High Nucleotide Diversity in the *para*-Like Voltage-Sensitive Sodium Channel Gene Sequence in the Western Flower Thrips (Thysanoptera: Thripidae)

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ABSTRACT In a search for a pyrethroid resistance diagnostic marker, a partial sequence of the para-like sodium channel gene was obtained from 78 diploid females of the arrhenotokous insect pest species *Frankliniella occidentalis* (Pergande), the western flower thrips. Although all the insects analyzed came from a single laboratory population, nine different haplotypes were obtained. Two haplotypes did have the well-known L to F *kdr* mutation, but only one of these could be statistically linked to pyrethroid resistance in our population. This haplotype did not have the *superkdr* mutation, but did have a unique mutation a few amino acids downstream, at a position already linked to resistance in *Plutella.* Although this *para*-like locus seemed to have a role in pyrethroid resistance in our population, other resistance mechanisms were also probably involved. The fact that our laboratory population, open to migration, contained a high genetic diversity for this selected gene shows that "pest tourism" is a major factor for resistance dynamics in this greenhouse pest. This, with the possible occurrence of an original resistance mutation, might preclude the use of very specific approaches for resistance monitoring in the field in this species.

KEY WORDS western flower thrips, *para* sodium channel gene, *kdr* mutation, molecular diagnostic, pyrethroid, insecticide resistance

ONE PARAMETER RELEVANT to the development of pesticide resistance is the rate of introduction of new genetic variants into local pest populations. This is especially important in horticultural greenhouse production where local pest populations are to be kept at the lowest possible level. This is a problem because arthropod pests can travel over long distances on plants by taking advantage of the international plant trade network through so-called "pest tourism" (Frey 1993, Vierbergen 1995). Therefore, there is an urgent need for tools to monitor the genetic make-up of these pest populations for insecticide resistance management.

To address this problem, we initiated a search for molecular markers for pyrethroid resistance in the western flower thrips, *Frankliniella occidentalis* (Pergande). This arrhenotokous species, although introduced only recently from California, has already infested greenhouses world-wide (Brodsgaard 1989), and it has recently become a serious pest in Southern Europe. *F. occidentalis* not only feeds on a wide variety of plants species, inducing direct damage but it is also a very efficient vector for plant viruses. Thus, it led to a very low pest tolerance level of one thrips per plant per day in greenhouse production. The western flower thrips has already acquired resistance mechanisms to all major insecticide classes and most notably to pyrethroids. However, resistance profiles can differ dramatically among greenhouse populations, which is one of the main reasons for the need of a resistance management program for this species (Helyer and Brobyn 1992, Immajaru et al. 1992, Brodsgaard 1994, Zhao et al. 1995, Herron et al. 1996, Seaton et al. 1997).

The molecular basis of resistance to pyrethroids has already been characterized in a number of insect species, for example in *Musca domestica* (Williamson et al. 1996), *Hematobia irritans* (Guerrero et al. 1997), *Heliothis virescens* (Park and Taylor 1997, Park et al. 1997, Head et al. 1998), *Plutella xylostella* (Schuler et al. 1998), *Blatella germanica* (Miyazaki et al. 1996, Dong 1997), and *Leptinotarsa decemlineata* (Lee et al. 1999). Thus far, the resistance to pyrethroids has always been at least partly linked to the *para*-like voltage dependent sodium channel (but see Stokes et al. 1997). This ion channel, responsible for the propagation of the nerve influx, is the direct target of pyrethroids (Zlotkin 1999). Although several other mutations have been linked to resistance in some species (Park et al. 1997, Head et al. 1998, Schuler et al. 1998, Vais et al. 2001), two point mutations at homologous positions in

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the coding sequence of this gene have consistently been associated to a lower affinity to the pyrethroids at the molecular level and to accrued pyrethroid tolerance for the insects bearing them (Scott and Dong 1994, Smith et al. 1997, Schuler et al. 1998, Vais et al. 2001). The *kdr* mutation (for knockdown resistance) is a leucine to phenylalanine mutation in the hydrophobic IIs6 transmembrane segment of the sodium channel gene and has been found in quite all the resistant species studied so far (but see Park and Taylor 1997). The other point mutation, *superkdr*, always associated to *kdr* for a higher resistance level, has been identified in dipterans only so far (Williamson et al. 1996, Guerrero et al. 1997).

As these resistance conferring point mutations seem to be quite conserved throughout arthropod pests, and as pyrethroid resistance is common in thrips, we initiated a study of this *para*-like gene in *Frankliniella occidentalis.* More precisely, we investigated the genetic diversity that could be found within a representative greenhouse population for a genomic fragment of the *para*-like fragment encompassing the homologous positions of the *kdr* and *superkdr* point mutations. We also tried to characterize in which ways this genetic diversity could be linked to pyrethroid tolerance in this species and thus assess the potential for molecular diagnostic tools that could be used for resistance management in this species.

Materials and Methods

Insect Material. As our main aim was to see if the voltage dependent sodium channel gene could be used for pyrethroid resistance diagnostics in western flower thrips, we first screened a greenhouse population polymorphic for that trait. Diploid *Frankliniella occidentalis* females were sampled from an "open" laboratory population that has not been directly submitted to selection to any pesticide for at least 120 generations. Thrips were cultivated on French beans (*Phaseolus vulgaris* L.) that were grown in small greenhouses c. 50 m apart from the station's greenhouses. It is said to be an "open" population because a small number of migrants from the crops grown in surrounding greenhouses is apparently able to immigrate into the laboratory culture every summer, as sometimes traces of typical feeding damage can be observed on the plants brought into the culture cages. As these migrants originate from treated areas, they likely are tolerant to various pesticides. Species status of random individuals is checked regularly, and no contamination by thrips other than *F. occidentalis* has been detected so far. Moreover, very low divergence exists among our population and *F. occidentalis* from independent commercial greenhouses for a species-specific marker (unpublished data). The proportion of insects tolerant to high doses of deltamethrin has been constant in our laboratory population over the past few years.

