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Identification of mutations associated with pyrethroid resistance in the voltage-gated sodium channel of the tomato leaf miner (*Tuta absoluta*)

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ABSTRACT

The tomato leaf miner, *Tuta absoluta* (Lepidoptera) is a significant pest of tomatoes that has undergone a rapid expansion in its range during the past six years and is now present across Europe, North Africa and parts of Asia. One of the main means of controlling this pest is through the use of chemical insecticides. In the current study insecticide bioassays were used to determine the susceptibility of five *T. absoluta* strains established from field collections from Europe and Brazil to pyrethroids. High levels of resistance to λ cyhalothrin and tau fluvalinate were observed in all five strains tested. To investigate whether pyrethroid resistance was mediated by mutation of the *para*-type sodium channel in *T. absoluta* the IIS4–IIS6 region of the *para* gene, which contains many of the mutation sites previously shown to confer knock down (kdr)-type resistance to pyrethroids across a range of different arthropod species, was cloned and sequenced. This revealed that three kdr/super-kdr-type mutations (M918T, T929I and L1014F), were present at high frequencies within all five resistant strains at known resistance 'hot-spots'. This is the first description of these mutations together in any insect population. High-throughput DNA-based diagnostic assays were developed and used to assess the prevalence of these mutations in 27 field strains from 12 countries. Overall mutant allele frequencies were high (L1014F 0.98, M918T 0.35, T929I 0.60) and remarkably no individual was observed that did not carry kdr in combination with either M918T or T929I. The presence of these mutations at high frequency in *T. absoluta* populations across much of its range suggests pyrethroids are likely to be ineffective for control and supports the idea that the rapid expansion of this species over the last six years may be in part mediated by the resistance of this pest to chemical insecticides.

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1. Introduction

The tomato leaf miner, *Tuta absoluta* is an economically significant pest of tomatoes that is currently undergoing a rapid expansion in its geographical distribution. The larvae cause damage by feeding on the leaves, stem and fruit leading to significant yield losses of up to 100% if the pest is not controlled (Desneux et al., 2010). *T. absoluta* is native to Central America but has spread to South America and more recently to North Africa and the Middle East. It was first detected in Europe (Spain) in 2006 and has since become a major problem to tomato growers in many European countries (Desneux et al., 2010). The main method of control of *T. absoluta* relies on the application of chemical insecticides (Picancëo et al., 1995). Unfortunately their intensive use has led to the development of resistance with tolerance to organophosphates, carbamates, synthetic pyrethroids, benzoylureas, avermectin and indoxacarb reported in *T. absoluta* populations from Brazil, Chile and Argentina (Guedes et al., 1994; Lietti et al., 2005; Salazar and Araya, 2001; Silva et al., 2011; Siqueira et al., 2001, 2000; Souza et al., 1992).

Pyrethroids are a major class of neurotoxic insecticides that have been used extensively to control a wide range of agricultural and human health pests. They act on the insect nervous system by modifying the gating kinetics of voltage-gated sodium channels to slow activation and inactivation, resulting in continuous nerve stimulation leading to paralysis and death (Soderlund and Bloomquist, 1989). A common mechanism of resistance to pyrethroids, termed knock down resistance (kdr), has been shown to

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arise through alterations (point mutations) in the *para*-type sodium channel protein leading to reduced sensitivity of the insect nervous system to these compounds. The most common mutation identified in a range of arthropod pests, often referred to simply as 'kdr' results from a single point mutation in the S6 segment of domain II of the sodium channel gene usually resulting in a leucine to phenylalanine (L1014F) substitution (Davies et al., 2007; Williamson et al., 1996). This mutation typically confers a 10–20 fold reduction in sensitivity to pyrethroids, however, secondary mutations giving enhanced (super-kdr) resistance have also been identified in pyrethroid resistant arthropods either singly or in combination with kdr (reviewed in Davies and Williamson, 2009). These most commonly occur in domains II or III of the channel protein and include M918T, first described in resistant housefly (*Musca domestica*), and T929I first described in diamondback moth, *Plutella xylostella* (Schuler et al., 1998; Williamson et al., 1996).

To date, the molecular mechanisms underlying pyrethroid resistance in *T. absoluta* have not been characterised. Knowledge of the underlying mechanisms involved and their distribution in world-wide *T. absoluta* populations is essential for effective control of this pest and to minimise the wasteful application of ineffective chemistry. Towards this goal we have carried out leaf-dip bioassays to evaluate the susceptibility of five field strains of *T. absoluta* collected in Europe and Brazil to two pyrethroid insecticides. We describe the cloning and sequencing of a 420 bp fragment (domains IIS4-S6) of the *para* gene from the five *T. absoluta* strains and the identification of three mutations that have previously been reported to confer reduced sensitivity to pyrethroids in several other arthropod pests. We then developed diagnostic tools that allow sensitive detection of these mutations in individual *T. absoluta* larvae and adults. These were used to screen field-collected samples of diverse geographic origin to examine the frequency and distribution of these mutations in global populations of this pest species.

