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A single nucleotide polymorphism in the acetylcholinesterase gene of the predatory mite *Kampimodromus aberrans* (Acari: Phytoseiidae) is associated with chlorpyrifos resistance

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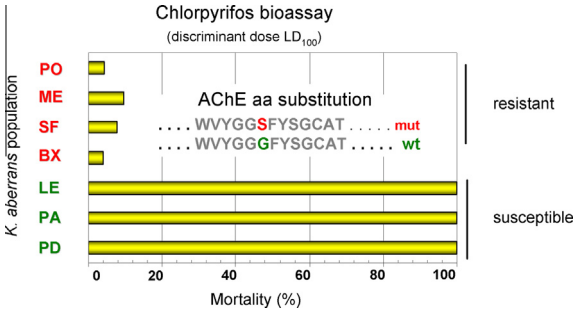
HIGHLIGHTS

- Pesticide treatments strongly affect the success of predatory mites releases.
- Insensitivity to pesticides is an advantageous trait for biocontrol agents.
- Some *Kampimodromus aberrans* strains were highly resistant to chlorpyrifos.
- AChE genotyping revealed a no silent mutation associated with chlorpyrifos insensitivity.
- The molecular marker could be used in field applications or in marker-assisted selection.

GRAPHICAL ABSTRACT



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ABSTRACT

The predatory mite *Kampimodromus aberrans* (Oudemans) (Acari: Phytoseiidae) is one of the most important biocontrol agents of herbivorous mites in European perennial crops. The use of pesticides, such as organophosphate insecticides (OPs), is a major threat to the success of biocontrol strategies based on predatory mites in these cropping systems. However, resistance to OPs in *K. aberrans* has recently been reported. The present study investigated the target site resistance mechanisms that are potentially involved in OP insensitivity. In the herbivorous mite *Tetranychus urticae* Koch (Acari: Tetranychidae), resistance to OPs is due to a modified and insensitive acetylcholinesterase (AChE; EC: 3.1.1.7) that bears amino acid substitution F331W (AChE Torpedo numbering). To determine whether the predators and prey have evolved analogous molecular mechanisms to withstand the same selective pressure, the AChE cDNA from a putative orthologous gene was cloned and sequenced from susceptible and resistant strains of *K. aberrans*. No synonymous mutation coding for a G119S substitution was determined to be strongly associated with the resistant phenotype instead of the alternative F331W. Because the same mutation in *T. urticae* AChE was not associated with comparable levels of chlorpyrifos resistance, the role of the G119S substitution in defining insensitive AChE in *K. aberrans* remains unclear. G119S AChE genotyping can be useful in ecological studies that trace the fate of resistant strains after field release or in marker-assisted selection of improved populations of *K. aberrans* to achieve multiple resistance

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phenotypes through gene pyramiding. The latent complexity of the target site resistance in *K. aberrans* vs. that of *T. urticae* is also discussed in the context of data from the genome project of the predatory mite *Metaseiulus occidentalis* (Nesbitt) (Acari: Phytoseiidae).

1. Introduction

Kampimodromus aberrans (Oudemans) (Acari: Phytoseiidae) is a generalist predator used for biocontrol of tetranychid and eriophyoid mites in European vineyards (Duso, 1989; Kreiter et al., 2002; Lorenzon et al., 2012; Duso et al., 2012). *K. aberrans* occurs in various European cropping systems, such as grapevines, apples and hazelnuts (Ivancich Gambaro, 1973; El Borolossy and Fischer-Colbrie, 1989) as well as in surrounding uncultivated plants which thus represent a potential reservoir of biocontrol agents (Tixier et al., 2002, 2006). In addition to several ecological factors, insecticide and fungicide applications strongly affect naturally occurring and artificially introduced *K. aberrans* populations (Ivancich Gambaro, 1973; Girolami, 1987; Pozzebon et al., 2002). Nevertheless a *K. aberrans* strain, detected in North Italian vineyards under conditions of integrated pest management strategies (IPM) (Posenato, 1994), was also successfully released in other vineyards and apple orchards following organic or IPM strategies (Duso et al., 2007, 2009; Ahmad et al., 2013). Recently, bioassay studies have confirmed significant levels of chlorpyrifos resistance in this strain (Tirello et al., 2012) but molecular markers associated with this insensitivity are still unknown.

