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Bactrocera oleae (Diptera: Tephritidae) organophosphate resistance alleles in Iberia: Recent expansion and variable frequencies

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Abstract. The olive fly, *Bactrocera oleae* (Rossi, 1790) (Diptera: Tephritidae), is the most important pest of olive trees globally, causing losses that, in the absence of control measures, can exceed 90% of the crop. In the Mediterranean basin, where the overwhelming majority of production is concentrated (~98%), organophosphate insecticides (OPs) have been the main tool for *B. oleae* control for the last four decades, leading to the development of resistance to these compounds. Mutations of the *Ace* gene, which codes for acetylcholinesterase, the target enzyme of OPs and other insecticides, have been identified as the underlying cause, with studies reporting mid to very high frequencies of resistance alleles in several countries. Interestingly, no resistance alleles were detected in Portugal, at the Western end of the Mediterranean basin. As the original study was done almost a decade ago and did not include many samples, we decided to re-evaluate the situation, by analysing a larger number of individuals from multiple locations in Western and Southern Iberia (Portugal and Spain). In our present study, resistance-associated *Ace* alleles were found to have become widespread in both regions, but with highly varying frequencies. Together with the observed haplotype distributions, this finding is consistent with previous suggestions of a recent, selection-driven spread and has implications for the importance of *Ace* mutations in organophosphate resistance in the field as well as the importance of gene flow between Mediterranean populations of *B. oleae*.

INTRODUCTION

The olive fly, *Bactrocera oleae* (Rossi, 1790) (Diptera: Tephritidae), is the most important pest of olive trees, causing losses estimated to average some 15% of production – or more than a billion US dollars per annum. However, in the absence of control measures, losses can be much higher, in some cases exceeding 90% of the crop (Montiel Bueno & Jones, 2002; Skouras et al., 2007). In the Mediterranean basin, where the overwhelming majority of the world's production is concentrated (~98%), chemical insecticides, particularly organophosphates (OPs), have been the main tool for *B. oleae* control for the last four decades (Tsakas & Krimbas, 1970; Vontas et al., 2001). As with other widespread pests, such usage would be expected to lead to the development of resistance to these compounds (Feyereisen, 1995), and indeed this was observed, both in the laboratory and on the field, more than a decade ago (Vontas et al., 2001).

Biochemical and molecular analysis of acteylcholinesterase (AChE), the target enzyme of OPs and other insecticides, and the *Ace* gene, which encodes it, has led to the identification of mutations in that gene as the underlying cause of OP resistance in olive fly (Vontas et al., 2001, 2002; Kakani et al., 2008). Two of these alterations (I214V and G488S) affect amino-acids close to the active site of the enzyme, and are thought to cause topological alterations that decrease the effectiveness of the action of OPs (Mutero et al., 1994; Walsh et al., 2001; Vontas et al., 2002). Interestingly, identical mutations were found in laboratory-developed OP-resistant Oriental fruit fly, *Bactrocera dorsalis* (Hendel), and I214V is equivalent to the I199V mutation found earlier in field resistant strains of *Drosophila melanogaster* (Meigen) (Mutero et al., 1994; Hsu et al., 2006), providing support for the notion of parallel evolution of resistance-associated mutations (ffrench-Constant et al., 2004). Both *Ace* I214V and G488S substitutions were found at high or very high frequencies in natural *B. oleae* populations in Greece, Albania and Italy, and less frequently in populations in France, Spain and Turkey (Hawkes et al., 2005; Nardi et al., 2006; Baskurt et al., 2011). A third mutation of a completely different type was later discovered in natural *B. oleae* populations, also in the *Ace* gene, in association with high levels of OP resistance: a 9 bp-deletion causing the loss of three consecutive glutamine residues (Δ3Q) near the carboxyl-terminus of the protein (Kakani et al., 2008). This is thought to increase the efficiency of AChE GPI-anchoring, leading to a higher number of enzyme molecules in the synaptic cleft and consequently to a reduced sensitivity to OPs (Kakani et al., 2008, 2011). The Δ3Q alteration has subsequently been found in natural *B. oleae* populations in several countries of the Mediterranean basin, albeit at lower frequen-

