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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ABSTRACT

The predatory mite *Kampimodromus aberrans* (Oudemans) (Acari Phytoseiidae) is one of the most important biocontrol agents for herbivorous mites in European perennial crops. The use of pesticides, such as organophosphate insecticides (OP), is a major threat to the success of biocontrol strategies based on predatory mites in these cropping systems. However, resistance to OP 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,* resistance to OP is due to a modified and insensitive acetylcholinesterase (AChE) that bears amino acid substitution F331W (AChE Torpedo numbering). To determine whether the predators and prey had developed 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 phenotypes through gene pyramiding. The latent complexity of the target site resistance in *K. aberrans* vs. that of *T. urticae* is also discussed by exploiting data from the genome project of the predatory mite *Metaseiulus occidentalis* (Nesbitt).

1. Introduction

Kampimodromus aberrans (Oudemans) (Acari: Phytoseiidae) is a predatory mite that occurs in various European cropping systems, such as grapevines, apples and hazelnuts (Ivancich Gambaro, 1973; El Borolossy and Fischer-Colbrie, 1989; Tsolakis et al., 2000; Ozman-Sullivan, 2006). This predatory mite is also common on the uncultivated plants that surround crops and represent a potential reservoir for biocontrol agents (Tixier et al., 2002, 2006). *K. aberrans* is considered to be generalist predator (McMurtry and Croft, 1997; Kreiter et al., 2002; Broufas et al., 2007; Lorenzon et al., 2012) and an effective biocontrol agent of tetranychid and eriophyoid mites in European vineyards (Duso 1989; Girolami et al., 1992; Duso and Pasqualetto, 1993; Duso et al., 2012). 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; Peverieri et al., 2009). However, a *K. aberrans* strain has been detected in North Italian vineyards under conditions of integrated pest management strategies (IPM) that rely on common ethylene-bis-dithiocarbamate (EBCD) fungicides and organophosphate (OP) insecticides (Posenato 1994). This strain was also successfully released in other vineyards and apple orchards following organic or IPM strategies (Duso et al., 2007; Duso et al., 2009; Ahmad et al., 2013). Recently, laboratory studies have confirmed significant levels of chlorpyrifos resistance in this same strain (Tirello et al., 2012). 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 resistance phenotype might rely on high detoxifying enzyme activities and/or on a modified and insensitive target AChE. For example, laboratory selection with methidathion in *Amblyseius womersleyi* (Schicha) leads to increases in monooxygenase activity and CYP4-d isoform overexpression (Sato et al., 2001, 2006, 2007), while in *Phytoseiulus persimilis* (Athias-Henriot), this selection results in an enhancement of glutathione transferase (Fournier et al., 1987). High rate *in-vitro* degradation of azinphos-methyl has been observed under both polygenic and monogenic control in OP-resistant strains of *Amblyseius fallacis* (Garman) (Motoyama et al., 1971; Croft et al., 1976). Target

site resistance to OP has also been detected biochemically either in isolation or combination with enhanced OP detoxifying pathways. Resistance to certain OP and carbamate compounds, such as parathion and propoxur, in a Dutch strain of *Typhlodromus pyri* Scheuten has been found to be under monogenic control and to be associated with an insensitive target AChE (Overmeer and van Zon, 1983). In a paraoxon-resistant strain of *Amblyseius andersoni*, (Chant) the resistant phenotype has been revealed to be due to an insensitive AChE coupled with modified carboxylesterases (Anber et al., 1988, 1989).

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 levels of chlorpyrifos resistance in *Tetranychus urticae* Koch have been found to be due to a F331W amino acid substitution in the target enzyme acetylcholinesterase (AChE) (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 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 *M. occidentalis*.

2. Materials and Methods

2.1. Kampimodromus aberrans populations

This study was performed on seven *K. aberrans* strains collected in North-eastern 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 the 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 the effects of OP was available for only two strains; specifically, the PO strain is resistant to chlorpyrifos, and the LE strain is highly susceptible to this insecticide (Tirello et al., 2012).