Two independent samples from our "open" population were used in two successive bioassays. A first sample of insects was selected at 7.5 ppm Deltamethrin (75% of the specified field rate) and split in two groups according to their tolerance phenotype (dead or alive 4 h after treatment). All the insects that survived treatment but only a subset of the ones that died at this dose were subsequently genetically analyzed for a total of 32 insects (16 of each phenotype). A second sample was then selected at 2.5 ppm Deltamethrin (25% of the specified field rate), and split in two groups as previously. All the insects of this second sample were analyzed, so pooling the insects alive and dead after exposure to 2.5 ppm Deltamethrin forms a random sample from the "open" population of 46 individuals (which split into 30 alive and 16 dead insects after treatment).

Resistance Bioassay. Batches of ten to twelve thrips were immersed for 20 min in an aqueous solution of either 2.5 or 7.5 ppm Deltamethrin and 0.005% Ajutol (or in water and Ajutol for controls). Ajutol is a wetting agent used to enhance the activity of the insecticides and is manufactured by Siegfried AG (Zofingen, Switzerland).

The thrips were then gently poured on a 4 × 4cm closed plastic cylinder 3/4 filled with plaster. Mortality was assessed after 4 h by probing insects for reaction. None of the controls displayed >5% mortality. These two doses of insecticide were chosen as they consistently resulted in respectively 30% and 85% mortality after 4 h in samples taken from our "open" laboratory culture. No general oxydase inhibitor such as piperonyl butoxide was used, as we wanted to check if mutations within the *para*-like gene sequence could be used as general diagnostic markers for pyrethroid resistance in *Frankliniella occidentalis.*

Genomic DNA Extraction and polymerase chain reaction (PCR) Amplification. Genomic DNA was obtained from single thrips by a proteinase K extraction in 50 μl extraction buffer, modified after Kawasaki (1990), following Dilworth and Frey (2000). Approximately 150–300 ng total DNA at the most could thus be obtained from a single diploid female thrips.

Together with degenerate primers (provided by M. Williamson, IACR-Rothamsted, UK), consensus primers derived from the sequence information available on Genebank for other insect species were first used. The sequences used were the *para* homologous sequences from *Haematobia irritans* (Gene bank accessions HIU83871, HIU83872, HIU83874, HIU83873), *Musca domestica* (accessions MDPARA, MD38813, MD38814), *Drosophila melanogaster* (DM2616), *Drosophila viridis* (DV26343), *Anopheles gambiae* (AGY13592), *Culex pipiens* (CP1012476, CP1012476, CP1012476), *Leptinotarsa decemlineata* (AF114489), *Myzus persicae* (MPE131759, MPE131760), *Plutella xylostella* (PXAJ2379, PXAJ2378), and *Blatella germanica* (BGU73584, BGU73583, BGU71083). The primers were chosen so that the amplified fragment should encompass the homologous positions to the two point mutations *kdr* and *superkdr.* Once the first *para*-like sequences were obtained for *F. occidentalis*, thrips specific primers were designed, for a better PCR yield (see Table 1). Some thrips specific primer pairs had to be redesigned during the subsequent popula-

Table 1. Primers and PCR protocols

Primer pairs	PCR cycles	Comments
skdr/Dg1-F	With HotStarTaq	"universal primers"
GCCCBAAGTACTAYTTCCAGGARGG	15′ 95°C	*super kdr* and *kdr* regions
kdr/2-R	45×(40′′ 95°C/40′′ 55°C/1′30′′ 72°C)	(around 760bp in *F. o.*)
GGAGAGATTGGAAGACCCAAAA	7′ 72°C	
skdr/2-F	With HotStarTaq	*Frankliniella* primers
CAGGARGGCTGGAACATCTTCGACT	15′ 95°C	*superkdr* and *kdr* regions (around 730bp in *F.o.*)
kdr/4-R	45×(40′′ 95°C/40′′ 58°C/1′30′′ 72°C)	
GACCCAAAATTGGACAGGAGCAAGG	7′ 72°C	
skdr/Dg1-F	With HotStarTaq	Thrips specific
GCCCBAAGTACTAYTTCCAGGARGG	15′ 95°C	First intron region (around 200bp in *F.o.*)
ikdr/4-R	45×(40′′ 95°C/40′′ 48°C/1′30′′ 72°C)	
GCCACGACTTGGCWAGCTTGAAC	7′ 72°C	
ikdr/4-F	With HotStarTaq	Thrips specific
GTTCAAGCTWGCCAAGTCGTGGC	15′ 95°C	*superkdr* and *kdr* regions (around 570 bp in *F.o.*)
kdr/4-R	45×(40′′ 95°C/40′′ 52°C/1′30′′ 72°C)	
GACCCAAAATTGGACAGGAGCAAGG	7′ 72°C	
Sequencing primers	**Linear amplification**	**Comments**
skdr/1-F		Up to 600 bp readable
GCTGGAACATCTTCGACTTC	28×(5′′ 95°C/5′′ 40°C/3′ 60°C)	
kdr/2-R		Up to 600 bp readable
GGAGAGATTGGAAGACCCAAAA	28×(5′′ 95°C/5′′ 40°C/3′ 60°C)	
ikdr/3-F (internal primer)		Product of around 360 bp
TGTCATTCAACAACGCCTGT	28×(5′′ 95°C/5′′ 40°C/3′ 60°C)	
ikdr/3-R (internal primer)		Product of around 380 bp
ACAGGCGTTGTTGAATGACA	28×(5′′ 95°C/5′′ 40°C/3′ 60°C)	
ikdr/4-F and kdr/4-R		Depends on the PCR template
as above	28×(5′′ 95°C/5′′ 50°C/3′ 60°C)	

tion screening, as their hybridization sites were disrupted by within species polymorphism, and are not presented here.

PCR reactions were optimized to 20 μl total reaction volume using the HotStarTaq Mastermix from Qiagen (Qiagen Gmbh, Hilden, Germany) with five pmol of each primer and 1–3 μl of template genomic DNA (for ≈3–10 ng total DNA). PCR cycling conditions are reported in Table 1. PCR were performed either on an Eppendorf Mastercycler Gradient (Eppendorf AG, Hamburg, Germany) or Techne Genius PCR (Techne, Cambridge, UK) machines.

