

# A PCR Diagnostic For Cyclodiene Insecticide Resistance in the Red Flour Beetle *Tribolium castaneum*

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**Abstract:** A molecular diagnostic was used to examine the conservation of cyclodiene resistance associated mutations between different strains of *Tribolium castaneum* (Herbst.). An improved insecticide bioassay for discrimination between resistant genotypes was developed and seven resistant strains were established from five different continents. In order to develop a molecular diagnostic a partial cDNA of the cyclodiene insecticide resistance gene *Rdl*, a  $\gamma$ -aminobutyric-acid-gated chloride-ion channel, was cloned and sequenced. This cDNA spans exon 7, the region containing the resistance-associated mutation, and part of exon 8. An 'allele-specific' oligonucleotide primer, carrying the resistance-associated mutation at its 3' end, was used in combination with a flanking 'allele-independent' primer in the polymerase chain reaction to selectively amplify a single resistance-associated mutation from all seven strains collected worldwide. The implications of these findings for the population genetics of insecticide resistance and its management in pest insects via quarantine are discussed.

## 1 INTRODUCTION

Cyclodiene resistance has accounted for approximately 60% of reported cases of insecticide resistance.<sup>1</sup> Therefore, although the cyclodienes themselves have largely been withdrawn from use, resistance to these compounds forms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.<sup>2</sup> 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.

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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.<sup>3</sup> Detailed studies on the mechanism of resistance revealed insensitivity of the nervous system to dieldrin but no differences in penetration or metabolism between susceptible and resistant strains.<sup>4</sup> This target site insensitivity is consistent with the observed mechanism of resistance in *Drosophila melanogaster* Meig. where a single amino acid replacement (alanine 302 to serine) in the proposed lining of the  $\gamma$ -aminobutyric acid (GABA)-gated chloride-ion channel *Rdl* causes insensitivity to cyclodienes.<sup>5,6</sup>

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.<sup>7</sup> In this paper, we describe the use of the polymerase chain reaction to amplify selectively a specific resistance 'allele' (PCR amplification of specific alleles or PASA) from a number of strains collected worldwide. The design of this diagnostic will allow the correlation of the presence of the mutation with resistance in a number of strains. Further, in the longer term, in combination with detailed restriction enzyme and nucleotide sequence analysis of a number of alleles, it will facilitate an analysis of the number of probable independent origins of cyclodiene resistance in *Tribolium* populations.

## 2 EXPERIMENTAL METHODS

### 2.1 *Tribolium* strains

Reference laboratory susceptible and resistant strains were *Lab-S* (strain 1) and the isogenic *lin-R* (strain 2) whose origins have been described previously.<sup>3</sup> 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 Fig. 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 organophosphorus 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. All work with *SS* susceptibles was performed with *Lab-S*.

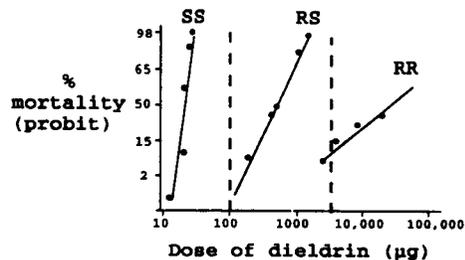


Fig. 1. Dose response curves of homozygous susceptible (*SS*), homozygous resistant (*RR*) and heterozygous (*RS*) cyclodiene-resistant *Tribolium castaneum*. The dotted lines show the doses that can be used to discriminate between *SS* and *RS* (100 µg with no *RS* mortality) and *RR* and *RS* (4000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

### 2.2 Insecticide bioassays

Insecticide bioassays were performed in 100 × 15 mm plastic Petri dishes (Falcon). Varying concentrations of dieldrin in ethanol (4 ml) were applied to the both halves of the dishes (2 ml per half) and evaporated until dry. 30–50 adult *Tribolium* were added to each dish and kept at 25°C. Mortality, defined as inability to move when disturbed, was assessed at 48 h. Concentration–mortality responses were estimated by probit analysis<sup>8</sup> and calculated using the software POLO-PC (Le Ora Software 1987).