2.2. Insecticide bioassays

Laboratory bioassays were conducted for the preliminary screening for resistant and susceptible phenotypes. The bioassays were performed with a commercial formulate (Dursban® 75WG, 75% a.i., Dow AgroSciences). The discriminant concentration for the 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 and leafhoppers. The pesticide formulate was diluted in distilled water before the toxicological test procedures (Tirello et al., 2013). The latter procedures were performed using rectangular leaf sections (approximately 6 cm^2). The sections were immersed in the insecticide solution for 30 s, and distilled water was used in the control treatments. When the pesticide residues completely dried out, the leaf sections were placed on wet cotton pads, and cotton barriers were created along their perimeters to prevent predatory 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 the treatments. The females that drowned or escaped were removed from the initial test number. In total, we assessed 40-45 females per strain. The 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 *Metaseiulus occidentalis* (Nesbitt) (WOPM genome project) was used to search for putative AChE-like proteins with the tBlastn algorithm using the AChE sequence that was amplified from the susceptible strain of *T. urticae* (GenBank accession n. ADK12697.1) as the query sequence.

Transcripts predicted to code for putative AChE-like proteins were extracted from the scaffolds, and their open reading frames (ORFs) were compared to the *T. urticae* AChE

protein using Lasergene sequence analysis tools EditSeq and MegAlign 5.0 (DNASTAR, Inc., Madison, WI, USA).

Degenerate primers were designed by manual inspection of the conserved domains after the alignments of *T. urticae* AChE and putative orthologous AChE-like proteins in *M. occidentalis*. 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 and 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), according to the manufacturer's instructions. The sample integrities were examined by electrophoresis in 1.2% agarose and 2.2 M formamide/formaldehyde denaturing gel. Quality and quantity assessments of the extracted RNA were performed in a Nanodrop ND-1000 Spectrophotometer (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) 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. The PCR mixtures (25 µl) contained GoTaq Flexi 1x buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 30 pmol forward and reverse degenerate primers, 1 U GoTaq and 2.5 µl of cDNA. The degenerate primers were based on partially conserved functional domains of homologous AChEs proteins in *T. urticae* and *M. occidentalis*. For the first RT-PCR round, the forward and reverse primers were KaAChEF1d (GIPYAKP domain) and KaAChER1d or KaAChER2d (WVYGGSF motif) (Table 2). The PCR product was then diluted 10-fold and used as the template for a second RT-PCR in which the KaAChEF1 primer was replaced with the more internal primer KaAChEF2d, which was designed based on the PYAKPP domain (Table 2). 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 purified from 1% (w/v) agarose/TBE 1x gel using a EuroGOLD Gel Extraction Kit (Euroclone) and cloned using a pGEM-T easy vector

(Promega). The plasmids were purified with a EuroGOLD Plasmid Miniprep Kit (Euroclone) and 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 forward primer that was designed based on the first cloned cDNA fragment in *K. aberrans* (KaAChEF3) and a reverse primer (KaAChER3) that was designed based on the sequence coding for the conserved domain CAFWKNFL in both of the 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 prime and 5' rapid amplification of cDNA ends reactions (RACEs) were performed to complete the *AChE* cDNA sequences. In the RACE reactions, the first strand cDNAs were synthesised using total RNA and polyT-adaptor primer for 3' RACE or KaAChE-R4 for 5' RACE (Table 2) according to the manufacturer's protocol (5´ RACE System for Rapid Amplification of cDNA Ends, Invitrogen). The 3' RACE product spanning across the unknown 3'-end of the *AChE* cDNA was amplified in two consecutive PCR rounds with the KaAChEF4-Adaptor1 and KaAChEF5-Adaptor2 primer pairs. To obtain the 5' end of the AChE transcript, the cDNA was subjected to polyC-tailing of its 3'-end with terminal deoxynucleotidyl-transferase (TdT) following the protocol of the kit (5´ RACE System for Rapid Amplification of cDNA Ends, Invitrogen). The upstream cDNA sequence encompassing the 5' untranslated region was amplified with two PCR rounds using the coupled primers KaAChER5-TS-primer and KaAChER6-TS-PCR (Table 2). The first 5' RACE round was performed as follows: 94 °C for 2 min (1 cycle); 5 cycles at 94 °C, 56 °C for 30 s and 72 °C for 60 s; 5 cycles at 94 °C, 57 °C for 30 s and 72 °C for 60 s; and 20 cycles at 94 °C for 30 s, 58 °C for 20 s and 72 °C for 60 s. The second 5' RACE round consisted of the following: 1 cycle at 94 °C for 2 min; and 30 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s. The 5' RACE fragment was purified from the agarose gel and sequenced as previously described.