Polymerase chain reaction products were cleaned for direct sequencing using Geneclean spinfilter kit from Bio101 (Qbiogene, Illkirch, France). PCR yield was measured after purification either by spectrophotometry on a Genequant II (Amersham Biosciences AB, Uppsala, Sweden) or by fluorometry using the dsDNA specific pigment Picogreen (Molecular Probes, Eugene, OR) on a Turner Design TD700 fluorometer (Turner Designs, Sunnyvale, CA), following manufacturer's recommendations.

Direct Sequencing. Linear amplifications for direct sequencing were performed in 8 μl final reaction volume on 30 ng of PCR product with five pmol of the sequencing primer and 2 μl of Ready Reaction Mix (ABI PRISM BigDye Terminator Cycle Sequencing Kit, Applied Biosystems, Foster City, CA). Cycling conditions are described in Table 1. Four different sequencing primers were used for each individual (two distal and two internal primers) thus allowing at least two to three readings in both orientations for each nucleotide position in the sequence. Sequences were run on an capillary-based automated sequencer (ABI310 Genetic Analyzer, Applied Biosystems) and then proofread and assembled using the Sequencher 3.0 software from Gene Codes Corporation (Gene Codes, Ann Arbor, MI).

Data Analysis. Basic genetic diversity calculations (π) were performed using the analysis software DNAsp (Rozas and Rozas 1999). Any genetic association between the *para*-like sodium channel and pyrethroid resistance in *F. occidentalis* should appear in the frequencies of its different haplotypes. The Tajima test of neutrality (Tajima 1989, Tajima 1993), based on the comparison of the mean pair wise number of mutations and the number of segregating sites, was computed to detect an eventual effect of selection on the haplotype frequencies within the reconstructed random sample, using DNAsp (Rozas and Rozas 1999). As this test was developed for diploid organisms, and as selection acts faster on haplodiploid loci (Hedrick and Parker 1997), it should be conservative when applied to diploid females of an arrhenotokous species.

All the insects for which the *para*-like sequence was obtained had been previously assigned to one of four phenotypic groups according to their deltamethrin tolerance. Insects were thus classified as: alive at 7.5 ppm Deltamethrin, dead at 7.5 ppm, alive at 2.5 ppm and dead at 2.5 ppm. Any haplotype somehow linked to the insecticide tolerance should vary in frequency among these phenotypic classes. Moreover, the comparison of the genetic composition of the first and the last of these groups should be decisive in determining the association between pyrethroid resistance and genetic diversity at the *para*-like locus in the western flower thrips. Therefore, the effect of selection on

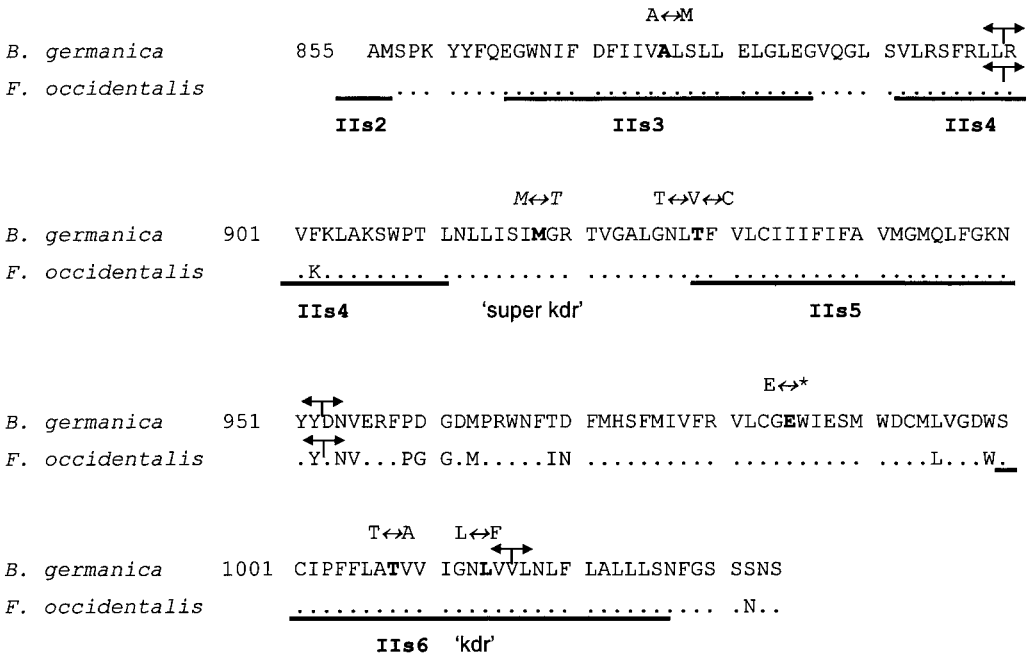

Fig. 1. Domain II region amino acid sequence of the *para*-like sodium channel of *Blatella germanica* and *Frankliniella occidentalis.* The position of the pyrethroid resistance conferring point mutations *kdr* (position 1014, numbering according to *Musca* sequence) and *superkdr* (position 918) and the coding mutations found in *F. occidentalis* are indicated in bold and the amino acid changes are shown. Position of the introns are shown by arrows above the corresponding sequences. Transmembrane protein domains are shown by bold lines under the sequence.

each haplotype at the two doses tested was assessed by considering each phenotypic class as a repeated sample of the whole population and testing for replicated goodness-of-fit, taking the reconstructed random sample of the population as reference. Association to the tolerance phenotype of each haplotype against all others was further tested independently, by comparing its distribution among individuals dead at 2.5 ppm and those alive at 7.5 ppm, using independence tests. All the statistical tests were performed following Sokal and Rohlf (1995).