Field strains were screened at a dose of 400 µg dieldrin (total dieldrin in both halves of the Petri dish) 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 µg at which only *RR* beetles survive (10% mortality). Resistance was recorded in 37 (31%) of 119 strains examined worldwide.

### 2.3 Isolation and sequencing of cDNA

The PCR amplification and cloning of exon 7 of *Tribolium Rdl* has been described elsewhere.<sup>7</sup> The resulting 80 bp probe was used to screen an adult *Tribolium* cDNA  $\lambda$ gt22 library (16 h hybridization 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 Erase-a-Base System (Promega) and the resulting clones sequenced by the dideoxy chain termination method<sup>9</sup> using the Sequenase II kit (United States Biochemical).

### 2.4 PCR amplification of a specific resistance allele

Genomic DNA was prepared from 10–20 beetles by standard procedures.<sup>10</sup> 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 method 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- $\mu$ l reaction containing 0.2  $\mu$ M of the allele-specific primer, 0.2  $\mu$ M 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 magnesium chloride) in order to determine the conditions under which specific resistance alleles were amplified. PCR products were electrophoresed in a 4% agarose gel and visualized by staining with ethidium bromide.

### 3 RESULTS AND DISCUSSION

The LD<sub>50</sub>, in  $\mu$ g of dieldrin per dish (95% CL; number of beetles tested [*n*], and slope  $\pm$  SE), for the *Lab-S* strain (*SS*) was 23.2 (21.0–25.7; *n* = 744; 11.5  $\pm$  0.8), and for the *lin-R* (*RR*) strain 47, 170 (20 920–106 314; *n* = 683; 1.1  $\pm$  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.<sup>3</sup> However it should be noted that confidence limits on the LD<sub>50</sub> 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 LD<sub>50</sub> for the F1 (*RS*) progeny was 638.5 (557.1–724.5; *n* = 727; 4.0  $\pm$  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  $\mu$ g discriminates unambiguously between *SS* and *RS* genotypes, while a dose of 4000  $\mu$ g kills only 10% of *RR* individuals. Further, the contact test is simpler and quicker to perform on large numbers of insects.

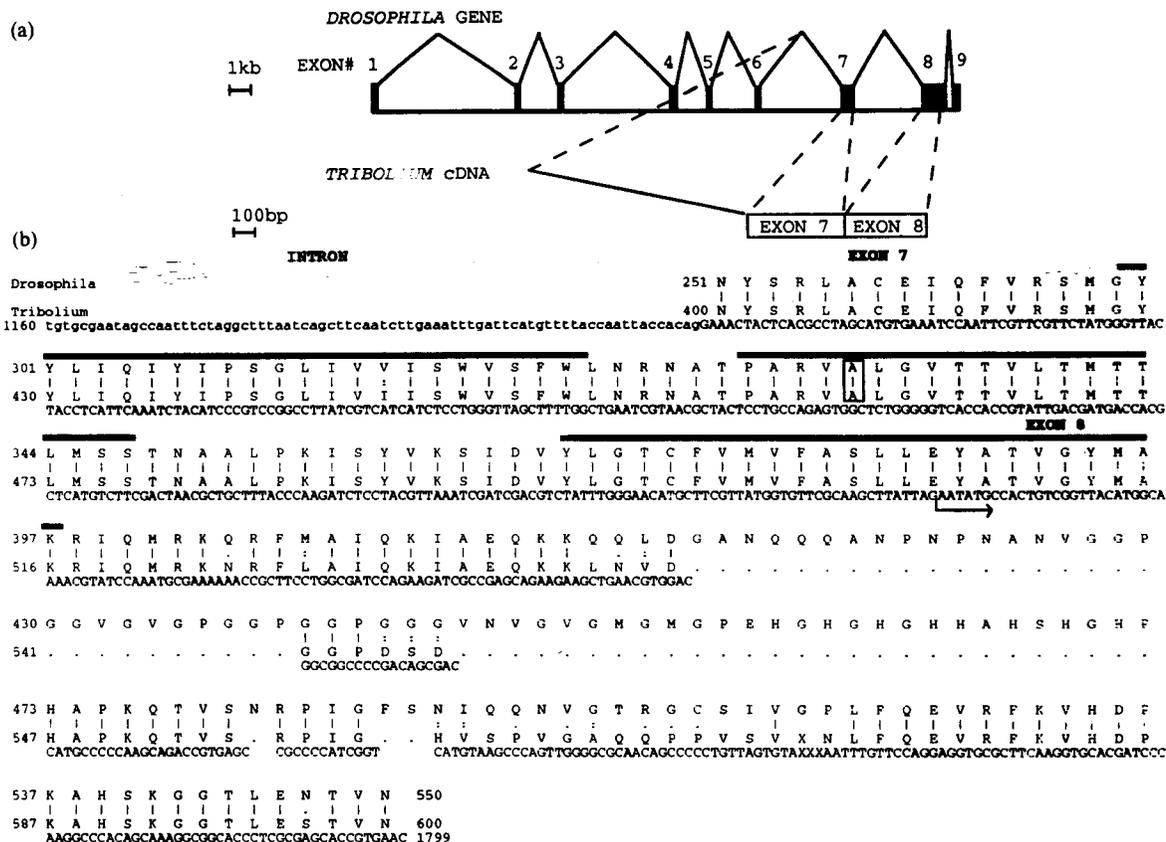
The 80 bp probe, previously isolated via the use of degenerate PCR primers,<sup>7</sup> hybridized to an 11 kb *Eco*RI restriction fragment on a genomic Southern blot (data not shown), confirming that the probe corresponds to a *Tribolium* gene. The same probe was used to isolate a 1799 bp clone from 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. 2(a)). The finding of unspliced introns in cDNAs is not without precedent for genes associated with the nervous system,<sup>11</sup> and a large number of incompletely processed *Rdl* cDNAs were also isolated in screens of *Drosophila* libraries.<sup>12</sup> Of the 188 predicted