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205 *2.5. Full length AChE cDNA sequencing*

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207 Total RNA was extracted from adults of both sensitive (LE) and resistant (PO) strains with
208 TRI-Reagent described for the cDNA cloning. First-strand cDNA was synthesised from
209 total RNA with Improm-II reverse transcriptase (Promega) and random primers as
210 indicated by manufacturer's protocol. To sequence the ORF of the cloned cDNA, three RT-
211 PCR fragments that partially overlapped were generated using the following primer
212 couples: KaAChEF6-R7, KaAChEF7-R8, and KaAChEF8-R9 (Table 2). The PCR reaction
213 (25 µl) included 2 µl of cDNA, a final concentration of GoTaq Flexi 1x buffer, 1.5 mM $MgCl_2$,
214 0.2 mM dNTPs, 0.6 µM of each primer and 0.625 U/µl GoTaq (Promega). The thermal
215 profile adopted was as follows: 94°C for 2 min (1 cycle); 30 cycles of 94 °C for 30 s, 56 °C
216 for 30 s and 72 °C 60 s; and a final extension step at 72 °C for 10 min. The PCR products
217 were checked by electrophoresis on 1% agarose in TBE 0.5x buffer, purified with the
218 EuroGOLD Cycle-Pure Kit (Euroclone) and sent to BMR genomics (Padua, Italy) for
219 sequencing. To this aim, the same primers used for the RT-PCR amplifications and new
220 internal primers (KaAChEF9, R10, and F10) were used (Table 2). Chromatograms were
221 assembled with SeqMan tools (DNAstar, Lasergene), and the alignments of the cDNA
222 consensus sequences from sensitive and resistant strains were manually inspected for
223 non-synonymous SNPs with the MegAlign program (DNAstar, Lasergene).

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225 *2.6. DNA extraction and exon-intron junction amplification*

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227 DNA extraction was performed according to the methods described by Tixier et al. (2008)
228 while scaling up the reagents. Two hundred frozen adults of each strain were
229 homogenised in 150 µl of extraction buffer (2% CTAB, 1.4 M NaCl, 0.2% 2-mercaptoethaol,
230 100 mM EDTA, and 100 mM Tris-HCl at pH 8.0) using a micro tissue grinder (Wheaton,
231 Millville, NJ). The homogenate was transferred to a 0.5-ml test tube and incubated for one
232 hour at 65°C with periodic hand mixing. One hundred and fifty microliters of a
233 chloroform:isoamyl alcohol mixture (24:1) was added, the solution was mixed by inversion,
234 and tubes were centrifuged at 6°C for 5 min at 1000 g. The aqueous solution was collected
235 in a new test tube, and 80 µl of isopropanol was added to the decanted aqueous phase,
236 which was then chilled at –20 °C for 20 min for DNA precipitation. After centrifugation (15
237 min, 6 °C, 1000 g), the pellet was suspended in 100 µl of 96% alcohol at 4 °C. After a final

centrifugation of 10 min (6 °C, 1000 g), the dried pellet was suspended in 30 µl of de-ionised water. 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 0.5x 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 accession n. AFFJ01003151.1). 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. The PCR products were purified and sequenced as described for the cDNA sequencing using the same primers that were employed for the DNA amplifications.