Results

PCR/Sequence and Polymorphism. Consensus primers derived from the sequence information available for other insect species were identified (Table 1). As mentioned earlier, several sets of primers had to be developed during this study, as there was enough diversity within this species to disrupt sequences otherwise conserved among species. Moreover, the consistently low PCR amplification yields for this DNA fragment when compared with other nuclear sequences (unpublished data) pushed us, for reproducibility's sake, to devise thrips specific primers and abandon even slightly degenerate primers. The exact reason for this low amplification yield is still unclear.

A 760-bp long fragment of amplified genomic DNA was obtained for *Frankliniella occidentalis* that was homologous to the *para*-like sodium channel gene. This stretch of DNA contained the homologous positions to the two point mutations that had been linked to pyrethroid resistance in many insect species (*kdr* for knock-down resistance and *super-kdr*) (Fig. 1). There was slightly <10% divergence at the amino acid level between the *F. occidentalis* and *Musca domestica*, and around 5% between *F. occidentalis* and *Blatella germanica.* The sequences obtained have been deposited in EMBL (accessions AJ408849, AJ408850, AJ408851, AJ408852, AJ408853, AJ408854, AJ408855, AJ408856, and AJ408858).

By comparing the amplified *F. occidentalis* genomic DNA fragment to the cDNA sequences of other species, three exons or fragments of exons of respectively 124, 163, and 245 bp and two putative introns of resp. 73 and 155 bp could be identified in the thrips sequence (Fig. 1). *F. occidentalis* introns are found at homologous positions to the *B. germanica* introns, but are around ten times shorter (Dong et al. 1998). However, *F. occidentalis* seems to have lost the intron in the IIs6 domain that has been found in the other species for which genomic data were available (Thackeray and Ganetzky 1994, Dong et al. 1998, Lee et al. 1999, Park et al. 1999).

From the 78 *F. occidentalis* diploid females sequenced so far, 22 mutations were identified on this genomic fragment (Fig. 2). Eleven mutations, including two indels, were found in the introns, and 11 were found in the exons, out of which six were nonsynonymous substitutions. One of these mutations was ho-

mologous to the *kdr* point mutation, resulting in a leucine to phenylalanine mutation at position 1014, following the *Musca domestica para*-like amino acid sequence indexation. The *superkdr* mutation was not found in our sample. However, a "mutation hot spot" with four different amino acids possible (Fig. 2) was found in our sample at a position that had been linked to pyrethroid resistance in *Plutella xylostella* (Schuler et al. 1998) and *Pediculus humanus capitis* Deg (Vais et al. 2001) (amino acid position 929 in *M. domestica*).

This polymorphism could be organized in nine haplotypes (Fig. 2). As small indels were present in the sequences from most heterozygote individuals, this allowed the unambiguous definition of their haplotypes. For heterozygotes without such indels, complete linkage disequilibrium was postulated to extract the haplotypes. This seemed legitimate as no recombined haplotype has been identified in the homozygotes or the more numerous heterozygotes for indels. Only the A1 haplotype could possibly be a product of recombination, albeit an old one, as it would imply a double recombination and as it had been found in an other unrelated population (unpublished data). The haplotypes thus defined could be further organized in three groups (A., B. and C.) differing among each other by as much as thirteen mutations, while the haplotypes within each group differed by only a few substitutions (Fig. 2).

The A haplotype was by far the most frequent haplotype in our laboratory population, as more than half of the sampled individuals carried at least one copy of it, and was followed in ranking order by the C., B. and A1 haplotypes (Tables 2 and 3). The A0 haplotypes, found only in two heterozygous individuals (Table 3) carried a stop mutation (Fig. 2). This could indicate the existence of parthenogenetic female lines in *F. occidentalis*, as this would correspond otherwise to a relatively high frequency for such a probably lethal mutation. However, as this haplotype was not found in subsequent independent samplings, this relatively high frequency could also be due to the accidental sampling of two descendants of the same mutated mother. The haplotypes B1, C1, and C? were found only once each (Table 2), and could also correspond to relatively recent mutations from the more frequent B and C haplotypes respectively. The C? haplotype was found only once in an apparently C/C? heterozygous female. As these two haplotypes share the same indels (Fig. 2), the exact nature of this haplotype could not be resolved. It was not included in the subsequent diversity analysis.

This polymorphism resulted in a high nucleotide diversity value, π (as defined in Nei 1987). The observed nucleotide diversity was higher within our "open" laboratory population, and even within the phenotypic groups obtained after selection, than among several *Drosophila* strains of worldwide origin (Moriyama and Powell 1996) (Table 4).

Insecticide Tolerance and Genetic Diversity. Tajima's D value (Tajima 1989) computed over the reconstructed random sample was significantly positive with D = 2.397 ($P < 0.05$). This indicates that the