The biochemical basis of OP resistance in phytoseiid mites depends on the active ingredients involved in the selective pressure and on species/strain-specific genetic backgrounds. The resistant phenotype might rely on high activities of detoxifying enzymes and/or on a modified and insensitive target site on AChE. Laboratory selection with methidathion leads to enhanced monooxygenases in *Amblyseius womersleyi* (Schicha) (Acari: Phytoseiidae) (Sato et al., 2006, 2007), and glutathione transferases in *Phytoseiulus persimilis* (Athias-Henriot) (Acari: Phytoseiidae) (Fournier et al., 1987). OP insensitive AChE has also been detected biochemically either in isolation or combination with modified carboxylesterases in *Typhlodromus pyri* (Scheuten) (Acari: Phytoseiidae) (Overmeer and van Zon, 1983) or in *Amblyseius potentillae* (Garman) (= *Amblyseius andersoni* (Chant)) (Acari: Phytoseiidae) (Anber and Overmeer, 1988; Anber and Oppenoorth, 1989) respectively. Although reductions in chlorpyrifos susceptibility have been reported in other predatory mites, e.g., *T. pyri* (Fitzgerald and Solomon, 1999; Cross and Berrie, 1994; Bonafos et al., 2008), little is known about the underlying molecular mechanisms. Among the Acari, high AChE insensitivity to chlorpyrifos was strongly associated to a F331W amino acid substitution in *Tetranychus urticae* Koch (Acari: Tetranychidae) (Khajehali et al., 2010).

Knowledge of a genetic marker associated with chlorpyrifos insensitivity in *K. aberrans* could be useful for understanding the amplitude of this phenomenon and managing predatory mite populations with IPM strategies. Therefore, we report here the cloning and sequencing of a *T. urticae*-like acetylcholinesterase cDNA in *K. aberrans* and its genotyping in chlorpyrifos-susceptible and resistant strains. The potential complexity of the target site resistance that occurs in predatory mites was also inferred by inspecting the annotated genome of *Metaseiulus occidentalis* (Nesbitt) (Acari: Phytoseiidae).

2. Material and methods

2.1. K. aberrans populations

This study was performed on seven *K. aberrans* strains collected in Northeastern Italy (Veneto Region). Four strains were collected from commercial vineyards, and three strains were collected from untreated European nettle trees (*Celtis australis* L.) (Table 1).

All strains were reared without insecticide exposure in separate rearing units at the Department of Agronomy, Food, Natural Resources, Animals and Environment of the University of Padova, Italy. Grapevine leaves on pads of wet cotton were used as a substrate for the predatory mites, and small pieces of PVC were placed for shelter and oviposition. *Typha latifolia* L. pollen was provided as food (Lorenzon et al., 2012).

Information about OP susceptibility was available for only two strains; specifically, the LE strain is highly susceptible to this insecticide and the PO strain is resistant to chlorpyrifos (resistance ratio at LD50, RR_{50} = 539,602) (Tirello et al., 2012).

2.2. Insecticide bioassays

Laboratory bioassays were conducted for preliminary screening of resistant and susceptible phenotypes with a commercial formulation (Dursban® 75WG, 75% a.i., Dow AgroSciences). The discriminant concentration for resistant and susceptible phenotypes was set at 70 g/hl of formulate, which is the recommended field dose for use in vineyards against grape berry moths (e.g., *Lobesia botrana* Denis & Schiffermüller) and leafhoppers (e.g., *Empoasca vitis* Goethe). The pesticide formulation was diluted in distilled water before toxicological procedures were performed that immersed rectangular leaf sections (approximately 6 cm^2) in the insecticide solution for 30 s (Tirello et al., 2013); distilled water was used in controls. When pesticide residues dried completely, leaf sections were placed on wet cotton pads, and cotton barriers were created along their perimeters to prevent mite escape. Two 12-d-old *K. aberrans* females were gently transferred to each leaf section, and fresh pollen was provided as food. The experimental units were maintained in a climate chamber at 25 ± 2 °C and 70 ± 10% relative humidity with a 16L:8D photoperiod. Female mortality was assessed 72 h after treatment. Females that drowned or escaped were excluded from analysis. In total, we assessed 40–45 females (20–24 replicates) per strain per treatment. Corrected mortalities (Abbott, 1925) were calculated.

2.3. Primer design for cloning AChE cDNA in K. aberrans

The annotated version of the genome assembly (release Mocc_1.0, March 2012) of the predatory mite *M. occidentalis*

Table 1
Strains of *Kampimodromus aberrans* and their origin.