3. Results

3.1. Insecticide bioassays

Laboratory trials confirmed the findings reported by Tirello et al. (2012). At the discriminant dose, 100% corrected mortalities were observed for the LE, PA and PD strains, which originated from untreated nettles, and high survival 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. T. 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 codes for putative AChEs and had sequences that were significant similar to that of the query (XR_145413 and XR_145279; identity 54%, positive 69%, e-value 0.0). These mRNAs originated from genes in partially overlapping contigs (AFFJ01003151 and AFFJ01002402). The corresponding open reading frames differed primarily in the amino terminal due to a diverse prediction of the first splicing site, while 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,

272 which did not differ in the intronic sequences with the exception of the first intron that
273 originated from alternative splicing paths. Because the algorithms used for automatic
274 splicing site predictions often fail to identify splicing sites at the 5' end of putative
275 transcripts and because of the low level of sequence divergence between the two genes, it
276 was unclear whether there were two copies per genome or if they were derived from *in*
277 *silico* mis-assembling of the high-throughput sequencing reads. In any case, when the *M.*
278 *occidentalis* transcriptome shotgun assembly was interrogated with Blastn with the two
279 putative transcripts, a pair of cDNA fragments were retrieved that covered both mRNAs
280 (JL046593.1 and JL050556.1; identities 99% and 98%), which confirmed that they were
281 actually transcribed. Altogether, these findings suggested that the two very similar
282 predicted mRNAs could be informative for cloning *T. urticae*-like AChE cDNA in *K.*
283 *aberrans*.
284
285 *3.3. AChE cDNA in the susceptible strains of K. aberrans*
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287 cDNA of 2329 was isolated from the susceptible LE strain (Genbank accession number:
288 HF934042). The deduced precursor was composed of 655 amino acids (Fig. 1) with a
289 signal peptide that was predicted to encompass the first 32 amino acids from the amino
290 terminal (Shen et al. 2007). The cloned KaAChE displayed most of the amino acids
291 responsible for the functional integrity of the enzyme that are typically well conserved both
292 in insect and mite AChEs; i.e., the KaAChE residues involved in the intramolecular
293 disulphide bonds (C139, C166, C325, C336, C471, and C593), the catalytic triad (S271,
294 E395, and H509), the anionic subsite (W156), the oxianion-hole (G189, G190, and A274),
295 and the acyl pocket (W304, F360, and F399) (Fig. 1). The highest identity (> 93%) was
296 observed for the AChE that was annotated in *M. occidentalis* from the transcript
297 XR_145413 because the first splicing path was consistent with that predicted in this
298 putative mRNA. No alternative cDNA sequence similar to the *M. occidentalis* transcript
299 XR_145279 was detected in *K. aberrans*. As expected, the greatest divergences in the
300 amino acid sequences between the KaAChE and XR_145413 predicted AChEs were
301 restricted to the amino and carboxy terminals of the protein outside of the functional
302 domains. The amino acid identities with the other cloned and predicted AChEs in the Acari
303 genomes that carry multiple AChE loci ranged from 61% (*Ixodes scapularis* putative AChE,
304 XP_002413212) to 33% (*Rhipicephalus microplus*, AChE3, AAP92139). The amino acid
305 identity was 52% between the AChEs coded by single copy genes in the *T. urticae* and *T.*