2. Materials and methods

2.1. Tuta absoluta strains

Live *T. absoluta* strains were provided by Dr Pablo Bielza (Departamento de Produccion Vegetal, Universidad Politecnica de Cartagena, Spain) and designated TA1 (Spain), TA2 (Spain), TA3 (Italy) and TA4 (Portugal). They were all collected from the field during the year 2010. The strain GA was provided by Bayer CropScience laboratories. This is a mixed field population collected in 2008 from different areas of Brazil (São Paulo and Minas Gerais states) and reared since then without insecticide selection. The five strains were reared on tomato plants inside insect proof cages and maintained under controlled environment conditions (26 ± 2 °C and 16 h daylength). Additionally, field-collected *T. absoluta* stored in 70% ethanol, originating from 27 locations (Table 1), were provided by Dr. Pablo Bielza.

2.2. Insecticide bioassays

A leaf-dip bioassay protocol, as recommended by the Insecticide Resistance Action Committee (IRAC), was used to evaluate the susceptibility of the five strains of *T. absoluta* to λ cyhalothrin (CS 10%, Syngenta) and tau fluvalinate (TEC 91.7%, Makhteshim). Tomato leaflets were immersed in serial dilutions of insecticide or control solutions, allowed to air dry for 1 h and then supplied as the sole food source to larvae. All insecticide solutions were prepared using an aqueous diluent (0.01% Agral, Syngenta); control solutions consisted of the diluent only. Three replicates at each of six different concentrations were used for each insecticide. Replicates consisted of a Petri dish (90 mm × 20 mm) containing a lightly moistened filter paper, onto which one or two tomato leaves (dependent upon size) were placed and inoculated with 15–20

Table 1
Kdr/skdr mutation frequencies in *Tuta absoluta* populations collected from different locations around the world and stored in 70% ethanol for molecular analysis. *N* = number of individuals.

ID N°	Country	Location	Sampled from	*N*	Mutation frequency		
					L1014F	M918T	T929I
1	Canary Islands	Tejina, (La Laguna) Tenerife	Tomato	12	1.00	0.54	0.46
2	Canary Islands	Guía de Isora, Tenerife	Tomato	10	1.00	0.35	0.60
3	Canary Islands	Granadilla, Tenerife	Tomato	10	1.00	0.25	0.60
4	Canary Islands	Arico,Tenerife	Tomato	8	1.00	0.25	0.69
16	Canary Islands	La Palma	Unknown	7	1.00	0.36	0.64
5	Baleares Islands	Teulera, Mallorca	Tomato	3	1.00	0.33	0.67
6	Baleares Islands	San Fangos, Mallorca	Tomato	3	1.00	0.33	0.67
7&8	Algeria	Mostaganem	Tomato under-protection	9	1.00	0.17	0.83
9	Italy	Turín	Unknown	3	1.00	0.33	0.50
15	Italy	Cagliari.S.Margherita di Pula	Tomato under-protection	5	1.00	0.20	0.80
17	Italy	Sicilia	Aubergine	14	1.00	0.43	0.54
28	Italy	Sele valley.Salerno.Campania	Tomato	17	1.00	0.44	0.41
20	Colombia	Antioquía/Rionegro	Tomato	10	0.60	0.50	0.45
10	Ecuador	La Tola, Pichincha	Tomato	11	1.00	0.55	0.41
11	Spain	Tudela, Navarra	Tomato	8	1.00	0.44	0.44
18	Spain	Ramonete/Lorca, MU	Tomato	6	1.00	0.25	0.75
22	Spain	Mazarrón, Murcia	Tomato	16	1.00	0.13	0.81
25	Spain	Valencia	Unknown	9	1.00	0.44	0.44
26	Spain	Maresme, Cataluña	Tomato	12	1.00	0.50	0.50
12	Argentina	Barrancas,Santa Fé	Tomato under-protection	7	1.00	0.00	1.00
13	Argentina	LaPrimavera, Mendoza	Tomato	8	1.00	0.13	0.75
14	Argentina	La Plata, Bs.As	Tomato under-protection	3	1.00	0.00	1.00
21	Crete	Heraklion	wild plants	3	1.00	0.50	0.50
24	Peru	Chulacanas, Piura	Tomato	7	1.00	0.43	0.50
27	Portugal	Silveira. Concello Torres Vedres	Tomato	5	1.00	0.20	0.80
29a	Israel	Beit hashita, Israel valley	Unknown	5	1.00	0.40	0.50
29b	Israel	Ein hmifraz, Western galilee	Unknown	6	1.00	0.50	0.50
Total				217	0.98	0.35	0.60

L2/L3 stage larvae. These were maintained under controlled environmental conditions (26 ± 2 °C, 16 h daylength) and mortality was assessed after 48 h. Larvae were counted as dead if when stimulated with a fine paintbrush, there was either no movement, or if movement was uncoordinated and they were unable to move a distance equal to double their body length. Statistical analysis of data from bioassays was carried out to estimate LC_{50} values (concentration of insecticide required to kill 50% of the tested insects) using GenStat 13th Edition software (VSN International), which corrects for control mortality prior to performing probit transformations.