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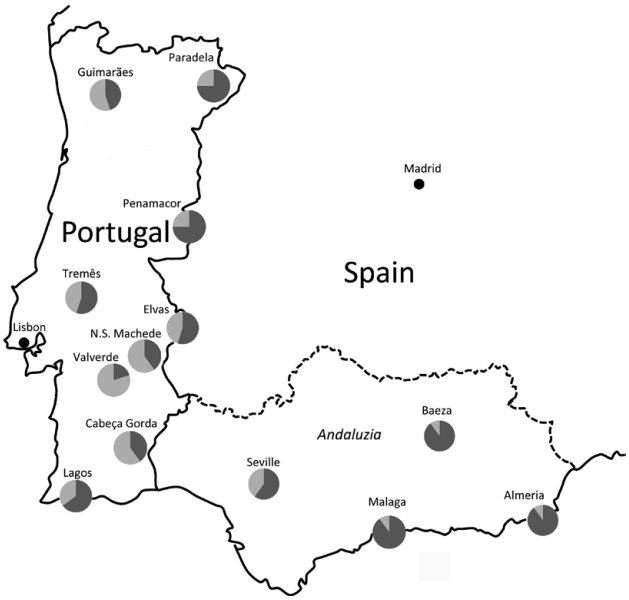

Fig. 1. Olive fly (*Bactrocera oleae*) collection sites in the Iberian Peninsula. Pie charts indicate the proportions of OP resistance-associated (dark grey) and sensitivity-associated (light grey) alleles.

cies than I214V or G488S (Baskurt et al., 2011; Kakani et al., 2014).

Despite this widespread presence of OP-resistant olive flies in Europe, neither I214V and G488S (Nardi et al., 2006), nor, more recently, Δ3Q (Kakani et al., 2014) were detected in Portugal, at the Western end of the Mediterranean basin. However, the sample size used for screening for I214V and G488S was small, and the flies tested were collected in 2001. Additionally, in Andalusia (southern Spain), the most intensive region of olive production (a third of the world's olives in fact), the frequencies of the I214V and G488S *Ace* substitutions were limited in natural populations assayed to about 30% in 2002, in contrast to 80–90% and 60–90% for Greek and Italian populations, respectively (Hawkes et al., 2005; Nardi et al., 2006). Because in Greece the frequencies of resistance alleles had been found to change from 0–2.5% to >90% in the period 1987–1999 (Hawkes et al., 2005; Margaritopoulos et al., 2008), we hypothesized that a similar increase in OP-resistance alleles had occurred in the Iberian Peninsula since 2001. To test this hypothesis, 100+ individual olive flies collected from thirteen locations in Portugal and Spain (Andalusia) were screened for the I214V, G88S and Δ3Q alterations in the *Ace* gene.

MATERIAL AND METHODS

Olive fly samples

Olive flies were collected at or near 13 different localities, of which nine were in Portugal and four in Andalusia, Spain (Fig. 1, Table 1). At most collection sites, olives were picked and stored in plastic bags, with emerging larvae, pupae and adults being collected periodically, whereas at others, McPhail traps baited with a 5% solution of diammonium hydrogen phosphate (Sigma-Aldrich, Sintra, Portugal) were placed in olive groves for periods of 1–2 weeks and inspected every 1–2 days. Trapped adults were washed once in 70% ethanol before storage. All individuals were stored at –20°C in 70% ethanol prior to DNA extraction.

DNA extraction, amplification and sequencing

Total DNA was extracted using a standard SDS/Proteinase K method. PCR amplifications (primers used in PCR amplification and sequencing are listed in Table 2) of the three fragments (Intron III/Exon IV, Exon VII and Exon X) of the *Ace* gene screened were performed in 25 μL reactions containing 10 ng of genomic DNA, 75 mM Tris-HCl, pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01 % (v/v) Tween 20 (Fermentas, Vilnius, Lithuania), 1.5 mM $MgCl_2$ (Fermentas), 0.25 mM of each deoxy-NTP (Fermentas), 175 ng of each primer (Metabion International, Steinkirchen, Bavaria, Germany and Macrogen, Seoul, Republic of Korea) and 1.0 U of *Taq* DNA polymerase (Fermentas). The thermocycling protocol was: 95°C for 3 min; 38–40 cycles of 95°C for 30 s, 56°C for 45 s and 72°C for 30 s; and 72°C for 3 min. In general, primer pairs used for amplifications were: Bo12-009/BoAce_1040R, BoAce_1424F/BoAce_1519R and Bo12-023/Bo14-056, and sequencing reactions were performed with primers Bo12-027, BoAce_1519R and Bo14-057 (Fig. 2). For the I214V screen, amplification from a few samples was performed using twice the concentrations of *Taq*, dNTPs and $MgCl_2$ in the PCR mixture and primers BoAce_518F/BoAce_1040R or Boace3F/Boace3R (Fig. 2). In the latter case, Boace3R was also used for sequencing. To determine the sequences of intron III and intron IV, PCR was performed with the primer pair Bo12-009/Bo12-010 and the duration of the extension step increased to 1 min and 15 s. Sequencing was