306 *evansi* that carry mutations associated with reduced chlorpyrifos sensitivity (GQ461344,
307 ADK12694, and AFS60097) This divergence was compatible with that observed in the
308 AChEs from different species of Acari and even between AChEs from multiple loci in the *I.*
309 *scapularis* or *R. microplus* genomes. AChEs of insects are divided in two groups, i.e.,
310 those orthologous and those paralogous to the *D. melanogaster* AChE (Kim et al., 2012),
311 and KaAChE exhibited a high level of similarity to the paralogous AChEs found in
312 *Nephotettix cincticeps* (Hemiptera: Deltocephalidae) and *Blattella germanica* (Blattodea:
313 Blattellidae) with an amino acid identity of approximately 57% (ADZ15146; ABB89946).
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315 *3.4. Organisation of the clone AChE locus in K. aberrans*
316
317 The intron-spanning amplifications of the *K. aberrans* AChE locus confirmed the exon-
318 intron junctions that were predicted *in silico* in the *M. occidentalis* genome scaffold
319 AFFJ01003151, which lead to XR_145413 transcript annotation and coding for a putative
320 M. occidentalis AChE (MoAChE). However, the first 106 nucleotides of the 5' UTR region
321 of the KaAChE cDNA did not match with any portion of the scaffold sequence
322 AFFJ01003151. In contrast, the unmatched 5'UTR portion of the KaAChE cDNA exhibited
323 an 81% identity with segments of two partially overlapping scaffolds in the *M. occidentalis*
324 genome (Genbank accession n. AFFJ01002403 and AFFJ01002403). The GT-AG
325 consensus rule for donor and acceptor splice sites was also respected using the KaAChE
326 cDNA sequence to guide the joining of the putative and still unannotated 5'UTR portion of
327 MoAChE on the scaffolds AFFJ01002403 and AFFJ01002403 to the 5' end of the
328 remaining open reading frame relying on the AFFJ01003151 scaffold. Because the
329 AFFJ01002403 and AFFJ01002403 scaffolds do not overlap with AFFJ01003151, a long
330 intronic sequence has to be envisaged in the MoAChE locus and is likely excluded from
331 the assembly step. Assuming intron size conservation between the two phytoseiids, this
332 hypothesis was supported by the unsuccessful amplification of this intron in the *K.*
333 *aberrans* AChE locus. Although we were able to characterise 5 introns experimentally and
334 an additional putative splicing site bioinformatically, we suggest that the KaAChE gene
335 includes seven exons (I-VII) that are separated by 6 introns (Table 3, (Genbank accession
336 n. HG328327). Exon I is non-coding, whereas exon II contains the initiation codon (ATG),
337 which is similar to the observations of the majority of the AChE gene loci that have been
338 annotated in insects and mites. Exons III-VI formed the catalytic domain and exhibited
339 partially amino acid conservation across the AChEs that were cloned from the mites. Exon

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 homologous 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). Sequence inspection of the third intron in the *K. aberrans AChE* locus revealed the presence of short microsatellite repeats and a long inverted repeat (LIR) (Wang et al., 2006). These nucleotide motifs can cause sliding of the intron sequences during DNA replication and might account for the different sizes of the third intron in the KaAChE gene.

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). This residue is involved in the oxianion hole, which is one of the functional domains of AChE activity (Zhang et al. 2002). 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 still 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 for spider mites in perennial crops. Indeed, strains of this predatory mite that are apparently resistant to OP have successfully been released in vineyards and apple orchards in which the pest control strategies included chlorpyrifos and many other pesticides (Duso et al. 2009; Duso et al., 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 about this issue came from the chlorpyrifos-resistant strain of *T. pyri* that exhibited a lower level of AChE activity that the susceptible strain, which suggests that the reduced substrate affinity observed in the biochemical assay might be associated with a modified AChE (Fitzgerald and Solomon, 1999). In Acari, target site resistance due to a modified AChE that confers high levels of insensitivity to OPs, including chlorpyrifos, has been described in *T. urticae* and *Tetranychus kanzawai* Kishida (Aiki et al., 2004; 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 the moderate decreases in chlorpyrifos susceptibility between the resistant compared to the sensitive strains of *T. urticae* (resistance ratio at LD50, RR_{50} = 31), and a greater resistance ratio has been detected in cases of F331W replacement (RR_{50} > 400). *In vitro* expression of the AChE isoforms of *T. urticae* that carry F331W and/or G119S substitutions has revealed a reduction in sensitivity to another organophosphate (monocrotophos) and a decrease in the catalytic efficiency of the enzyme (Kwon et al., 2012); however, no data had been reported for chlorpyrifos. Although these features appeared much more evident in the F331W-mutated AChE, the two substitutions acted synergistically when they were associated *in vitro* and thus were favourably co-selected in the resistant strains in vivo (Kwon et al., 2010b; Ilias et