2.3. Cloning of sequences encoding domain II

To clone and sequence the domain II region of the *T. absoluta* sodium channel gene PCR reactions were initially carried out on cDNA prepared from pools of 15–20 individuals from each strain using degenerate primers designed against conserved motifs within the IIS4 and IIS6/II–III linker regions of the channel protein as described previously (Martinez-Torres et al., 1997). A nested PCR approach was employed using primers DgN1 and DgN3 in a primary PCR and primers DgN2 and DgN3 in a secondary reaction (primer sequences are given in Table 2). Once the *T. absoluta* sodium channel gene sequence had been determined, specific primers were designed to perform direct PCR analysis of genomic DNA. To amplify a PCR fragment encompassing the L1014F mutation position a nested PCR was performed using primers TAF3 and TAR1 in the first reaction and primers TAF4 and TAR2 in the second reaction. To amplify the region containing the M918T and T929I mutations a single PCR using TAF2 and TARouter was performed (Table 2). To determine the positions and sizes of two introns within this region of the sodium channel gene nested PCR was performed using the primers TAF1 and TAR4 followed by TAF2 and TAR3 for the first intron and primers TAF5 and TAR1 followed by TAF4 and TAR2 for the second intron (Table 2).

Total RNA was extracted from pools of 15–20 individuals using Trizol and following the manufacturer's instructions. Genomic DNA was removed by DNase I digestion using DNA-free DNase treatment and removal reagent (Ambion). The quality and quantity of RNA pools were assessed by spectrophotometry (Nanodrop Technologies) and by running an aliquot on a 1.2% agarose gel. A quantity of 4 μg of RNA sample was then used for cDNA synthesis using Superscript III and random hexamers (Invitrogen) according to the manufacturer's instructions.

PCR reactions (20 μl) consisted of 1 μl of template DNA, 1 μl of each primer (10 μM), 10 μl of GreenTaq (Fermentas) and 7 μl of sterile distilled water. Temperature cycling conditions were: 35 cycles of 95 °C for 30 s 48–58 °C for 60 s and 72 °C for 90–120 s. Agarose gel electrophoresis (1.2%) of PCR products was carried out in 1× TBE buffer and the Wizard SV gel and PCR clean up System from Promega was used to recover DNA from gel slices according to manufacturer's recommendations. PCR products were either direct sequenced (using the same primers used in PCR) or cloned using the Strataclone PCR Cloning kit (Stratagene) and plasmids sequenced with standard T3/T7 primers. PCR products or plasmid DNA was sent to Eurofins MWG/Operon for sequencing.

Table 2
Oligonucleotide primers used to amplify the *Tuta absoluta para*-type sodium channel gene and for TaqMan assays, all primers are shown 5′–3′. Degenerate bases are represented using standard IUB codes.

Name	Sequence
TAkdr_F	CTTCTTAGCCACCGTCGTCATT
TAkdr_R	CGCTTTTACTGGTATATTGCAATAAAAAGCT
TAkdr VIC	AACCACAAGATTACC
TAkdr FAM	ACCACAAAATTACC
TAT929I_F	ACGATGGGTGCCTTGGG
TAT929I_R	TGCATACCCATCACGGCAAATAT
TAT929IVIC	CACAATACGAAGGTCAGGTT
TAT929IFAM	CACAATACGAAGATCAGGTT
TAM918T_F	TGGCCGACGTTTAATTTACTCATCT
TAM918T_R	TGCCCAAGGCACCCATC
TAM918TVIC	TCCTACCCATAATCG
TAM918TFAM	TCCTACCCGTAATCG
DgN1	GCNAARTCNTGGCCNACNYT
DgN2	GCNAARTCNTGGCCNAC
DgN3	YTTRTTNGTNTCRTTRTCRGC
TAF1	GAAATCGTGGCCGAC
TAF2	GGCCGACGTTTAATTTACTC
TAF3	AGAATGGATTGAGAGTATGTGG
TAF4	GTATGTGGGACTGTATGTTGG
TAF5	TACCACGATGGAACTTTACG
TAR1	GGTGTCGTTATCGGCAGTAG
TAR2	GTTATCGGCAGTAGGTGTCGA
TAR3	AAGTTCCATCGTGGTAGGTC
TAR4	CGGTGGCTAAGAAGAATGG
TARouter	TGTTTCAACAGAATGACGATACTA

2.4. TaqMan diagnostic assays

Forward and reverse primers and two probes were designed using the Custom TaqMan Assay Design Tool (Applied Biosystems). The primers TAM918T_F + TAM918T_R, TAT929I_F + TAT929I_R and TAkdr_F + TAkdr_R were used to amplify the regions encompassing the M918T, T929I and L1014F sites respectively. For all assays the probe labelled with VIC, was specific for the wild-type allele, while a second probe, labelled with FAM was specific for the mutant allele. Each probe also carried a 3′ non-fluorescent quencher. The probes TAM918TVIC + TAM918TFAM, TAT929IVIC + TAT929IFAM, and TAkdr VIC + TAkdr FAM were used in the M198T, T929I and L1014F assays respectively. The sequences of the primers and probes used in the TaqMan assays are given in Table 2.

