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Single-stranded DNA conformation polymorphism at the *Rdl* locus in *Hypothenemus hampei* (Coleoptera: Scolytidae)

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The homologue of the resistance to dieldrin gene (*Rdl*) in *Drosophila melanogaster* was cloned and sequenced in the scolytid beetle *Hypothenemus hampei*, a coffee pest resistant to cyclodiene insecticides in New Caledonia. The amino acid sequence of the *Rdl* exon no. 7 protein product in *H. hampei* was identical to that in *D. melanogaster* and showed the same amino acid change as that characterizing susceptible vs. resistant *D. melanogaster*. Samples from natural *H. hampei* populations (from Asia, the Pacific Islands, Africa and Central America), from reference susceptible (S) and resistant (R) laboratory strains, and from their hybrid progenies, were analysed at the *Rdl* locus using single-stranded DNA conformation polymorphism on polymerase chain reaction products. The susceptible allele was the only allele present in all samples from natural populations except in the only resistant population known to date (Ponerihouen, New Caledonia). Females and some males obtained as F_1 from R × S crosses were heterozygous at the *Rdl* locus, confirming that this local mate competing species is diplo-diploid.

Keywords: *Hypothenemus hampei*, insecticide resistance, local mate competition, pseudoarrhenotoky, SSCP.

Introduction

One single base-pair substitution in exon no. 7 of the resistance to dieldrin (*Rdl*) locus (encoding a γ-aminobutyric acid (GABA) receptor/gated ion channel) is responsible for 1000-fold resistance to cyclodiene insecticides in *Drosophila melanogaster* (ffrench-Constant *et al.*, 1993a). This mutation causes an alanine 302 (Ala) to serine (Ser) substitution which presumably affects the tertiary structure of the membrane spanning domain 2 of the GABA receptor, reducing its binding affinity to the insecticide. The same Ala to Ser point mutation has been identified in resistant vs. susceptible *D. simulans*, *Tribolium castaneum* and other insects (ffrench-Constant *et al.*, 1993b; Thompson *et al.*, 1993).

Cyclodiene resistance has been reported in the scolytid *Hypothenemus hampei* in New Caledonia (Brun *et al.*, 1989). Three resistance phenotypes have so far been observed in insecticide resistance tests on samples of females from natural *H. hampei* populations. These were the resistant phenotype (hereafter referred to as [R]), the susceptible phenotype [S] and an intermediate phenotype [I] for which the response curve was about halfway between those of [S] and [R] on a log-probit scale (Brun *et al.*, 1995b; P. Borsa and V. Gaudichon, unpublished data). Laboratory strains have been constituted from, respectively, [R]-only and [S]-only samples from natural populations, then inbred for several generations. Female F_1 progeny from [R] strain × [S] strain crosses invariably exhibited the phenotype [I] (Brun *et al.*, 1995b). These results suggest that resistance to endosulfan is determined by a single locus with codominant alleles in *H. hampei*, as has been demonstrated in *D. melanogaster* (ffrench-Constant *et al.*, 1993a).

ffrench-Constant *et al.* (1994) observed that the homologue in *D. melanogaster* of the Ser 302 amino acid in the GABA receptor's membrane spanning domain 2 was present in resistant *H. hampei*, making the *Rdl* gene a likely candidate for the determination of cyclodiene resistance in this species. It was

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thus interesting to study the population genetics of the *Rdl* gene in *H. hampei* and, in particular, to determine its level of polymorphism in natural populations and in resistant and susceptible laboratory strains.

The objectives of the present study were: (i) to obtain the sequence of a large portion of *Rdl* exon no. 7 in both resistant and susceptible *H. hampei*; (ii) to obtain a simple molecular method for determining genotypes at the *Rdl* locus in *H. hampei*; and (iii) to estimate the amount of polymorphism at the *Rdl* locus in populations of *H. hampei* from various localities worldwide and in laboratory strains. For this, single-stranded DNA conformation polymorphism (SSCP; Lessa & Applebaum, 1993) was developed on polymerase chain reaction (PCR) products of DNA extracts from individual *H. hampei*. In the process, heterozygosity was detected at the *Rdl* locus in both females and males, confirming that this local mate competing species (Hamilton, 1967; Kirkendall, 1993) is diplo-diploid. This result, together with karyological and resistance phenotype data suggested that pseudoarrhenotoky (Schulten, 1985) is the system of sex determination in *H. hampei*.

Materials and methods

Reference H. hampei *strains*

The reference resistant strain (R) was derived from a sample (sample size, N = about 1000 fertilized females) from Ponerihouen in New Caledonia (Brun *et al.*, 1989). It was inbred in the laboratory for 11 generations, subjected to selection by endosulfan insecticide every generation, with 100 to 1000 [R] fertilized females retained for the next generation. The reference susceptible strain (S) was derived from a sample (N = about 200 fertilized females) from La Foa in New Caledonia (Brun *et al.*, 1989). It was inbred in the laboratory for four generations. Every generation, all 100–200 mothers were tested for endosulphan resistance and all exhibited the [S] phenotype. One hundred to 200 of their fertilized female progeny, not subjected to the test, were used for making up the next generation.