Strain	Origin	Geographic coordinates
PO	Commercial vineyard at Monteforte d'Alpone (Verona province)	45°26′41.80″N; 11°18′46.77″E
SF	Commercial vineyard at S. Floriano (Verona province)	45°30′55.85″N; 10°54′28.41″E
BX	Commercial vineyard at Soave (Verona province)	45°24′18.14″N; 11°13′39.19″E
ME	Commercial vineyard at Valdobbiadene (Treviso province)	45°53′46.54″N; 11°57′43.30″E
LE	Untreated European nettle trees (*Celtis australi* L.) at Legnaro (Padova province)	45°20′49.77″N; 11°57′38.01″E
PD	Untreated European nettle trees (*Celtis australi* L.) at Padova	45°24′22.58″N; 11°53′39.59″E
PA	Untreated European nettle trees (*Celtis australi* L.) at Paese (Treviso province)	45°40′55.46″N; 12°09′55.28″E

(Nesbitt) (WOPM genome project) was used to search for putative AChE-like proteins with the tBlastn algorithm exploiting the AChE sequence from the susceptible strain of *T. urticae* (GenBank: ADK12697.1) as the query sequence. Transcripts predicted to code for putative AChE-like proteins were extracted from the scaffolds. After the alignments of *T. urticae* AChE and putative orthologous AChE-like proteins in *M. occidentalis*, degenerate primers were designed by manual inspection of the conserved domains (EditSeq and MegAlign 5.0; DNASTAR, Inc., Madison, WI, USA). The resulting primers were used to amplify the cDNA core fragments of the orthologous AChE in *K. aberrans*. To complete the cloning, walking steps and 3′-5′ RACEs, were performed using no degenerate primers outlined with PrimerSelect 5.0 (DNASTAR, Inc., Madison, WI, USA).

2.4. mRNA extraction and AChE cDNA cloning

Total RNA was extracted by homogenising 200 adults in 500 µl Tri-Reagent (Sigma, St. Louis, Mo.), according to the manufacturer's instructions. After quality and quantity assessments of the extracted RNA by formamide/formaldehyde denaturing gel electrophoresis (Sambrook et al., 1989) and spectrophotometric reading (NanoDrop, Fisher Thermo, Wilmington, DE, USA), first-strand cDNA was synthesised according to the protocol recommended by the supplier using Improm-II reverse transcriptase (Promega, Madison, WI) and random primers. Amplification of a cDNA fragment for a putative AChE in *K. aberrans* was achieved through two consecutive rounds of reverse-transcription PCR (RT-PCR) with degenerate primers (Table 2) designed on partially conserved functional domains. To this aim, the GoTaq Flexi PCR system (Promega, Madison, WI) was provided with 30 pmol forward and reverse degenerate oligonucleotides and 2.5 µl of cDNA as templates. The two PCR rounds shared the following profile: an initial denaturation step of 3 min at 94 °C; 5 cycles at 94 °C for 30 s, 45 °C for 30 s and 72 °C for 60 s; 5 cycles at 94 °C for 30 s, 45 °C plus +1 °C/cycle and 72 °C for 60 s; 25 cycles at 94 °C for 30 s, 50 °C for 30 s and 72 °C for 60 s; and a final extension step at 72 °C for 10 min. PCR products of the expected size (approximately 300 bp) were cloned using a pGEM-T easy vector (Promega, Madison, WI) sent for sequencing at BMR genomics (Padua, Italy). The sequences were assembled and analysed using SeqMan 5.0 (DNASTAR, Inc., Madison, WI, USA). Identification of the AChE-like sequences was performed via a BLASTX search in GenBank (http://www.ncbi.nkm.nih.gov) using the ORFs deduced from the cloned cDNA fragments. The cDNA clones were further extended in the 3′ direction by performing an RT-PCR that used a specific forward primer for the first cloned cDNA fragment in *K. aberrans* and a reverse primer that was designed based on the sequence coding for the conserved domain CAFWKNFL in AChE transcripts found in *M. occidentalis*, without any primer degeneration (Table 2). The RT-PCR mixture had the same composition described above except that the primer concentration was reduced to 15 pmol. The PCR was performed as follows: 1 cycle of 94 °C for 2 min; 5 cycles that included the three steps of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 60 s; 5 cycles of 94 °C for 30 s, 50 °C for 30 s (+1 °C/cycle) and 72 °C for 60 s; 20 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s; and a final extension step at 72 °C for 10 min. The PCR product was purified, sequenced and analysed as described above. Three primed and 5′ rapid amplification of cDNA ends reactions (RACEs) were performed to complete the *AChE* cDNA sequences exploiting specific forward and reverse primers respectively in combination with oligonucleotide adaptors according to the manufacturer's protocol (5′ RACE System for Rapid Amplification of cDNA Ends, Invitrogen) (Table 2). The RACE fragments were purified, sequenced and assembled as previously described.

2.5. Full length AChE cDNA sequencing

Total RNA was extracted from adults of both sensitive and resistant strains with TRI-Reagent described for the cDNA cloning. First-strand cDNA was synthesised from total RNA with Improm-II reverse transcriptase (Promega, Madison, WI) and

Table 2
Primer sequences.