PCR reactions (15 μl) contained 2 μl of genomic DNA extracted from individual insects using DNAzol reagent, 7.5 μl of SensiMix DNA kit (Quantace), 800 nM of each primer and 200 nM of each probe. Samples were run on a Rotor-Gene 6000™ (Corbett Research) using the temperature cycling conditions of: 10 min at 95 °C followed by 40 cycles of 95 °C for 10 s and 60 °C for 45 s. The increase in fluorescence of the two probes was monitored in real time by acquiring each cycle on the yellow (530 nm excitation and 555 nm emission) and green channel (470 nm excitation and 510 nm emission) of the Rotor-Gene respectively. In order to optimize and validate the TaqMan method, and before applying it to the screening of *T. absoluta* collections stored in ethanol, genomic DNA templates of known genotype from the five laboratory strains was used to optimise initial PCRs. During all runs template controls (a wild-type homozygous, a mutant homozygous and a mutant heterozygous sample) were included in each run to aid genotype scoring.

3. Results

3.1. Bioassays

Significant variation in susceptibility between the five strains to λ cyhalothrin and tau fluvalinate was observed (Table 3). For λ cyhalothrin GA and TA1 exhibited the highest and lowest LC_{50} values of 1514 mg l^{-1} and 85 mg l^{-1} respectively (17-fold difference). There was no overlap between the confidence intervals of GA or TA1 with the other three strains (TA2, TA3 and TA4), whose LC_{50} values grouped closely with less than 2-fold separation (351–700 mg l^{-1}). For tau fluvalinate TA3 had the lowest LC_{50} value of 821 mg l^{-1}. The other four strains had overlapping confidence

Table 3
Relative toxicity of λ cyhalothrin and tau fluvalinate to five laboratory strains of *Tuta absoluta*.

	Strain	LC50 (mg l^{-1})	Lower 95% CL	Upper 95% CL	Slope	SE
λ cyhalothrin	TA1	85	36.7	159	0.904	0.132
	TA2	351	176	507	2.256	0.577
	TA3	631	456	837	2.452	0.458
	TA4	700	472	957	1.859	0.34
	GA	1514	1137	2106	2.106	0.371
Tau fluvalinate	TA1	2047	1235	4483	1.8	0.5
	TA2	1952	1365	2841	2.4	0.6
	TA3	821	544	1177	1.765	0.358
	TA4	3716	1443	29,334	0.7	0.2
	GA	9259	1403	61,099	2	0.3

intervals with the highest LC_{50} value of 9259 mg l^{-1} being obtained with GA. We were unable to obtain a known susceptible standard strain to use as a reference in these studies, nevertheless, the recommended field rate of λ cyhalothrin is around 25 mg l^{-1} and for tau fluvalinate is around 50 mg l^{-1}. Therefore the resistance exhibited by these strains would compromise control using these insecticides.

3.2. Sequencing of domain II of the voltage-gated sodium channel

Using degenerate primers designed against conserved sequences within the domain II region of several insect *para* sodium channel gene sequences a ~420 bp fragment of the *T. absoluta para* gene was PCR amplified, cloned and sequenced (Genbank accession number JQ701800). The encoded amino acid sequence of this fragment is shown in Fig. 1. As expected this sequence showed high similarity to other insects from the Lepidoptera order including diamondback moth (*P. xylostella*), silkworm (*Bombyx mori*), corn earworm (*Helicoverpa zea*), cotton leafworm (*Spodoptera litura*) and tobacco budworm (*Heliothis virescens*). The sequenced region obtained (IIS4–IIS6) contains many of the mutation sites previously reported to be implicated in conferring kdr-type resistance to pyrethroids across a range of different insects (Davies et al., 2007). Preliminary sequencing of RT-PCR cDNA fragments from pools of 20 individuals of the five strains (four clones per strain) revealed three point mutations within this region that result in a leucine to phenylalanine amino acid substitution in IIS6 (L1014F, all numbering throughout this manuscript is based on the housefly *para* sequence, GenBank accession: X96668), a methionine to threonine substitution upstream of IIS5 (M918T) and a threonine to isoleucine mutation (T929I) within IIS5. In this sequencing of pooled samples the L1014F mutation appeared to be homozygous and present in all strains and was found in combination with either the M918T and/or the T929I mutations. No other nucleotide polymorphisms were observed in the sequence obtained from different clones of the same strain or between strains.

Specific primers were designed based on the obtained cDNA sequence for use in the analysis of *T. absoluta* genomic DNA to determine the positions and sizes of introns within this region of the sodium channel gene. Previous work, characterizing the domain IIS4–S6 region of the *para* gene from different insect species, has shown that this region contains two introns that are highly conserved in their position but vary widely in size. The position and sequence of the two introns is shown in Fig. 2. The size of the introns was 862 and 106 nucleotides respectively with the sequence of both introns highly conserved across the different strains with no polymorphic bases observed. To assess the frequency of the kdr and skdr mutations within the five strains of

