Research Article

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Tracking malathion resistance in Spanish *Ceratitis capitata* populations: prevalence of resistance alleles/haplotypes before and after the withdrawal of malathion

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Abstract

BACKGROUND: Field resistance to malathion was reported for Mediterranean fruit fly (medfly), *Ceratitis capitata*, populations collected in Spain in 2004 and 2005, when medfly control mainly relied on malathion bait sprays. The mutation G328A in the acetylcholinesterase (AChE) gene (*Ccace2*) was then identified as the main resistance mechanism in a field-derived resistant strain. However, outdoor plant protection products containing malathion were withdrawn from the European Union in 2009 and other insecticides gained importance, such as spinosad and pyrethroids, though other organophosphates were occasionally used for medfly control for a few years.

RESULTS: We have: (i) provided evidences of a novel malathion resistant mechanism in *Ceratitis capitata*, mediated by a heterogeneous duplication of the *Ccace2* gene (RS haplotype, one of the copies bearing the mutation G328A and the other copy non-mutated); (ii) found that individuals bearing the G328A mutation (R allele) and/or the RS haplotype were widely distributed in Spanish medfly populations during the years that malathion was used; and (iii) showed that malathion resistance reverted in field populations when analysed 8–13 years after malathion was withdrawn, but the frequencies of the genotypes containing the RS haplotype remained stable (RS/RS) or declined less (S/RS) than those containing the R allele (R/R, R/S, R/RS).

CONCLUSION: This represents a scenario where the R allele and the RS haplotype are present in the field at low frequencies, but resistance may rapidly evolve if malathion or other organophosphates were used in the absence of appropriate management resistance strategies.

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

Keywords: medfly; AChE; resistance mechanism; duplicated haplotype; resistance management

1 INTRODUCTION

Resistance to malathion was reported in Spanish field populations of the Mediterranean fruit fly (medfly), *Ceratitis capitata* (Diptera: Tephritidae), collected in 2004 and 2005.[1] At that time, medfly control in citrus crops mainly relied on ground and aerial treatments with malathion bait sprays. A mutation in the acetylcholinesterase (AChE) gene (*Ccace2*) was then identified as the main resistance mechanism in a field-derived resistant strain.[2] A single nucleotide polymorphism (SNP) produced a substitution of a glycine for an alanine at position 328 (G328A, *Torpedo californica* numbering), adjacent to the residue N327 of the AChE catalytic triad. Medfly adults homozygous for this mutation showed both a low AChE activity and a reduced sensitivity to the active form of malathion, malaoxon, compared to susceptible individuals.[2] The mutation G328A has been detected in natural medfly populations from Spain, Brazil and Tunisia, but not in individuals collected in Greece, South Africa, Guatemala, and Australia.[3] The same residue substitution in AChE has been associated to

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organophosphate (OP) resistance in other flies, such as *Drosophila melanogaster* and *Musca domestica*.[4,5]

Resistance associated to point mutations in AChE often compromise the kinetics of acetylcholine hydrolysis,[6] which can lead to fitness costs for resistant phenotypes, as reported for *Culex pipiens*,[7] *Culex quinquefasciatus*,[8] and *Anopheles gambiae*.[9] As a consequence, the frequency of both, resistant phenotypes and altered AChE resistance alleles, have been reported to decline over time when the populations are no longer exposed to the insecticide.[10–12] However, absence of trade-offs between fitness and insecticide resistance could evolve in field populations as a result of the selection of compensatory mutations (modifiers) that ameliorate the fitness of resistant genotypes, or the replacement of the original resistance alleles by less costly resistance alleles.[13] Remarkably, duplications of the AChE gene, that resulted in one susceptible and one resistant copy (heterogeneous AChE duplications), have been found in *Culex pipiens* and *Anopheles gambiae*.[14,15] These heterogeneous AChE duplications compensate for reduced catalytic activity, overcoming some of the fitness cost associated with the single copy mutated alleles.[16–18] It has been suggested that the resistant and the susceptible copies of the gene in heterogeneous AChE duplications are closely linked, hence producing 'duplicated haplotypes' (also called 'permanent heterozygotes'), that avoid segregation load.[13,16] They are advantaged because they maintain resistance while restoring part of the fitness cost associated to mutations in the AChE, conferring a more favourable resistance/cost balance across treated and non-treated areas.[19]

Since 2009, outdoor plant protection products containing malathion were withdrawn from the European Union (EU) market because of their identified acute and long-term risks to birds.[20] Other control strategies gained importance for medfly control in citrus crops in Spain, such as spinosad and lambda-cyhalothrin bait sprays, the deployment of bait stations coated with pirethroids (deltamethrin, lambda-cyhalothrin or esfenvalerate), and the liberation of sterile males in some areas of eastern Spain.[21] However, resistance to lambda-cyhalothrin was shortly detected in 2009–2010,[22] and resistance to MagnetMED™ traps (coated with deltamethrin) has been recently reported.[23] In both cases, resistance was already widespread when first detected, probably related to the high rates of gene flow among Spanish medfly populations.[24] Etofenprox and cyantraniliprole are also registered for medfly control in citrus crops, although their use is very limited. Other OPs (phosmet, triclorphon and methyl-chlorpyrifos) were also occasionally used for medfly control in citrus and other crops, but their use was discontinued after a few years. Remarkably, a field-derived malathion resistant strain showed moderate cross-resistance to these three OPs,[25] which could have continued exercising selection pressure on the malathion resistance alleles present in the field populations. Moreover, other OPs such as chlorpyrifos have been used in Spanish citrus growing areas against the California red scale, *Aonidiella aurantii*, potentially imposing an additional selection pressure, since formulations of this insecticide have shown residual toxicity against *Ceratitis capitata*.[26] At present, insecticide resistance management (IRM) strategies are required to ensure the viability of medfly control programmes. This includes the development of diagnostic tools to monitor resistance. The use of malathion in the EU is restricted to applications in glasshouses with a permanent structure,[20] but plant protection products containing malathion or other OPs as active substance could be exceptionally granted.

In this article, we have: (i) developed monitoring tools for resistance alleles/haplotypes in *Ceratitis capitata*; (ii) found that the G328A mutation and a heterogeneous duplication of the *Ccace2* gene (RS haplotype, one of the copies bearing the mutation G328A and the other copy non-mutated) were widely distributed in Spanish *Ceratitis capitata* populations when malathion was in use; and (iii) showed that malathion resistance reverted in field populations when analysed 8–13 years after malathion was withdrawn from the EU, but the frequencies of the genotypes containing the RS haplotype remained stable (RS/RS) or declined less (S/RS) than those containing the R allele (R/R, R/S, R/RS).