Table 1. Distributions of the I214V and G488S *Bactrocera oleae Ace* gene substitutions at the 13 locations sampled. [1]S – OP-sensitive alleles (coding for 214I and 488G); R – OP-resistant alleles (coding for 214V and 488S). *Also screened for Δ3Q.

Collection site (nearest locality)	Geographic coordinates	Collection date	Genotype			R allele frequency
			SS/SS[1]	SR/SR[1]	RR/RR[1]	
Paradela*	41.58N; 6.25W	Oct 2012	0	5	5	0.75
Guimarães	41.46N; 8.31W	Dec 2011/Nov 2012	3	5	2	0.45
Penamacor*	40.16N; 7.10W	Jan 2013	1	3	6	0.75
Tremês	39.36N; 8.75W	Nov 2009	1	7	2	0.55
Elvas	38.86N; 7.27W	Jul 2009	2	5	3	0.55
Nossa Senhora de Machede	38.58N; 7.78W	Aug 2009	3	6	1	0.40
Valverde	38.53N; 8.02W	Aug 2009	6	4	0	0.20
Cabeça Gorda	37.91N; 7.82W	Sep 2009	3	6	1	0.40
Lagos*	37.13N; 8.68W	Dec 2012	1	5	4	0.65
Seville*	37.27N; 5.50W	Oct 2012	1	6	3	0.60
Malaga*	36.73N; 4.71W	Oct 2012	0	2	8	0.90
Baeza	38.05N; 3.37W	Aug 2009	0	2	8	0.90
Almeria*	36.89N; 2.44W	Oct 2012	0	2	8	0.90

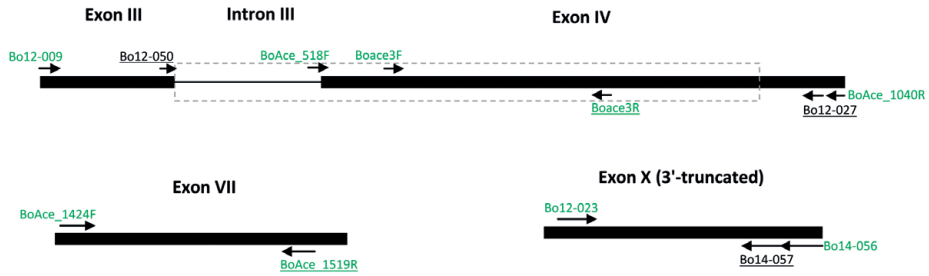

Fig. 2. Schematic representation of the segments of the *Bactrocera oleae Ace* gene analysed. Thick lines represent exons, the thin line an intron. Primers used for PCR are in green; those used for sequencing are underlined. The boxed region is shown in greater detail in Fig. 3. Elements in each segment are drawn to scale, but the representation for the smaller segments is twice enlarged relative to that of the bigger one.

