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Widespread pyrethroid resistance in Australian diamondback moth, *Plutella xylostella* (L.), is related to multiple mutations in the *para* sodium channel gene

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Abstract

Populations of *Plutella xylostella*, extending over 3800 km in southern Australia, show no genetic structure as assessed by microsatellite markers; yet outbreaks of pyrethroid resistance occur sporadically in cropping areas. Since mutations in the *para* voltage-gated sodium channel gene have been implicated in pyrethroid resistance, we looked for DNA sequence variation at this target among Australian moths. We found two resistance mutations previously reported for this species (L1014F and T929I), as well as a novel substitution (F1020S). Of the eight possible haplotypes formed by combinations of these three biallelic polymorphisms, only four were found in Australian populations: the *wild-type* allele (*w*), the *kdr* mutation allele (*kdr*) with only L1014F, the *super-kdr-like* combination of L1014F and T929I (*skdrl*), and the *crashdown* allele with only F1020S (*cdr*). Comparison of genotype frequencies among survivors of permethrin assays with those from untreated controls identified three resistant genotypes: *skdrl* homozygotes, *cdr* homozygotes and the corresponding heterozygote, *cdr*/*skrdl* – the heterozygote being at least as resistant as either homozygote. Spatial heterogeneity of allele frequencies was conspicuous, both across the continent and among local collections, consistent with reported spatial heterogeneity of pyrethroid resistance. Further, high resistance samples were sometimes associated with high frequency of *cdr*, sometimes high frequency of *skdrl*, or sometimes with a high combined *cdr* + *skdrl* frequency. The *skdrl* and *cdr* alleles explain a high proportion of the Australia-wide resistance variation. These data add to evidence that nerve insensitivity by mutations in the *para-sodium channel* gene is a common pyrethroid resistance mechanism in *P. xylostella*.

Keywords: *Plutella xylostella*, sodium channel, *kdr*, pyrethroid, insecticide resistance

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Introduction

Knockdown resistance (*kdr*) in insects is caused by reduced sensitivity of the nervous system to pyrethroid insecticides and to DDT (Busvine, 1951; Soderlund & Knipple, 2003). The target site for pyrethroids is the α subunit of the voltage-gated sodium channel encoded by the *para* gene (Williamson *et al*., 1996). In susceptible insects, pyrethroid insecticides selectively affect the dynamics of sodium channel inactivation (Soderlund & Bloomquist, 1989) leading to paralysis and death. In resistant individuals, mutations in the binding pocket render the sodium channel insensitive to pyrethroids (Davies & Williamson, 2009). Point mutations leading to amino acid substitutions within this subunit have been associated with knockdown resistance in more than twenty species of insect, including cockroach, aphids, mosquitoes, beetles, thrips, fleas, hornflies, houseflies, leafminers, whiteflies and moths (Heckel, 2008; Davies & Williamson, 2009 and table 2 therein).

The first knockdown resistance mutation to be discovered was in the *kdr* strain of housefly (Williamson *et al*., 1996). It is a point mutation, also referred to as *kdr*, that causes a change from leucine to phenylalanine (CTT → TTT) at amino acid residue 1014 in the Vssc1 sodium channel α subunit, and is designated L1014F. This change occurs in transmembrane segment 6 of domain II of this long protein (the designating code here, and throughout the manuscript for all species, refers to the amino acid position number in the housefly protein (Smith *et al*., 1997) (GenBank Accession X96668: Williamson *et al*., 1996).

A second mutation in the housefly gene, M918T, was associated with very high levels of resistance and is referred to as *super-kdr* (Williamson *et al*., 1996). The mutation at position M918 is an ATG-ATC substitution and is located in the middle of the IIS4-S5 linker (Williamson *et al*., 1996). This mutation has been observed in *Musca domestica* (Williamson *et al*., 1996), the horn fly, *Haematobia irritans* (L.) (Jamroz *et al*., 1998) and *Myzus persicae* (Anstead *et al*., 2005) and has not been found independently of *kdr* (Anstead *et al*., 2005: the M918 T mutation likely occurred in a copy of the gene that already harboured the L1014F mutation). When the M918 T mutation was introduced alone to the *Drosophila para* gene, it provided little protection from the action of DDT (Usherwood *et al*., 2005).

The first strong evidence for the knockdown resistance mechanism, nerve insensitivity, was demonstrated in diamondback moth, *Plutella xylostella* (L.), using a pyrethroid-resistant strain from Taiwan and an electrophysiological assay (Schuler *et al*., 1998). In this strain, mutation L1014F is associated with a second mutation, T929I (Schuler *et al*., 1998). T929I is a threonine to isoleucine mutation (ACC → ATC) that occurs in the middle of segment 5 in domain II and is located 11 amino acids downstream of the position where in the housefly the M918 T mutation (*super-kdr*) occurred. Schuler *et al*. (1998) suggested that this mutation may function in a similar manner to *super-kdr* and so has been referred to as *super-kdr-like*. As a single mutation expressed in oocytes, it causes a low level of resistance to pyrethroids (Vais *et al*., 2001) and a high level of resistance to DDT (Usherwood *et al*., 2007); but, when present in the same copy of the gene as L1014F, it is thought to produce additive or synergistic enhancement of pyrethroid resistance (Soderlund & Knipple, 2003; Vais *et al*., 2003).

In a fenvalerate selection experiment with *P. xylostella*, Tsukahara *et al*. (2003) found evidence of an association between the two mutations (L1014F and T929I) and resistance to pyrethroids, though the resistance mutations also occurred at low frequency in non-selected susceptible strains. To complicate matters, in *Drosophila*, mosquitoes and several Lepidoptera, there is alternate splicing in which either exon 18a or 18b (*P. xylostella* nomenclature of Sonoda *et al*. (2008b)) may be utilized in the mature protein. This exon contains the region encoding T929; and, in a resistant strain of *P. xylostella* from Korea, exon 18b harbours the T929I mutation while exon 18a encodes the unchanged T929 (Sonoda *et al*., 2006). This is likely to result in two alternate copies of the protein being produced in a moth – in the susceptible strain both exons encode threonine (Sonoda *et al*., 2008b).

The exact relationships between these different mutations, the mutant combinations, nerve insensitivity and resistance are not fully understood but have been investigated in several species. For example, Forcioli *et al*. (2002), working with the western flower thrips, *Frankliniella occidentalis* (Pergande), found no clear association between the *kdr* mutation, various mutations at position T929I and resistance to pyrethroids. In the Colorado potato beetle, *Leptinotarsa decemlineata*, the L1014F mutation is present; and, although there is some correlation between increased LD_{50} and increased frequency of the resistance allele, a robust association with pyrethroid resistance was not observed in all field populations tested (Kim *et al*., 2005).

A lack of association means that other factors may cause resistance, such as further point mutations in the sodium channel gene that have not yet been discovered (Tsukahara *et al*., 2003) or completely different mechanisms such as degradation of insecticides by cytochrome P_{450} oxidases. There is also the possibility that more than one mechanism gives rise to the resistance phenotype in an individual.

The genetic basis of resistance to pyrethroids in Australian populations of *P. xylostella* has not yet been characterized. This species is a destructive horticultural pest due mainly to its propensity to develop resistance to insecticides, a rapid lifecycle and lack of natural enemies in newly colonised areas or where insecticide application has led to their destruction (Talekar & Shelton, 1993). Pyrethroid insecticides became available in the 1970s and have since been used extensively for control of *P. xylostella* in *Brassica* vegetables in Australia and throughout the world. More recently in Australia, pyrethroid insecticides have been used against *P. xylostella* in canola (oilseed rape) with varying degrees of success, and now there is sporadic and widespread resistance across the country (Endersby *et al*., 2008). Understanding the nature of pyrethroid resistance in Australian populations would be valuable for the horticultural and grains industries for future efforts to control this pest species.