amino acids coded for by the partial *Tribolium* cDNA, those that align with the *Drosophila* sequence show 85% identity (Fig. 2(b)) (the *Drosophila* gene being significantly longer in this region). Interestingly, in common with *Rdl* from the yellow fever mosquito *Aedes aegypti* L.,<sup>13</sup> 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*<sup>14</sup> and the pupal cuticular protein EDG 91,<sup>15</sup> 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.

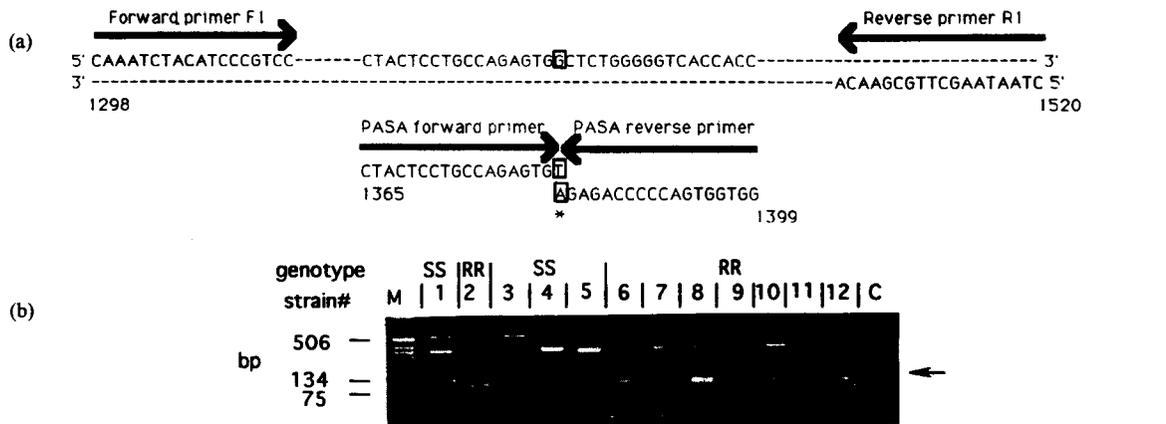
The reverse PASA primer in combination with forward primer F1 (Fig. 3(a)) gave the correct-sized 101 bp product and was also associated with fewer non-specific products at varying magnesium concentrations than the forward PASA primer. The reverse PASA primer was therefore used in all further analyses. 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. Analysis of seven homozygous strains collected from five different continents showed selective amplification of the same resistance 'allele' from all the strains examined (Fig. 3(b)).