done with the same two primers and BoAce_518F. Haplotype determinations in PCR products with more than one heterozygosity were made by sequencing with allele-specific primers (Bo12-051, Bo12-052, Bo14-118 or Bo14-147), or by allele-specific re-amplification of a 1 : 100 dilution of the original PCR product (Fig. 3). This was done using Bo12-009 and Bo12-051 or Bo14-125 (Figs 2 and 3) and a touch-down cycling protocol: 95°C for 1 min; 14 cycles of 95°C for 30 s, T_{hib} for 30 s and 72°C for 30 s; and 72°C for 1 min, in which T_{hib} was 2 × 68°C, 2 × 66°C, 2 × 64°C, 2 × 62°C, 2 × 60°C and 4 × 58°C. Re-amplification products were sequenced with primers Bo12-050 and BoAce_518F. PCR products were used in sequencing reactions after purification with ExoI (Fermentas) and SAP (Fermentas). Part of the sequencing was outsourced to Macrogen Europe (Amsterdam, The Netherlands), and part was performed with the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Porto, Portugal) using the following cycling protocol: 96°C for 3 min; 35 cycles of 95°C for 15 s, 56°C for 15 s and 60°C for 2 min; and 60°C for 5 min. Sequencing reaction products were purified using Sephadex G-50 micro-spin columns (GE Healthcare, Carnaxide, Portugal) and sequenced using a ABI Prism 3130 XL sequence analyzer (Applied Biosystems). Sequence variants were detected by visually inspecting electropherograms using Chromas 1.45 (Technelysium Pty Ltd.), while editing and alignments were performed with CLC Main Workbench 6 (CLC Bio).

Table 2. Sequences of PCR and sequencing primers used in this study.

Primer	Sequence (5'-3')
BoAce_518F**	TACTCAATTTCACTTTCAGCACTC
BoAce_1040R**	CAACTCACCGACAATAGCG
BoAce_1424F**	CAGCTGGGTTGGTAATCC
BoAce_1519R**	TAGTGCACGGAAGCTCC
Bo12-009	CTAGATATGAATATTTTCCCGG
Bo12-010	ACCTTCGTCTCTGACATTTC
Boace3F*	GCTTACCCGTGCTTATTTGG
Boace3R*	AGCCAACGTATAGCCAATGC
Bo12-023	TGAAGTCAAACCATCATCCG
Bo12-027	GCATTACAATTGCAGTCGTTG
Bo12-050	GCGCTAATGGCGGTGAG
Bo12-051	CCCGATTGCATCATGCCC
Bo12-052	CCCGATTGCATCATGCCA
Bo14-056	TAGAAAAATGCATTTACTGACG
Bo14-057	GACGGAATTTTTAAAACCAGTG
Bo14-118	TCCTTTCATAATAAATTTCATTTC
Bo14-125	CCCGATTGCATCATGCGC
Bo14-147	TAGAACACAAGCTAACTAACTG

* From Hawkes et al. (2005). ** From Nardi et al. (2006).

RESULTS

The primary goal of this study was to assess how the distribution of *Ace* OP-resistant alleles in the Western and Southern Iberian Peninsula had evolved since the first studies on the subject almost a decade ago (i.e. Hawkes et al., 2005; Nardi et al., 2006). We therefore focused on the two substitutions earlier described, I214V and G488S, involving screening a total of 130 Iberian flies for their presence. Searching for the Δ3Q variant was restricted to the six locations most recently sampled (Table 1). To try to avoid potential local biases, and therefore obtain reasonable representations of the two regions studied, multiple, widely separated locations were sampled (Fig. 1). More sites were selected from Portugal, both because of the absence of resistance alleles during sampling in 2001 (Nardi et al., 2006) and because preliminary data suggested higher heterogeneity in populations from this region.

In terms of the resistance genotypes detected from our surveying regime, both I214V and G488S were found at medium to high frequencies in all locations, demonstrating they are now widespread, even in Portugal (Table 1). Interestingly, their frequencies vary widely, from 90% at various locations in Andalusia, to as low as 20% in Valverde, in the neighboring Portuguese region of Alentejo. In contrast, no alleles carrying the Δ3Q variant were found in the 60 samples analysed, a result consistent with recently published data from the same regions (Kakani et al., 2014).