2 MATERIALS AND METHODS

2.1 Laboratory strains and field populations

The resistant strains described here derive from a malathion-resistant (120-fold) field population collected in 2004 from citrus orchards in Castellón (Spain).[1] Individuals from this population were selected by exposing adults to artificial diet containing increasing concentrations of malathion during 48 h in successive generations, until fixing selection conditions to 2000, 4000 and 10 000 ppm to obtain sequentially the W (79-fold),[1,2] W-4Km (178-fold)[25] and W-10Km (403-fold) strains. A control strain (C strain) was established from wild *Ceratitis capitata*, collected in 2001 at non-treated experimental fields (Instituto Valenciano de Investigaciones Agrarias, València, Spain), and maintained in our laboratory without exposure to insecticides. All the strains have been maintained at laboratory conditions as described by Magaña *et al.*[1]

The isoline Wace2m (homozygous for the resistant R allele carrying the mutation G328A in homozygosis, genotype R/R) was obtained from the malathion resistant strain W-4Km. For the generation of this isoline (progeny from one female and one male), sex was determined immediately after adult emergence, and males and females were placed separately into ventilated plastic dishes (89 mm in diameter, 23 mm in height) and fed with water and rearing diet (4:1:0.1, powdered sugar/hydrolysed yeast/water). After 5 days, about 100 males and 100 females were placed together to allow mating, being steadily observed until couples were established. Coupled pairs were placed independently into ventilated plastic dishes and fed with water and rearing diet. The eggs from each couple were collected and seeded every day. The progeny was kept if both parents had the genotype of interest. The process was repeated in successive generations until the genotype of interest was present.

Adult flies from field populations were collected at 49 localities in Spain (Supporting Information, Table S1 and Fig. S1). Sampling of field populations was: (i) performed in north-eastern Spain (Girona and Tarragona), eastern Spain (Castelló, València and Alacant), southern Spain (Granada, Málaga and Huelva), central Spain (La Rioja) and insular Spain (Balearic and Canary Islands) for the period 2003–2008 (when malathion was still in use), to cover the most representative areas for medfly distribution in Spain; and (ii) focussed on three provinces in eastern Spain (Castelló, València and Alacant) in 2017–2023 (after malathion was banned from the EU in 2009), due to their importance in citrus production and to the results obtained in the former period. Flies were obtained from infested fruits and preserved at −80 °C, or from vapona fly killer traps and preserved in ethanol at 70%. Only females were analysed when individuals were captured in traps at areas where sterile males were being released as a control method.

2.2 Malathion susceptibility assays

The susceptibility to malathion of field populations collected in the period 2003–2005 was evaluated by Magaña *et al.*[1] (6–201-fold) using concentration-mortality feeding assays with Malafin 50 EC (malathion 50% *w/v*; Agrodan, Madrid Spain). For those collected in 2023, concentration-mortality feeding assays were performed with SMART®EV (malathion 44% *w/v*; FMC Corporation, Philadelphia, PA, USA). We used a different malathion formulation than that used by Magaña *et al.*[1] because of availability issues. Resistance ratios were calculated in both cases with respect to the same susceptible laboratory C strain for cross-validation. Malathion was administered mixed with rearing diet (0.9 g of powdered sugar and hydrolysed yeast (4:1) and 100 µL of the insecticide solution). Three experimental replicates of 10–15 flies each (3–5 day old adults) were performed at each of the four concentrations tested (30, 100, 300, and 1000 ppm). Untreated controls consisted of rearing diet mixed with water. Assays were performed in ventilated boxes (2.5 cm high × 9 cm in diameter), that were kept in an environmentally controlled chamber at 25 ± 1 °C and 16-h:8-h light/dark photoperiod (Sanyo MLR-350-H; Sanyo, Osaka, Japan) as previously described.[1] Flies were considered dead if they were ataxic after 48 h.

2.3 Detection method for the mutation G328A in the *Ccace2* gene by PCR-PIRA

A polymerase chain reaction-primer introduced restriction analysis (PCR-PIRA) system[27] was developed for the detection of the mutation G328A in the *Ccace2* gene conferring malathion resistance. The system requires the use of the forward primer FG328A_27 (5′ TCAACATGTTTTTCATTTTCGTTCCAG 3′) that was designed based on the sequence of the 3′ end of intron 5 of the *Ccace2* gene. The last two nucleotides of the primer correspond to the AG consensus acceptor splice site, located immediately before the SNP (a G/C substitution) associated to the mutation G328A. After PCR amplification, a T/C mismatch at the fourth nucleotide from the 3′ end (underlined in the sequence above) creates a BstNI restriction site (CCWGG) specifically in the absence of the mutation.

DNA was extracted from the head of adult flies, in order to avoid the possible interference of male sperm on females, using the DNeasy® Blood & Tissue Kit (Qiagen, Foster City, CA, USA), following the manufacturer instructions. PCR reactions were performed in a GeneAmp® PCR System 2700 Thermocycler (Applied Biosystem, Foster City, CA, USA), using 0.6 µM of primers FG328A_27 and RiAChE_CcE60 (5′ CCGAAAAATGATGGCCTCGCGTTCCG 3′), 0.5 units of Amplitaq Gold® (Roche Molecular Systems, Inc., Branchburg, NJ, USA), 0.2 mM dNTPs (deoxynucleoside triphosphates deoxyribose), 2 mM magnesium chloride ($MgCl_2$), and 50–100 ng of template DNA, in final volume of 10 µL with buffer II. Thermocycler conditions were: one hold of 95 °C for 10 min; 40 cycles of 95 °C for 30 s, 48 °C for 30 s, and 72 °C for 15 s; and a final hold of 72 °C for 7 min. PCR products were digested with BstNI at 60 °C for 2 h in the same thermocycler, adding 2 µL of a master mix [that included 2 units of the restriction enzyme BstNI (New England Biolabs, Ipswich, MA, USA), buffer NE-2 (1×) and bovine serum albumin (BSA, 100 µg/mL)] to 10 µL of the PCR product. The digested PCR products were migrated in a 3% agarose (1:1 mixture of agarose D2 Lab. Conda, Madrid, Spain/NuSieveR GTGR Agarose Lonza, Rockland, ME, USA) gels (Tris 40 mM, EDTA 1 mM, pH 8.0) and visualized using ethidium bromide. A single digested band of 145 bp indicates absence of the mutation, whereas a single non-digested band of 172 bp indicates that the individual carries the G328A mutation (Fig. S2). Individuals genotyped in a previous study,[2] by direct sequencing of *Ccace2* complementary DNA (cDNA), as homozygous for the G328A mutation (from the resistant W strain) and homozygous for the wild-type allele (from the susceptible C strain) were used as positive and negative controls for the presence of the mutation, respectively.

2.4 Crossing experiments

Reciprocal crosses between flies from C and W-10Km strains were performed by confining one male and one unfertilized female of each strain in ventilated Petri dishes (9 cm diameter). The eggs from 20 couples (ten couples from each reciprocal cross) were collected and the F1 progeny reared until adult emergence. Three couples of each reciprocal cross were selected (all with more than 15 pupae at F1). The parents and 10–15 F1 descendants from each of the six crosses were analysed to identify the mutation G328A in the *Ccace2* gene by PCR-PIRA, as detailed earlier.

2.5 Quantification of the relative copy number of the *Ccace2* gene by qPCR on genomic DNA

Genomic DNA extraction from single adult individuals was carried out as previously described (see Section 2.3). Real-time PCR (qPCR) was performed with genomic DNA using specific forward FAChE4qPCR (5′ GAAATCCGCAAAACACCACA 3′) and reverse RAChE4qPCR (5′ CGGTCATAAAGCCACCAC 3′) primers for *Ccace2* gene. The ribosomal protein P0 (*Ccp0*, Ref. GenBank Y18444) [forward primer FP0_2 (5′ TCCAGGCTCTCTCCATACCA 3′), reverse primer RP0 (5′ CGACTTTGTCACCAGGCTTC 3′)] was used as reference gene. The qPCR was carried out in a Corbett Rotor Gene 6000 real-time cycler (Qiagen). The reactions contained 7.5 µL of Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (1×) (Agilent Technologies, Santa Clara, CA, USA), 0.3 µM of each primer, 1 ng DNA template and ultrapure water to a total volume of 15 µL. Thermocycler conditions were: 10 min at 95 °C; 40 cycles of 20 s at 95 °C, 20 s at 58 °C and 20 s at 72 °C. The relative copy number of the *CCace2* gene in sampled individuals was determined with respect to the reference gene using the ΔΔCt method.[28,29]

2.6 Expression of the *Ccace2* gene by qPCR, AChE activity and inhibition by malaoxon

Adult flies of 3–5 days from C and W-10Km strains were used. The thorax and abdomens were used for expression analysis and the heads for enzymatic assays (five pools of six adults, three males and three females).