	Forward primer	Sequence 5′ → 3′	Reverse primer	Sequence 5′ → 3′
Degenerate				
Primers	KaAChEF1d	GGNATHCCNTAYGCNAARCC	KaAChER1d	RAANSWNCCNCCRTANACCCA
	KaAChEF2d	CCNTAYGCZAARCCNCC	KaAChER2d	RAARCTNCCNCCRTANACCCA
3′ cDNA extension				
	KaAChEF3	TGGAATGCCAACACTAATA	KaAChER3	AGGAAGTTCTTCCAAAACGCGCA
3′ RACE				
			oligdTadapter	ACAGCAGGTCAGTCAAGCAGTAGCAGCAGTTCGATA AGCGGCCGCCATGGAT$_{12}$ DN
	KaAChEF4	AGGTGGTGAACGAAGCCATCAT	Adapter1	ACAGCAGGTCAAGTCAAG
	KaAChEF5	TTGAGTACACGGACTGGTTGAACCCT	Adapter2	AGCAGTAGCAGCAGTTCGATA
5′ RACE				
			KaAChER4	CTCCGTAAACCCATAGAAGGA
	TS-Primer	CACCATCGATGTCGACACGCGTCGGGIGGIG	KaAChER5	CCACATTGTTGAACCCTCAAAGTCG
	TS-PCR	CATCGATGTCGACACGCGTC	KaAChER6	TGTTGAACCCTCAAAGTCGTCG
cDNA sequencing				
	KaAChEF6	ACCTTGATAAACTGTCGCTGTGGC	KaAChER7	AAAGTCACGTTGTTCGGGTTGCCT
	KaAChEF7	CAATGCAGGCATGATGGACCAAGT	KaAChER8	AGGAAATTCTTCCAGAATGCGCA
	KaAChEF8	GTTGAACCCTGACGATCCGATCAA	KaAChER9	TCCTAGTTCGCTCCTTCAGTTGGA
	KaAChEF9	AAATGCGATTTCGACATCCTGTGCC	KaAChER10	CCAACAATCTTGTCGACGGCATCT
	KaAChEF10	GCGCTATCGGGCAACAACACAAACA		
G119S screening				
	KaAChEF11	AATGCGATTTCGACATCCTGTGCC	KaAChER11	AAAGTCCGTTGTTCGGGTTGCC
F331W screening				
	KaAChEF12	AAACTCGCGGAGGAAGTCAAGTGT	KaAChER12	CCAACAATCTTGTCGACGGCATCT

random primers as indicated by manufacturer's protocol. To sequence the ORF of the cloned cDNA, three RT-PCR fragments that partially overlapped were generated using primer pairs: KaAChEF6-R7, KaAChEF7-R8, and KaAChEF8-R9 as well as two additional internal primers (KaAChEF9, R10, and F10) for direct sequencing (Table 2). The PCR reaction (25 µl) included 2 µl of cDNA and 0.6 µM of each primer as final concentration in GoTaq Flexi reaction mix (Promega, Madison, WI). The thermal profile adopted was as follows: 94 °C for 2 min (1 cycle); 30 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 60 s; and a final extension step at 72 °C for 10 min. The PCR products were checked by electrophoresis and sent to sequencing service as described above. Chromatograms were assembled with SeqMan tools (DNAstar, Lasergene), and the alignments of the cDNA consensus sequences from sensitive and resistant strains were manually inspected for non-synonymous SNPs with the MegAlign program (DNAstar, Lasergene).

2.6. DNA extraction for exon–intron junction amplification and mutation screenings

DNA extraction was performed according to the methods described by Tixier et al. (2008); two hundred frozen adults of each strain were used as starting material after homogenisation in 150 µl of extraction buffer. The quality and quantity of the extracted DNA were assayed by spectrophotometric analyses with a Nanodrop ND-1000, and the integrities were verified through electrophoresis on 1% agarose/TBE 0.5× gel. Exon–intron boundary predictions were made by aligning the AChE cDNA sequence cloned in *K. aberrans* with the scaffold form the *M. occidentalis* genome project from which the transcript XR_145413 had been predicted (GenBank: AFFJ01003151). Relying on hypothetical gene structure conservation, the primers were designed on the exon sequences to generate partially overlapping PCR fragments that encompassed the putative introns in the *K. aberrans* AChE gene. Two primer pairs were also designed for G119S and F331W mutation screenings by direct sequencing of amplified genomic fragments (Table 2).

3. Results

3.1. Insecticide bioassays

At the discriminant dose, 100% corrected mortalities were observed for the LE, PA and PD strains, which originated from untreated nettles, and low mortality rates were observed for the PO, ME, SF and BX strains (4.08%, 9.57%, 7.69% and 4.26% corrected mortalities, respectively), which were collected from commercial vineyards.