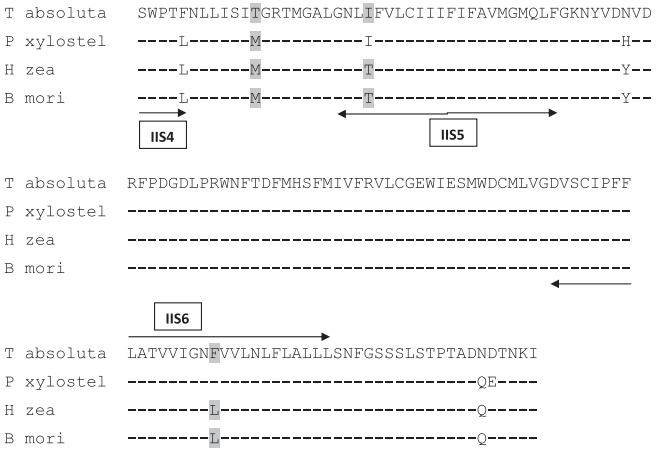

Fig. 1. Amino acid alignment of domains IIS4-IIS6 of the *Tuta absoluta* sodium channel with the corresponding sequence of *Plutella xylostella* (AB265178.2), *Helicoverpa zea* (ADF80418.1) and *Bombyx mori* (NP_001136084). Transmembrane segments (S4, S5 and S6) are indicated by arrows. The positions of the L1014F, M918T and T929I mutations are highlighted.

T. absoluta, genomic DNA was extracted from 10 individual adults of each strain and used as template to amplify the IIS4–IIS6 region of the *para* gene using the specific primers designed from the cDNA sequence. No polymorphic bases were observed in the coding/non-coding sequence obtained between any individuals apart from at the two skdr positions. All ten individuals of all five strains were homozygous for the L1014F mutation. For the two skdr mutations (M918T and T929I), the frequency of the T929I mutation was present at higher frequency than M918T in TA1, TA3 and GA and for TA2 and TA4 the two mutations were present at the same frequency (Table 4). Although for most strains all potential genotypes at each mutation site were present (homozygous wild-type, homozygous mutant and heterozygous), the two mutations were only found in combination in individual larvae when both mutations were in the heterozygous form. To test if the two mutations were on separate alleles or found on the same allele, several individuals of this genotype were cloned and sequenced. Sequencing of ten colonies derived from each individual showed that the two mutations were only ever observed on separate alleles. Therefore two 'resistance alleles' are present in the *T. absoluta* strains the M918T allele which has the amino acid residues T918 + T929 + F1014 and the T929I allele which has the amino acid residues M918 + I929 + F1014.

3.3. TaqMan assays

DNA was extracted from 220 individuals, from 27 locations spanning a substantial part of the geographical range of *T. absoluta* (Table 1). Separate TaqMan assays were performed for each of the three mutations. Using samples of known genotypes as controls, the assays allowed homozygous resistant (R/R), heterozygous (R/S) and homozygous susceptible individuals (S/S) to be distinguished. The TaqMan assays use two probes, one specific for the resistant (mutant) allele labelled with FAM and the other specific for the susceptible (wild-type) allele labelled with VIC. A homozygous resistant individual will display a strong increase in FAM fluorescence, whilst a homozygous wild-type individual will show a strong increase in VIC fluorescence. Heterozygous individuals show an intermediate increase in both channels (Fig. 3). To facilitate genotyping, a scatter plot comparing VIC and FAM fluorescence values at the end of the 40 cycles was created using the Rotor-Gene analysis option. Of the 220 samples analysed three samples failed to amplify a product in PCR, and 17 required confirmation by DNA

Fig. 2. Sequence of domain IIS4-S6 of the *Tuta absoluta para*-type sodium channel gene. Positions of known kdr/super-kdr mutations are boxed. Lower cases indicate intron sequence.

sequencing as the TaqMan results for these samples were ambiguous.

Overall mutation frequencies were high (L1014F 0.98, M918T 0.35, T929I 0.60; Table 1). No individual was observed that did not carry kdr in combination with either M918T or T929I. For the L1014F (kdr) mutation, no individuals were homozygous wild-type, and only eight were heterozygous (from Colombia); samples from all other countries in this study were homozygous mutant. A map comparing mutation frequencies is shown in Fig. 4. Overall no strong geographic trend for the distribution of M198T and T929I was observed, however the frequency of T929I was generally highest and the frequency of M918T correspondingly low where tomato was grown under-protection (see samples 7 and 8, 12, 14 and 15, Table 1).

4. Discussion

Five laboratory strains of *T. absoluta* established from field collections from Europe and Brazil showed significant differences in their susceptibility to two pyrethroid insecticides in leaf-dip bioassays. Although we were unable to source a fully pyrethroid susceptible strain for use as a reference in these experiments (probably for the reasons discussed below) comparison of the LC_{50} values obtained with the recommended field rates of λ cyhalothrin and tau fluvalinate for *T. absoluta* control strongly suggest that all five strains would exhibit resistance to both compounds in the field. In the absence of a known susceptible population, comparisons with the most susceptible strain for each compound (TA1 for λ cyhalothrin and TA3 for tau fluvalinate) gave resistance factors of 4–17-fold for λ cyhalothrin and 2–11-fold for tau fluvalinate. Interestingly, for both pyrethroids the GA strain that was derived from several *T. absoluta* populations collected in Brazil exhibited a higher resistance factor than the four strains collected from Europe. As mentioned above, resistance to pyrethroid insecticides has previously been documented for *T. absoluta*, encompassing multiple reports of decreased susceptibilities to a range of pyrethroid chemistries including deltamethrin, λ cyhalothin, bifenthrin and permethrin (Branco et al., 2001; Guedes et al., 1994; Salazar and Araya, 1997; Silva et al., 2011; Siqueira et al., 2000).

