex10wt3′F-IMP amplify a 76 bp fragment in wild type and heterozygote individuals, whereas the homozygote mutants are not amplified.

3.2 PCR-RFLP

An alternative PCR-RFLP diagnostic assay was also developed in which a PCR product was generated by primers amplifying almost the entire exon 10 and consequently flanking the Δ3Q mutation site. The presence or absence of the Δ3Q mutation was diagnosed by incubation of the PCR amplification product with a restriction endonuclease where the Δ3Q mutation affects its recognition sequence. An *Mwo*I restriction site (GCNNNNNNNGC) is present in the wild type noucleotide sequence. The 9 bp deletion of the Δ3Q mutation deletes the last two GC residues of the *Mwo*I site. Therefore, the *Mwo*I site of exon 10 was used to determine the genotype of different olive fruit flies in a diagnostic PCR-RFLP test. Figure 1 shows the sequence of the primers, the amplified product of wild type and mutant allele, as well as the recognition

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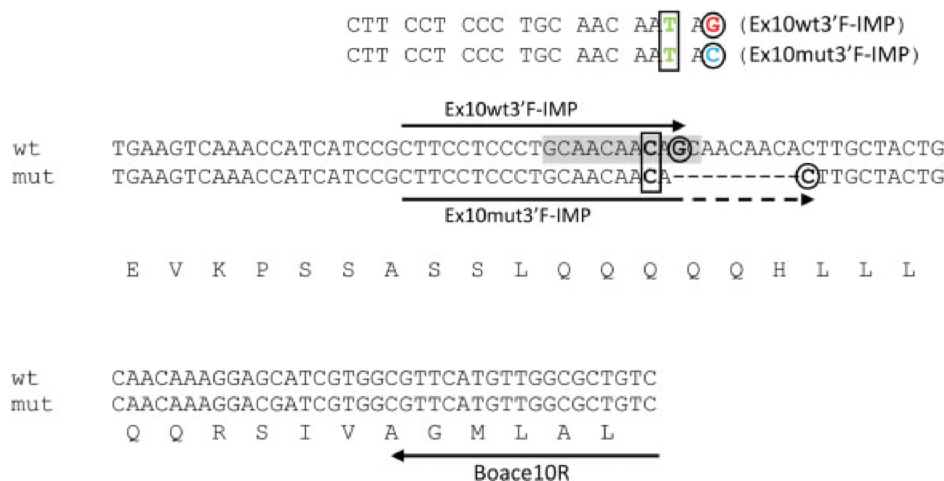

Figure 1. Diagrammatic representation of exon 10 of *Bactrocera oleae ace* that contains the Δ3Q mutation. Arrows indicate the position of the primers on the sequence. Primer Ex10wt3′F-IMP in combination with Boace10R amplifies the wild type allele. Primer Ex10mut3′F-IMP in combination with Boace10R amplifies the mutant allele. The intentional mismatch for both target alleles is colored green (in the online version) and placed in a box, where the terminal bases are colored red for the wild type allele and blue for the mutated one (in the online version) and placed in circles. The shaded area includes the recognition site for *Mwo*I that is used in the PCR-RFLP diagnostic test.

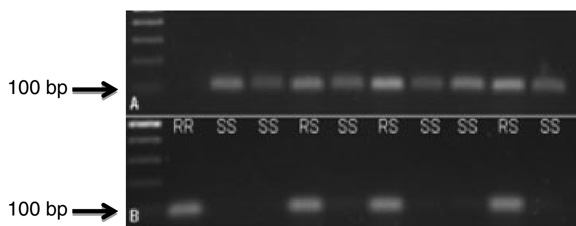

Figure 2. Allele-specific PCR test in 1.5% agarose gel. (A) Corresponds to PCR products obtained by primers Ex10wt3′F-IMP and Boace10R. (B) Corresponds to PCR products obtained by Ex10mut3′F-IMP and Boace10R. The RR lane is the amplification product of the cloned Δ3Q allele in a plasmid vector and represents a hypothetical Δ3Q homozygous genotype. The RS lanes are amplification products of field-collected flies of Δ3Q heterozygous genotype, whereas the SS lane represents the null Δ3Q genotype. The leftmost lane is the molecular weight marker.

