

Using a PCR diagnostic for detection of insecticide resistance in *Tribolium castaneum* populations

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Abstract

Tribolium castaneum Herbst is a multiply resistant pest for which novel pesticides are desirable. The study of genetic structure in insect populations is important for understanding the spread of insecticide resistance. A PCR-based resistance-monitoring technique has been developed and used for screening worldwide collections of *T. castaneum* field strains. The technique provides a rapid and precise method of documenting the presence of resistance-associated mutations in a single gene, *Rdl*, that confers resistance to cyclodienes. PCR can be performed on pooled samples to detect the presence of an altered *Rdl* gene in a population, or individuals can be analysed to obtain frequency data. In a parallel study *T. castaneum* field strains were subjected to a conventional insecticide bioassay. We screened 119 strains from 23 countries and found resistance in 37 (31%) strains originating from 11 countries. The PCR diagnostic described above has been performed on 7 homozygous resistant strains from different continents to test if they carry the same resistance associated mutation. Preliminary analysis revealed that all these strains, widely separated by origin, have the same resistance associated mutation of alanine to serine. We will continue testing the remaining resistant strains for presence of this mutation.

Introduction

Cyclodiene resistance has accounted for over 60% of reported cases of insecticide resistance (Georghiou 1986). Therefore, although the cyclodienes themselves have largely been withdrawn from use, resistance to these compounds forms a highly representative and useful system in which to answer fundamental questions relating to the molecular and population genetics of insecticide resistance (ffrench-Constant 1994). In order to facilitate the analysis of resistance in red flour beetle, *Tribolium castaneum* (Herbst), populations we sought to design a molecular diagnostic for cyclodiene-resistant genotypes and to correlate this technique with discriminating doses of insecticide.

The *lin-R* strain of *T. castaneum* is resistant to lindane and cyclodiene insecticides. Repeated backcrossing of the *lin-R* strain to a susceptible strain *Lab-S* revealed that resistance was associated with a single major gene. This gene was mapped to the far left end of the third linkage group (Beeman and Stuart 1990). Detailed studies on the mechanism of resist-

ance revealed insensitivity of the nervous system to dieldrin but no differences in penetration or metabolism between susceptible and resistant strains (Lin et al. 1993). This target site insensitivity is consistent with the observed mechanism of resistance in *Drosophila melanogaster*, where a single amino acid replacement (alanine302 to serine) in the proposed lining of the γ -aminobutyric acid (GABA) gated chloride ion channel *Rdl*, causes insensitivity to cyclodienes (ffrench-Constant et al. 1993a,b).

Recently we have reported that the cyclodiene-resistant *Tribolium* strain *lin-R* also carries the same alanine to serine mutation as *Drosophila* and a number of other resistant insects (Thompson et al. 1993b). In this paper, we describe the use of the polymerase chain reaction to selectively amplify a specific resistance 'allele' (PCR amplification of specific alleles or PASA) from a number of strains collected worldwide. The design of this diagnostic will not only allow for the correlation of the presence of the mutation with resistance in a number of strains but also in the longer term will facilitate an analysis of the number of independent origins of cyclodiene resistance in *Tribolium* populations.

Experimental Methods

Tribolium strains

Reference laboratory susceptible and resistant strains were *Lab-S* (strain 1) and the isogenic *lin-R* (strain 2) whose origins have been previously described (Beeman and Stuart 1990). The code names, town and country of origin of the other field collected strains used were as follows. Collection dates are given when known (strains are numbered in reference to Figure 3). 3) NDG-14, St John, New Brunswick, Canada, 1976. 4) WI-1, Madison, Wisconsin, USA, laboratory strain. 5) GER-1, Germany, Kunast strain resistant to organophosphorous insecticides, in laboratory for >10 years. 6) COL-2, Palmira, Columbia, 1989. 7) GW-9, Millmerran, Australia. 8) Z-5, Lake City, Minnesota, USA, 1988. 9) Heng-2, Chiang Mai University, Thailand, in laboratory for >3 years. 10) Tiw-5, Ballia, India, 1989. 11) GW-14, Mikelson, Australia. 12) BRM, Beaumont, Texas, 1988. F₁ crosses to generate resistant heterozygotes (*RS*) were performed by isolating individual pupae and then reciprocally mating resistant (*RR*) and susceptible (*SS*) males and females.