To investigate whether pyrethroid resistance was mediated by mutation of the *para*-type sodium channel in *T. absoluta* we cloned and sequenced the IIS4–IIS6 region of the *para* gene which contains

Table 4
Genotypes of 10 individuals from each of the five laboratory strains of *Tuta absoluta* at mutation positions 918 and 929 and overall mutation frequency in each strain.

Sample N°	TA1		TA2		TA3		TA4		GA	
	M918T	T929I	M918T	T929I	M918T	T929I	M918T	T929I	M918T	T929I
1	S/S	R/R	S/S	R/R	S/S	R/R	R/S	R/S	S/S	R/R
2	S/S	R/R	S/R	S/R	S/S	R/R	R/S	R/S	S/S	R/R
3	R/S	R/S	R/R	S/S	S/S	R/R	R/S	S/S	R/S	R/S
4	R/S	R/S	R/S	R/S	S/S	R/R	S/S	R/R	S/S	R/R
5	R/S	R/S	R/R	S/S	S/S	R/R	R/S	R/S	S/S	R/R
6	R/S	R/S	S/S	R/R	R/S	R/S	R/S	R/S	R/S	R/S
7	S/S	R/R	S/S	R/R	R/S	R/S	R/S	R/S	S/S	R/R
8	S/S	R/R	R/S	R/S	R/S	R/S	R/S	R/S	R/S	R/S
9	R/S	R/S	R/S	R/S	S/S	R/R	R/S	R/S	S/S	R/R
10	R/R	S/S	R/R	S/S	R/S	R/S	R/S	S/S	R/S	R/S
Frequency	0.35	0.65	0.5	0.5	0.2	0.8	0.45	0.45	0.2	0.8

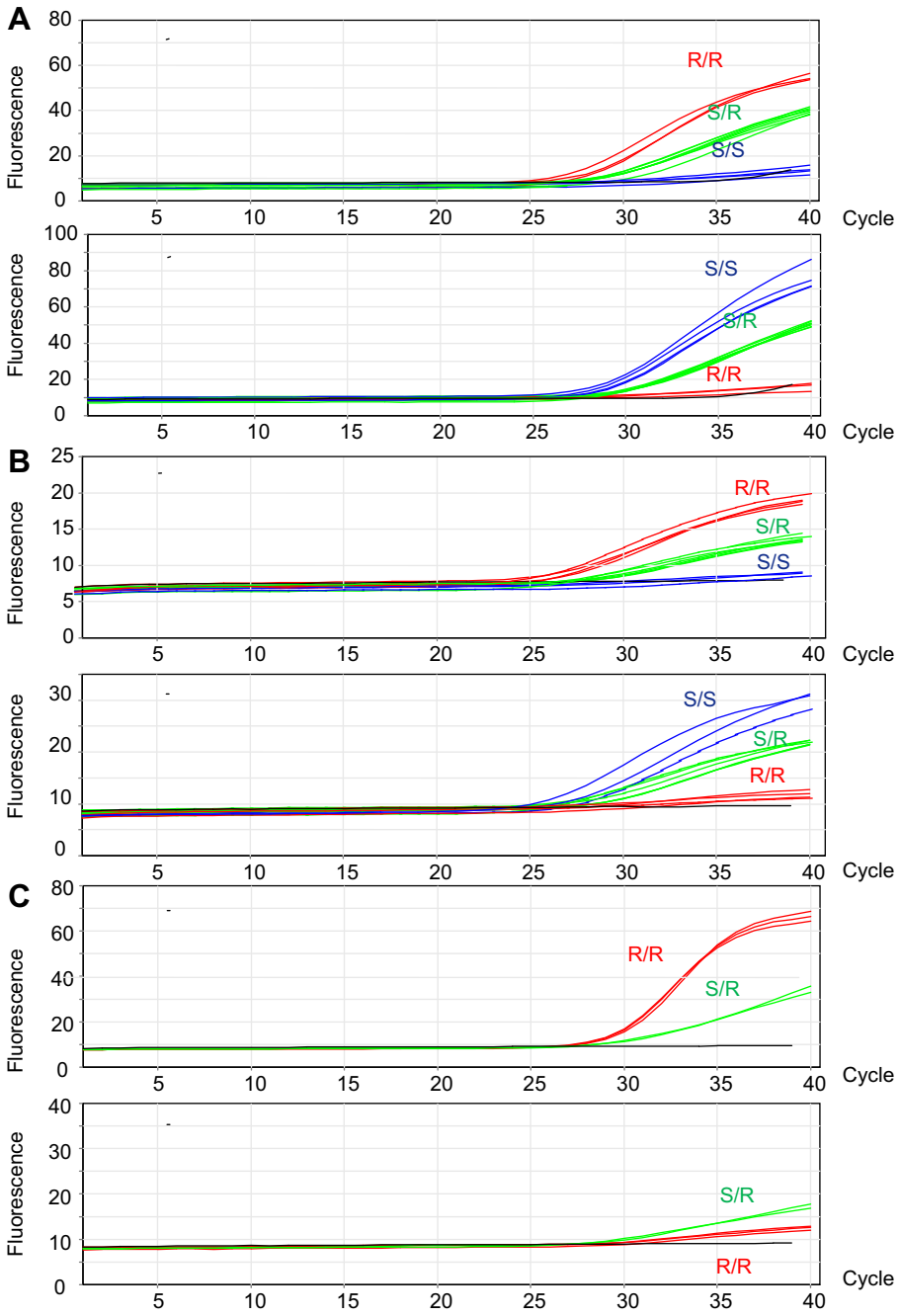

Fig. 3. Real-time TaqMan detection of the kdr and super kdr mutations in *Tuta absoluta*. (A) M918T, (B) T929I, (C) L1014F. For each pair of graphs, the top graph shows the FAM-labelled probe specific for the mutant allele, and the bottom graph shows the VIC-labelled probe specific for the wild-type allele. S: wild-type allele; R: resistant allele.