Total RNA from thorax and abdomens was extracted using TRIzol® reagent (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. RNA concentration was measured with NanoDrop (Thermo Scientific, Waltham, MA, USA). The cDNA was synthesized from 1 µg RNA using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) following manufacturer instructions. The qPCR was performed with cDNA using the primers FAChE4qPCR and RAChE4qPCR for *Ccace2* gene and FP0_2, and RP0 for the reference gene *Ccp0*. The qPCR was carried out in a Corbett Rotor Gene 6000 real-time cycler (Qiagen) using 7.5 µL of Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (1×) (Agilent Technologies), 0.3 µM of each primer, about 8 ng of cDNA and ultrapure water to a total volume of 15 µL. Thermocycler conditions were: 10 min at 95 °C; followed by 40 cycles of 20 s at 95 °C, 20 s at 58 °C, and 20 s at 72 °C. The expression of the *CCace2* gene was determined using the ΔΔCt method, normalized with the *Ccp0* reference gene.

The heads were homogenized in 900 µL 0.1 M sodium phosphate buffer pH 7 that contained 1% Triton X-100 (*v/v*). The homogenates were centrifuged at 20 000 × *g* for 10 min at 4 °C (Universal 32 R; Hettich, Tuttlingen, Germany), and the supernatants were collected and used as enzyme source. The protein concentration was determined according to the procedure of Bradford.[30] AChE activity was determined by the spectrophotometric method described by Ellman *et al.*[31] The reaction mixture consisted of 5 µL of heads homogenate, 2 mM acetylthiocholine iodide (ATChI), 1 mM 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB), and 0.1 M sodium phosphate buffer pH 7 up to 100 µL. The reaction was incubated at 30 °C and the increment in absorbance at 412 nm was recorded during 5 min in a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA, USA). To transform absorbance units to concentration, a molecular extinction coefficient ($\varepsilon_{412} = 1.36 \times 10^4$ M^{-1} cm^{-1}) was used.[32] Each replica was repeated three times. Except otherwise stated, all the products were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Stock solutions of malaoxon (Pestanal®, Riedel de Haën, Seelze, Germany) were prepared in ethanol (Merck, Darmstadt, Germany). Head extracts were incubated with different concentrations of malaoxon (1×10^{-4}, 3×10^{-5}, 1×10^{-5}, 3×10^{-6}, 1×10^{-6}, and 3×10^{-7} M) at 30 °C. The reaction was started by the addition of the substrate (ATChI) and the reagent (DTNB), and AChE activity was measured as described earlier. The experiment was performed three times at each concentration. The bimolecular rate constant of inhibition (Ki) was determined according to Main.[33]

2.7 Molecular detection of genotypes with the G328A mutation and/or the heterogeneous duplication of the *Ccace2* gene in field populations of *Ceratitis capitata*

The wild-type allele (from here on referred to as S allele), G328A mutated allele (from here on referred to as R allele) and the heterogeneous duplication (from here on referred to as RS haplotype) of the *Ccace2* gene were detected in specimens collected from field populations by one of these two strategies: (i) the combination of a double PCR-PIRA method and confirmation when needed by qPCR on genomic DNA; and (ii) TaqMan genotyping assay and confirmation when needed by qPCR on genomic DNA.

The double PCR-PIRA method consisted in two PCR reactions performed on the same genomic DNA sample using a unique reverse primer RiAChE_CcE60 and two different forward primers: (i) FG328A_27, previously described, modified to create a BstNI restriction site in the absence of the G328A mutation; and (ii) FG328A_27TseI (5′ TCAACATGTTTTTCATTTTCGTTGCAG 3′), modified to create an ApeKI restriction site in the presence of the mutation. PCR products of the first reaction were digested with BstNI as previously described. The PCR products of the second reaction were digested with ApeKI at 75 °C for 2 h in a GeneAmp® PCR System 2700 Thermocycler, in a final volume of 12 µL containing 10 µL of the PCR product and 2 µL of a master mix composed of 0.05 units of the restriction enzyme ApeKI (New England Biolabs) and buffer NE-3.1 (1×). The low amount of restriction enzyme units in this reaction was necessary to avoid the unspecific digestion of heterodimers. Digested products were visualized in 2% agarose gels as described earlier. The double PCR-PIRA was designed to generate a characteristic pattern of bands depending on the genotype, given by: (i) the initial proportion of mutated and non-mutated copies of the *Ccace2* gene in the PCR reaction for each genotype (depending on the presence/absence of the mutation and the duplication); and (ii) the formation of heterodimers of mutated and non-mutated DNA chains during the PCR reaction, which were inefficiently digested by the restriction enzymes (Table S2). Thus, the double PCR-PIRA allowed to differentiate the following genotypes: R/R (a single band of 172 bp in the first PCR-PIRA digested with BstNI, and a single band of 145 bp in the second PCR-PIRA digested with ApeKI); S/S (a band of 145 bp with BstNI, and a band of 172 bp with ApeKI); RS/R (two bands of 145 and 172 bp, whose intensity is similar with ApeKI and higher at 172 bp with BstNI); and RS/S (two bands of 145 and 172 bp, whose intensity is similar with BstNI and higher at 172 bp with ApeKI) (Fig. S3(A)). Genotypes RS/RS and R/S presented the same profile (two bands of 145 bp and 172 bp, of higher intensity at 172 bp with both BstNI and ApeKI) (Fig. S3(A)). In those cases, when the genotype was not clearly assessed by the two reactions or assigned to the RS/RS or R/S genotypes, qPCR with genomic DNA was performed, as previously described. The copy number of the *CCace2* gene in sampled individuals was determined using the ΔΔCt method, normalized to the reference gene, as the ratio with respect to individuals of the C strain with S/S genotype (two copies), which allow differentiate between genotypes with two (R/R and R/S), three (RS/R and RS/S) and four (RS/RS) copies (Fig. S3(B)).