al., 2014). In mosquitoes, two acetylcholinesterase genes are present, both substitutions affect the paralogous AChE in the highly OP- and carbamate-resistant strains of *Culex pipiens* L. and *Anopheles gambiae* Giles (G119S; Weill et al., 2004a) and in *Culex tritaeniorhynchus* Giles (F331W; Alout et al., 2007). The role of these mutations in reducing the AChE sensitivity to OP was confirmed via inhibition analysis of the expression of AChE from mutated mosquitos S2 cells (Weill et al., 2003; Oh et al., 2006). The F331W substitution has also been detected in AChE1 of a chlorpyrifos-resistant strain of the sweet potato whitefly *Bemisia tabaci* Gennadius (Alon et al., 2008). 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* and *B. tabaci* populations with the F331W AChE genotype. There a target site resistance might be present in that strain. Because no AChE sequences for *K. aberrans* are stored in databases, the annotated genome project of the predatory mite *M. occidentalis* was inspected. More than a dozen AChEs-like sequences were predicted by the curators of the *M. occidentalis* genome project using an automated computational analysis, although some of the transcripts represented uncompleted open reading frames or differed only in their splicing paths. 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 probing the annotated genome of *M. occidentalis*. 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*. Full sequencing of the cloned AChE cDNA revealed that the resistant strain (PO) differed from the susceptible strain (LE) in terms of non-synonymous G to A mutation that introduced a G191S substitution in the AChE open reading frame. That mutation corresponds to the aforementioned G119S substitution in AChE Torpedo numbering. Strangely, the corresponding amino acid position in the homologous AChE that was found in the annotated genome of *M. occidentalis* is occupied

427 by a serine. Unfortunately no information is available concerning the chlorpyrifos
428 susceptibilities of the *M. occidentalis* strains employed for the genome project. Notably, the
429 G119 in the *K. aberrans* AChE is encoded by a GGC codon, which could easily be
430 converted to the AGC codon for serine. This substitution in the mosquito paralogous
431 AChE seems not to be neutral under the selective pressure produced by organophosphate
432 and carbamate treatments (Weill et al. 2004b). Indeed, when KaAChE cDNA from an
433 additional two chlorpyrifos-susceptible and three chlorpyrifos-resistant unrelated strains of
434 *K. aberrans,* the G191S substitution was absent only in the resistant strains in the in
435 homozygous condition. The silent nucleotide polymorphisms found in the KaAChE cDNA
436 of the resistant strains might may also suggest that different G119S mutation events
437 occurred independently. Although, in *Culex quinquefasciatus* Say, a chlorpyrifos inhibition
438 study of a paralogous AChE bearing the G119S substitution revealed a reduced sensitivity
439 to the insecticide (Liu et al., 2005) that likely resulted from the reduced accessibility of the
440 catalytic site (Weill et al., 2004a). *In vivo*, *T. urticae* strains with the same mutated AChE
441 genotype display only a moderate resistance to chlorpyrifos. In contrast, all examined
442 resistant strains of *K. aberrans* are highly resistant to the insecticide (Tirello et al., 2012).
443 Thus, the role of the G119S remains unclear, although the possibility that the same
444 mutation has different effects on chlorpyrifos AChE sensitivity in the predatory mite cannot
445 be ruled out. Nevertheless, the non-silent nucleotide polymorphism responsible for the
446 G119S substitution represents a reliable molecular marker that is associated with the
447 detected resistant phenotype. Alternative target site resistance mechanisms not explored
448 in this study might also rely on the amplification of modified AChEs as occurs in *T. urticae*
449 and *T. evansi* (Kwon et al., 2010a; Carvalho et al., 2012) or on mutations that affect
450 multiple AChE loci with additive effects such as has been observed in *R. microplus*
451 (Temeyer et al., 2009, 2010, 2012). In either case, the co-expression of sensitive and
452 insensitive AChEs might contribute to reducing the fitness costs associated with OP