Insecticide bioassays

Insecticide bioassays were performed in 100 × 15 mm plastic petri dishes (Falcon). Varying concentrations of dieldrin in 4 mL of ethanol were applied to the both halves of the dishes (2 mL per half) and evaporated until dry. Approximately 30–50 adult *Tribolium* were added to each dish and kept at 25°C. Mortality, defined as inability to locomote when disturbed, was assessed at 48 hours. Concentration-mortality

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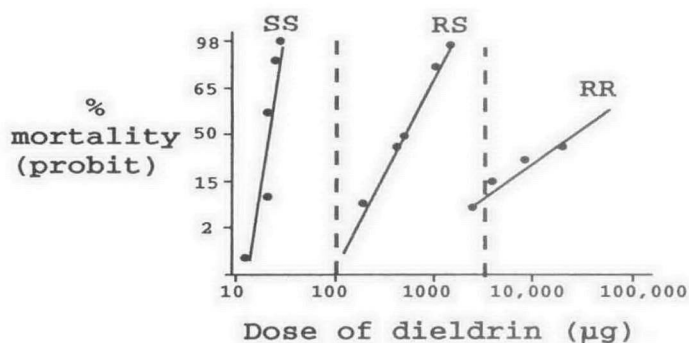


Fig. 1. Dose response curves of homozygous susceptible (*SS*), homozygous resistant (*RR*) and heterozygous (*RS*) cyclodiene resistant *T. castaneum*. The dotted lines show the doses that can be used to discriminate between *SS* and *RS* (100 mg with no *RS* mortality) and *RR* and *RS* (4000 mg with 10% *RR* mortality) genotypes.

responses were estimated by probit analysis (POLO-PC; Le Ora Software 1987).

Field strains were screened at a dose of 400 mg dieldrin which kills all *SS* individuals and allows survival of 85% of *RS* beetles (Fig. 1). Surviving putative resistant heterozygotes were then intermated and rescreened at a dose of 4000 mg at which only *RR* beetles survive (10% mortality).

Isolation and sequencing of cDNA

The PCR amplification and cloning of exon 7 of *Tribolium Rdl* has been described elsewhere (Thompson et al. 1993a). The resulting 80 bp probe was used to screen an adult *Tribolium* cDNA lgt22 library (16 hr hybridisation at 65°C in 6X SSC, 1X Denhardt's and 0.5% SDS; four washes at 65°C in 2X SSC and 0.5% SDS for 20 min), a kind gift of R. Dennell, Kansas State University. A single positive phage clone was purified and the 1799 bp insert subcloned into pBluescript. A series of nested deletions was generated across the clone using exonuclease III in the Erasea-Base System (Promega) and the resulting clones sequenced by the dideoxy chain termination method (Sanger et al. 1977) using the Sequenase II kit (United States Biochemical).

PCR amplification of a specific resistance allele

Genomic DNA was prepared from 10–20 beetles by standard procedures (Sambrook et al. 1989). Allele specific PCR primers were made by placing the resistance-associated single base mutation at the 3' end of the oligonucleotide. Both forward and reverse allele specific primers were made in order to test which best discriminates between alleles. PCR was performed between these primers and flanking primers predicted to be nested within exon 7 of *Rdl* which contains the resistance associated mutation. For PCR, approximately 100 ng of genomic DNA was added to a 50 µL reaction containing 0.2 mM of the allele specific primer, 0.2 mM of the allele independent primer, 0.2 mM dNTPs and 1.5 units of *Taq* polymerase. PCR was performed for 30 cycles with 1' denaturation at 94°C, 2' annealing at 50°C and 3' extension at 72°C. The concentration of magnesium in the reaction was varied (1.5, 2.0 or 2.5 mM MgCl₂) in order to determine the conditions under which specific resistance alleles were ampli-

fied. PCR products were electrophoresed in a 4% agarose gel and visualised by staining with ethidium bromide.