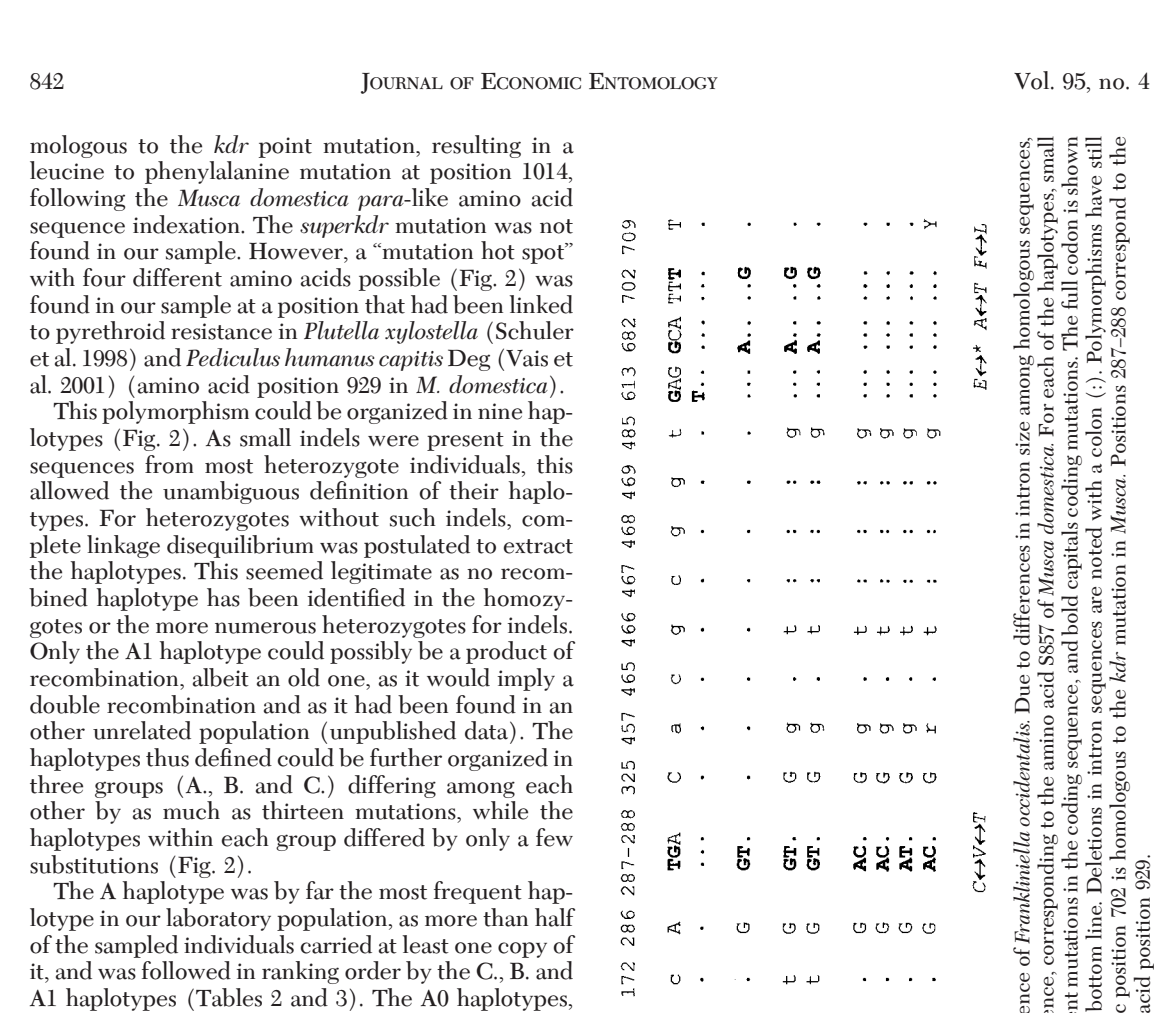

Fig. 2. Variable positions in the domains IIs2 to IIs6 genomic *para*-like gene sequence of *Frankliniella occidentalis*. Due to differences in intron size among homologous sequences, nucleotide positions are based on the beginning of the amplified *F. occidentalis* sequence, corresponding to the amino acid S857 of *Musca domestica*. For each of the haplotypes, small case letters indicate polymorphic positions in the intron sequences, capital letters silent mutations in the coding sequence, and bold capitals coding mutations. The full codon is shown for these mutations and the corresponding amino acid changes are reported on the bottom line. Deletions in intron sequences are noted with a colon (:). Polymorphisms have still to be confirmed by independent sequencing for the haplotype C?. The polymorphic position 702 is homologous to the *kdr* mutation in *Musca*. Positions 287–288 correspond to the mutation "hotspot" mentioned in the text and are homologous to the *Musca* amino acid position 929.

Table 2. Distribution of the haplotypes among phenotypic classes within a single population

Haplotypes	A	Ax		B.		C				Total
		A0	A1	B	B1	C	C1	C2	C?	
Phenotypic classes										
Alive at 7.5 ppm	23	—	4	2	—	2	—	1	—	
Deltamethrin	*0.72*		*0.13*	*0.06*		*0.06*		*0.03*		*32*
Dead at 7.5 ppm	17	—	1	5	1	8	—	—	—	
Deltamethrin	*0.53*		*0.03*	*0.16*	*0.03*	*0.25*				*32*
Alive at 2.5 ppm	32	—	2	7	—	16	—	3	—	
Deltamethrin	*0.53*		*0.03*	*0.12*		*0.27*		*0.05*		*60*
Dead at 2.5 ppm	10	2	3	7	—	7	1	1	1	
Deltamethrin	*0.31*	*0.06*	*0.09*	*0.22*		*0.22*	*0.03*	*0.03*	*0.03*	*32*
Random sample	42	2	5	14	—	23	1	4	1	
	0.46	*0.02*	*0.06*	*0.15*		*0.25*	*0.01*	*0.04*	*0.01*	*92*

This table shows the total number of each haplotype that was found within the phenotypic classes. The frequency of the haplotypes within each phenotypic class is shown in small case italic. Haplotype Ax stands for A1 and A0, B. for B1 and B2 and C. for C, C?, C1 and C2. It should be noted that 85% of the individuals from a random sample of the laboratory population here tested die after exposure to an aqueous solution of 7.5 ppm of Deltamethrin.

observed haplotype frequencies do not fit with the neutrality expectations, and that they are shaped by some form of positive selection (Tajima 1993).

Furthermore, both the A haplotype and the pooled C. haplotypes frequencies were significantly affected by selection with deltamethrin (Tables 5 and 6). The A haplotype is statistically associated to deltamethrin resistance ($G = 10.84, P < 0.001$), as can be confirmed by comparing its respective frequency among the individuals dead at 2.5 ppm Deltamethrin and to the ones alive at 7.5 ppm (Table 7). Nearly 95% of the females which survived an exposure to a high dose of this insecticide (alive at 7.5 ppm) were at least heterozygotes for this haplotype, whereas this proportion was only 44% in the individuals that died after exposure to a low dose of insecticide (dead at 2.5 ppm) (and 56% in a random sample of the population, Table 3). This mortality was due to pyrethroid toxicity, as the mortality in the control bioassays was <5%. However, the association between the A haplotype and pyrethroid resistance was not strict, as this haplotype was still the most frequent among the individuals dead at low dose (Table 7). However, the C. haplotypes significantly diminished in frequency after treatment at a high dose of insecticide as can be seen in Table 8 (frequency shift from individuals dead at 2.5 ppm to the ones alive at 7.5 ppm, $G = 4.91, P < 0.05$).