TaqMan genotyping assay was performed at Secugen S.L facilities (Madrid, Spain). The assay included two locus-specific PCR primers that flank the SNP of interest [SNP_FW (5′ CCGTTAGTTTTCACAACAGATTTG 3′) and SNP_RV (5′ GGTCGCCTCATCCTTATCAA3 ′)], and two allele-specific oligonucleotide TaqMan® probes to discriminate between the wild-type susceptible (S) allele [SNP_WT_P (/5SUN/TTT + CA + G + G + C + A CT + T A/3IABkFQ/)] and the resistant G328A mutated (R) allele [SNP_MUT_P (/56-FAM/TTT + CA + G + C + C + A C + TT AC/3IABkFQ/)]. These probes have a fluorescent reporter dye at the 5′ end, and a 3′ Iowa Black FQ quencher. The assay also included a set of primers (FP0_2 and RP0, previously described) and probe (/5Cy5/CCA A + GA TT + T CCA A + GG GTA + CTA TTG /3IAbRQSp/) to amplify an internal reference gene (ribosomal protein P0). PCR reactions were performed in a QuantStudio™ 5 System (Applied Biosystem), and DNA samples were genotyped simultaneously on 384-well plates, that included reference individuals of the different possible genotypes. Data were analysed for allelic discrimination and relative quantification using the Design and Analysis 2.4.3 software (Applied Biosystem), which normalized the number of *Ccace2* gene copies to the internal reference gene and estimated the number of copies of each allele as the ratio (Rq-target R and Rq-target S) with respect to an individual with one copy of each allele (R/S genotype). The representation of these ratios allowed to differentiate five separated clusters, that corresponded to the following genotypes: R/R (Rq-target S = 0, on the *X*-axis); S/S (Rq-target R = 0, on the *Y*-axis); R/S and RS/RS (Rq-target S = Rq-target R, on the diagonal); RS/R (Rq-target R > Rq-target S, below the diagonal); and RS/S (Rq-target S > Rq-target R, over the diagonal) Fig. S4). To differentiate the RS/RS and R/S genotypes, that overlap on the diagonal, qPCR with genomic DNA was further performed, as previously described (Fig. S3(B)).

Specimens collected from field populations in the period 2003–2008 (25 localities) were genotyped by the combination of a double PCR-PIRA method and qPCR with genomic DNA, and those collected in the period 2017–2022 (21 localities) by TaqMan and qPCR with genomic DNA when required. The consistency of both methods was established by genotyping three individuals of each of the six possible genotypes with the two methods.

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2.8 Statistics

Susceptibility to malathion was analysed using mortality data to estimate the concentration needed to cause 50% mortality (LC_{50}) by Probit analysis (program POLO-PC, LeOra Software14; LeOra, Berkeley, CA, USA, which corrects samples' mortality by control mortality using Abbott's transformation). Resistance ratio (RR = LC_{50} field population/LC_{50} C strain) was considered significant if the 95% fiducial limit (FL) did not include 1.[34]

Gene expression, gene copy numbers, AChE enzymatic activity and Ki in different strains and genotypes were compared by *t*-student test or analysis of variance (ANOVA) followed by Tukey *post hoc* test (SPSS Statistics 24.0 software; SPSS Inc., Chicago, IL, USA).

3 RESULTS

3.1 Evidences for a heterogeneous duplication of the *Ccace2* gene in a malathion selected strain

Analysis of the presence of the mutation G328A in the *Ccace2* gene by PCR-PIRA (BstNI digestion) along the selection process of the malathion resistant strains W, W-4Km and W-10Km revealed that both, homozygous individuals for the mutated or non-mutated alleles were gradually phased out (Fig. 1). At generation F2 (W), 56% of the individuals were heterozygous for the mutation, 38% homozygous for the mutation and 6% did not bear the mutation. However, the percentage of individuals heterozygous for the mutation increased in parallel with the increase in selection pressure [70% and 96% at F10 (W) and F37 (W-4Km), respectively], and became fixed at F52 (W-10Km). Given that two alleles (wild and mutated) of one gene following the Mendelian inheritance should segregate in the progeny, and assuming that homozygosis for the wild or mutated alleles would not be associated to mortality, we explored the possibility of the existence of a heterogeneous duplication of the *Ccace2* gene. In this case, one of the copies bearing the mutation G328A and the other copy non-mutated would segregate together resulting in a 'permanent heterozygous' genotype, as reported in mosquitos.[14,35]

Firstly, reciprocal crosses between individuals of the W-10Km strain and the susceptible C strain (homozygous for the non-mutated wild-type S allele) were performed. Parents and descendants of these couples were analysed for the presence of the mutation G328A in the *Ccace2* gene by PCR-PIRA using the restriction enzyme BstNI (Fig. 2(A)). The males and females of the C strain used in the crosses did not bear the mutation (a single 145 bp band), whilst all individuals of the W-10Km strain were heterozygous for the G238A mutation (two bands of 145 and 172 bp). Remarkably, none of the F1 descendants from the six couples (n = 80 in total) showed the wild genotype, being all heterozygous for the G238A mutation. These results were compatible with the hypothesis of the 'permanent heterozygous' in the parent W-10Km. Secondly, the relative copy number of the *Ccace2* gene in individuals of the W-10Km and C strains with respect to the reference gene *Ccp0* was analysed by qPCR on genomic DNA (Fig. 2 (B)). The results confirmed the existence of a duplication in the *Ccace2* gene in W-10Km, since the relative number of copies was double in individuals of this strain when compared to individuals of the laboratory C strain. Taken together, these results provide strong evidence for the selection and fixation of a heterogeneous duplication of the *Ccace2* gene that segregate as a unique allele (RS haplotype) in the malathion resistant strain W-10Km. Nevertheless, other genetic mechanisms, such as gene conversion, unequal crossing over, or the presence of transposable elements may be ruled out for an undoubted demonstration of the heterogeneous duplication nature of the RS haplotype.

To further study the implication of the heterogeneous duplication, the expression of *Ccace2* was quantified in individuals of the W-10Km and C strains by means of qPCR (Fig. 2(C)). This experiment demonstrated that the expression of *Ccace2* in W-10Km was about two-fold compared to the C strain. We also determined implications of the heterogeneous duplication of the *Ccace2* gene in AChE specific activity and its inhibition by malaoxon (Fig. 2(D)). Individuals from the W-10Km strain showed about 30% higher AChE activity than those of the C strain. In addition, the Ki of this activity by malaoxon was significantly lower for the W-10Km strain than for the C strain. These results indicate that the expression of the two copies of the *Ccace-2* gene resulted in augmented levels of *Ccace2* expression and AChE activity in the W-10Km strain, whereas the lower susceptibility to malaoxon may be explained because the mutated copy of the duplicated *Ccace2* gene may produce an altered AChE enzyme that is less sensitive to malaoxon inhibition.

3.2 Fate of the G328A mutation and the heterogeneous duplication of the *Ccace2* gene in Spanish field populations of *Ceratitis capitata* in the last 20 years

The analysis of field populations showed that the G328A mutation (R allele) and the heterogeneous duplication (RS haplotype, assuming that the two copies of the *Ccace2* gene also segregate

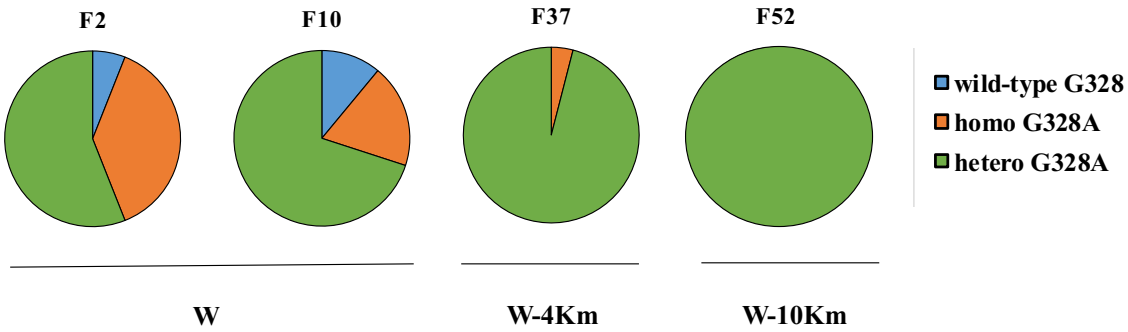

Figure 1. Percentage of individuals bearing the mutation G328A in the *Ccace2* gene along the selection process. The W, W-4Km and W-10Km strains were sequentially obtained by selection with increasing concentrations of malathion (1500 ppm at F2, 2000 ppm at F10, 4000 ppm at F37 and 10 000 ppm at F52). The percentage of individuals that do not carry the mutation (wild-type G328), and of those carrying the mutation in homozygosis (homo G328A) or heterozygosis (hetero G328A) during the course of selection (generations F2–F52) is indicated. A minimum of 20 individuals were analysed each generation by PCR-PIRA using the restriction enzyme BstNI.