453 resistance (Carvalho et al., 2012, Temeyer et al. 2013a). Alternatively, the overexpression
454 of distinct AChEs from different loci it is thought to result in to bio-scavenging due to the
455 supply of excess targets for xenobiotics, including OP and carbamate insecticides (Lee et
456 al. 2014, in press). While the detection of homozygosity for the G119S substitution argues
457 against the co-existence of duplicated sensitive and insensitive AChEs in chlorpyrifos-
458 resistant strains, the role of multiple AChE loci in *K. aberrans* cannot be not excluded.
459 Intriguingly, the genome of the closely related phytoseides *M. occidentalis* harbours at
460 least six AChE-like candidates (GenBank accession numbers XP_003743019,
461 XP_003747509, XP_003739584, XP_003738701, XP_003744479, and XP_003745369).
462 Although these putative AChEs have lower amino acid identities (< 34%) to those found in
463 *T. urticae*, they exhibit conserved functional residues for acetylcholinesterase activity (i.e.,
464 the form the catalytic triad and the acetylcholine binding pocket), exhibit conserved amino
465 acid positions, are potentially involved in substitutions that affect AChE sensitivity to
466 organophosphates and carbamates, have and received support from transcriptomics
467 analyses (Hoy et al., 2013). Together, these findings suggest that, in predatory mites,
468 multiple AChEs resemble the composite picture observed in ticks (Temeyer et al. 2013b).
469 Thus, herbivorous and predatory mites can differ not only in detoxification pathways
470 (Mullin et al., 1982; Grbic et al., 2001; Dermauw et al., 2012) but also in AChE repertoires,
471 which potentially offers alternative solutions for the development of target site resistance.
472 However, chlorpyrifos inhibition assays of AChE activity should be performed to support
473 this hypothesis in examined OP resistant strains. Although, the contribution of enhanced
474 detoxifying activities to chlorpyrifos resistance in predatory mites has not yet been reported
475 as it has for other organophosphates (Sato et al., 2001, Fournier et al., 1987; Motoyama et
476 al., 1971; Anber et al., 1988), the use of synergists in bioassays should be combined with
477 detoxification enzyme assays to confirm or deny the involvement of metabolic resistance.

In summary, the 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 that is highly insensitive to chlorpyrifos in Tetranychidae was absent in a putative homologous gene that was cloned from the resistant strain *K. aberrans*. However, a G119S 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 release (Ahmad et al., 2013), 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.

References

Not included

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

Strain	Origin
PO	Commercial vineyard at Monteforte d'Alpone (Verona province)
SF	Commercial vineyard at S. Pietro Incariano (Verona province)
BX	Commercial vineyard at Soave (Verona province)
ME	Commercial vineyard at Valdobbiadene (Treviso province)
LE	Untreated European nettle trees (*Celtis australi* L.) at Legnaro (Padova province)
PD	Untreated European nettle trees (*Celtis australi* L.) at Padova
PA	Untreated European nettle trees (*Celtis australi* L.) at Paese (Treviso province)

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	ACAGCAGGTCAGTCAAGCAGTAGC AGCAGTTCGATAAGCGGCCGCCAT $GGAT_{12}$ DN
	KaAChEF4	AGGTGGTGAACGAAGCCATCAT	Adapter1	ACAGCAGGTCAAGTCAAG
	KaAChEF5	TTGAGTACACGGACTGGTTGAACCCT	Adapter2	AGCAGTAGCAGCAGTTCGATA
5' RACE				
			KaAChER4	CTCCGTAAACCCATAGAAGGA
	TS-Primer	CACCATCGATGTCGACACGCGTCGGG IGGIG	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

Table 3. Genome organisation of the *K. aberrans* ace locus

Exon	Position (nt)[a]	Exon size (bp)	Splice junction	Intron size (bp)	phase	amino acid 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

The numbering of nucleotides is based on the *K. aberrans* AChE cDNA in which +1 corresponds to start codon.