In this study of Australian populations of *P. xylostella*, we report on three mutations in the *para*-type sodium channel gene, namely the two previously characterized (L1014F, T929I) and a new mutation (F1020S) that we report here for the first time. We look at the frequency of all mutations singly and in combination in populations from throughout southern Australia over a three-year period and compare these frequencies with resistance to the pyrethroid, permethrin, using a leaf-dip bioassay. A clear picture of association between these mutations, resistance and outbreaks of resistance emerges. We also discuss the utility of screening for sodium channel point mutations as a method for early detection of this type of pyrethroid resistance in populations of *P. xylostella*.

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Materials and methods

Insect collection

Eggs, larvae or pupae of *P. xylostella* were collected from 16 field sites in southern Australia, some on more than one occasion and some from closely adjacent sub-sites, from September 2003 to September 2005 (table 1). To minimize obtaining related individuals, collection was from dispersed field plants at each site where possible. Larvae were reared on seedling leaves of cabbage (*Brassica oleracea* var. *oleracea* cv. Green Coronet) at 25°C (16 h:8 h, L:D) at ambient relative humidity for one to four generations. A population (Waite) of *P. xylostella* that is susceptible to synthetic pyrethroid insecticides (Baker & Kovaliski, 1999) was used as a reference in each assay.

Insecticide bioassays

Third instar larvae of *P. xylostella* were tested for susceptibility to permethrin using a leaf dip bioassay (after Tabashnik & Cushing, 1987). Cabbage leaf discs of 4.5 cm diameter were dipped for five seconds in distilled water solutions of formulated insecticide (Ambush® Emulsifiable Concentrate Insecticide, Crop Care Australasia Pty Ltd, Brisbane, Australia) and hung vertically to dry in a fume hood for two hours. Control discs were dipped in distilled water. No wetting agents were used. Discs were placed into Gelman® 50 mm diameter × 9 mm plastic Petri dishes. Ten larvae were placed on each disc and four replicates of seven or eight concentrations were set up for the field populations tested and for the susceptible laboratory population. Mortality was assessed after 48 h at 28°C. Larvae were considered dead if they did not move when touched with a paintbrush.

Concentration-mortality data were analysed using the probit analysis program, POLO-PC (LeOra Software) (Russell *et al.*, 1977). The lethal concentration expected to cause 50% mortality (LC_{50}) of each insecticide for each sample of *P. xylostella*, the 95% confidence intervals for these concentrations and the slope (+standard error) of the probit line was estimated.

Mutation assays

Extraction of genomic DNA

Genomic DNA was extracted from whole moths using CTAB-(hexadecyltrimethylammonium bromide)/phenol/ chloroform, RNase, proteinase K and ethanol precipitation with 5M NaCl (based on Sambrook *et al.* (1989)). Each extract was used for the three separate SNP (single nucleotide polymorphism) mutation assays.

Bi-PASA assay protocol for L1014F mutation detection

To screen Australian populations of *P. xylostella* for the L1014F mutation, we modified the single-tube bi-PASA technique used by Kim *et al.* (2005) for the Colorado potato beetle. PASA (PCR amplification of specific alleles) requires two separate PCR reactions per individual, each with primers that amplify a specific allele. Bi-PASA (bidirectional PASA: Liu *et al.*, 1997) employs a single reaction with a pair of outer primers P and Q amplifying a region containing the mutation of interest, and two allele-specific inner primers facing in opposite directions, each of which terminates with its 3′-OH end on the nucleotide position to be screened. This position is closer to one of the outer primers than the other, producing allele-specific products of different sizes. The polymerase chain reaction in 25 µl was performed using primers P (5′ ATCAC GTGGA CCGCT TCCC 3′) in exon 19 and Q (5′ TTCGG CTACT TTGTT GGTCT CCTG 3′) in exon 20, A (5′ GCGGG GCTCG TCATT GGCAA CC 3′), the forward primer specific for wild-type L1014 in exon 19, and B (5′ GCGGG CGACA ACAGC TTACC ACAAA 3′), the reverse primer specific for the *kdr* L1014F substitution straddling the exon 19-intron 19 junction. Numbering of exons and introns follows Sonoda *et al.* (2008b). The A and B primers had short GC-clamps at their 5′ ends to control the annealing temperatures. The reaction mixture contained 2.5 µl 10× Reaction Buffer, 2.5 µl 2 mM dNTPs, 0.25 µl 10 µM of each primer, 0.5 units *Taq* DNA polymerase (HotMaster–Eppendorf) and 16.9 µl ddH_2O. The PCR profile was initial denaturation at 95° C for 2 min, 35 cycles of 94°C for 20 s, 52°C for 20 s, 72°C for 1 min and a final extension at 72°C for 10 min. After PCR, the products were separated by electrophoresis in a 2.5% agarose gel for 1.5 h at 110 V. Primers P and Q produced a 394-bp amplicon from both alleles, A and Q produced a 233-bp fragment from the wild-type allele, and P and B generated a 207-bp fragment from the *kdr*-type-allele. Heterozygotes displayed all three bands. These conditions were developed after extensive optimization of primer sequences and PCR conditions, which was necessary to balance the reliable production of all three amplicons from heterozygotes. Amplicons from a subset of individuals were sequenced to validate the bi-PASA technique and confirm genotypes.

Table 1. Australian samples of *Plutella xylostella* screened for *para* genotype variation.

Locality	Collected	Crop
Garvoc VIC (38° 17′ S 142° 50′E)	Nov. 03	weeds
Garvoc VIC (38° 17′ S 142° 50′ E)	Dec. 04	weeds
Gatton QLD (27° 34′ S 152° 20′ E)	Aug. 04	vegetable
Gatton Research Station QLD (27° 34′ S 152° 19′ E)	Aug. 04	vegetable
Geraldton WA (27° 52′ S 114° 45′ E)	Sept. 04	canola
Glenore Grove QLD (27° 31′ S 154° 24′ E)	Aug. 04	vegetable
Grantham QLD (27° 38′ S 152° 15′ E)	Aug. 04	vegetable
Knoxfield VIC (37° 52′ S 145° 15′ E)	Dec. 03	cabbage
Lindenow VIC (37° 48′ S 147° 28′ E)	Apr. 04	vegetable
Moree NSW (29° 14′ S 150° 02′ E)	Sept. 03	canola
Stanthorpe, B QLD (28° 37′ S 151° 58′ E)	Dec. 04	vegetable
Stanthorpe, G QLD (28° 39′ S 151° 55′ E)	Dec. 04	vegetable
Stanthorpe, H QLD (28° 39′ S 151° 55′ E)	Dec. 04	vegetable
Tenthill QLD (27° 45′ S 152° 10′ E)	Aug. 04	vegetable
Three Springs WA (29° 24′ S 115° 47′ E)	Sept. 04	canola
Walkaway WA (28° 56′ S 114° 48′ E)	Sept. 03	weeds
Walkaway WA (28° 58′ S 114° 50′ E)	Sept. 05	canola
Werribee South, K VIC (37° 58′ S 144° 41′ E)	Dec. 03	vegetable
Werribee South, M VIC (37° 56′ S 144° 41′ E)	Dec. 03	vegetable
Werribee South, S VIC (37° 56′ S 144° 42′ E)	Dec. 03	weeds
White Peak WA (28° 37′ S 114° 39′ E)	Sept. 05	canola
Yuna WA (28° 20′ S 115° 00′ E)	Sept. 03	canola

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PCR-RFLP assay protocol for T929I mutation detection

To screen populations for the T929I mutation, we exploited the presence of an MboI site in the region. PCR amplification took place in a volume of 25 µl with 2 µl of genomic DNA diluted 1:4. Primers used were: SuperKDR_F1 (5′ CATGG CCGAC ACTTA ATTTA CTCAT C 3′) in exon 18b and SuperKDR_R1 (5′ CATGC AACAC TCCCA CCAA 3′) in intron 18b. These primers are specific for the region containing the T929I mutation (termed the A2 exon by Sonoda *et al.* (2006)) and do not amplify from the corresponding region of exon 18a (A1 exon). The reaction mixture contained 2.5 µl 10× Reaction Buffer (Fisher Biotech), 2.0 µl 25 mM $MgCl_2$, 2.5 µl 2 mM dNTPs, 0.25 µl 10 µM of each primer, 0.5 units *Taq* DNA polymerase (Fisher Biotech) made up to 23 µl ddH_2O per sample. The PCR profile was initial denaturation at 95°C for 2 min, 35 cycles of 94°C for 20 s, 52°C for 20 s, 72°C for 1 min and a final extension at 72°C for 10 min; 5 µl of PCR product was incubated in a restriction enzyme digestion containing 0.35 µl MboI (New England Biolabs), 2 µl 10× Buffer 3, 2 µl 10× BSA and ddH_2O. After digestion, the products were separated by electrophoresis in a 2.0% agarose gel for 1 h at 110 V. The allele bearing the wild-type T929 produced an uncut amplicon of 167 bp, and the mutant allele with the T929I substitution produced two restriction fragments of 66 and 101 bp. Heterozygotes displayed all three bands.