Data on haplotype frequencies and distributions at the *Ace* locus was also obtained. Unfortunately, the chromosomal distance between the two substitutions screened for (at least 13 kb, and likely >16 kb; Kakani et al., 2013) and the lack of genomic sequence information made it unpractical to ascertain the gametic phase for all individuals. Nevertheless, it could be inferred for homozygous individuals in at least one of the two sites, representing 52% of the Iberian flies tested (68/130). Interestingly, both variants had the same zygosity status in all cases, suggesting that, in Iberia at least, I214V and G488S are always found in the same chromosome. A more complete analysis was performed for haplotypes associated with OP-resistance and OP-sensitivity *Ace* alleles. To do this, the intronic sequences flanking the exon IV of the *Ace* gene, where the

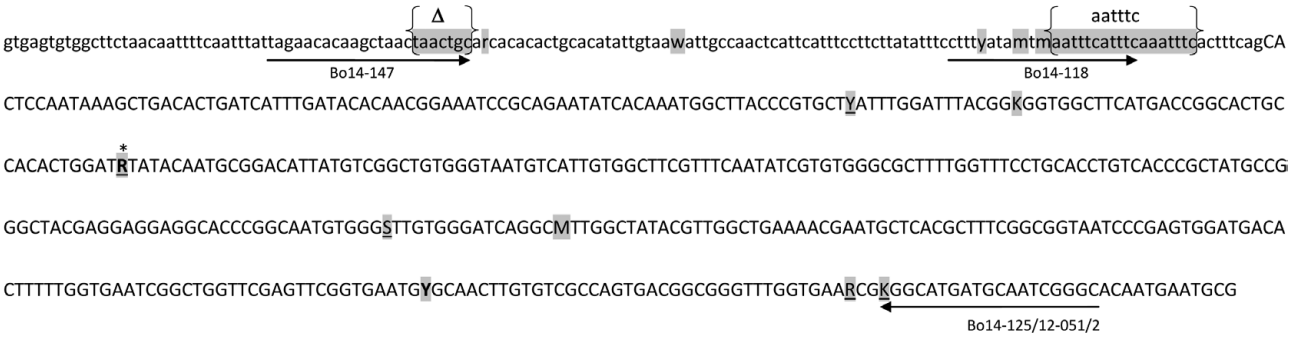

Fig. 3. Details of the sequence analysis of the *Ace* gene segment of *Bactrocera oleae* encompassing intron III and exon IV. Sequence variants (shaded) are labeled as follows: previously described – underlined; affecting protein sequence – bold; OP-resistance associated – marked with an asterisk. Primers indicated were used for allele-specific PCR or allele-specific sequencing. Bo12-051 and Bo12-052 differ in the last base, whereas Bo14-125 differs from Bo12-051 in having a sequence mismatch in the penultimate base.

I214V substitution is located, were obtained. Preliminary experiments had shown intron III to be polymorphic, and therefore the screen for I214V was designed to include amplification and sequencing of that intron as well (Fig. 2). Individual allele sequences were obtained for 116 of the Iberian samples, yielding a total of nine haplotypes (GenBank accession numbers KM204136–KM204145) defined by 15 sequence variants (Table 3 and Fig. 3). A stark contrast was found between chromosomes carrying OP-sensitive *Ace* alleles, 90% of which had Hap01, and those with OP-resistant *Ace* alleles, that in >99% of the cases (146/147) had Hap02, suggesting that virtually every case of olive fly OP-resistance in Iberia can be traced to the same genetic origin (Table 3). Of special interest was the finding that, though nine of the variants other than that causing I214V were either intronic or silent, one causes an Alanine to Valine substitution at residue 298. Because this residue is conserved in all the homologous fly proteins in a dataset including species from five different families (Table 4) and no other alteration was detected in this particular sample, the possibility that A298V represents a novel *Ace* mutation with functional consequences cannot be ruled out.

DISCUSSION AND CONCLUSIONS

As seen from the present results, it may firstly be accepted that a major alteration in the distribution of OP-associated *Ace* alleles has occurred in Iberia during the past decade. Indeed, the data show that average frequencies for both I214V and G488S have reached >50% in Portugal and >80% in Andalusia, in contrast with the previously reported values of zero and 33%, respectively (Hawkes et al., 2005; Nardi et al., 2006). Even considering that those values were estimated from smaller sample sets, such alterations clearly reflect profound changes in the genetic make-up of populations, and cannot be ascribed simply to geographic variation and sampling effects. For example, although substantial frequency differences were found in Portugal, OP-resistant alleles were detected at every location sampled from, and in Paradela, closest to the site sampled by Nardi et al., in 2001 (Nardi et al., 2006), their frequency reached 75%, the highest in the country.