Results and Discussion

The LD₅₀, in mg of dieldrin per dish (95% CL; number of beetles tested [n], and slope±SE), for the *Lab-S* strain (*SS*) was 23.2 (21.0–25.7; n = 744; 11.5 ± 0.8) and for the *lin-R* (*RR*) strain 47,170 (29,920–106,314; n = 683; 1.1 ± 0.2). This yields a resistance ratio of 2033. This is higher than that previously observed with these strains (>190) following topical application of dieldrin (Beeman and Stuart 1990). However, it should be noted that confidence limits on the LD₅₀ estimate for the *lin-R* strain are large due to the difficulty in killing more than 50% of *RR* insects at any dose (Fig. 1). The LD₅₀ for the F₁ (*RS*) progeny was 638.5 (557.1–724.5; n = 727; 4.0 ± 0.3). Although both the contact exposure method used here and the topical application used previously show the same semi-dominant resistance phenotype, the dose response curves obtained here are steeper, allowing for superior discrimination between genotypes (Fig. 1). Thus a dose of 100 mg discriminates unambiguously between *SS* and *RS* genotypes, while a dose of 400 mg kills only 10% of *RR* individuals. Further, the contact test is simpler and quicker to perform on large numbers of insects.

The 80 bp probe, previously isolated via the use of degenerate PCR primers (Thompson et al. 1993a), hybridised to an 11kb *EcoRI* restriction fragment on a genomic Southern blot (data not shown), confirming that the probe corresponds to a *Tribolium* gene. The same probe was used to isolate a 1799 bp clone from the cDNA library. Sequencing of this clone revealed that it comprises all of exon 7 and part of exon 8 of *Rdl*, as well as a large portion of the intron prior to exon 7 (Fig. 2a). The finding of unspliced introns in cDNAs is not without precedent for genes associated with the nervous system (Beall and Hirsh 1984), and a large number of incompletely processed *Rdl* cDNAs were also isolated in screens of *Drosophila* libraries (French-Constant and Rocheleau 1993). Of the 188 predicted amino acids coded for by the partial *Tribolium* cDNA, those that align with the *Drosophila* sequence show 85% identity (Fig. 2b) (the *Drosophila* gene being significantly longer in this region). Interestingly, in common with *Rdl* from the yellow fever mosquito *Aedes aegypti* (Thompson et al. 1993a), the *Tribolium* cDNA lacks most of the repetitive glycine and proline-rich sequence within the predicted intracellular domain of the GABA receptor. A number of roles have been proposed for such glycine repeats including 'hinge regions' connecting two domains of a protein and regions involved in protein-protein interactions, as in the *Drosophila* genes for *Ultrabithorax* (Beachy et al. 1985) and the pupal cuticular protein EDG 91 (Apple and Fristrom 1991), respectively. However, the much reduced presence of repeated glycines and prolines in the non *Drosophilids* may suggest that not all of this simple sequence is highly functional in the *Drosophila* cDNA.

The reverse PASA primer in combination with forward primer F1 (Fig. 3a) gave the correct sized 101 bp product and was also associated with fewer non-specific products at varying magnesium concentrations than the forward PASA primer. The reverse PASA primer was therefore used in all further analyses. Varying the concentration of magnesium in the PCR reaction revealed that the resistance allele was selectively amplified at the normal recommended concentration of 1.5 mM while no products were seen in susceptible control reactions (Fig. 3b). Analysis of a seven homozygous strains collected from five different continents showed selective amplification of the same resistance 'allele' from all the strains examined.