PCR-RFLP assay protocol for F1020S mutation detection

In the process of optimizing the bi-PASA method for the L1014F detection, we sequenced amplicons generated from genomic DNA with the primers SuperKDR_F1 and Q. After detection of the F1020S mutation by sequencing, we scored it using a PCR-RFLP polymorphism with StyI. The polymerase chain reaction was performed using primer pair PxSci 18F (5′ TGATC GTGTT CCGCG TGCTG TG 3′) within exon 19 and PxSci 18R (5′ GGCTT CCGCT ACTTT GTTGG TCTCC TG 3′) within exon 20. The reaction mixture contained 22.5 µl 10 × Reaction Buffer (HotMaster, Eppendorf, Hamburg, Germany), 2.5 µl of 2 mM dNTPs, 0.25 µl 10µM of each primer, 0.5 units HotMaster *Taq* DNA polymerase (Eppendorf), 2 µl of genomic DNA diluted 1:4, and autoclaved milliQ H_2O. The PCR profile was initial denaturation at 94°C for 1 min, 35 cycles of 94°C for 20 s, 60°C for 30 s, 72°C for 1 min and a final extension at 72°C for 10 min. 10 µl of PCR product was incubated in a restriction enzyme digestion containing 0.1 µl StyI (Promega), 2 µl 10× Buffer F, 0.2 µl 100× BSA and ddH_2O. After digestion, the products were separated by electrophoresis in a 2.5% agarose gel for 1 h at 110 V. The allele bearing the wild-type F1020 produced an uncut amplicon of 328 bp and the mutant allele with the F1020S substitution produced two restriction fragments of 83 and 245 bp. Heterozygotes displayed all three bands.

Sequence analysis

PCR products generated from genomic DNA with the primers SuperKDR_F1 and Q using the conditions described above for L1014F were cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) and at least five clones per haplotype were sequenced on an ABI 3730 at the Australian Genome Research Facility in Melbourne. Sequences for the four haplotypes have been deposited in GenBank with the following accession numbers: *w*: GU797896, *kdr*: GU797897, *skdrl*: GU797898, *cdr*: GU797899. Coding sequences from exons 18b, 19, and 20 are shown in fig. 1b.

Population genetic analyses

Genotype scores were converted to SNP allelic frequencies by direct count and using Genepop version 4.0.10 (Raymond & Rousset, 1995a). Linkage disequilibrium was assessed using LinkDos (Garrier-Géré & Dillmann, 1992). Haplotype frequencies were estimated by maximum likelihood (Lin & Zeng, 2006) using Hapstat version 3.0. Assessing fit to Hardy-Weinberg equilibrium (Guo & Thompson, 1992), assessing differentiation of haplotype frequencies among locations using an exact G tests (Raymond & Rousset, 1995b), estimating F_{ST} (Weir & Cockerham, 1984) and assessing differentiation between control and permethrin survivors with an exact genotypic χ^2 test (Goudet *et al.*, 1996) were also facilitated using Genepop.

Results

Genotype determinations

Single-nucleotide polymorphisms (SNPs) in the *para* sodium channel gene were scored by PCR-RFLP and bi-PASA assays (figs 1a and 2) in 1076 individuals and verified by sequencing a random sample (fig. 1b). The 'mutant' variants of all three SNPs were detected in all locations sampled across Australia at high frequencies, averaging 0.139 for T921I, 0.609 for L1014F and 0.305 for the new F1020S (table 2). At these frequencies, the SNP mutations were not distributed randomly with respect to each other in each of the sampled field populations since, among 1076 field-collected individuals, only ten genotypes were detected of 27 three-SNP genotypes possible under an assumption of random chromosomal association of the mutations. This strong linkage disequilibrium was confirmed between the T929I and L1014F sites, the T929I and F1020S sites and the L1014F and F1020S sites using genotypic data that takes population subdivision into account across 19 field samples (Black & Krafsur, 1985), with $\chi^2_{(1df)}$ values of 178.13, 122.43 and 846.66 (all $P \leq 0.0001$), respectively.

When the genotype data were used to estimate haplotype frequencies in each of the field samples, of eight possible haplotypes involving the three mutation sites (fig. 3a), only four were indicated to be present (fig. 3b). This facilitated a multiple-allele single locus interpretation, and the excellent fit of field genotype frequencies to Hardy-Weinberg equilibrium under the four-haplotype assumption (table 2) supported this approach. While we cannot exclude the possibility that one or more of the four additional haplotypes, depicted in fig. 3a, occur in Australian *P. xylostella* populations, if they are present, they would be at low frequency. Complete linkage association is also consistent with previous reports in Korean *P. xylostella* populations that only found the T921I substitution along with L1014F within a single haplotype, although L1014F may occur alone (Kwon *et al.*, 2004). Thus, our survey of Australian populations of *P. xylostella* indicates that only the four haplotypes, which we call *w*, *cdr*, *kdr* and *skdrl* (figs 1b and 3b), are present, *cdr* being an acronym for crash down resistance by analogy to knock down resistance (*c* also representing the mutant base cytosine of this resistant haplotype). We interpret the distribution and bioassay data accordingly.

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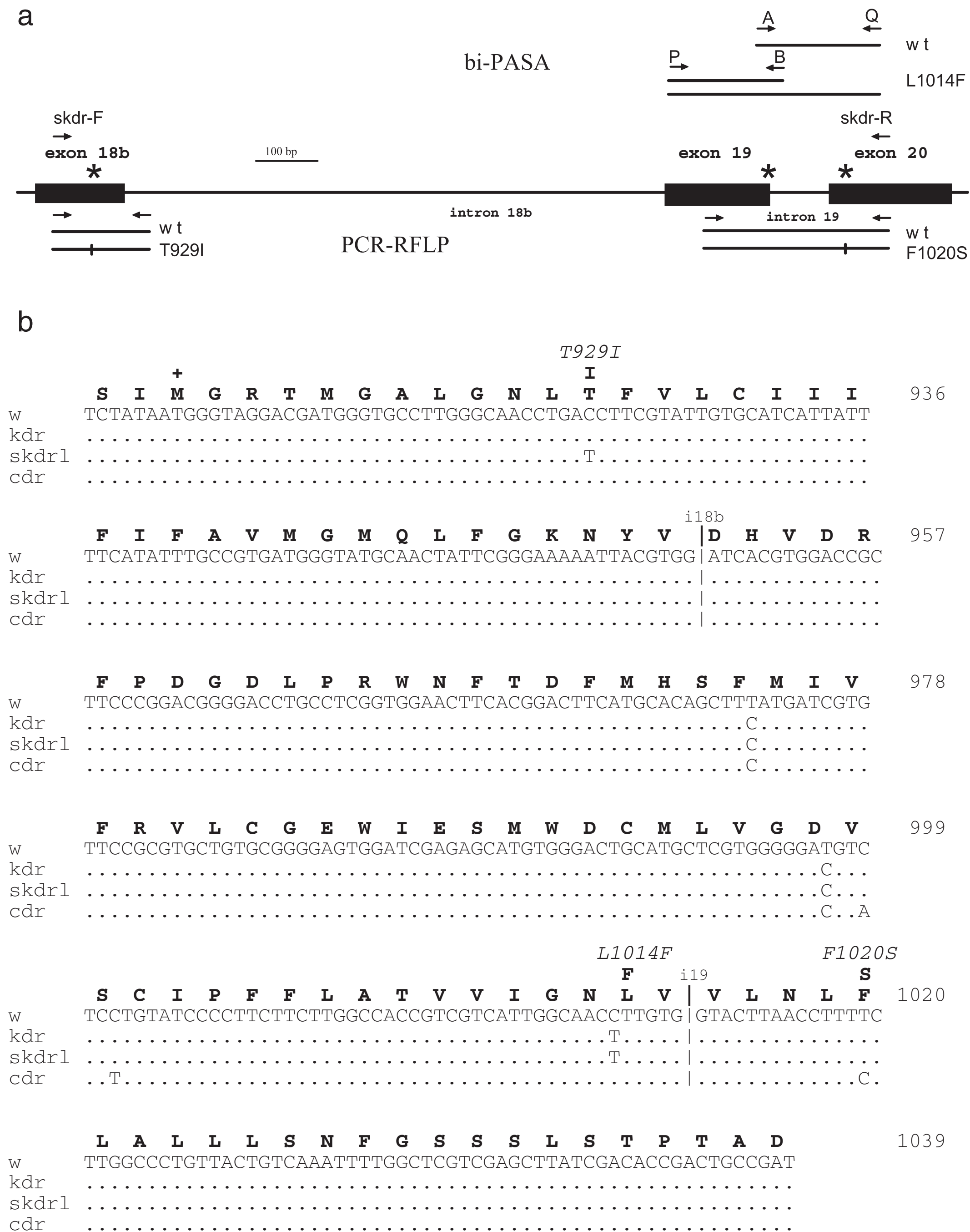