These haplotypes A and C. all carried a mutation homologous to the *kdr* mutation known to confer pyrethroid resistance in other species. The opposite consequences on the frequency of these haplotypes led to an apparent lack of effect of a treatment at a high dose of the insecticide on the frequency of the *kdr* mutation itself (Table 9). But, as such a treatment resulted in a significantly lower frequency of the C haplotype, which bears this mutation, the *kdr* mutation by itself is probably not able to confer a high level of resistance to its bearer. However, this mutation seemed to provide some kind of protection to low doses of insecticide. This mutation was found at a significantly higher frequency among the individuals alive at 2.5 ppm than among the ones dead at 2.5 ppm ($G = 5.74, P < 0.05$) (calculated on pooled A and C. haplotype frequencies from Table 2). Our proposition that the *kdr* mutation confers nothing more than a protection to low doses of pyrethroids in western flower thrips is further confirmed by the comparison of the individuals alive at 7.5 ppm and the ones alive at 2.5 ppm (Tables 2 and 3). Selection at a higher dose led to a significant loss of the C. haplotypes among the

Table 3. Genotypic distribution among phenotypic classes within a population

Genotypes	A/A	A/Ax	A/B.	A/C.	B./B.	B./C.	C./C.	C./Ax	N
Phenotypic classes									
Alive at 7.5 ppm	8	4	1	2	—	1	—	—	
Deltamethrin	*0.50*	*0.25*	*0.06*	*0.13*		*0.06*			*16*
Dead at 7.5 ppm	6	—	2	3	1	2	1	1	
Deltamethrin	*0.37*		*0.13*	*0.19*	*0.06*	*0.13*	*0.06*	*0.06*	*16*
Alive at 2.5 ppm	11	2	4	4	—	3	6	—	
Deltamethrin	*0.37*	*0.07*	*0.13*	*0.13*		*0.10*	*0.20*		*30*
Dead at 2.5 ppm	3	2	2	—	1	3	2	3	
Deltamethrin	*0.19*	*0.12*	*0.12*		*0.06*	*0.19*	*0.12*	*0.19*	*16*
Random sample	14	4	6	4	1	6	8	3	
	0.30	*0.09*	*0.13*	*0.09*	*0.02*	*0.13*	*0.17*	*0.07*	*46*

This table shows the total number of each genotype that was found within the phenotypic classes. The frequency of the genotypes within each phenotypic class is noted in small case italic. The genotype of each individual could be assessed unambiguously because of the presence of indels in the sequence studied. "Ax" stands for the pooled counts of haplotypes A0 and A1, "B." stands for the haplotypes B, B1 and B2, "C." for haplotypes C, C?, C1 and C2. It should be noted again that 85% of the individuals from a random sample of the laboratory population here tested die after exposure to an aqueous solution of 7.5 ppm of Deltamethrin.

Table 4. Observed nucleotide diversity π within a single greenhouse population of *Frankliniella occidentalis* after deltamethrin selection

	$\pi.10^3$	
	Coding	Noncoding
Frankliniella occidentalis		
Random sample[a]	5.86	14.96
Alive at 7.5 ppm[b]	4.09	8.50
Dead at 7.5 ppm[c]	5.49	14.48
Dead at 2.5 ppm[d]	6.86	15.99
Drosophila melanogaster		
X chromosome[e]	0.22–4.95[i]	0.97–20.65[i]
Autosomes[f]	1.22–9.75[i]	2.67–24.50[i]
Drosophila simulans		
X chromosome[g]	0.83–10.72[i]	5.24–15.35[i]
Autosomes[h]	3.95–22.24[i]	5.22–24.98[i]

Observed nucleotide diversities in *Drosophila* are given for a comparison basis (from Moryiama and Powell 1996). Only the X chromosome is in an haplodiploid state in an heterogametic diploid species.

[a] From 46 diploid females of the same population.
[b] From 16 females.
[c] From 16 females.
[d] From 30 females.
[e] Seven genes sampled.
[f] 16 genes sampled.
[g] Six genes sampled.
[h] Six genes sampled.
[i] Minimum and maximum values over the genes sampled.

survivors, even if they carried the *kdr* mutation ($G = 6.35$, $P < 0.01$). Only the A haplotype seemed to be significantly linked to resistance to high doses of insecticides.

Discussion

We have obtained a 760-bp long fragment of genomic DNA of the *para* sodium channel gene in *Frankliniella occidentalis* (Fig. 1). Nine different haplotypes were identified from 78 diploid females sampled in a single greenhouse population (Fig. 2), indicating a high nucleotide diversity (Table 4). These haplotypes could be further classified in three divergent haplotype groups (A., B. and C.).

Selective effects were detected on the *para*-like sodium channel gene haplotype frequencies within a random sample of our laboratory population and one of the haplotypes (the A haplotype) was associated with survival after exposure to a high dose of pyrethroid and hence with pyrethroid resistance (Tables 2, 3, 5, 6, and 7), indicating that the *para* locus may be linked to insecticide resistance in *Frankliniella occidentalis* as well. Because our laboratory culture has not been subjected to any insecticide treatment for many generations, this finding suggests that either immigrants from the station's greenhouses that carried resistance alleles were detected, or that under our rearing conditions, the mutations conferring pyrethroid resistance do not suffer from a selective disadvantage and are not lost. This second hypothesis is more probable in our case as the observed migration level from the greenhouses to the laboratory population was too low to explain by itself the relatively high frequency of resistant insects.