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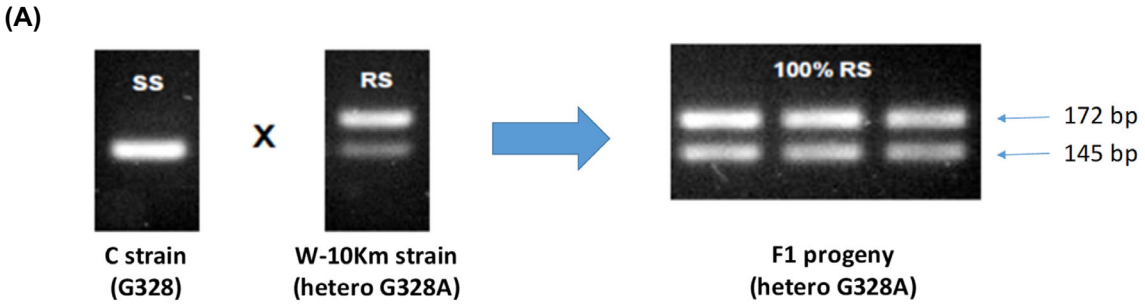

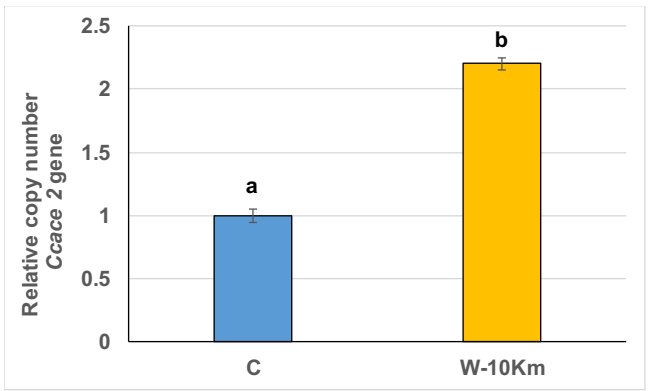

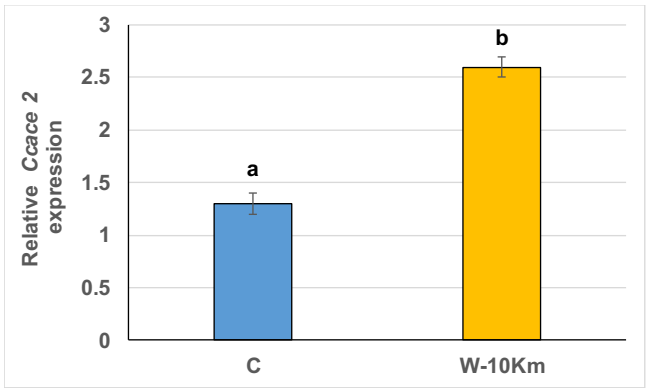

(D)

Strain	AChE activity ±SE (µmol/min/mg protein)	Inhibition by malaoxon Ki ± SE (x 10^6 M^{-1} min^{-1})
C	0.38 ± 0.01 a	2.09 ± 0.21 a
W-10Km	0.49 ± 0.01 b	1.36 ± 0.14 b

Figure 2. Experiments that provide evidences for a heterogeneous duplication of the *Ccace2* gene in the W-10Km strain. (A) Crossing experiments with flies from the C and W-10Km strains. The presence (172 bp band) or absence (145 bp band) of the mutation G328A in the *Ccace2* gene in the parents (three couples per reciprocal cross) and F1 progeny (n = 80, 10–15 from each of the six crosses) was determined by PCR-PIRA using the restriction enzyme BstNI (a representative case is shown). (B) Quantification of the relative copy number of the *Ccace2* gene with respect to the reference gene *Ccp0* by qPCR with genomic DNA. Data are mean ± standard error of six individuals of the W-10Km and C strains. (C) Expression of the *Ccace2* gene in adults from the W-10Km and C strains. Data are mean ± standard error of five pools of six individuals (thorax and abdomen of three males and three females) normalized to the reference gene *Ccp0*. (D) AChE specific activity in head extracts of 3–5-day-old adults from the W-10Km and C strains and its inhibition (Ki, bimolecular rate constant of inhibition) by malaoxon. Data are mean ± standard error of five pools of six individuals (three males and three females). Different lowercase letters account for significant differences between strains (*t*-student test, $P < 0.05$).

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as a unique allele in field populations) of the *Ccace2* gene were widely distributed throughout Spain when malathion was used for medfly control (period 2003–2008, Table 1). The R allele was found in 21 of the 25 populations analysed, located in different provinces distributed along north-eastern Spain (Girona and Tarragona), eastern Spain (Castelló, València and Alacant), southern Spain (Granada, Málaga and Huelva), central Spain (La Rioja) and insular Spain (Balearic and Canary Islands). The RS haplotype was also found in 17 of the populations analysed, including all the provinces in north-eastern and eastern Spain, and a province in central Spain (Zaragoza), but it was not found in La Rioja and in those provinces located in southern Spain. Populations with 100% wild-type S allele were only found in two of the 25 analysed, one located at Balearic Islands (Marratxi) and the other at southern Spain (Torre de Benagalbón, Málaga). Both, the R allele and the RS haplotype were present in fields where malathion was heavily used (e.g., Alcanar, Burriana, Alcúdia, etc.), but also in fields that had not been treated with malathion the year that the flies were collected (Xavia and Muro) and even in urban areas (Vila-real) non-treated with insecticides for at least the last 3 years (Table S1). Total allelic frequencies were calculated as: 75.8% S, 17.8% R and 6.3% RS; with the population from Valle de Guerra (Canary Islands) presenting the highest frequency (50%) for the R allele, and the population sampled in Les Coves de Vinromá (Castelló) the highest frequency (20%) for the RS haplotype. All possible genotypes were identified, the genotypic frequency being on average 62.2% S/S, 18.8% S/R, 7.0% R/R, 8.5% S/RS, 2.8% R/RS and 0.7% RS/RS (Table 1). Those genotypes containing the R allele (R/R or S/R) were spread throughout Spain, though the highest added frequencies (over 40%) were only found in populations from north-eastern and eastern Spain and Canary Islands. The genotypes with the RS haplotype (RS/RS, S/RS or R/RS) were mostly restricted to north-eastern and eastern Spain and their added frequencies were always below 35%.