Did these alleles arise in Iberia, or were they imported? The fact that the substitutions involved are identical and (with a single exception) occur in the same haplotypic background where they were found in other geographical locations points to the latter explanation, yet is not conclusive by itself since identical (or equivalent) *Ace* substitutions have been shown to arise independently, even in different species (Fournier, 2005; Hsu et al., 2006; Nardi et al., 2006). However, if the OP-resistant *Ace* alleles observed were of local origin, at least some individuals homozygous at the 214 position and heterozygous at 488, or vice versa, would be expected to be found, as previously described in populations from several countries (Hawkes et al., 2005; Nardi et al., 2006; Baskurt et al., 2011). In

Table 3. *Ace* intron III / exon IV *Bactrocera oleae* haplotypes found in Iberia, and their absolute frequencies among 116 samples. Intron position numbering is that of the 149 bp and 137 bp variants.

	Intron III						Exon IV								
	g57a	t79a	c117t	a121c	a123c	Size (bp)	78T/C*	93T/G	127A/G**	264G/C*	279A/C	380C/T	417G/A*	420G/T*	Frequency
Hap01	g	t	c	a	a	149	T	T	A	G	A	C	G	G	77
Hap02	g	t	c	c	c	137	C	T	G	C	A	C	A	T	146
Hap03	g	t	c	c	c	137	C	T	A	C	A	C	A	T	3
Hap04	g	t	c	c	c	137	C	G	A	C	C	C	A	T	1
Hap05	g	t	c	a	a	149	T	G	A	G	A	C	G	T	1
Hap06	g	t	c	a	a	149	T	T	A	G	A	T	G	G	1
Hap07	a	t	t	c	c	137	T	G	A	G	A	C	G	G	1
Hap08	g	t	c	a	a	149	T	T	G	G	A	C	G	G	1
Hap09	a	a	t	c	c	130	T	T	A	G	C	C	G	T	1

*Variants already described in Nardi et al. (2006). **Corresponds to 214 I/V.

Table 4. Fly species for which *Ace* sequences are available used for comparison with *Bactrocera oleae* in this study.

Species	Family	Superfamily	GenBank (nt)	GenBank (P)
Bactrocera dorsalis	Tephritidae	Tephritoidea	JN112509	AAO06900
Ceratitis capitata	Tephritidae	Tephritoidea	NM_001279434	NP_001266363
Drosophila virilis	Drosophilidae	Ephydroidea	XM_002056133	XP_002056169
Drosophila willistoni	Drosophilidae	Ephydroidea	XM_002072251	XP_002072287
Drosophila erecta	Drosophilidae	Ephydroidea	XM_001980267	XP_001980303
Drosophila mojavensis	Drosophilidae	Ephydroidea	XM_001998863	XP_001998899
Drosophila yakuba	Drosophilidae	Ephydroidea	XM_002097460	XP_002097496
Drosophila melanogaster	Drosophilidae	Ephydroidea	X05893	P07140
Drosophila sechellia	Drosophilidae	Ephydroidea	XM_002031326	XP_002031362
Drosophila simulans	Drosophilidae	Ephydroidea	XM_002103562	XP_002103598
Drosophila persimilis	Drosophilidae	Ephydroidea	XM_002013876	XP_002013912
Drosophila grimshawi	Drosophilidae	Ephydroidea	XM_001990127	XP_001990163
Drosophila pseudoobscura	Drosophilidae	Ephydroidea	XM_001358452	XP_001358489
Drosophila ananassae	Drosophilidae	Ephydroidea	XM_001953033	XP_001953068
Lucilia cuprina	Calliphoridae	Oestroidea	U88631.1	AAC02779
Cochliomyia hominivorax	Calliphoridae	Oestroidea	FJ830868	ACZ59082
Exorista sorbillans	Tachinidae	Oestroidea	HM028669	ADN37668
Haematobia irritans	Muscidae	Muscoidea	AY466160	AAS45645
Musca domestica	Muscidae	Muscoidea	AJ310134	CAC39209
Stomoxys calcitrans	Muscidae	Muscoidea	HM125963	ADJ67805