Fig. 1. Mutations in the *para* sodium channel gene of *Plutella xylostella*. (a) Genomic region scored for mutations using PCR-RFLP and bi-PASA techniques. Asterisks mark the positions of the mutations surveyed. Primers and amplicons for bi-PASA, and restriction sites for PCR-RFLP, are indicated. (b) Nucleotide and deduced amino acid sequences of four haplotypes in the IIS4-IIS6 coding region, bounded by the primers skdr-F and skdr-R. Vertical lines denote positions of introns 18b and 19. The plus sign marks the position of the *super-kdr* mutation M918T in housefly.

Spatial and temporal variation

The Australian samples are widespread, being separated by distances of up to 3800 km, and were taken over three consecutive seasons. However, as indicated, some of the samples were clustered in cropping areas on opposite sides of the continent. The four haplotypes were each present in all the field populations sampled across Australia (table 2, fig. 4). The most common was the *kdr* haplotype at an average frequency of 0.47 and ranging between a low of 0.153 at Tenthill to a high of 0.651 at Gatton Research Station, both sites being sampled in Queensland in 2004 and being separated by only 25 km.

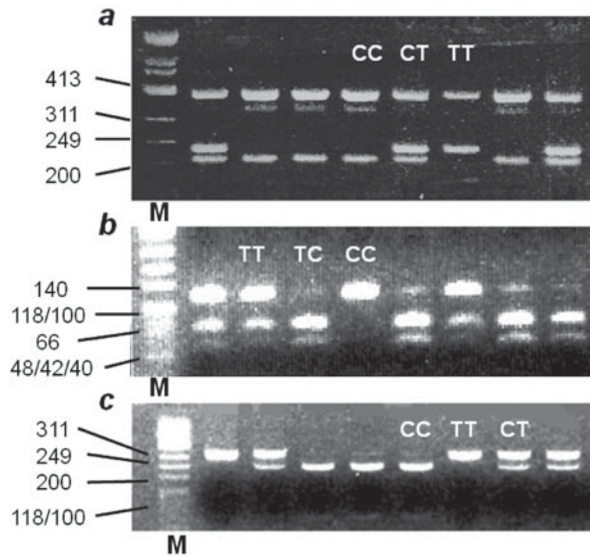

Fig. 2. Genotype gel assays for three *para*-sodium channel mutations in *Plutella xylostella*: (a) L1014F, (b) T929I and (c) F1020S. Scored genotypes (TT, CT or CC) are indicated for representative sample lanes and DNA size markers (φX174/Hinf1) are indicated in the M lane.

The rarest haplotype, *w*, averaged a frequency of 0.085, with a range from 0.013 at White Peak in WA to 0.183 at Moree in NSW.

When all 19 field samples are compared, clear differentiation in haplotype frequencies occurred. Variation was measured by F_{ST} (table 3), with an average value of 0.074 across all pair-wise sample comparisons. This is noticeably higher than the overall average F_{ST} of 0.005 obtained for six microsatellite loci across a similar geographic set of Australian *P. xylostella* samples taken between 2001 and 2003 (Endersby *et al.*, 2006). The average level of *para* haplotype differentiation was of similar magnitude and remained highly significant, even if comparisons were confined to particular regions. Thus, heterogeneity remained, though slightly reduced in magnitude, if the comparisons were confined to the cluster of six samples in close proximity in Western Australia, or was exacerbated if the comparisons were among the five sample cluster in Queensland within a single season, with average F_{ST} reaching a high of 0.15 (table 3). However, such a high level of differentiation of clustered samples did not occur among the seven Victorian samples where the average F_{ST} was reduced to 0.012.

Strong spatial differentiation was the rule rather than the exception, and it was not accounted for by changes across seasons. Thus, the strong average level of differentiation persisted when comparisons were only between samples taken within single seasons (table 2), even within seasons within States, except for the two Western Australian samples taken in 2004 and among the six Victorian samples in 2003. No consistent pattern of differentiation was detected in space or time. Thus, whereas no differentiation was detected among three samples from adjacent growers in 2003 at Werribee in Victoria (average $F_{ST}=0.004$), significant local differentiation ($F_{ST}=0.03$) occurred between two adjacent sites taken from Stanthorpe in Queensland in 2004 (fig. 4, table 3). Likewise, between-season differentiation was absent in Victoria at Garvoc when the 2003 and 2004 seasons are compared ($F_{ST}=0.002$), but differentiation was marked between the 2003 and 2005 seasons at Walkaway in Western Australia ($F_{ST}=0.03$).

Pyrethroid resistance assays

Over a 30 month period from November 2003 to March 2006, 25 permethrin resistance leaf-dip bioassays were carried out on either field-collected individuals or on mass-reared early-laboratory-generation individuals from a geographically diverse group of populations. Each population assay involved an estimate of the level of permethrin resistance and the genotype determination of the three *para* SNPs, both in a sample of control individuals and of adult survivors of the permethrin treatment (at concentrations in excess of 56 ppm).

Many assays (12 of 25) showed marked and significant differentiation between the control samples and the survivors in the relative numbers of the ten genotypes (table 4), indicating differential genotype mortality following permethrin treatment. Across all assays, the pattern of excess survivors was clearly associated with three genotypes CCC/CCC, TTT/TTT and CCC/TTT, corresponding to the *cdr*/*cdr* homozygote, the *skdrl*/*skdrl* homozygote and the *cdr*/*skdrl* heterozygote, respectively. For each genotype, a ratio of its genotype frequency among survivors to its frequency in the control sample, over all trials, provides a rough measure of its relative survival under pyrethroid stress. Thus, these three genotypes had relative survival levels of 2.41, 2.99 and 3.14, respectively.

While these ratios for a given genotype were highly variable across the assessable samples (mainly due to low numbers of a given genotype within samples), we would expect 50% of samples to exceed a ratio of 1.00 and 50% to be less than 1.00 if there was no differential survival following insecticide treatment. For each of these three genotypes, a significant excess of samples with a ratio greater than 1.00 occurred, indicating that they conferred a significant survival advantage (table 4). While not significantly higher, it is worth noting that the *skdrl*/*cdr* heterozygote had the highest survival ratio. Among genotypes that were particularly poor survivors were the *w*/*kdr* heterozygote (with a survival ratio of 0.05), the *w*/*skdrl* heterozygote (with a ratio of 0.24), the *kdr*/*kdr* homozygote (with a ratio of 0.48) and the *w*/*cdr* heterozygote (with a ratio of 0.71), all of these showing a significant excess of samples with a ratio less than 1.00. While the wildtype homozygote *w* had a low survival ratio of 0.21, it did not have a significant excess of samples with a ratio less than one, likely due to the rarity of the genotype and the resulting insensitivity of the χ^2 test when fewer samples were assessable. No indication of an advantage or disadvantage under permethrin stress occurred for the remaining two genotypes, *cdr*/*kdr* (with a ratio of 1.09) and *kdr skdrl* (with a ratio of 0.90).