Table 1. Genotypic and allelic frequencies for *Ccace2* in field populations of *Ceratitis capitata* collected in Spain 2003–2008 (when malathion was still in use)

			Genotypic frequency (%)[‡]						Allelic/haplotype frequency (%)[§]		
Field populations (2003–2008)	Year	*n*[†]	S/S	S/R	R/R	S/RS	R/RS	RS/RS	S	R	RS
North-eastern Spain											
Baix Empordà (Girona)	2004	20	35.0	30.0	15.0	20.0	0.0	0.0	60.0	30.0	10.0
Tortosa (Tarragona)	2003	20	25.0	25.0	20.0	20.0	10.0	0.0	47.5	37.5	15.0
Amposta (Tarragona)	2003	20	15.0	40.0	15.0	20.0	10.0	0.0	45.0	40.0	15.0
Alcanar (Tarragona)	2007	20	60.0	25.0	0.0	10.0	0.0	5.0	77.5	12.5	10.0
Eastern Spain											
Les Coves de Vinromà (Castelló)	2003	20	10.0	35.0	20.0	15.0	15.0	5.0	35.0	45.0	20.0
Vila-real (Castelló)	2005	20	50.0	25.0	10.0	15.0	0.0	0.0	70.0	22.5	7.5
Burriana (Castelló)	2004	20	55.0	25.0	10.0	5.0	0.0	5.0	70.0	22.5	7.5
Alcúdia (València)	2004	12	58.3	25.0	0.0	16.7	0.0	0.0	79.2	12.5	8.3
Serra (València)	2005	20	40.0	25.0	5.0	25.0	5.0	0.0	65.0	20.0	15.0
Moncada (València)	2003	20	30.0	20.0	20.0	20.0	10.0	0.0	50.0	35.0	15.0
Carlet (València)	2004	20	80.0	15.0	0.0	5.0	0.0	0.0	90.0	7.5	2.5
Xàbia (Alacant)	2007	10	40.0	10.0	20.0	30.0	0.0	0.0	60.0	25.0	15.0
Vila Joiosa (Alacant)	2007	10	90.0	0.0	0.0	10.0	0.0	0.0	95.0	0.0	5.0
Alacant (Alacant)	2004	23	73.9	13.0	4.3	0.0	8.7	0.0	80.4	15.2	4.3
Orihuela (Alicant)	2005	20	55.0	40	5.0	0.0	0.0	0.0	75.0	25.0	0.0
Total eastern Spain		195	51.8	22.6	8.7	11.8	4.1	1.0	69.0	22.1	9.0
Southern Spain											
Almuñécar (Granada)	2007	20	95.0	5.0	0.0	0.0	0.0	0.0	97.5	2.5	0.0
Algarrobo Costa (Málaga)	2008	20	95.0	5.0	0.0	0.0	0.0	0.0	97.5	2.5	0.0
Torre de Benagalbón (Málaga)	2003	20	100.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
Gibraleón (Huelva)	2008	20	95.0	5.0	0.0	0.0	0.0	0.0	97.5	2.5	0.0
Ayamonte (Huelva)	2008	20	90.0	10.0	0.0	0.0	0.0	0.0	95.0	5.0	0.0
Central Spain											
Albelda (La Rioja)	2004	9	55.6	44.4	0.0	0.0	0.0	0.0	77.8	22.2	0.0
Villalengua (Zaragoza)	2007	12	91.7	0.0	0.0	8.3	0.0	0.0	95.8	0.0	4.2
Insular Spain											
Marratxí (Balearic Islands)	2007	20	100.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
Muro (Balearic Islands)	2007	10	80.0	10.0	0.0	10.0	0.0	0.0	90.0	5.0	5.0
Valle de Guerra (Canary Islands)	2004	20	25.0	40.0	30.0	5.0	0.0	0.0	47.5	50.0	2.5
Total Spain		426	62.2	18.8	7.0	8.5	2.8	0.7	75.8	17.8	6.3

[†] Number of adults analysed per field population or laboratory strain or crossing.
[‡] The genotypes were determined by double PCR-PIRA and qPCR when needed for the field populations sampled in the period 2003–2008.
[§] Assuming that the heterogeneous duplication of the *Ccace2* gene (with one of the copies bearing the mutation G328A and the other copy non-mutated) also segregates as a unique allele (RS haplotype) in field populations.

For the period 2017–2022, 8–13 years after malathion was banned from the EU in 2009, we analysed populations from three provinces in eastern Spain (Castelló, València and Alacant) (Table 2), due to their importance in citrus production in Spain and because they were among those with the highest frequencies of resistant alleles and genotypes in former years. We have found that the R allele and the RS haplotype were present, respectively, in 18 and 14 of the 21 populations analysed. Populations with 100% wild-type S allele were only found in two of the 21 locations, one at Castelló (Castelló) and the other at València (Oliva). Total allelic frequencies were calculated as: 88.3% S, 6.8% R and 4.9% RS; which represents a reduction in the resistant allele R and the haplotype RS with respect to the period 2003–2008 in populations from the same three provinces in eastern Spain (69.0% S, 22.1% R and 8.9% RS). Besides, the highest frequency obtained for the R allele (17.5% at Carcaixent) was also lower than in the previous period (45.0% at Les Coves de Vinromà), whereas the highest frequency obtained for the RS haplotype (21.1% at Onda) was similar (20.0% at Les Coves de Vinromà). All possible genotypes were found, the genotypic frequency being on average 80.7% S/S, 9.3% S/R, 1.8% R/R, 6% S/RS, 0.8% R/RS and 1.5% RS/RS (Table 2). This represents a reduction in the frequencies of all genotypes containing the R allele and/or the RS haplotype, except for the RS/RS genotype that remained stable, when compared with the results obtained in the same three provinces in eastern Spain for the period 2003–2008 (51.8% S/S, 22.6% S/R, 8.7% R/R, 11.8% S/RS, 4.1% R/RS and 1.0% RS/RS) (Table 1).

The susceptibility to malathion of Spanish field populations, assessed at the time when malathion was in use (2003–2005) and 14 years after it was banned from the EU (2023), were also compared (Table 3). All populations sampled in the period 2003–2005 showed moderate to high levels of resistance to malathion (RR = 30–201), except the population from Vila-real that showed low level of resistance (RR = 6) when compared to the susceptible C strain. However, the levels of resistance to malathion of all the populations sampled in 2023 were low (RR = 3–4). Our results indicate that the decline in the frequency of resistant alleles was associated with a decline in malathion resistance.