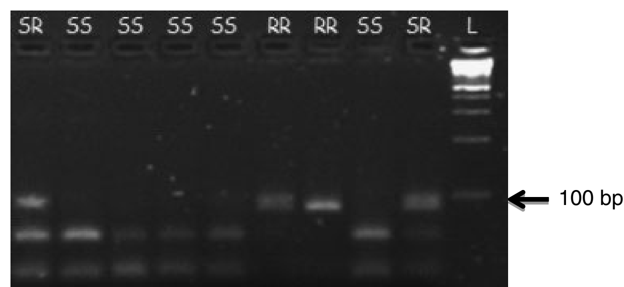

Figure 3. PCR-RFLP analysis of the Δ3Q mutation in a 2% low range agarose gel. The PCR product of the null Δ3Q genotype (SS) is digested into a 59 bp and a 37 bp fragment, whereas the homozygous Δ3Q genotype (RR) is not digested and presents a band at 87 bp, as the Δ3Q mutation deletes the last two GC residues of the *Mwo*I recognition site. The RR lane is the amplification product of the cloned Δ3Q allele in a plasmid vector that is used as a control. The heterozygote individual (SR) presents all three bands, 86 bp, 59 bp and 37 bp. The rightmost lane, indicated L, is the molecular weight marker.

sequence of *Mwo*I. The PCR of the wild type and mutant alleles with the primers Boace10F and Boace10R produces a 96 bp and 87 bp products, respectively. As shown in Figure 3, *Mwo*I digestion of 96 bp PCR product results in two fragments of 59 bp and 37 bp, whereas the 87 bp PCR product containing the Δ3Q mutations remains undigested, as indicated by the 87 bp band. However, individuals heterogyzous for the Δ3Q mutation present all three fragments (87 bp, 59 bp and 37 bp).

3.3 Taq-Man assay

A Taq-Man assay was also designed in order to discriminate the wild-type and mutant Δ3Q allele. During the assay a probe that amplifies the wild type allele but not the homozygous mutant allele was used. As shown in Figure 4, no amplification indicates a homozygous (RR) individual mutant carrying the Δ3Q deletion. Substantial increase in FAM fluorescence signal indicates a wild type individual and an intermediate increase indicates a heterozygote individual.

3.4 Duplex qPCR assay for G488S and I214V mutation detection

A new diagnostic test based on a single duplex qPCR reaction was developed for G488S and I214V mutations. The presence or absence of the mutations was identified by a melting curve analysis as base changes cause a lowered melting temperature, which can read out in genotypes. As shown in Figure 5, a high melting point (66 °C) at 640 nm indicates the homozygote individuals (RR) for the G488S mutation, while the wild type homozygote SS is observed at a melting point of 60 °C in the same fluorescence channel. Heterozygous individuals are discriminated from RR and SS genotypes by a two-peak melting point (60 and 66 °C). However, the I214V mutation is detected at 705 nm with homozygote wild type individuals (SS) observed at a melting point of 66 °C and homozygote mutant (RR) at 60 °C. Heterozygous discriminated from RR and SS genotypes by a two-peak melting point (60 and 66 °C).

3.5 Frequency of Δ3Q in Mediterranean populations

The distribution of Δ3Q mutation in Mediterranean populations was monitored by the PCR-RFLP diagnostic test developed in this study. A total of ten populations (∼30 individuals for each population) from different regions of eight countries (two from Israel and France and one from Cyprus, Greece, Italy, Spain, Portugal and Morocco) were analyzed for the presence of the resistant Δ3Q

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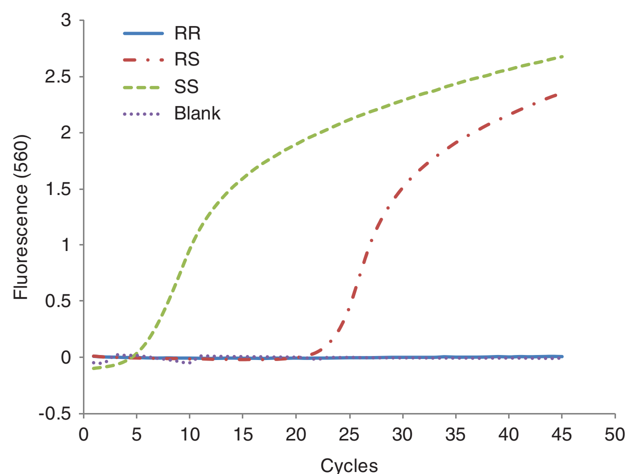