3.2. Tetranychus urticae AChE-like gene in the M. occidentalis genome

The tBlastn search on the annotated genome of the predator mite *M. occidentalis* using the AChE cloned from *T. urticae* as the query sequence resulted in two predicted mRNAs that code for putative AChEs and had sequences that were significantly similar to that of the query (GenBank: XR_145413 and XR_145279; identity 54%, positive 69%, *e*-value 0.0). These mRNAs originated from genes in partially overlapping contigs (GenBank: AFFJ01003151 and AFFJ01002402). The corresponding open reading frames differed primarily in the amino terminal due to a diverse prediction of the first splicing site, whereas they shared the remaining five, which resulted in only 8 mismatches out of the 593 conserved amino acid residues. These mismatches arose from indels in the coding regions of the two genes, which did not differ in the intronic sequences with the exception of the first intron that originated from alternative splicing paths. Because the algorithms used for automatic splicing site predictions often fail to identify splicing sites at the 5′ end of putative transcripts and because of the low level of sequence divergence between the two genes, it was unclear whether there were two copies per genome or if they were derived from *in silico* mis-assembling of the high-throughput sequencing reads. In any case, when the *M. occidentalis* transcriptome shotgun assembly was interrogated with Blastn with the two putative transcripts, a pair of cDNA fragments were retrieved that covered both mRNAs (GenBank: JL046593.1 and JL050556.1; identities 99% and 98%), which confirmed that they were actually transcribed.

3.3. AChE cDNA in the susceptible strains of K. aberrans

cDNA of 2329 was isolated from the susceptible LE strain (GenBank: HF934042). The deduced precursor was composed of 655 amino acids (Fig. 1) with a signal peptide that was predicted to encompass the first 32 amino acids from the amino terminal (Shen and Cho, 2007). The cloned KaAChE displayed most of the amino acids responsible for the functional integrity of the enzyme that are typically well conserved both in insect and mite AChEs; i.e., the KaAChE residues involved in the intramolecular disulphide bonds (C139, C166, C325, C336, C471, and C593), the catalytic triad (S271, E395, and H509), the anionic subsite (W156), the oxianion-hole (G189, G190, and A274), and the acyl pocket (W304, F360, and F399) (Fig. 1). The highest identity (>93%) was observed for the AChE that was annotated in *M. occidentalis* from the transcript XR_145413 because the first splicing path was consistent with that predicted in this putative mRNA. No alternative cDNA sequence similar to the *M. occidentalis* transcript XR_145279 was detected in *K. aberrans*. As expected, the greatest divergences in the amino acid sequences between the KaAChE and XR_145413 predicted AChEs were restricted to the amino and carboxy terminals of the protein outside of the functional domains.

3.4. Organisation of the clone AChE locus in K. aberrans

The intron-spanning amplifications of the *K. aberrans* AChE locus confirmed the exon–intron junctions that were predicted *in silico* in the *M. occidentalis* genome scaffold AFFJ01003151, which lead to XR_145413 transcript annotation and coding for a putative *M. occidentalis* AChE (MoAChE). However, the first 106 nucleotides of the 5′ UTR region of the KaAChE cDNA did not match any portion of the scaffold sequence AFFJ01003151. In contrast, the unmatched 5′ UTR portion of the KaAChE cDNA exhibited an 81% identity with segments of two partially overlapping scaffolds in the *M. occidentalis* genome (GenBank: AFFJ01002403 and AFFJ01002403). The GT-AG consensus rule for donor and acceptor splice sites was also respected using the KaAChE cDNA sequence to guide the joining of the putative and still unannotated 5′ UTR portion of MoAChE on the scaffolds AFFJ01002403 and AFFJ01002403 to the 5′ end of the remaining open reading frame relying on the AFFJ01003151 scaffold. Because the AFFJ01002403 and AFFJ01002403 scaffolds do not overlap with AFFJ01003151, a long intronic sequence has to be envisaged in the MoAChE locus and is likely excluded from the assembly step. Assuming intron size conservation between the two orthologous AChE genes, this hypothesis was supported by the unsuccessful amplification of this intron in the *K. aberrans* AChE locus. Although we were able to characterise 5 introns experimentally and an additional putative splicing site bioinformatically, we suggest that the KaAChE gene includes seven exons (I–VII) that are separated by 6 introns (Table 3, GenBank: HG328327). Exon I is non-coding, whereas exon II contains the initiation codon (ATG),