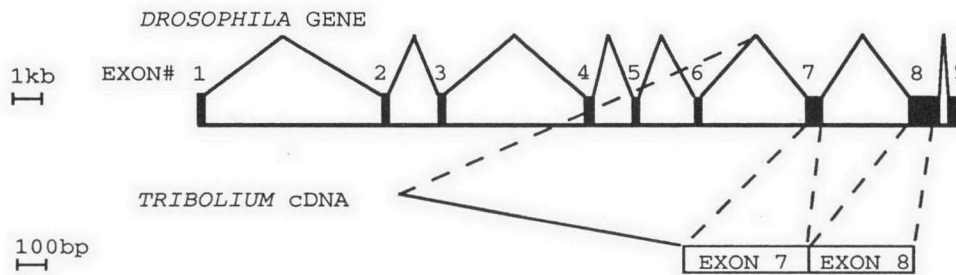


Fig. 2a. Location and sequence of the *T. castaneum Rdl* cDNA. Linear representation of the *Drosophila* GABA gated chloride ion channel gene showing its genomic organisation. The relative location of the *T. castaneum* cDNA is shown below.

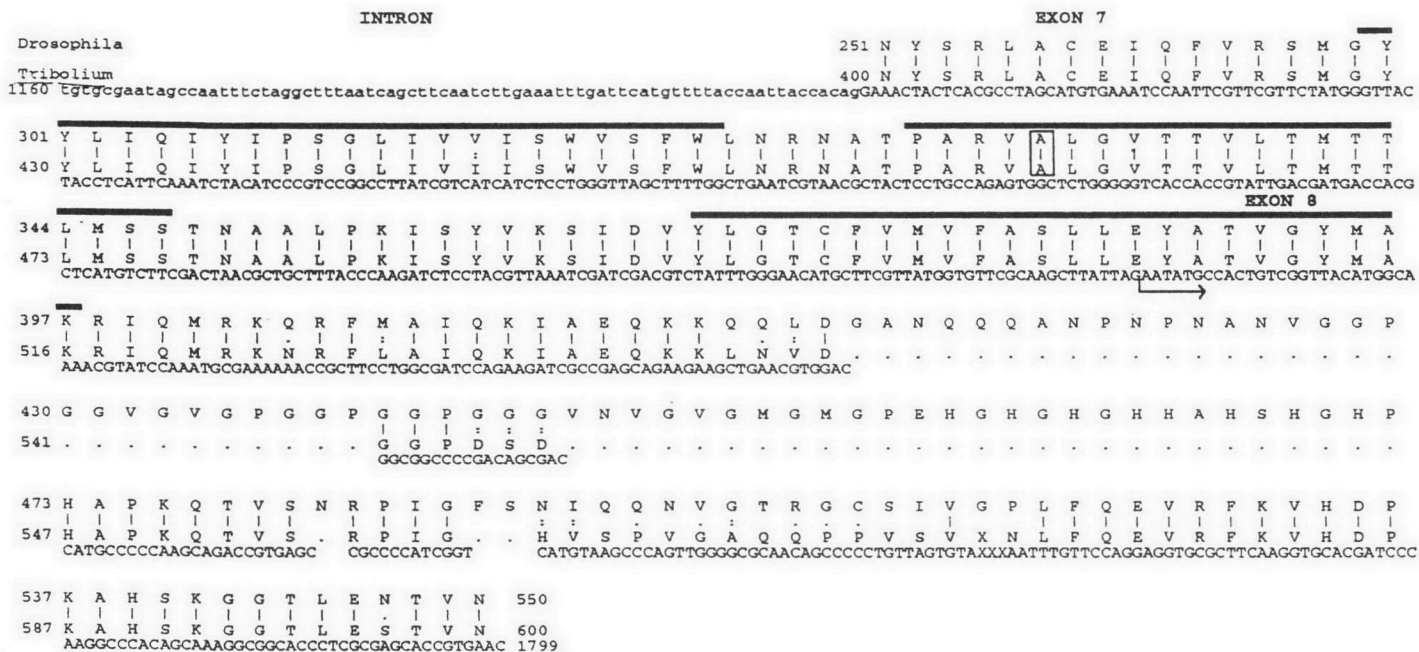


Fig. 2b. Location and sequence of the *T. castaneum Rdl* cDNA. Nucleotide and predicted amino acid sequence of the *T. castaneum* cDNA aligned to the *Drosophila* amino acid sequence. The proposed membrane spanning regions of the receptor are indicated above the sequence by solid lines. The position of the boundary between exon 7 and 8, predicted from the *Drosophila* sequence, is shown by an arrow. Nucleotide sequence within exons is uppercase and lowercase within introns.

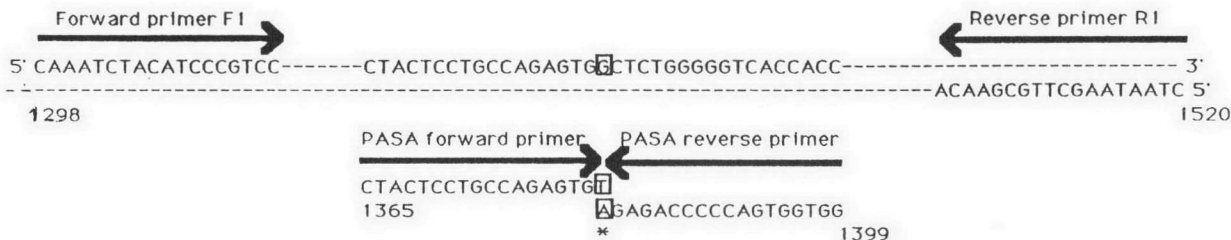


Fig. 3a. PCR mediated amplification of a specific cyclodiene resistance allele from *T. castaneum*. Sequence and relative location of allele specific (PASA) and allele independent (forward and reverse) primers used in selective amplification of the cyclodiene resistance allele. The nucleotide in susceptible sequence (top) substituted by the resistance associated mutation (G>T) and the complementary nucleotides at the 3' ends of the PASA primers are boxed.



Fig. 3b. PCR mediated amplification of a specific cyclodiene resistance allele from *T. castaneum*. PASA PCR products from a number of homozygous susceptible (SS) and resistant strains (RR) cyclodiene resistant strains. Strain numbers refer to those given in the text. Sizes of molecular markers are shown in base pairs (lane M) and a negative no DNA control is included (lane C).