Figure 4. Cycling of FAM-labeled probe specific for the wild-type (SS) and the heterozygous (RS) alleles of Δ3Q mutation using Taq-Man chemistry. No amplification indicates a homozygous (RR) individual mutant carrying the Δ3Q deletion. Substantial increase in FAM fluorescence signal indicates a wild type individual and an intermediate increase indicates a heterozygote individual.

allele. The number of individuals and the frequencies of the Δ3Q allele for each of the ten populations are given in Table 1. The Δ3Q allele was found in nine out of the ten natural populations tested and frequencies ranged from 2 to 12.5%. The Δ3Q allele was not detected in Portugal. The highest frequencies > 10% were found in Greece and Italy, whereas a gradual decrease of Δ3Q frequency towards the western Mediterranean was also noted (Figure 6). Interestingly, homozygote individuals for the R allele were not found in any of the populations examined. Additionally, all populations were in Hardy–Weinberg equilibrium, apart from the population of Portugal that was monomorphic.

4 DISCUSSION

Since the first reported case of pesticide resistance by Melander in 1914,[41] the incidence of resistance has proliferated exponentially, becoming a challenging problem for applied entomology. In olive fly, three mutations have been associated with resistance to OPs: two point mutations in the active site of the enzyme that are predominantly responsible for the resistance and a short deletion in the C-terminal of the enzyme that appears associated with resistance at higher OP doses but always in combination with the two aforementioned mutations. While geographical distribution of the two point mutations has been analyzed in a number of studies,[31–33] there is little information for the Δ3Q mutation in that aspect. On that account, we developed three new diagnostic tests to detect the presence of Δ3Q *ace* mutation in olive fly and used them to monitor its frequency in natural populations.

In a previous study, we had proposed a direct PCR method for Δ3Q detection in which the 9 bp difference between the wild type and the mutant PCR product was visualized either in high-resolution > 3% agarose gel electrophoresis or in a 10% acrylamide gel electrophoresis.[28] However, high-resolution agarose or acrylamide gel electrophoresis increases the cost and the processing time. Thus, together with the fact that handling acrylamide requires special caution, we developed an allele-specific PCR and a PCR-RFLP diagnostic test, as well as a real-time Taq-Man assay in order to simplify the Δ3Q detection. In addition, we developed a duplex qPCR assay for the detection of the two point mutations. While all the tests are robust, simple to perform, easy to interpret and can be implemented in routine monitoring resistance plans, there are some factors that should be taken into consideration in test choice. On the basis of cost, the direct PCR developed previously and the allele-specific PCR are the front-runners. The direct PCR was based on regular primers, while the development of an allele-specific PCR necessitated the introduction of an internal mismatch (IMP: internal mismatch primer) in order to increase the specificity. Guo et al.[39] and Wilkins et al.[40] showed that an arbitrary mutation introduced in the third before the last nucleotide can increase the specificity and sensitivity of the primers. However, both of them were frequently problematic and unreliable. Concerning the specificity and sensitivity, the duplex qPCR approach shows excellent discrimination of the resistance alleles in two point mutations (G488S and I214V).

The Taq-Man assay is a PCR method using oligonucleotide probes that are labelled with a fluorescent reporter dye and a quencher molecule. In the current study the developed Taq-Man assay could unambiguously indicate the presence of the Δ3Q deletion. However, whether an individual fly contains two (RR), one (RS) or zero (SS) copies of Δ3Q is judged upon the time (cycle) of appearance of the fluorescence signal during the qPCR. This is confounded by other factors as well (e.g. DNA quantity and/or purity). Therefore, this reaction can only be used with caution and laboratory experience, or could only be used to indicate presence or absence of the mutation but not to decide the heterozygote state of an individual. Moreover, the running cost of Taq-Man method is higher comparable to the others. However, the PCR-RFLP and the allele-specific PCR assays for the Δ3Q mutation are relatively cheap and provide acceptable sensitivity. Overall, where the cost is a deciding factor in diagnostic test choice, the PCR-RFLP tests are preferable based on cost, sensitivity and ease of use. However, the duplex qPCR assay for the two point mutations is the preferred method where cost is not a restriction and specificity, sensitivity and quick throughput are required.