4 DISCUSSION

We report here a new malathion resistant mechanism in *Ceratitis capitata*, mediated by a heterogeneous duplication of the *Ccace2* gene coding for AChE (RS haplotype, one of the copies bearing the mutation G328A and the other copy non-mutated). Empirical evidences that support this finding include: (i) crossing experiments and offspring phenotyping that demonstrated that both copies segregated together; (ii) quantification by qPCR of double relative number of gene copies in individuals of the W-10Km strain (RS/RS genotype) when compared to individuals of the C strain (S/S genotype); and (iii) gene expression of *Ccace2* in W-10Km was about two-fold compared to the C strain. The importance of gene amplification in driving the evolution of insecticide resistance is well recognized.[36] However, examples of

Table 2. Genotypic and allelic frequencies for *Ccace2* in field populations of *Ceratitis capitata* collected in Spain 2017–2022 (after malathion was banned from the EU in 2009)

			Genotypic frequency (%)[‡]						Allelic/haplotype frequency (%)[§]		
Field populations (2017–2022)	Year	*n*[†]	S/S	S/R	R/R	S/RS	R/RS	RS/RS	S	R	RS
Eastern Spain											
Onda (Castelló)	2017	19	63.2	5.3	0.0	21.1	0.0	10.5	76.3	2.6	21.1
Vila-real (Castelló)	2017	19	89.5	0.0	0.0	10.5	0.0	0.0	94.7	0.0	5.3
Betxí (Castelló)	2017	20	60.0	20.0	0.0	15.0	0.0	5.0	77.5	10.0	12.5
Onda (Castelló)	2022	20	95.0	5.0	0.0	0.0	0.0	0.0	97.5	2.5	0.0
Castelló (Castelló)	2022	20	100.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
Ador (València)	2017	20	85.0	15.0	0.0	0.0	0.0	0.0	92.5	7.5	0.0
La Pobla de Farnals (València)	2017	20	75.0	5.0	0.0	10.0	5.0	5.0	82.5	5.0	12.5
Monserrat (València)	2017	20	75.0	15.0	0.0	10.0	0.0	0.0	87.5	7.5	5.0
Montroy (València)	2017	20	90.0	5.0	5.0	0.0	0.0	0.0	92.5	7.5	0.0
Picassent (València)	2017	20	70.0	15.0	5.0	10.0	0.0	0.0	82.5	12.5	5.0
Puig (València)	2017	17	82.4	5.9	5.9	0.0	0.0	5.9	85.3	8.8	5.9
Turis (València)	2017	19	89.5	5.3	0.0	5.3	0.0	0.0	94.7	2.6	2.6
Godelleta (València)	2017	20	75.0	15.0	0.0	10.0	0.0	0.0	87.5	7.5	5.0
Palma de Gandia (València)	2017	20	75.0	5.0	10.0	5.0	0.0	5.0	80.0	12.5	7.5
Tavernes de la Valldigna (València)	2017	10	70.0	0.0	0.0	30.0	0.0	0.0	85.0	0.0	15.0
Chiva (València)	2022	20	90.0	10.0	0.0	0.0	0.0	0.0	95.0	5.0	0.0
Carcaixent (València)	2022	20	70.0	25.0	5.0	0.0	0.0	0.0	82.5	17.5	0.0
Oliva (València)	2022	20	85.0	15.0	0.0	0.0	0.0	0.0	92.5	7.5	0.0
Nàquera (València)	2022	20	100.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
L'Atzubia (Alacant)	2017	15	73.3	6.7	0.0	6.7	13.3	0.0	80.0	10.0	10.0
Pego (Alacant)	2017	20	75.0	15.0	5.0	5.0	0.0	0.0	85.0	12.5	2.5
Total *eastern Spain*		399	80.7	9.3	1.8	6.0	0.8	1.5	88.3	6.8	4.9

[†] Number of adults analysed per field population or laboratory strain or crossing.
[‡] The genotypes were determined by TaqMan and qPCR when needed for those sampled in the period 2017–2023.
[§] Assuming that the heterogeneous duplication of the *Ccace2* gene (with one of the copies bearing the mutation G328A and the other copy non-mutated) also segregates as a unique allele (RS haplotype) in field populations.

Table 3. Susceptibility to malathion of Spanish field populations of *Ceratitis capitata*

Period	Locality (region)	LC_{50}[†] (95% FL)	RR[‡] (95% FL)
2003–2005[§]	Baix Empordà (Girona)	519 (130–1169)	33 (11–100)*
	Albelda (La Rioja)	472 (163–1084)	30 (13–72)*
	Vila-real (Castelló)	102 (44–160)	6 (4–11)*
	Burriana (Castelló)	1376 (668–3126)	88 (47–166)*
	Alcúdia (València)	1029 (503–2074)	66 (34–127)*
	Serra (València)	938 (278–2735)	60 (25–143)*
	Carlet (València)	3137 (2182–4850)	201 (129–314)*
	Orihuela (Alacant)	1636 (763–3629)	105 (46–241)*
	Laboratory C strain	16 (11–19)	-
2023	Simat de Valldigna (València)	114 (77–160)	4 (2–5)*
	La Pobla Llarga (València)	88 (50–136)	3 (2–4)*
	L´Énova (València)	116 (79–164)	4 (3–5)*
	Llíria (València)	117 (73–187)	4 (3–5)*
	Nàquera (València)	122 (63–209)	4 (3–6)*
	Laboratory C strain	33 (23–43)	-

Note: * RR is significant different ($P < 0.05$) if the 95% FL does not include 1.
[†] Lethal concentration (LC_{50}) and 95% fiducial limit (FL) in ppm of malathion in the diet.
[‡] Resistance ratio (RR) and 95% FL at LC_{50} level of each population with respect to the laboratory C strain for each period. Calculated according to Robertson and Preisler.[34]
[§] Data from Magaña *et al.*[1]

heterogeneous duplications remains scarce and restricted to the order Diptera: AChE gene *ace1* bearing wild-type and resistant G119S copies in *Culex pipiens*,[14] *Anopheles gambiae*[15] and *Anopheles coluzzii*[37]; *ace1* bearing wild-type and resistant F290V copies in *Culex pipiens*[38]; voltage-gated sodium channel (VGSC) gene bearing wild-type and resistant L1014F copies in *Culex quinquefasciatus*[39]; VGSC gene bearing wild-type and resistant I1011M copies in *Aedes aegypti*[40]; GABA-gated chloride channel subunit *Rdl* bearing one wild-type copy and a second copy with two point mutations (A301S and M360I) in *Drosophila melanogaster*[41]; and glutathione S transferase *Gste2* combining a resistance-associated mutation 119V with its wild-type counterpart in *Anopheles gambiae*.[42] In the case of the AChE G119S heterogeneous duplication in *Anopheles gambiae*, it has been possible to demonstrate that the two copies are in tandem, and separated by a distance lower than 500 kb, by fluorescence *in situ* hybridization[17] and genomic structure analysis.[18] Indeed, an amplicon of 203 kb encompassing the *ace-1* gene and 11 other genes is amplified in both heterogeneous and homogeneous (identical resistant copies) duplications in *Anopheles gambiae* and organized strictly in tandem.[18] Most insects, including mosquitoes, have two AChE genes (*ace1* and *ace2*), but higher Diptera have only one (*ace2*).[43] The organization of *ace2* locus, conserved among Diptera, is composed of ten exons, the first of them being at the 5′ non-coding region.[44] The SNP responsible for the G328A mutation associated to malathion resistance in *Ceratitis capitata* was located at the first base of the exon 6 in the *Ccace2* gene.[3,45] *In situ* hybridization on medfly polythene chromosomes showed that *Ccace2* is placed at the autosomal chromosome 2L.[46] More importantly, a single signal at the same location was obtained for the genotypes S/S, R/R and RS/RS, suggesting that both copies of the gene might be located very close in the same chromosome.[46] However, full-length sequence of both copies to demonstrate that they are in tandem is lacking.