many of the mutation sites previously reported to be implicated in conferring kdr-type resistance to pyrethroids across a range of different insects. This revealed three kdr/skdr-type mutations within the resistant strains at known resistance 'hot-spots' within this gene region, M918T, T929I and L1014F.

All five strains were fixed for the common kdr mutation L1014F (present at 100% frequency). This mutation, initially identified in *M. domestica* has since been reported in resistant strains of over 20 different arthropod species (Davies and Williamson, 2009). Functional expression studies of cloned insect sodium channels harbouring the L1014F mutation using *Xenopus laevis* oocytes has confirmed the effect of this substitution in conferring up to a17-fold reduction in sensitivity to certain pyrethroids (Soderlund and Knipple, 2003; Tan et al., 2002; Vais et al., 2000). More recently molecular modelling of insect sodium channels (O'Reilly et al., 2006), based on the crystal structure of the rat brain Kv1.2 potassium channel (Long et al., 2005) has revealed that the L1014F mutation does not make physical contact with pyrethroid insecticides and may confer resistance via a conformational effect that makes the sodium channel less prone to open (Davies and Williamson, 2009). Sequencing ten individuals of the five *T. absoluta* strains revealed that the L1014F mutation was always associated with two additional mutations M918T or T929I.

M918T was the first 'skdr'-type mutation identified in insects (*M. domestica*) where it was associated with an enhanced knock down resistant phenotype (Williamson et al., 1996). It has

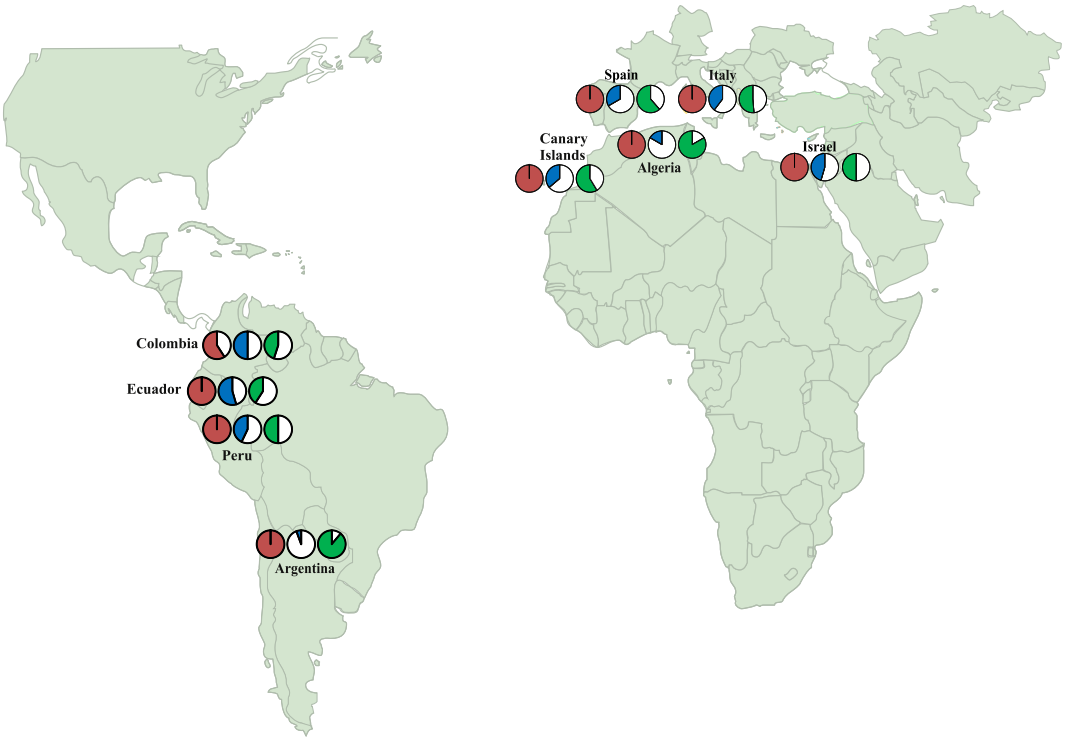

Fig. 4. Map of kdr/skdr mutation frequencies in *T. absoluta* populations from countries where the sample size was >6. The red pie charts show the frequency of L1014F, blue pie charts the frequency of M918T and green pie charts the frequency of T929I. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

subsequently been described for horn fly, *Haematobia irritans*, the peach potato aphid, *Myzus persicae* and the South American leaf miner, *Liriomyza huidobrensis* where it was always associated with L1014F (Davies et al., 2007; Eleftherianos et al., 2008; Guerrero et al., 1997). However, more recently M918T has been found in isolation in bifenthrin resistant tomato red spider mite, *Tetranychus evansi* (Nyoni et al., 2011). The M918T mutation has been shown to confer highest resistance to type II pyrethroids such as λ cyhalothrin and deltamethrin and in *M. domestica* gave 560-fold resistance to the latter compound (in association with L1014F) (Khambay et al., 1994). Functional expression of the *Drosophila melanogaster para* gene containing the M918T + L1014F mutations reduced the sensitivity of sodium channels by approximately 100 fold (Vais et al., 2000).