The new PCR-RFLP method developed in this study for the Δ3Q mutation was used to genotype ten olive fly populations that were collected from widely separated locations around the Mediterranean basin. It is important to monitor resistant alleles at regional scales, so that the resistance management strategies can be adjusted appropriately for the local conditions. The resistant allele was detected in nine out of ten populations, confirming that resistant genotypes associated with OP resistance are widespread in the Mediterranean region. Remarkably, the distribution of Δ3Q follows the spread of the OP insecticides across the Mediterranean basin, showing that the strong selective pressure imposed by insecticide treatments has driven the spread of resistant alleles both in frequency and geographic range, as ffrench Constant et al.[42] demonstrated in 2004. The highest frequency was found in Greece and Italy, where the extensive and continuous use of OPs is an important selection factor.[30,43] In Greece, dimethoate and fenthion have been used against olive fly for about four decades.[33] The lowest frequencies were found in western Mediterranean countries, where the use of OP insecticides is limited. In Spain, only one Δ3Q allele was detected, whereas in Portugal Δ3Q was not found. In 1996, in Portugal the total use of OP insecticides was just 225 tonnes in contrast to Greece, where the annual use was 1437 tonnes. A year later, Greece almost doubled this quantity (2275 tonnes) in contrast to Portugal that remained at similar levels.[44] This distribution of Δ3Q mutation is also consistent with the Mediterranean pattern of the two other resistance associated

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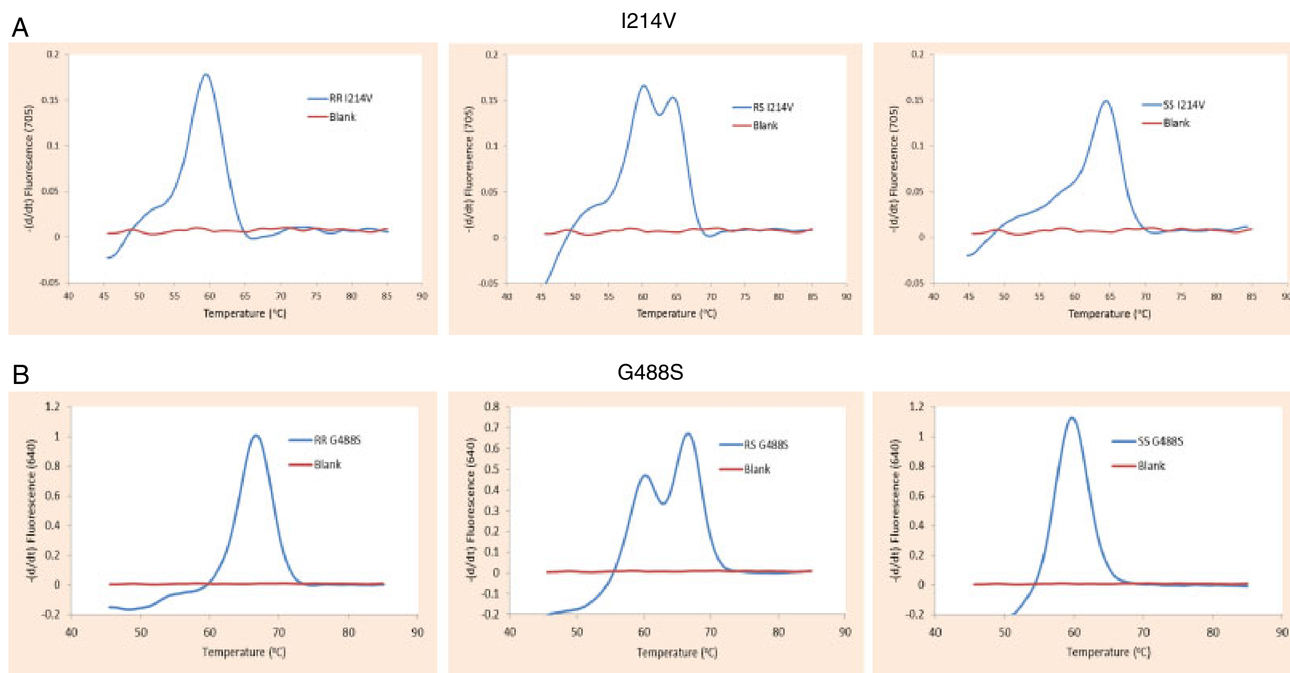