A similar analysis was carried out for each haplotype within each assay by comparing its frequency among the survivors to its frequency in the controls and asking whether it increased or decreased as a result of the insecticide (table 4). As expected from the genotype results, the *cdr* and *skdrl* alleles increased in frequency among survivors in the majority of assays at the expense of *w* and *kdr*, the differentiation being highly significant in most cases. The relative 'fitness' of *skdrl* at 1.68 is indicated to be higher than *cdr* with a ratio of 1.39, with *kdr* (at 0.60) and *w* (at 0.43) having reduced performance levels.

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Table 2. Australian samples of *Plutella xylostella para* SNP frequencies, and haplotype frequencies assessed for fit to Hardy-Weinberg equilibrium.

Location and Collection Date	*n*	SNP mutant frequencies			Haplotype frequencies						
		freq T T929I	freq T L1014F	freq C F1020S	CCT *w*	CCC *cdr*	CTT *kdr*	TTT *skdrl*	H_O	H_E	*P*
Garvoc VIC Nov. 03	65	0.131	0.677	0.185	0.138	0.185	0.546	0.131	44	41.36	0.039*
Garvoc VIC Dec. 04	27	0.222	0.796	0.13	0.074	0.13	0.574	0.222	21	16.47	0.398
Gatton QLD Aug. 04	24	0.146	0.521	0.396	0.083	0.396	0.375	0.146	17	16.53	1.000
Gatton Research Station QLD Sept. 04	66	0.007	0.658	0.257	0.083	0.257	0.651	0.007	39	33.4	0.413
Geraldton WA Sept. 04	123	0.094	0.675	0.264	0.061	0.264	0.585	0.09	68	71.11	0.017*
Knoxfield VIC Dec. 03	28	0.179	0.64	0.321	0.036	0.321	0.461	0.179	20	18.47	0.129
Lindenow VIC 22 Apr. 04	144	0.139	0.729	0.136	0.135	0.136	0.59	0.139	84	86.07	0.436
Moree NSW Sept. 03	41	0.061	0.354	0.463	0.183	0.463	0.293	0.061	32	27.49	0.364
Stanthorpe, B QLD Dec. 04	61	0.172	0.549	0.418	0.033	0.418	0.377	0.172	40	40.12	0.522
Stanthorpe, G QLD Dec. 04	54	0.019	0.346	0.539	0.115	0.539	0.327	0.019	33	30.73	0.143
Tenthill QLD Aug. 04	45	0.578	0.734	0.233	0.033	0.233	0.156	0.578	28	26.69	0.294
Three Springs WA Sept. 04	62	0.097	0.589	0.322	0.089	0.322	0.492	0.097	48	39.80	0.089
Walkaway WA Sept. 03	53	0.075	0.585	0.292	0.123	0.292	0.51	0.075	37	33.93	0.889
Walkaway WA Sept. 05	66	0.167	0.803	0.174	0.023	0.174	0.636	0.167	40	35.67	0.053
Werribee Sth, K VIC Dec. 03	21	0.119	0.69	0.262	0.048	0.262	0.571	0.119	13	12.66	0.629
Werribee Sth, M VIC Dec. 03	21	0.191	0.548	0.262	0.19	0.262	0.357	0.191	17	15.73	0.514
Werribee Sth, S VIC Dec. 03	32	0.125	0.609	0.281	0.11	0.281	0.484	0.125	22	21.41	0.006**
White Peak WA Sept. 05	78	0.109	0.686	0.301	0.013	0.301	0.577	0.109	47	44.30	0.166
Yuna WA Sept. 03	65	0.015	0.384	0.562	0.054	0.562	0.369	0.015	26	24.20	0.116
Average		0.139	0.609	0.305	0.085	0.305	0.470	0.139			

n, number genotyped; H_O, observed number of heterozygotes; H_E, expected number of heterozygotes;
P, probability of fit to Hardy-Weinberg proportions of four alleles (exact test of Guo & Thompson, 1992).
The table only includes field samples with where $n>20$, with mean $n=56.6$. * $P<0.05$, ** $P<0.01$.

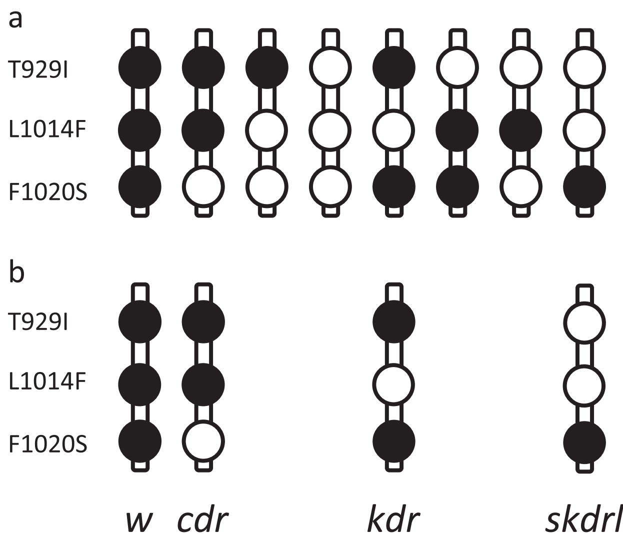

Fig. 3. (a) Theoretical and (b) observed haplotype configurations for three *para sodium channel* mutations of *Plutella xylostella* in Australian populations (*w* = wildtype C-C-T; *cdr* = C-C-C; *kdr* = C-T-T; *skdrl* = T-T-T; these are not representations of codons but instead indicate the variant wild-type or mutant base within each of the three codons at positions 929, 1014 and 1020). Closed circles represent the susceptible variant; open circles the resistant variant.

Extensive spatial and temporal variation of pyrethroid resistance of *P. xylostella* among field locations across Australia has recently been reported (Endersby *et al.*, 2008). Does the molecular variation in the *para* gene help explain the resistance variation? A plot of the combined frequency of the three resistant genotypes against the permethrin resistance ratio indicates that it does (fig. 5a). A significant regression analysis indicates that ~66% of the field variation in resistance ratio is accounted for by the observed frequency of these genotypes at each site. This association is equally accounted for if the Hardy-Weinberg expected frequency of these genotypes is used as the predictor ($y=84.847x+0.9025$, $P<0.0001$, $R^2=0.66$) or if the LC_{50} measure of resistance is used instead ($y=439.69x+31.523$, $P<0.0001$, $R^2=0.66$). Our data suggest that if the combined allelic frequency of the two 'resistant' alleles (*cdr* + *skdrl*) is used as the predictor, the associations are not as high, but remain highly significant, explaining ~60% of the variation in resistance ratio ($y=85.174x–18.574$, $P<0.001$) and ~63% of the variation in LC_{50} (fig. 5b). Using the individual SNP mutant frequencies separately as predictors of resistance variation is far less informative and of borderline significance.