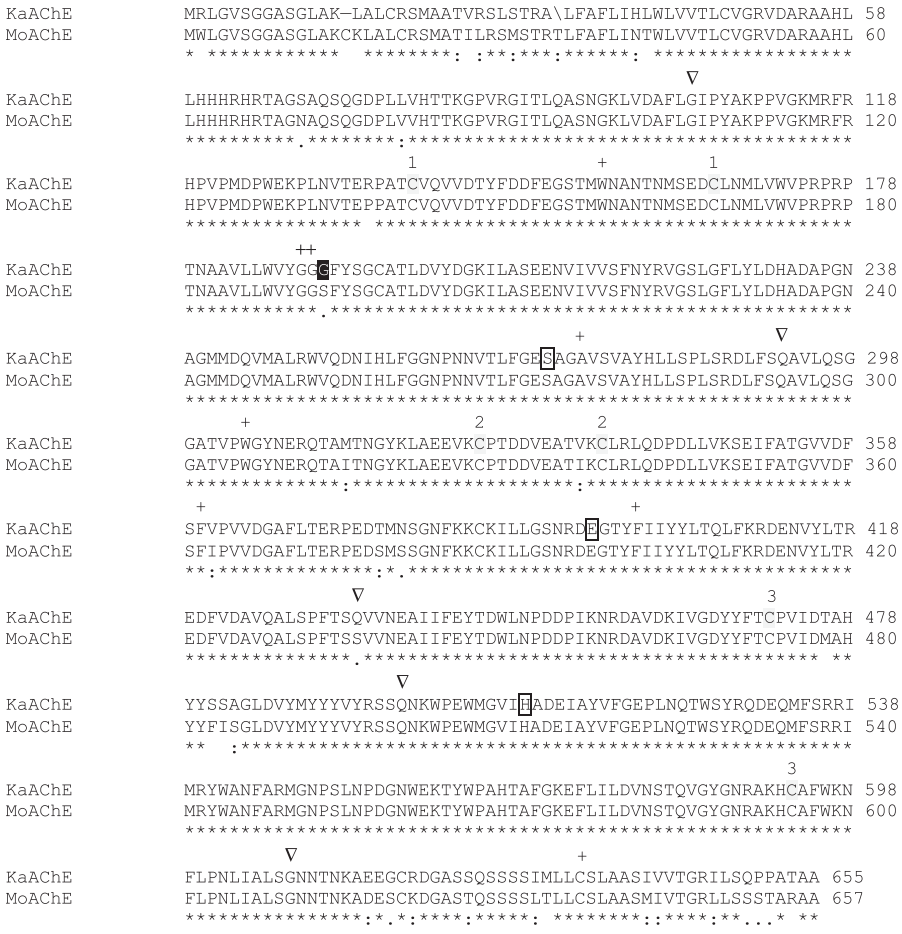

Fig. 1. Alignment of the AChEs cloned in from the chlorpyrifos susceptible strain of *Kampimodromus aberrans* (KaAChE) and predicted from *Metaseiulus occidentalis* transcript XR_145413 (MoAChE). Identical amino acids are indicated by asterisks, and conservative substitutions are indicated by dots. The cleavage site of signal peptide is indicated by a slash. The mutated residue (G191S) in the chlopyrifos-resistant strain is in reverse in the background, the cysteine residues that form the intramolecular disulphide bonds are numbered and on the light-gray background (C139-C166, C325-C336, C471-C593), the catalytic triad residues are boxed (S271, E395, H509), and the following conserved residues are indicated with plus signs: anionic subsite (W156), oxianion-hole (G189, G190, A274), acyl pocket (W304, F360, F399), and cysteine residue forming intermolecular disulphide bond (C634). The inverted triangles indicate the exon boundaries in the cDNA open reading frame.

Table 3
Genome organization of *Kampimodromus aberrans* AChE locus.

Exon	Position (nt)[a]	Exon size (bp)	Splice junction	Intron size (bp)	Phase	Aminoacid involved
Exon I (inferred)	(-106)-(-11)	106	TCGGAGGACTGG***gt***...	Unknown	–	5′ UTR
Exon II (ATG)	(-10)-310	320	***ag***ATGATTGGATAT... ACGCATTCCTCG**gt**...	113	I	G104
Exon III	311–876	566	**ag**GTATTCCGTATG... CTATTTTCTCAA**gt**...	1162	0	Q292
Exon IV	877–1302	426	**ag**GCCGTGCTGCAA... TTTACCTCGCAG**gt**...	194	0	Q434
Exon V	1303–1494	192	**ag**GTGGTGAACGAA... CGCTCGTCTCAG**gt**...	402	0	Q498
Exon VI	1495–1822	328	**ag**AATAAATGGCCG... TCGCGCTATCGG**gt**...	145	I	G608
Exon VII (TAA)	1823–2213	391	**ag**GCAACAACACAA...			3′UTR

[a] Numbering of nucleotides is based on the *K. aberrans* AChE cDNA where +1 corresponds to start codon.

which is similar to the observations of the majority of the AChE gene loci that have been annotated in insects and mites. Exons III–VI formed the catalytic domain and exhibited partial amino acid conservation across the AChEs that were cloned from the mites. Exon seven contains the stop codon (TAG) and the 3′ UTR region. All intron–exon boundaries followed the GT-AG rule (Breathnach et al., 1978); furthermore, these boundaries contained the YTNAN consensus sequence for lariat formation at the branch point close to the 5′ end of the acceptor-splicing site. In addition to the positions, the lengths of the amplifiable introns were also conserved in the orthologous AChE loci from the two phytoseiidae species with the exception of the third intron, which was slightly longer in the *K. aberrans* than in the *M. occidentalis* AChE gene (1162 bp vs. 936 base pairs, respectively).