The finding of the same cyclodiene-resistance-associated mutation in a number of widely separated *Tribolium* populations has potential implications both for the molecular basis of insecticide resistance, the population genetics of stored product insects and resistance management. In *Drosophila melanogaster* we have previously shown that the same alanine to serine replacement is associated with resistance in world-wide populations. Further, detailed sequence analysis of resistant haplotypes revealed that the resistance-associated mutation probably had a single origin (R.T. Roush, R.H. ffrench-Constant and C. Aquadro, unpublished data). As *Drosophila* is not an important pest and because its population ecology differs significantly from most insects of economic importance, particularly that of stored products pests, it is therefore important to ascertain the number of resistance alleles present in pest insect populations and the number of times they have arisen.

It should be recognised that the finding of the same resistance associated mutation in different *Tribolium* populations is not necessarily an indication that they all share the same original resistance *allele*, as individual mutations may also be constrained to the same location by functional considerations. A detailed analysis of each of the resistant haplotypes isolated is therefore necessary in order to determine how many times resistance has arisen independently. Nevertheless, these results form the first indications that the population genetics of resistance, as well as the resistance mechanism itself, may be similar between *Drosophila* and pest insects, despite their very different population ecologies. If, as in *Drosophila*, resistance in *Tribolium* has arisen a single time and spread globally, management of future resistance genes by quarantine becomes directly feasible. Thus, examination of grain shipments or other agricultural produce would not only guard against the invasion of introduced pest insects but would also prevent the introduction of new resistance genes into endemic pest populations.

Acknowledgments

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