T929I was first reported in pyrethroid resistant diamondback moth, *P. xylostella* and has since been identified in human headlice, *Pediculosis capitis* and maize weevil, *Sitophilus zeamais* (Araujo et al., 2011; Lee et al., 2000; Schuler et al., 1998). The effect of T929I has also been examined in functional expression studies in oocytes, where in combination with L1014F it was found to make the sodium channel highly insensitive to a range of type I and type II pyrethroid insecticides and DDT (Usherwood et al., 2007; Vais et al., 2001). Modelling suggests that T929 is likely to be a key residue for binding of all pyrethroids as well as DDT as a result of hydrogen bond formation with this amino acid that stabilizes the bound insecticide (Davies and Williamson, 2009).

To date, the three mutations observed in this study have never been identified together within a single insect population and our findings suggests that *T. absoluta* has historically undergone strong selection with a range of pyrethroid insecticides. No correlation was observed between the frequency of the two skdr mutations in the five strains and their response to the two pyrethroids and it is likely that additional resistance mechanisms are present in at least some of these strains. For example the frequency of the two mutations was the same in TA3 and GA but the latter is 2 and 11-fold more resistant to λ cyhalothrin and tau fluvalinate respectively. It would be interesting, in future, to investigate if metabolic mechanisms play a role in the resistance of these strains as previous studies using a range of insecticide synergists on resistant populations in Brazil have implicated increased production of detoxification enzymes as a mechanism of resistance and suggested resistance is multigenic (Siqueira et al., 2001). However, although of academic interest, this may have limited implications for control with pyrethroids as work on other insect species has shown that the level of resistance typically conferred by the L1014F mutation in combination with either M918T or T929I compromises control in the field.

Three diagnostic assays have been developed in this study that can be used for accurate genotyping of large numbers of individual larvae or adults for the three mutations. These are based on TaqMan real-time PCR, a high-throughput 'closed-tube' approach that requires no post-PCR processing. These tools were used to examine the frequency and distribution of these resistance mutations in *T. absoluta* field strains collected from Europe and South America. This revealed that the mutations are at high frequency throughout the regions surveyed, indeed, no individual was found that did not carry at least two of the three mutations. The kdr mutation appeared to be fixed in all the populations analysed apart from collections from Colombia where individuals were identified with the mutation in the heterozygous form. The overall frequency of the two skdr-type mutations in the field samples tested was lower for M918T (0.35) than T929I (0.6) suggesting the latter may be being preferentially selected in *T. absoluta* populations. As described for the laboratory strains, no allele was observed that carries both skdr mutations. This is consistent with the situation in resistant populations of *Bemisia tabaci* from the Mediterranean basin where the L925I and T929V mutations were never found to occur in combination in the same haplotype (Alon et al., 2006; Roditakis et al., 2006). A common occurrence in both lab and field strains of

T. absoluta were individuals that had one M918T allele and one T929I allele (around 50% of all individuals). An insect of this genotype would have half of their *para*-type sodium channel component modified with the M918T mutation and half modified with the T929I mutation (and no 'susceptible' channels). As kdr and skdr mutations have been shown to be recessive in many resistant insect species heterozygous individuals are susceptible to pyrethroids, however, *T. absoluta* individuals with both the M918T and T929I alleles (in addition to L1014F) would be expected to be strongly resistant. Recently, a genetic study of *T. absoluta* using ribosomal and mitochondrial markers revealed high genetic homogeneity in *T. absoluta* populations from the Mediterranean Basin and South America with a single genetic type identified (Cifuentes et al., 2011). The authors proposed that a single genetically uniform and invasive population has been able to spread through South America, and then to the Mediterranean Basin. They also suggested that this may have occurred as a result of selective pressure resulting from human activities and a replacement of more susceptible populations by one of higher insecticide tolerance, capable of invading crops in new areas. The findings of the current study that the kdr mutation is almost fixed and that the two skdr mutations are both at relatively high frequency in *T. absoluta* populations across its range would support this hypothesis. Indeed, taken together these studies suggest that the rapid expansion of *T. absoluta* over the last six years may have been in part mediated by the resistance of this pest to chemical insecticides.

Clearly our findings have significant implications for the control of *T. absoluta* with pyrethroid insecticides. Both M918T and T929I in combination with L1014F are known to give strong resistance across the entire class of synthetic pyrethroids. The fact that one or other of these mutation combinations are found within all of the field samples that were tested suggests that pyrethroids are likely to be ineffective at controlling *T. absoluta* across its range and alternative control agents should be sought.

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