The heterogeneous duplication of the *Ccace2* gene adds to the two malathion resistance mechanisms previously identified in *Ceratitis capitata*: (i) a mutation G328A in the *Ccace2* gene (R allele); and (ii) metabolic resistance mediated by esterases.[2] Both, the R allele and the RS haplotype were widely distributed in field populations throughout Spain in the period 2003–2008 (when malathion was used), and present in the malathion resistant strain W derived from a field population collected in eastern Spain in 2004.[2] Interestingly, the frequency of the RS haplotype raised when the W-4Km strain was generated from W by increasing the selection pressure from 2000 to 4000 ppm[25] and was fixed in the laboratory strain W-10Km, obtained from W-4Km by further increasing the selection pressure with malathion to 10 000 ppm. Population evolution experiments in cages containing a mixture of *Culex pipiens* RR (homozygous for the G119S mutation), SS (homozygous for the wild-type allele) and DD (homozygous for the heterogeneous *ace-1* duplication) genotypes also resulted in the fixation of the DD genotypes.[19] It has been reported that the reduced AChE catalytic activity associated to point mutation G119S in *Culex pipiens*[47] and *Anopheles gambiae*[48] is compensated in the heterogeneous AChE duplications,[16,18] overcoming some of the fitness cost associated with the single copy mutated alleles.[16–18] As a result, heterogeneous duplications that avoid segregation load can be adaptive because they permanently associate over dominant (RS is the fittest genotype) alleles.[19] Likewise, we have shown that individuals from the W-10Km strain (genotype RS/RS) showed about 30% higher AChE activity and a reduced sensitivity to malaoxon than those of the C strain (genotype S/S). In a previous study, Magaña *et al.*[2] found two different phenotypes in the individuals of the W strain: (i) individuals with reduced AChE activity and no inhibition by malaoxon (named WR); and (ii) individuals with AChE activity and inhibition by malaoxon similar to that of the C strain (named WS). Besides, individuals with the WR phenotype were homozygous for the G328A mutation, whereas individuals with the WS phenotype were either heterozygous or wild-type at this position.[2] We have shown here that the R and S alleles and the RS haplotype were present in the W strain, supporting that the WR phenotype corresponded to individuals with the R/R genotype, whereas the WS phenotype was formed by the rest of the genotypes in an undetermined proportion. Thus, under continuous and increased selection pressure with malathion, the individuals with RS/RS genotype were selected until fixation because they have both, lower susceptibility to malaoxon than the genotypes containing the S allele, and higher AChE activity than the genotypes containing the R allele. On the contrary, the frequency of the R allele and the RS haplotype declined in field populations after outdoor plant protection products containing malathion were withdrawn from the EU market because of their identified acute and long-term risks to birds. Our study has focussed in the three provinces of the Comunitat Valenciana (Castelló, València and Alacant) in eastern Spain, because of their importance in citrus production in Spain. We found that the average frequency of the individuals carrying the R allele and/or the RS haplotype declined from 44% in the period 2003–2008 (when malathion was in use) to 19.3% in 2017–2022 (8–13 years after the withdrawal of malathion). However, remarkable differences were found for the fate of the different resistant genotypes: 1.5× increase for RS/RS, 1.9× decrease for S/RS, 2.4× decrease for S/R, 4.8× decrease for R/R, 5.1× decrease for

R/RS. This result is in accordance with a higher fitness cost for those genotypes containing the R allele in homozygosis (R/R) or in heterozygosis (R/S, R/RS) with respect to those that contain the haplotype RS in homozygosis (RS/RS) or in heterozygosis with the wild-type allele (S/RS). We cannot discard that geographical and other factors such as the treatment record of each particular field may also have contributed to the observed results, since the fields in eastern Spain where the sampling was carried out were not the same in the two periods. Nevertheless, the high rates of gene flow among Spanish medfly populations[23] favours the circulation and evenness of genotypes within the area of study. A similar pattern was observed for field populations of *Culex pipiens* from Lebanon, in which a high frequency of both G119S and F209V mutations and the presence of duplicated haplotypes for both mutations were found in areas with a heavy usage of OPs in 2005[38]; whereas the frequencies of both mutations were dramatically reduced between 2008 and 2009 after control practices switched to pyrethroids, though the duplicated haplotype for the G119S mutation was still present.[12] Interestingly, a number of studies have monitored the evolution of *Culex pipìens* resistant alleles in areas where OPs have been routinely used for mosquito control during several years. In this case, the G119S duplicated haplotype has been shown to spread, but did not replace the original resistance allele, as reported in southern France.[11,49,50] Thus, Lenormand *et al.*[49] proposed that the duplicated G119S haplotype, which confers a reduced fitness cost, and the original resistant ace-1R allele, with higher resistance to OPs, might coexist in the presence of insecticide treatments; whereas a reduction of OPs treatments may favour the spread of the duplicated haplotype. Our study provides additional evidences for the highest prevalence of heterogeneous duplications with respect to point mutations in the absence of selection pressure under field conditions. Besides, the diagnostic tools developed in this study (PCR-PIRA and TaqMan genotyping) could be adapted for routine monitoring of resistance in area-wide pest management programmes, such as the trap network comprised for more than 1000 sampling points that covers most of the citrus growing area in the Comunitat Valenciana.[20] Further experiments to determine the inheritance and fitness cost associated to the medfly R allele and the RS haplotype will be required for a more comprehensive understanding of our results with the laboratory strain and the field populations.

5 CONCLUSION

Current medfly control practices in citrus crops in Spain mostly rely on spinosad and lambda-cyhalothrin bait sprays, bait stations coated with pirethroids, and the liberation of sterile males in some areas of eastern Spain.[20] However, the viability of medfly control programmes is jeopardized by the development of resistance to lambda-cyhalothrin,[21,51] and MagnetMED™ traps coated with deltamethrin.[23] Besides, though field populations are highly susceptible to spinosad,[52] spinosad resistant alleles have already been detected in field populations.[53] This represents a scenario where effective IRM strategies are required. The use of evolutionary models predicted that the best option for maintaining the effectiveness of the available insecticides would be to combine those without cross-resistance, preferably a pyrethroid (deltamethrin, lambda-cyhalothrin or esfenvalerate) with spinosad, and harmonize their use with other control methods such as the liberation of sterile males and cultural practices.[51,53] Indeed, simulation experiments at laboratory conditions demonstrated that alternation of lambda-cyhalothrin with spinosad helped delay the development of resistance.[54] Nevertheless, it becomes evident that the range of available effective insecticides for medfly control is increasingly limited, and the switch to other insecticides may be needed in the future to avoid medfly outbreaks. The use malathion in the EU is currently restricted to glasshouses,[20] but plant protection products containing OPs could be exceptionally granted. Our study showed that resistance to malathion has been almost completely reverted (3–4-fold when compared with the susceptible C strain) in Spanish medfly field populations collected 14 years after malathion withdrawal. This result contrast with the high resistance levels reported in the same areas when malathion was used (6–201-fold).[1] However, both malathion resistant R allele and RS haplotype remained in the field at low frequencies. Thus, they could be rapidly selected if malathion or other OPs with cross-resistance are used without implementing appropriate management strategies. Indeed, the use of OPs for the control of medfly (phosmet, triclorphon and methyl-chlorpyrifos) and other pests (chlorpyrifos), after the withdrawn of malathion, may have contributed to maintaining these alleles, since some have shown moderate cross-resistance with malathion.[25] Our results indicated that, in order to ensure the successful management of this pest, an integrated IRM approach is essential, which must involve the implementation of different control strategies and take advantage of the development of diagnostic and modelling tools to monitor and forecast resistance.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPORTING INFORMATION

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

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