Discussion

The mutations in the *para* voltage-gated sodium channel that confer target-site pyrethroid resistance are limited in number, but many recur independently in a variety of different insect species, including Lepidoptera (Heckel, 2008). This pattern is caused by rigid structural constraints on the protein structure, causing a large portion of the amino acid sequence to be absolutely conserved among insects and even vertebrates, allowing very few sites to vary. The L1014F substitution, first discovered in the *kdr* strain of housefly, is one of these and has been found in many other resistant

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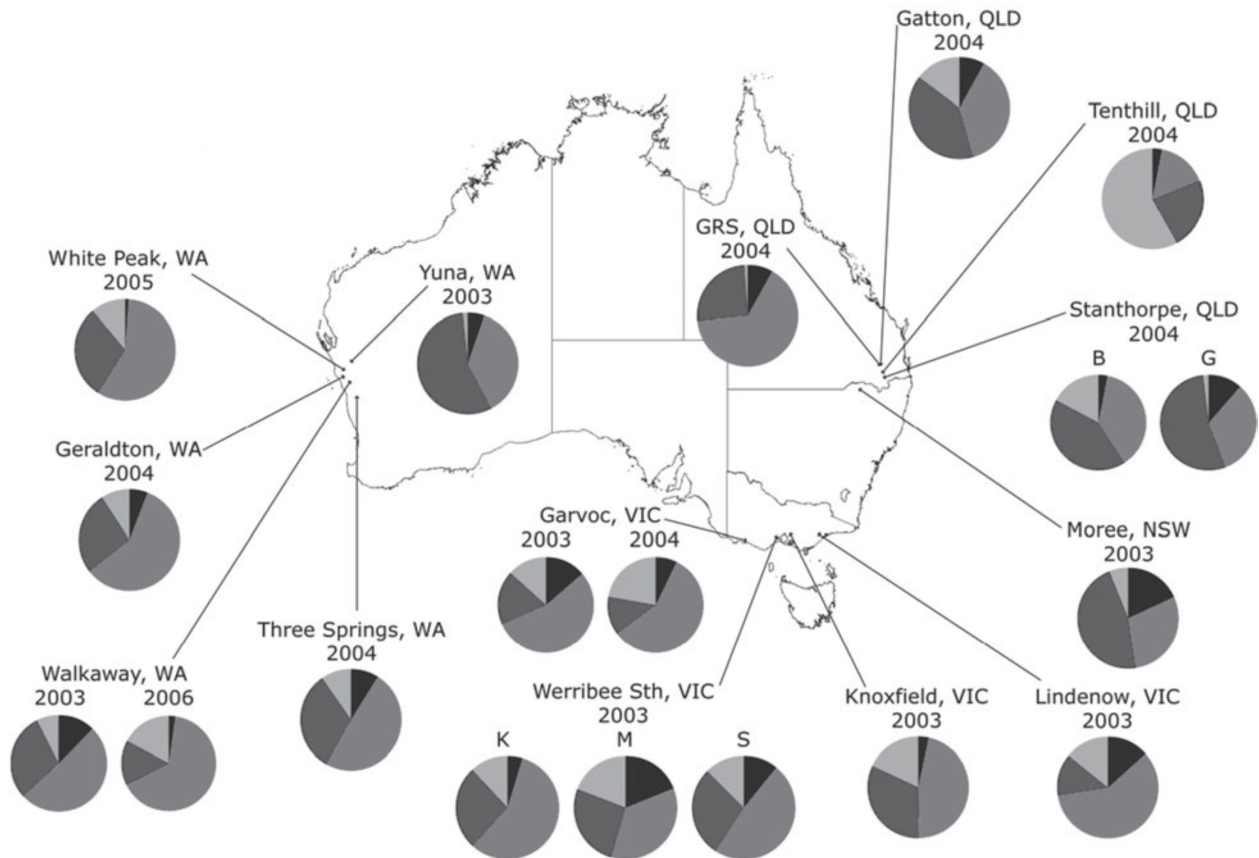

Fig. 4. Variation in frequency of the four *para* gene haplotypes of *Plutella xylostella* across Australia (Haplotype Key: ■, CCT (w); ■, CTT (kdr); ■, CCC (cdr); ■, TTT (skdrl))

species. Thus, it has been of interest to examine these variable sites in additional species with resistance problems, as has occurred in many population studies. However, a focus on known mutations can lead to a bias against discovering new ones, as illustrated by our fortuitous discovery of the novel F1020S substitution. The advent of new high-throughput sequencing technologies will offer the opportunity to survey population variation in the entire gene in a less biased manner.

The phenylalanine found at position 1020 is invariant in all animal species to our knowledge (Schuler *et al*., 1998, fig. 4 depicts eel, ascidian, squid, jellyfish, rat and housefly) and, as such, may be a key structural element. O'Reilly *et al*. (2006) used homology modelling of the housefly sodium channel with docking to various pyrethroid structures and predicted the pyrethroid binding site to occur between the IIS5 and IIIS6 helices. F1020 on the IIS6 helix does not appear to be a determinant of binding to pyrethroids, as it is far away from the predicted binding cavity, as is L1014. Assessment of the functional significance of the F1020S substitution will require heterologous expression, such as been performed for other substitutions.

The invariant nature of F1020 and flanking region, together with its proximity to L1014, may have paradoxically led to its being overlooked as a possible resistance site in previous surveys. This is because this region has been used as a target for the design of degenerate primers to screen for variation at L1014. Primer D3 of Martinez-Torres *et al*. (1997), which was used to amplify the L1014 region from eight previously-uncharacterized insect species, contains this site. Thus, the identity of positions 1015–1020 across these species depicted in fig. 2 of Martinez-Torres *et al*. (1997), including the invariant F at position 1020, derives not from the actual sequence of these species but from the D3 primer sequence itself. Consequently, additional sodium channel variants in other species, even quite close to known mutations, may have escaped detection.

Once mutations are identified, accurate and efficient methods must be used for population screening. Optimising assay conditions is especially important for the bi-PASA technique, in which up to three different amplicons are competing in the same PCR reaction. Kim *et al*. (2005) found that a bi-PASA screen for the L1014F substitution determined the correct genotype 98% of the time, compared to sequence-validated cases. Our sequencing of gel-scored individuals, including 24 that scored initially as containing the 'unnamed' haplotypes of fig. 3a, found a low error rate and confirmed that the 'unnamed' haplotypes were absent. We estimate one incorrect SNP genotype per 181 scored SNPs, assuming our mis-scoring occurred at random across all genotypes. This is a low and acceptable level, given the magnitude of the temporal and spatial allele frequency variations detected.

The *kdr* mutation is recognized as an 'old' mutation, having been detected in Korean *P. xylostella* collected prior to the widespread use of pyrethroids (Kwon *et al*., 2004). It was

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Table 3. Summary of spatial and temporal allelic differentiation and F_{ST} estimates among *para* haplotypes of Australian *P. xylostella* field samples.

Samples compared (collection dates)	Differentiation test		
	F_{ST}	P	SE
All 19 samples (May 03–Feb 06)	0.0738	<0.00001***	0.00000
All 9 samples of 2003 season	0.0676	<0.00001***	0.00000
All 8 samples of 2004 season	0.0952	<0.00001***	0.00000
Both samples of 2005 season (both were WA)	0.0646	0.00075***	0.00033
All 6 Western Australia samples (Sep 03–Feb 06)	0.0578	<0.00001***	0.00000
All 5 Queensland samples (Aug 04–Dec 04)	0.1476	<0.00001***	0.00000
All 7 Victorian samples (Nov 03–Dec 04)	0.0116	0.01065*	0.00327
All 6 Victorian samples of 2003 season	0.0127	0.01295*	0.00255
Local variation (3) at Werribee Vic. (Dec 03)	0.0043	0.34600	0.00790
Temporal variation at Garvoc Vic. (03–04)	0.0015	0.26120	0.00636
Both Western Australia samples in 2003 season	0.1275	<0.00001***	0.00000
Both Western Australia samples in 2004 season	−0.0059	0.9171	0.00307
Temporal variation at Walkway WA (03–06)	0.0327	0.0009***	0.00410
Local variation (2) at Stanthorpe Qld. (Dec 04)	0.0264	0.00019***	0.00014

Contingency test probability * $P<0.05$, *** $P<0.001$.

probably selected for in populations with early use of DDT. The T921I substitution is thought to be an additional mutation that originally occurred in a copy of the gene that already harboured the *kdr* mutation (Sonoda *et al.*, 2008a). Our data are consistent with this interpretation since, in the ~2700 *P. xylostella* genotyped in this study, we found no evidence of these two mutations occurring on different chromosomes.