Nucleotide diversity itself was high in our laboratory population, as we detected nine haplotypes on 78 females from this single population. This diversity was still high after the strongest selection was applied, even if this resulted in 85% mortality (Table 4). This result is striking for a selected gene in a haplodiploid species, as allele fixations should be quicker in such species (Hedrick and Parker 1997). Compared with species wide estimates on X chromosomes in *Drosophila* (Moriyama and Powell 1996), and even though the Drosophila estimates were obtained from laboratory

Table 5. Overall effect of deltamethrin selection on the frequency of the A haplotype

Test	df	*G*	*P*
Pooled	1	2.9893	0.08
Heterogeneity	3	10.9305	0.01
Total	4	13.9198	0.01
Alive at 7.5 ppm Deltamethrin	1	2.7625	0.10
Dead at 7.5 ppm Deltamethrin	1	1.4197	0.23
Alive at 2.5 ppm Deltamethrin	1	0.7167	0.40
Dead at 2.5 ppm Deltamethrin	1	9.0209	$p < 0.01$
Total	4	13.9198	0.01

A selective effect on a given haplotype frequency was detected when the different phenotypic classes could significantly not be considered as repeated samples of the whole population by a replicated goodness-of-fit test, taking the reconstructed random sample of the population as reference.

Table 6. Overall effect of deltamethrin selection on the frequencies of the pooled C. (i.e., C, C1, C2 and C?) haplotypes

Test	df	*G*	*P*
Pooled	1	2.5953	0.11
Heterogeneity	3	7.0408	0.07
Total	4	9.6361	0.04
Alive at 7.5 ppm Deltamethrin	1	0.0011	0.17
Dead at 7.5 ppm Deltamethrin	1	0.0006	0.10
Alive at 2.5 ppm Deltamethrin	1	0.6578	0.42
Dead at 2.5 ppm Deltamethrin	1	8.9766	$p < 0.01$
Total	4	9.6361	0.04

A selective effect on a given haplotype frequency was detected when the different phenotypic classes could significantly not be considered as repeated samples of the whole population by a replicated goodness-of-fit test, taking the reconstructed random sample of the population as reference.

Table 7. Effects of insecticide treatment on the frequencies of the A haplotype

	Genotype			Total no. of individuals
	A/A	A/.	./.	
Alive at 7.5 ppm	8	7	1	
Deltamethrin	*0.50*	*0.44*	*0.06*	16
Dead at 2.5 ppm	3	4	9	
Deltamethrin	*0.19*	*0.24*	*0.57*	16
Random sample	14	14	18	
	0.30	*0.30*	*0.40*	46

Genotypic distributions within the reconstructed random sample and two phenotypic classes, opposing A to all non A haplotypes. Non-A haplotypes are represented by a dot "." in the table. The frequency of the genotypes within each phenotypic class is noted in small case italic.

Table 8. Effects of insecticide treatment on the frequencies of the C. haplotypes

	Haplotype		Total no. of individuals
	C.	Non-C.	
Alive at 7.5 ppm	3	29	
Deltamethrin	*0.09*	*0.91*	32
Alive at 2.5 ppm	19	41	
Deltamethrin	*0.32*	*0.68*	60
Random sample	29	53	
	0.35	*0.64*	82

Haplotypic distributions within the reconstructed random sample and two phenotypic classes, opposing C. to all non-C. haplotypes. The frequency of the haplotypes within each phenotypic class is noted in small case italic.

Table 9. Distribution of the individuals within the phenotypic tolerance groups according to their number of *kdr* mutations

	No. of *kdr* mutations			Total no. of individuals
	2	1	0	
Alive at 7.5 ppm	10	6	—	
Deltamethrin	*0.63*	*0.37*		16
Dead at 7.5 ppm	10	5	1	
Deltamethrin	*0.63*	*0.31*	*0.06*	16
Alive at 2.5 ppm	21	9	—	
Deltamethrin	*0.70*	*0.30*		30
Dead at 2.5 ppm	5	10	1	
Deltamethrin	*0.31*	*0.63*	*0.06*	16
Random sample	26	19	1	
	0.57	*0.41*	*0.02*	46

This was done by pooling the A/A, A/C. and C./C. as having two *kdr* mutations, even in different genetic background. The B./B. individuals were the only ones found without any *kdr* mutation. All the other observed genotypes had one copy of the *kdr* mutation, either on a A or a C. haplotype. The A0/C individuals were counted as having only one *kdr* mutation, as a stop mutation occurred before the position of the *kdr* mutation on the A0 haplotypes. The frequency of the genotypes within each phenotypic class is noted in small case italic.

strains and not from natural populations for a proper comparison, the within population nucleotide diversity values in *F. occidentalis* for the *para*-like gene segment are high. This is further stressed by the fact that the *kdr* mutation, which was linked to low levels of pyrethroid resistance in our population, was found in two very different genetic contexts (on the A and C. haplotypes), in the absence of other apparent recombination events (Fig. 2). As the population size of our "open" population never reached more than a few thousand adult insects, this diversity is probably a direct consequence of pest migration. This "pest tourism" was observed for *Frankliniella* by direct counting of hitchhiking insects on imported plants (Frey 1993). The arrival of a batch of imported plants can possibly result in the replacement of a third of the local thrips population in a standard commercial greenhouse. Moreover, like the plants they are traveling on, these immigrant insects could be of worldwide origins.

In such a migration network, as migrants come from plant production sites or other greenhouses, they most probably already bear pesticide resistance genes. Resistance, as it was selected independently, could be due to different mechanisms, or as in the case of a highly constrained gene as *para*, could be due to the same mutations in different genetic backgrounds. Whatever the case, this seriously complicates the task for the molecular diagnostic of resistance, and definitely puts migration as a major parameter for any pesticide resistance management strategy in this species.

In the horticultural trade network, selection for pesticide resistance on thrips occurs first at the plant production centers, where heavy treatment is often the rule. This selection process is then followed by widespread distribution of the surviving insects, finally resulting in mixed populations in commercial greenhouses. Contrary to open field settings, where migrant pests come from untreated ruderal areas most of the time, in confined greenhouses, *F. occidentalis* migrants themselves or at least their very direct ancestors have already been exposed to pesticide treatment. These mixed populations are then submitted to continuous selection in situ. An important implication of such a system is a fast emergence of multiresistant individuals in greenhouses. The tedious task of adapting the treatments to the resistance profiles observed in the greenhouses may then be unavoidable. Contrary to the open field situation, the selection pressure for resistance is nearly constantly applied on the pest populations, so that even costly resistance mutations may be favored. This probably stresses even more the need for refuges where no treatment is applied.