Turkey, for example, such genotypic discrepancies were detected in all populations tested, at an average frequency of 20% (Baskurt et al., 2011). The observation of complete concordance between the zygosities of I214V and G488S in Iberian samples therefore strongly suggests that their presence in Iberia is due to the introduction of flies with a chromosome already carrying both substitutions. Nevertheless, the occurrence of endogenously-generated identical substitutions cannot be excluded. Indeed, Hap03, from which almost all the resistance alleles derive (compare haplotypes Hap02 and Hap03 in Table 3) was also found in Iberia, albeit at a low frequency, viz. 3.5% of OP-sensitive alleles. Furthermore, the single case where OP-resistance was found in the haplotype background usually associated with sensitivity alleles (Hap08, Table 3) might be an example of such a parallel process, as the alternative explanation would require a double recombination within a distance of less than 200 bp. Lastly, as mentioned above, the possibility that the A298V substitution found in one of the Portuguese samples alters sensitivity to OPs cannot be excluded. Indeed, not only is Alanine 298 conserved in all the fly species studied to date, but it is also very close to two residues with major roles in AChE protein function – Glu289 and Ser290, the latter a part of the catalytic triad.

Regardless of whether OP-resistance alleles have a regional origin or were imported, the data reported here has some bearing on whether the I214V/G488S combination plays an important role in the field. This has been a controversial issue: although there is no dispute as to the importance of the two substitutions in OP-resistance under controlled laboratory conditions (Vontas et al., 2002; Hsu et al., 2006), and initial field data indicated that selection played a major role in their geographic dispersal and distribution (Hawkes et al., 2005; Nardi et al., 2006), later studies failed to detect correlations between their frequencies and either levels of OP resistance (Kakani et al., 2008) or insecticide treatment regime (Baskurt et al., 2011). Given the large number of farms involved, many of them small, obtaining reliable information on OP use at all locations analysed in the present study over a decade-long period would be extremely difficult. For this reason, the study was not designed to evaluate those correlations in Iberia and therefore, it does not provide biochemical data on resistance levels or clear proof of a selection-driven OP resistance spread. Even so, the rapid spread of resistance-associated *Ace* alleles here reported, when contrasted with the observed differentiation between mitochondrial DNA sequences from Iberian and Italic populations (van Asch et al., 2012, Matallanas et al., 2013), seems to support that hypothesis. For example, in Andalusia, the world epicenter of olive production, and dominated by large, high yielding orchards, the frequencies of both the I214V and G488S *Ace* gene variants rose from values of about 30%, much lower than those found in Italy or Greece, to >80%, on par with those countries, in a 7–10 year period. By contrast, in Portugal, with a much lower orchard density and a varied production structure that includes many small and traditional farms, spread of these particular variants has also occurred, but average frequencies have remained significantly lower and, perhaps more importantly, display marked diversity. An extreme example of this diversity was found in the vicinity of Évora (Portugal), as OP-resistance alleles appeared to be twice as common in Nossa Senhora de Machede than in Valverde, only about 15 km away. Interestingly, there is a correlation between this difference and insecticide use: though in both cases collection took place in untreated groves, the former is a 1.0 ha plot surrounded by many treated olive farms, including an intensive orchard about 1 km away, whereas in Valverde, the olive trees are located within an area of "montado", a

semi-wild landscape dominated by holm oak and cork oak, and eucalyptus plantations. More generally, it is tempting to speculate that the heterogeneity in the frequencies of OP-resistant *Ace* alleles observed in Portugal results from different local selective pressures that contemporary gene flow has not yet masked, mainly because introduction is so recent. Hence, it is perhaps not a coincidence that the lowest frequencies of OP-resistance alleles in Andalusia were found in Seville, the closest sampled population to Portugal itself.

Finally, these results provide insights concerning the timeframe for agriculturally harmful gene-flow among Mediterranean populations of the olive fly. Indeed, they show that, despite evidence suggesting there is marked differentiation and relatively restricted genetic exchange among populations in the region (Augustinos et al., 2005; Nardi et al., 2005, 2010; Zygouridis et al., 2009; van Asch et al., 2012), genetically-based threats can disperse throughout the Mediterranean basin in less than twenty years. This highlights the need to reinforce programmes aimed at genetically characterizing olive fly populations and monitoring their evolution over time, with particular emphasis on insecticide resistance.

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