The F1020S substitution, on the other hand, occurs in field populations independently of *kdr* and *skdrl*, only being found in the *cdr* haplotype in the absence of the L1014F and T921I substitutions. Thus, it appears as if the F1020S mutation has arisen on a wild-type sequence of the gene, likely in recent times and possibly within Australia (unless it was present, but undetected, in other non-Australian studies due to primer design issues mentioned previously). It was not observed by Sonoda *et al.* (2008b) who looked thoroughly for mutations throughout the gene in *P. xylostella* from Japan.

Records of *P. xylostella* in Australia date from 1882 (Linnean Society of NSW, 1884; Tryon, 1889) and microsatellite and mtDNA data suggest that it is relatively isolated from SE Asian, African and Korean populations, that it has reduced variability, is genetically homogeneous and that it experienced a founder effect in recent times (Endersby *et al.*, 2006; Saw *et al.*, 2006). The L1014F and T921I mutations must have been present in the founding populations at low frequencies. The F1020S substitution may have been overlooked elsewhere or may be confined to Australian *P. xylostella*.

The genotype and haplotype survival patterns from the resistance bioassays strongly suggest that Australian *P. xylostella* populations harbour two alleles of the *para* gene that confer pyrethroid resistance, the *skdrl* allele and the *cdr* allele, that give rise to three resistant genotypes, the corresponding two homozygotes and individuals that are heterozygous for this pair of alleles. In general, excellent agreement of genotype frequencies in field samples, and in first laboratory-generation mass-reared individuals (data not shown), to Hardy-Weinberg expected frequencies suggest that there are no marked fitness costs (viability costs) to individuals bearing the resistance alleles, a point previously made about the *P. xylostella kdr* and *skdrl* haplotypes in Korean populations (Kwon *et al.*, 2004).

Selection for single-gene insecticide resistance is usually thought of in terms of dominance or recessive gene-substitution models. However, the patterns of genotype-resistance association among Australian *P. xylostella* suggest the possibility that a heterozygous advantage model is also appropriate. While not statistically significantly so, the survival ratio of the *cdr*/*skdrl* heterozygote was the highest of any genotype in our data set and suggests that it is at an advantage under pyrethroid stress. The mechanistic basis of any such heterosis is unknown. A heterozygous advantage fitness regime would help maintain both resistance alleles in a population that is repeatedly exposed to pyrethroid spraying, especially if there is a fitness cost (reproductive or subtle viability cost) associated with either homozygote.

Both of the resistance alleles are widespread in Australian field samples, their combined frequency falling between 25% and 59% (fig. 4, table 2). This has allowed a high resistance level to be attained by different means in different Australian populations, the most resistant sample (LC_{50} = 330 ppm) having come from Tenthill in Queensland where the *skdrl* haplotype is at particularly high frequency (fig. 4). Contrast this to the relatively high level of resistance at Yuna (LC_{50} = 272 ppm) more than 2500 km away in Western Australia where the *cdr* allele is at very high frequency, and *skdrl* is almost absent. This is not a function of distance apart, however, since Stanthorpe B, quite close by to Tenthill in Queensland, with high resistance (LC_{50} = 181 ppm) has a high frequency of both resistance alleles, particularly *cdr*, in contrast to Tenthill. Local patterns of insecticide use, in type and intensity, may be responsible for such close-by spatial disparity of resistance allele frequencies. This pattern contrasts with the generally low level of population structure seen in the analysis of microsatellite and mtDNA markers across Australia.

Another main mechanism of resistance to insecticides found in *P. xylostella* is the enhanced action of cytochrome P_{450} oxidases (Sun, 1992), but this has not been studied in detail in Australian populations. The *kdr* type resistance is unaffected by synergists (Soderlund & Knipple, 2003); therefore, any synergisable pyrethroid resistance identified in *P. xylostella* should be investigated further in case a P_{450} mechanism is acting.

Some of the LC_{50} data reported here were included in a recent spatial and temporal analysis of a much larger data set on pyrethroid resistance variation across Australia over an 11 year period (Endersby *et al.*, 2008). In that study, differences in the incidence of resistance among local populations were often stable over several years and local selection brought on by local patterns of insecticide use were postulated to be factors responsible for the spatial resistance heterogeneity. Our current data on spatial allelic variation in a major resistance gene are consistent with this idea. So, despite the known long-distance dispersal and colonisation behaviour of

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Table 4. Survival performance of *P. xylostella para-sodium channel* genotypes and haplotypes following leaf-dip bioassay of permethrin resistance carried out between 2003 and 2006 on field-collected or early generation laboratory-reared larval instars.

Collection site and date of bioassay	$n^{\dagger}$	$n^{\#}$	Bioassay data		Genotype increase (+) or decrease (–) among survivors [* indeterminate – no controls or no survivors]											Haplotype change				
			LC50 (ppm)	Resistance ratio	CCT/CTT *w/kdr*	CCC/CCT *w/cdr*	CTT/CTT *kdr/kdr*	CCT/TTT *w/skdrl*	CCT/CCT *w/w*	CCC/CTT *cdr/kdr*	CTT/TTT *kdr/skdrl*	CCC/CCC *cdr/cdr*	CCC/TTT *cdr/skdrl*	TTT/TTT *skdrl/skdrl*	Control vs Survivors differentiationψ χ^2	CCT *w*	CCC *cdr*	CTT *kdr*	TTT *skdrl*	Control vs Survivors differentiationψ χ^2
Garvoc VIC, Jan. 04	65	22	89.5§	11.2	–	–	–	–	*	+	+	+	+	+	23.03***	–	+	–	+	20.25***
Garvoc VIC, Jan. 05	27	8	29.2§	3.2	–	*	–	*	*	–	+	+	+	+	7.32*	–	+	–	+	6.95*
Gatton QLD, Sept. 04	24	39		Diagnostic dose only	–	–	–	–	*	–	+	+	+	+	13.45**	–	+	–	+	11.98**
Gatton Research Station QLD, Sept. 04	66	5	28.6§	5.8	–	–	+	*	*	–	–	+	+	*	32.8***	–	+	–	+	16.5***
Geraldton WA, Nov. 04	201	118	140.4§	12.6	–	–	–	–	–	–	–	+	+	+	40.1***	–	+	–	+	37.7***
Glenore Grove QLD, Sept. 04	8	15		Diagnostic dose only	*	*	–	*	*	–	+	+	+	*	4.11	*	+	–	+	2.81
Grantham QLD, Sept. 04	11	23		Diagnostic dose only	–	+	–	*	*	–	+	+	–	+	1.19	–	+	–	+	1.01
Knoxfield VIC, Dec. 03	28	16		Diagnostic dose only	*	*	–	–	*	–	+	–	+	+	6.37**	–	–	–	+	5.95
Lindenow VIC, Apr. 04	144	47	90.0	6.5	–	–	–	–	+	+	–	+	+	+	7.45*	–	+	–	+	8.39*
Moree NSW, Nov. 03	41	29	123§	12.9	–	–	–	–	*	–	+	+	+	*	18.63***	–	+	–	+	15.5***
Stanthorpe B QLD, Jan. 05	61	69	180.8§	25.1	*	–	–	+	*	–	–	–	+	+	5.15	+	+	–	+	5.27
Stanthorpe G QLD, Jan. 05	52	6		Diagnostic dose only	–	–	–	*	–	+	+	–	*	*	2.33	–	+	*	+	1.85
Stanthorpe H QLD, Jan. 05	7	1		Diagnostic dose only	*	*	*	+	*	*	*	–	+	*	2.76	*	*	–	+	2.02
Tenthill QLD, Sept.–Nov. 04	152	148	312.9§	63.1	*	–	–	–	*	–	–	+	+	+	19.81***	–	+	–	+	21.64***
Three Springs WA, Nov. 04	62	26	41.7§	9.0	–	–	–	*	–	–	–	+	+	*	16.40***	+	+	–	+	18.63***
Walkaway WA, Nov. 03	53	34	117.5§	12.3	–	–	+	*	+	+	–	–	+	*	4.77	–	+	–	+	4.34
Walkaway WA, Mar. 06	66	42	144.8	32.0	–	*	–	*	*	+	–	–	*	+	9.33**	+	–	–	+	9.06*
Werribee Sth K VIC, Dec. 03	21	15	106.7§	14.4	–	*	–	*	*	+	+	–	–	+	1.03	–	–	+	+	0.63
Werribee Sth M VIC, Dec. 03	21	14		Diagnostic dose only	–	–	–	–	–	–	–	+	+	+	13.61**	–	+	–	+	11.94**
Werribee Sth Sl VIC, Dec. 03	32	10		Diagnostic dose only	+	–	+	+	–	–	–	*	–	–	0.91	–	–	+	–	0.90
White Peak WA, Oct. 05	78	57	74.4	33.7	–	*	–	*	*	+	–	+	+	+	11.04**	–	+	–	+	11.00**
White Peak WA, Nov. 05	124	83	58.4§	15.5	–	+	–	–	*	+	–	+	+	+	3.83	+	+	–	+	3.51
Yuna WA, Nov. 03	51	35	272.2§	28.5	–	*	–	*	*	+	*	+	*	*	3.95	–	+	–	–	2.80
Yuna WA, Apr. 04	14	24	115.7§	8.3	–	–	–	–	*	+	+	+	+	*	7.22**	–	+	–	+	8.54*
Waite Colony Jan. 04 to Mar. 06 (10)	306	39			–	*	*	–	–	+	+	+	*	*	56.10***					20.51***
Controls-weighted average frequency					0.072	0.037	0.185	0.018	0.167	0.247	0.104	0.065	0.070	0.034		0.074	0.319	0.458	0.148	
Survivors-weighted average frequency					0.003	0.026	0.089	0.004	0.036	0.270	0.094	0.156	0.219	0.103		0.032	0.443	0.274	0.250	
Ratio Survivors/Controls					**0.045**	**0.706**	**0.478**	**0.239**	**0.214**	**1.093**	**0.901**	**2.405**	**3.136**	**2.985**		**0.432**	**1.387**	**0.599**	**1.681**	
Number of assessable samples					20	16	23	14	8	24	23	24	21	15						
Percentage of samples with genotype in excess					5.0	12.5	13.0	21.4	25.0	45.8	47.8	70.8	85.7	93.3						
c^2 Goodness-of-fit to 50:50 ratio					16.20***	9.00**	12.57***	4.57*	2.00	0.17	0.04	4.17*	10.71**	11.27***						