The other consequence of this particular migration network is the relative unpredictability of the genetic diversity to be found within a given greenhouse. Even for very conserved genes like *para*, the genetic diversity we observed in our laboratory population is very high (Table 4). As this population was not exposed to any treatment for several generations, and hence was not forced into population size bottlenecks, the observed diversity is probably higher than the one to be expected in treated commercial greenhouses. The composition of this laboratory "open" population reflects anyway the wide variety of the migrants arriving to one of these commercial greenhouses.

The *kdr* mutation, which was associated with pyrethroid resistance in several insect species (Scott and Dong 1994, Smith et al. 1997, Schuler et al. 1998), has also been found in our sample. In fact, nearly all the insects sampled in our laboratory population were at least heterozygous for that mutation (Table 9). However, *kdr* in itself seemed from our results to confer at the most a low resistance level in *F. occidentalis.*

Our population was not submitted to any pesticide treatment but was founded with insects coming from treated greenhouses, and occasionally received migrants coming from these same greenhouses. As the *kdr* mutation could be found at a high frequency in our population, composed of insects whose ancestors at least had been exposed to pyrethroid treatments, this reflects perhaps a basal tolerance level conferred by this mutation. This low resistance level could eventually be high enough to allow *F. occidentalis* to escape control. As *F. occidentalis* often lives hidden inside flower buds, it may escape exposure to heavy doses of

pesticides, thus explaining why a low effect mutation could eventually still be favored by selection. Alternatively, the low level of tolerance conferred by this mutation, when added to the effects of other unlinked insecticide resistance mutations, could still be enough to allow the pest species to escape control by pesticides, as seems to be the case with the citrus thrips (Immajaru et al. 1990).

Whatever the situation, the presence of *kdr* mutations in an individual did definitely not confer resistance to high doses of Deltamethrin (Table 8 and 9). However, this lack of a strong link between pyrethroid resistant phenotype and the *kdr* mutation has also been observed in natural populations of other species (Dong et al. 1998, Guerrero et al. 1998, Jamroz et al. 1998, Lee et al. 1999). This makes the *kdr* mutation an apparently poor diagnostic for pyrethroid tolerance in pest greenhouse populations.

Whereas *kdr* could not be linked to a high level of resistance, nearly 95% of the insects that survived an exposure to 75% of the field rate of deltamethrin were at least heterozygous for the A haplotype (Table 3). This haplotype bears the *kdr* mutation and also a Cys at a "mutation hotspot" where three other amino acids could be found in our population (Fig. 2). Substitutions at this position could possibly be involved in pyrethroid resistance. A Thr to Ile mutation on the homologous position in *Plutella xylostella* (Schuler et al. 1998) and a Leu to Phe mutation in human head louse (Vais et al. 2001) had been linked to resistance in association with the *kdr* mutation. The C2 haplotype found in our *F. occidentalis* population bears the same substitutions as *Plutella* (Ile at the "hotspot" and *kdr*, Fig. 2), but could not be linked to high levels of pyrethroid tolerance (Tables 2 and 3).

However, it is still not clear whether this association with the resistance is directly due to the Thr929Cys substitution or to a linkage disequilibrium between this *para* variant and some unidentified resistance conferring mutations. Half of the insects that died after exposure to a low dose of deltamethrin were at least heterozygous for the A haplotype, even if still virtually all the insects that survived 75% of the insecticide field rate were at least heterozygous for A (Table 3). To survive a high dose of pyrethroids, it seems thus necessary to have at least one copy of this haplotype, but it may not be enough, even if the Thr759Cys did confer some level of resistance by itself.

Furthermore, the *para*-like gene could still only be a major gene in an oligogenic resistance mechanism. The polymorphism on other resistance loci would then be needed to explain the lack of a perfect association between the A haplotype and the resistant phenotype. Alternatively, an imperfect dominance on the *para* locus could explain the distribution of the A/. heterozygotes among the phenotypic classes (see Lee et al. 1999 for such a situation).

Our results could also be obtained if the A haplotype in itself did not contribute to resistance but was tightly linked to a resistance locus. This other locus could either be a mutation in an other domain of the same gene as in *Heliothis virescens* (Park et al. 1997, Head et al. 1998) as the strong association of this haplotype to the resistance suggests, or eventually a totally different gene such as a general oxidases or even genes acting on cuticle thickness, close to the *para* locus. In that case, it would then be by pure chance that the resistant phenotype could be associated to a *para* haplotype bearing mutations linked to insecticide resistance in other species, which seems rather unlikely.

This first series of results illustrates the difficulties encountered when working on a pest species where pure strains are not readily available. Most of the uncertainties left at the end of this study, such as the exact implication of the *para* locus in pyrethroid resistance in *F. occidentalis* could be removed if pure lines for each haplotype were available. Efforts to develop such pure lines are difficult with such a small insect, but nevertheless are being undertaken at our research station. However, in parallel, a wider screening of real greenhouse populations could help test the association of the polymorphism at the *para* locus with pyrethroid resistance, and this line of research is also being pursued.

But we already can have some hints of the difficulties that will be encountered in the development of a molecular resistance diagnostic test in this species. When added to a possibly complex pattern of expression of the resistance genes (Lee et al. 1999), the high within population genetic diversity created by the "pest tourism" may preclude very specific approaches for diagnostic purposes (Guerrero et al. 1998, Jamroz et al. 1998), or at the very least may seriously increase the need for redundancy in any diagnostic test. This is why we would like to recommend the use of multilocus markers for these badly needed resistance monitoring purposes. It may eventually prove that the use of an array of neutral polymorphic genes may be more powerful than allele specific tests to monitor the distribution of insecticide resistance in natural pest populations. These markers would allow for a wider coverage of the genome, which should more than compensate for their eventual lower linkage to the most often unidentified resistance mutations.

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