3.5. Comparison the AChE cDNA sequences across different strains

Full-length sequencing of the KaAChE cDNA of the susceptible (LE) and resistant (PO) strains revealed a non-synonymous G to A mutation at position 687 that led to a G191S substitution in the protein sequence (G119S AChE *Torpedo* numbering; Fig. 1). The susceptible and resistant strains also differed in another single nucleotide polymorphism (SNP) at position 1499 of the cloned cDNA; this C to T transition did not affect the codon for the D461 residue. The resistant strain was homozygous at this site and carried only the T allele, while the sensitive strain exhibited both SNPs with a preference for C over T as indicated by the electropherograms. The phenylalanine residue (F339) that was replaced by a tryptophan in the chlorpyrifos-resistant stains of *T. urticae* (F439W mutation, or F331W AChE, Torpedo numbering) was conserved both in the susceptible and resistant strains of *K. aberrans*. The same was true for the glycine residue (G336) that was found to be replaced with alanine (G328A) in the F331W-bearing strains of *T. urticae*. The cDNA KaAChE sequences of two additional susceptible (PA and PD) and three resistant (ME, SF and BX) strains of *K. aberrans* were also examined. The resistant strains were all homozygous for the G191S substitution, while the susceptible strains carried only the G191 allele. The resistant BX strain sequence differed from the other strains in a SNP in the 3′ UTR that consisted of a G to A substitution.

4. Discussion

Resistance to pesticides can be a desirable feature in *K. aberrans* because this predatory mite is an effective biocontrol agent of spider mites on perennial crops. Indeed, resistant strains of this predatory mite have successfully been released in vineyards and apple orchards where pest control strategies included chlorpyrifos and many other pesticides (Duso et al., 2009, 2012; Ahmad et al., 2013). The resistance to chlorpyrifos of these strains has been definitively demonstrated (Tirello et al., 2012), but the underlying molecular mechanisms remain poorly understood. An initial clue came from the chlorpyrifos-resistant strain of *T. pyri* that exhibited a lower level of AChE activity than the susceptible strain, suggesting that the reduced substrate affinity observed in the biochemical assay might be associated with a modified AChE (Fitzgerald and Solomon, 1999). Target site resistance due to a modified AChE that confers high levels of insensitivity to OP, including chlorpyrifos, has been described in *T. urticae* and *T. kanzawai* Kishida (Acari: Tetranychidae) (Aiki et al., 2005; Van Leeuwen et al., 2010; Khajehali et al., 2010). A G119S substitution (AChE torpedo numbering) in the single copy AChE gene has been associated with moderate decreases in chlorpyrifos susceptibility between resistant and sensitive strains of *T. urticae* (resistance ratio at LD50, $RR_{50} = 31$), and a greater resistance ratio in cases of F331W replacement ($RR_{50} > 400$). The *K. aberrans* strain with the highest level of insensitivity to chlorpyrifos (PO strain) described by Tirello et al. (2012) has a $RR_{50} = 539{,}602$, and this ratio is even higher than that found in the *T. urticae* populations with the F331W AChE genotype.

To identify a suitable AChE candidate that is potentially responsible for target site resistance in *K. aberrans*, the AChE protein sequence from *T. urticae* was used to probe the annotated genome of *M. occidentalis* where more than a dozen AChE-like sequences were *in silico* predicted by the curators and supported by the predatory mite transcriptome (Hoy et al., 2013). Once a putative homologous AChE in *M. occidentalis* was found, its sequence was used to speed up the cloning of the corresponding AChE cDNA in *K. aberrans* (KaAChE). The amino acid identities of KaAChE with the other cloned and predicted AChEs in the Acari genomes that carry multiple AChE loci ranged from 61% (*Ixodes scapularis* Say (Acari: Ixodidae) putative AChE, GenBank: XP_002413212) to 33% (*Rhipicephalus microplus* Canestrini (Acari: Ixodidae) AChE3, GenBank: AAP92139). The amino acid identity was 52% with the AChEs coded by single copy genes in the *T. urticae* and *T. evansi* Baker and Prichard (Acari: Tetranychidae) populations that carry mutations associated with reduced chlorpyrifos sensitivity (GenBank: GQ461344, ADK12694, and AFS60097). This divergence was compatible with that observed in the AChEs from different species of Acari and even between AChEs from multiple loci in the *I. scapularis* or *R. microplus* genomes. AChEs of insects are divided in two groups, i.e., those orthologous and those paralogous to the *Drosophila melanogaster* AChE (Diptera: Drosophilidae) (Kim and Lee, 2012), and KaAChE exhibited a high level of similarity to the paralogous AChEs found in *Nephotettix cincticeps* (Hemiptera: Deltocephalidae) and *Blattella germanica* (Blattodea: Blattellidae) with an amino acid identity of approximately 57% (GenBank: ADZ15146; ABB89946). Moreover genomic amplification of five out of six intron–exon boundaries of KaAChE locus showed that the *in silico* predicted organization of the homologous locus in *M. occidentalis* was correct. Intron sizes and positions were highly conserved in putative homologus AChE loci between the two predatory mite species with the exception of the third intron which was longer in *K. aberrans* than in *M. occidentalis* AChE gene. Sequence inspection of this intron in the *K. aberrans* AChE locus revealed the presence of short microsatellite repeats and a long inverted repeat (LIR) (Wang and Leung, 2006). These nucleotide motifs can cause sliding of the intron sequences during DNA replication and might account for the different size of the third intron in the KaAChE gene. Altogether these findings suggest that even if multiple AChE genes have been assembled and annotated in *M. occidentalis* genome the *in silico* prediction are fairly reliable.