$n^{\dagger}$ = number of controls genotyped.
$n^{\#}$ = number of survivors genotyped.
§ LC50 previously reported in Endersby *et al.*, 2008.
ψ Contingency test probability.
* $P<0.05$, ** $P<0.01$, *** $P<0.001$.

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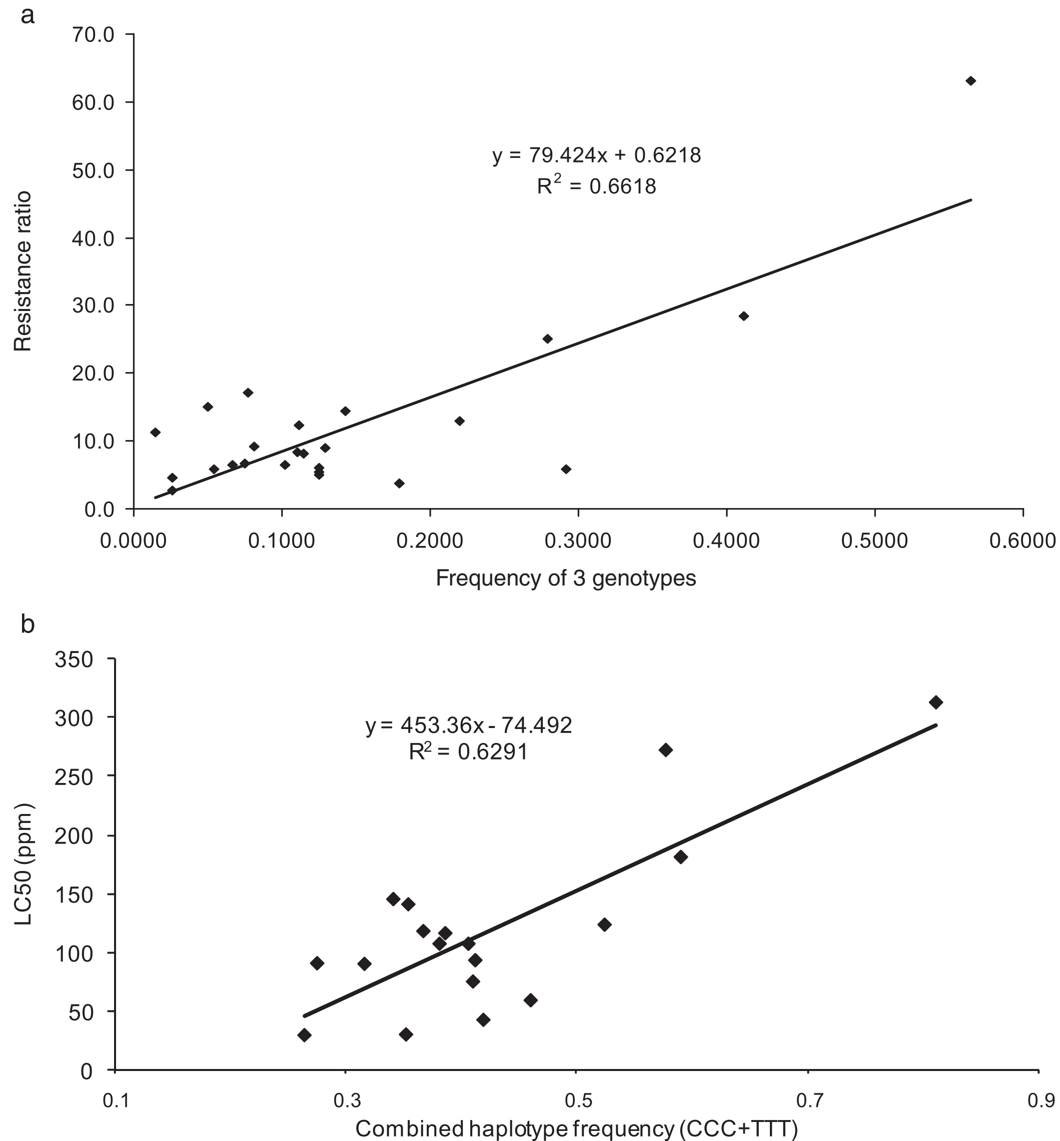

Fig. 5. Associations in *Plutella xylostella* between Australian geographic variation in permethrin resistance and genotype variation at the *para* gene locus. (a) observed combined frequency of the three resistant genotypes (*cdr/cdr + cdr/skdrl + skdrl/skdrl*) versus the resistance ratio; and (b) combined frequency of the two resistant haplotypes (*cdr + skdrl*) versus LC_{50}.

this species, in Australia (Endersby *et al*., 2006; Saw *et al*., 2006) and elsewhere in the world (Chapman *et al*., 2002; Honda, 1992), that effectively homogenizes genetic marker frequencies at 'neutral' loci, local contemporary selection maintains spatial allelic differentiation at a locus that has a major effect on an adaptive trait. In fact, several studies now interpret the heterogeneity in spatial variances between loci, as is the case here for microsatellite loci and the *para* gene variation, to be a signature of selection (Avise, 2004; and see Lewontin, 1974).

Finally, the capability to test for pyrethroid resistance may be valuable for the horticultural and grain industries, for a number of reasons: (i) to identify a resistance mechanism early in an outbreak, allowing for more informed treatment decisions; (ii) to confirm that resistance rather than defective insecticide contributed to spray failure; (iii) to help monitor change in resistance level over time, allowing for general advice to the industry; and (iv) to help to identify that new resistance mechanisms have arisen, thus adding to our understanding of resistance evolution and improving prospects for control. Once these are identified, simple and inexpensive SNP assays targeted to the most common resistance mutations, such as those used here, provide the most flexible way to track their variation in space and time.

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