Figure 5. Melting curve profiles for determining the presence of I214V (A) and G488S (B) alleles in *Bactrocera oleae* populations. The I214V mutation was detected at 705 nm with homozygote wild type flies (SS) observed at a melting point of 66 °C and homozygote mutants (RR) at 60 °C. Heterozygotes were discriminated from RR and SS genotypes by a two-peak melting point (60 and 66 °C). For G488S (B), the mutation was detected at 640 nm with homozygote mutants (RR) observed at a melting point of 66 °C and the wild type individuals (SS) at 60 °C. Heterozygous individuals were discriminated from RR and SS genotypes by a two-peak melting point (60 and 66 °C).

Figure 6. The spread of the Δ3Q mutation around the Mediterranean basin. In pie charts, yellow color represents the frequency of null Δ3Q allele while the orange color the frequency of R Δ3Q allele.

point mutations I214V and G488S in olive fly, since similar results were reported in the studies of Hawkes et al.,[31] Nardi et al.[32] and Skouras et al.[33] The highest frequency of I214V and G488S was recorded in Greece and Italy, confirming that the spread of *ace* mutant alleles largely follows OP use. Interestingly, all populations were under Hardy–Weinberg equilibrium, indicating that OPs constitute integral and steady-state elements of the Mediterranean agricultural environment.

Finally, it is worthy of note that homozygous Δ3Q individuals were not found in any of the populations examined, indicating a higher fitness cost of Δ3Q compared to that of I214V and G488S mutations, as also suggested in our previous study.[29] A notable feature is that OP resistance is achieved with few mutations that are generally costly and the cost is variable among them.[7,9] It is usually believed that the initial fitness cost would gradually decrease due to other mutations with a modifier effect.[45] Hawkes et al.[31] proposed that the tight linkage between I214V and G488S arose because this combination of mutations confers insecticide insensitivity whilst being less detrimental to the normal function of AChE than G488S alone. In such a context, the low frequency of the Δ3Q mutation could be due to a competition with the I214V and G488S alleles, which confer resistance in a certain level of OPs and consequently it is only needed when higher OPs pressure is applied. Thus, if a fitness cost is associated with the Δ3Q mutation, it could be due either to an excess of cholinergic activity leading to a deficit of ACh in the synapses or to another deleterious side effect of the mutation. This fitness cost may become higher when the Δ3Q allele is found in homozygosity or alone (not in combination with the others) and it may explain this scarcity. AChE enzyme was shown to be involved in the development of the nervous system in vertebrates and invertebrates and any AChE mutation could impair a developmental process and generate high fitness cost.[46,47] Be that as it may, the implications with regard to the fitness cost of Δ3Q remain an open question.

As prevention is better than cure, one of the most critical keys in control programs still remains monitoring of insecticide resistance.

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Knowledge of insecticide resistance status and changing trends of resistance are basic requirements to guide insecticide use in pest control programs. When the frequency of resistant phenotypes increases to a certain level in field populations, control efficacy with the concerned insecticide becomes economically unacceptable. It is therefore important to detect resistant alleles, especially highly resistant alleles such as the Δ3Q allele, when they are at incipient levels and monitor their increase and geographical spread so that appropriate measures can be employed in time, in order to curtail their increase. For example, detection of the Δ3Q mutation in a population could mean that resistance to OP insecticides is already at high levels, in comparison to detection of I214V and G488S mutations. In that sense, detection of I214V and G488S mutations could serve as an early warning of the impending problem of OP insecticide resistance and higher OP doses or change to a different OP could suffice. Instead, detection of Δ3Q mutation would indicate the need of a switch to a new insecticide class, since insect populations would possess substantially high OP resistance levels.

Nonetheless, as mentioned in the beginning, heavy and continuous use of insecticides inevitably leads to the development and spread of resistance. Apparently, spinosad use against *Bactrocera oleae* is following a similar OP-resistance development pattern.[48] Clearly, novel integrated pest management approaches are needed. Among them, the Sterile Insect Technique (SIT) is receiving renewed interest for the olive fly due to the development of more efficient transgenic tools[49] that appear promising in overcoming problems associated with earlier SIT efforts for olive fly control.[50,51]

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