Full sequencing of the cloned AChE cDNA revealed that the resistant strain (PO) differed from the susceptible strain (LE) in terms of a non-synonymous G to A mutation that introduced a G191S substitution in the AChE open reading frame. That mutation corresponds to the G119S substitution in the catalytic site of AChE Torpedo. This key residue is part of the oxianion hole, which is one of the functional domains required for acetylcholinesterase activity (Zhang et al., 2002). Strangely, the corresponding amino acid position in the homologous AChE that was found in the annotated genome of *M. occidentalis* is occupied by a serine. Unfortunately no information is available concerning the chlorpyrifos susceptibilities of the *M. occidentalis* strains employed for the genome project. Notably, the G119 in the *K. aberrans* AChE is encoded by a GGC codon, which could easily be converted to the AGC codon for serine. This substitution in the mosquito paralogous AChE seems not to be neutral under the selective pressure produced by organophosphate and carbamate treatments (Weill et al., 2004a). Indeed, when additional two chlorpyrifos-susceptible and three chlorpyrifos-resistant unrelated strains of *K. aberrans* were identified using a discriminant dose of chlorpyrifos (Tirello et al., 2012), KaAChE genotyping revealed the presence of the G191S substitution only in the resistant strains in the homozygous condition. The silent nucleotide polymorphisms found in the KaAChE cDNA of the resistant strains might also suggest that different G119S mutation events occurred independently. Although, in *Culex quinquefasciatus* Say (Diptera: Culicidae), a chlorpyrifos inhibition study of a paralogous AChE bearing the analogous amino acid substitution had a reduced sensitivity to the insecticide (Liu et al., 2005; Weill et al., 2004b), *T. urticae* strains with the same mutated AChE genotype display only a moderate resistance to chlorpyrifos *in vivo*. In contrast, all examined resistant strains of *K. aberrans* are highly resistant to the insecticide (Tirello et al., 2012). Thus, the role of the G191S remains unclear, although the possibility that the same mutation has a different impact on chlorpyrifos AChE sensitivity in the predatory mite cannot be excluded.

Alternative target site resistance mechanisms not explored in this study might also rely on the amplification of modified AChEs as occurs in *T. urticae* and *T. evansi* (Kwon et al., 2010; Carvalho et al., 2012) or on mutations that affect multiple AChE loci with additive effects such as those observed in *R. microplus* (Temeyer et al., 2009, 2010, 2012). While the detection of homozygosity for the G119S substitution argues against the co-existence of duplicated sensitive and insensitive AChEs in chlorpyrifos-resistant strains, the role of multiple AChE loci in *K. aberrans* cannot be ruled out since the genome of the closely related phytoseiid *M. occidentalis* harbors at least six putative AChEs with lower amino acid identities (<34%) to those found in *T. urticae*.

In conclusion, potential target site resistance to chlorpyrifos in *K. aberrans* has barely been dissected compared to that in *T. urticae*. The F331W substitution that is responsible for AChE insensitivity to chlorpyrifos in the Tetranychidae was absent in a putative homologous gene that was cloned from the resistant strain *K. aberrans*. However, a G191S mutation that was detected in the same gene appeared to be associated with the resistant phenotype. Because pesticide treatments strongly affect the success of predatory mites released in agroecosystems (Ahmad et al., 2013; Pozzebon et al., 2014), this polymorphism might be useful as a molecular marker for tracing the resistant phenotype in ecological studies or in gene pyramiding and marker-assisted selection of desirable traits for multiple insecticide resistance.

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