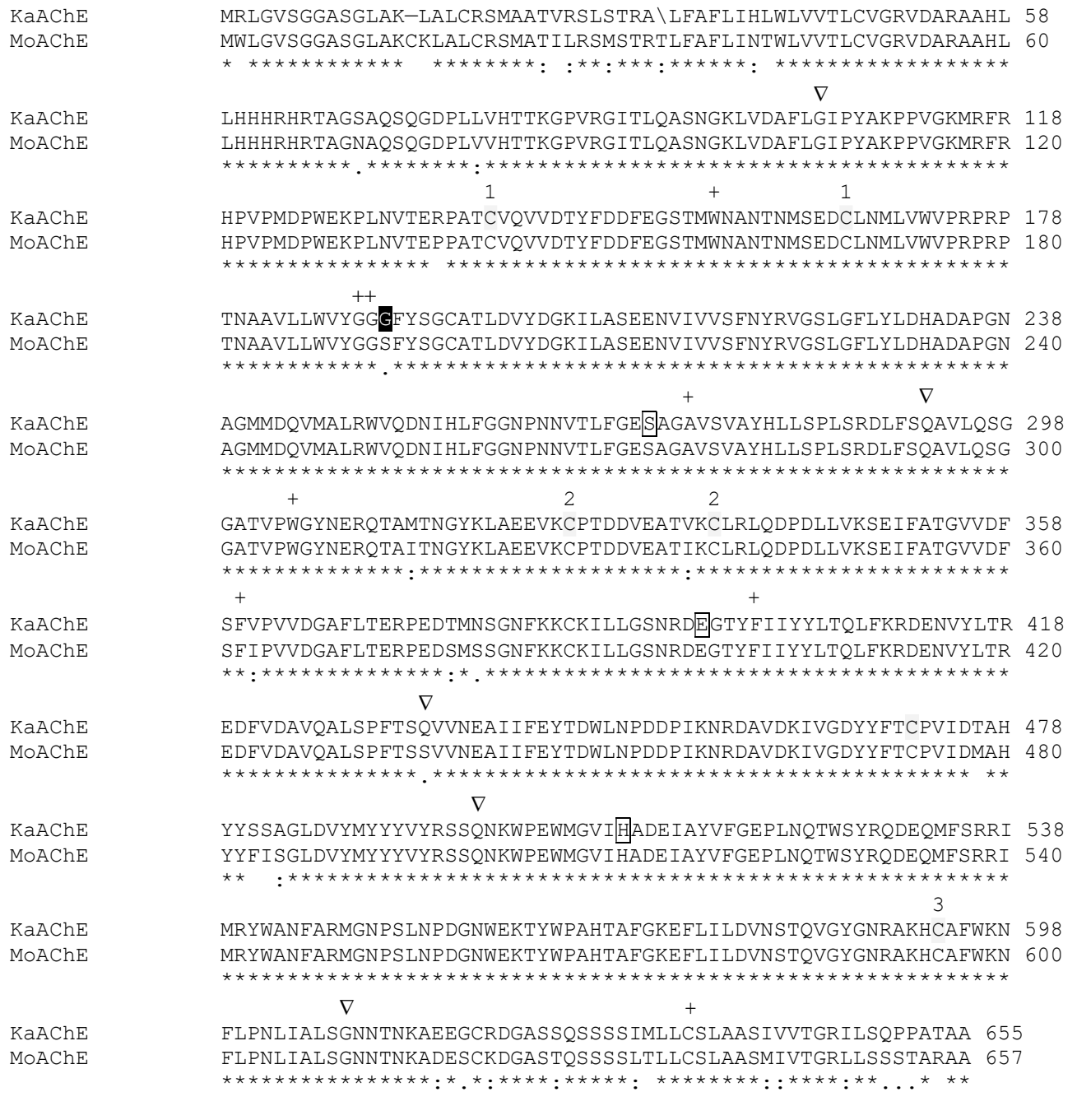

Fig. 1 Alignment of the AChEs cloned in from the chlorpyrifos -susceptible strain of *Kampimodromus aberrans* (KaAChE) and predicted from *Metaseiulus occidentalis* transcript XR_145413 (MoAChE). Identical amino acids are indicated by asterisks, and conservative substitutions are indicated by dots. The cleavage site of signal peptide is indicated by a slash. The mutated residue (G191S) in the chlopryrifos-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 indicates the exon boundaries in the cDNA open reading frame.