No direct comparisons were made of the efficiency of the PASA diagnostic with insecticide bioassays for determining resistance gene frequencies in *Tribolium*. However, previous work with *Drosophila* field samples has shown that resistance frequencies from PCR-based diagnostics could be estimated to an accuracy of 1% with only a 0.5% standard error, from samples of only 100 insects, whereas frequency estimates from the bioassay of several hundred insects ranged from 0 to 10% on the same field collections.<sup>16</sup> Thus, although this PCR-based diagnostic may miss minor variation imposed by genes of secondary effect (such as reduced penetration) or extraneous differences imposed by the physiological condition of the insects following collection, much more accurate information on single gene frequencies is available from a much smaller sample size using this molecular diagnostic. We will seek to confirm that PASA is similarly more efficient than insecticide bioassay in *Tribolium* by using these techniques in parallel on the same samples of individuals collected directly from the field.

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



**Fig. 2.** Location and sequence of the *Tribolium castaneum* Rdl cDNA. (a) Linear representation of the *Drosophila* GABA-gated chloride-ion channel gene showing its genomic organization. The relative location of the *T. castaneum* cDNA is shown below. (b) 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 by the *Drosophila* sequence, is shown by an arrow. Nucleotide sequence is uppercase within exons and lowercase within introns.



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

management. In *Drosophila melanogaster*, we have previously shown that the same alanine-to-serine replacement is associated with resistance in worldwide populations. Further, detailed sequence analysis of resistant haplotypes revealed that the resistance-associated mutation probably had a single origin (R. T. Roush, R. H. French-Constant & C. Aquadro, unpublished results). As *Drosophila* population ecology differs significantly from that of 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 recognized that the finding of the same resistance-associated mutation in different *Tribolium* populations is not necessarily an indication that they all have 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 resistance haplotypes isolated is therefore necessary in order to determine how many times resistance has arisen independently. However, 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 other pest insects, despite their very different population ecologies. If resistance in *Tribolium* has arisen at 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.

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#### REFERENCES

1. Georghiou, G. P. In *Pesticide Resistance: Strategies and Tactics for Management*, ed. National Academy of Sciences. 14-43 National Academy Press, Washington DC, 1986.
2. French-Constant, R. H., *Ins. Biochem. Mol. Biol.* **24** (1994) 335-45.
3. Beeman, R. W. & Stuart, J. J., *J. Econ. Entomol.*, **83** (1990) 1745-51.
4. Lin, H., Bloomquist, J. R., Beeman, R. W. & Clark, J. M., *Pestic. Biochem. Physiol.*, **45** (1993) 154-65.
5. French-Constant, R. H., Steichen, J., Rocheleau, T. A., Aronstein, K. & Roush, R. T., *Proc. Natl Acad. Sci. USA*, **90** (1993) 1957-61.
6. French-Constant, R. H., Rocheleau, T. A., Steichen, J. C. & Chalmers, A. E., *Nature (London)*, **363** (1993) 449-51.
7. Thompson, M., Steichen, J. C. & French-Constant, R. H., *Ins. Mol. Biol.*, **2** (1993) 149-54.
8. Finney, D. J., *Probit Analysis* 1-319. Cambridge University Press, Cambridge, UK, 1952.
9. Sanger, F., Nicklen, S. & Coulson, A. R., *Proc. Natl Acad. Sci. USA*, **74** (1977) 5463-7.
10. Sambrook, J., Fritsch, E. F. & Maniatis, T., *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Lab., Cold Spring Harbor, NY, 1989.
11. Beall, C. F. & Hirsh, J., *Molec. Cell. Biol.*, **4** (1984) 1669-74.
12. French-Constant, R. H. & Rocheleau, T., *Neurochem.*, **60** (1993) 2323-6.
13. Thompson, M., Shotkoski, F. & French-Constant, R., *FEBS Letters*, **313** (1985) 545-51.
14. Beachy, P. A., Helfand, S. L. & Hogness, D. S., *Nature*, **313** (1985) 545-51.
15. Apple, R. T. & Fristrom, J. W., *Dev. Biol.*, **146** (1991) 569-82.
16. Aronstein, K. & French-Constant, R. H., *Pestic. Biochem. Physiol.*, (1994) (in press).