The insecticide tests are presented in Brun *et al.* (1989, 1995b). The insects were reared on artificial diet. Methods for rearing and diet composition have been presented elsewhere (Brun *et al.*, 1993, 1995a). Some of the material used in the present work was the hybrid F_1 progeny of crosses between resistant and susceptible reference strains.

Samples from natural populations

Hypothenemus hampei females were obtained from samples of parasitized coffee berries collected in coffee fields from Labuhan Ratu (Sumatera Selatan, Indonesia), Sumbawa Besar (Nusa Tenggara Barat, Indonesia), Ngadirejo (Jawa Tengah, Indonesia), Cagayanat de Oro (Mindanao, Philippines), Sawee (Chumphon, Thailand), La Foa, Canala and Ponerihouen (New Caledonia), Tahiti-Nui and Tahiti-Iti (French Polynesia) and Man and Toubou (Côte d'Ivoire). Emerging females were drawn at random from each sample of parasitized berries and preserved in 70 per cent ethanol or frozen at −80°C or in liquid nitrogen for subsequent analyses. Two to 21 individuals of each sample were assayed by PCR/SSCP. Two additional individuals were taken from a laboratory strain from Tapachula (Chiapas, Mexico) and assayed.

Cloning of H. hampei Rdl *genes and PCR procedures*

Genomic DNA was extracted from *H. hampei* resistant and susceptible reference strains as previously described for *Drosophila* (ffrench-Constant *et al.*, 1993b). Degenerate primers were designed from the amino acid sequence encoded by *Rdl* exon no. 7 in *Drosophila* (ffrench-Constant *et al.*, 1991). The degenerate forward primer was primer F5 of ffrench-Constant *et al.* (1994). The degenerate reverse primer, R8 (Fig. 1) was designed from the amino acid sequence comprised between cytosine 338 and phenylalanine 343 of the *Drosophila* GABA receptor β subunit (ffrench-Constant *et al.*, 1991).

For degenerate PCR approximately 50–200 ng of genomic DNA was added to a 50 μL reaction mixture containing 1 μM of each primer, 0.4 mM dNTPs, 1–5 mM $MgCl_2$ and 2 units of Taq polymerase. Thirty-five cycles of PCR were performed (93°C for 1 min; 45°C for 1 min; 72°C for 1 min) following a 2 min start at 93°C. PCR products were subjected to electrophoresis in 2 per cent agarose gel and visualized by staining with ethidium bromide.

A clean, single-banded PCR product of the expected size (251 bp) was obtained at a $MgCl_2$ concentration of 4 mM. This first round PCR product was excised from the agarose gel, homogenized in an equal volume of distilled water, and extracted by three cycles of freezing/thawing/spinning. One μL of the supernatant was used as the DNA template for a second round of PCR at 4 mM $MgCl_2$. The second round PCR product was visualized as a single clean band of the expected size.

```
5' TGCGAGATTCAATTCGTTCGTTCGATGGGTTACTACTTAATCCAGATCTA
   C  E  I  Q  F  V  R  S  M  G  Y  Y  L  I  Q  I  Y
   260                                              276

   CATCCCATCCGGTCTGATTGTCATTATTTCCTGGGTGAGTTTCTGGCTGA
    I  P  S  G  L  I  V  I  I  S  W  V  S  F  W  L  N
    277                                              293

   ATAGGAACGCCACCCCGGCTCGTGTGTCTCTCGGAGTAACCACTGTACTT
     R  N  A  T  P  A  R  V  S  L  G  V  T  T  V  L
     294                                          309

   ACCATGACTACTTTGATGTCTTCTACGAATGCTGCCCTCCCAAAAATATC
   T  M  T  T  L  M  S  S  T  N  A  A  L  P  K  I  S
   310                                              326

   ATACGTAAAATCGATCGACGTCTATTTGGGTACCTGCTTCGTCATGGTCTT  3'
    Y  V  K  S  I  D  V  Y  L  G  T  C  F  V  M  V
    327                                          342
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Fig. 1 Nucleotide sequence of the 251 bp PCR product of the *Rdl* exon no. 7 in a resistant *Hypothenemus hampei* strain (PN106, Ponerihouen, New Caledonia) (first line), and corresponding amino acid sequence (second line), identical to homologous sequence in *Drosophila melanogaster* (ffrench-Constant *et al.*, 1991; numbering on third line). Underlined: degenerate primer regions F5 (5′ end) and R8 (3′ end). Bold: serine homologous to serine 302 characteristic of resistant *D. melanogaster*.

Hence it was cloned into the PCRII plasmid (Invitrogen, San Diego) according to the manufacturer's instructions. DNA mini-preparations from positive clones (Sambrook *et al.*, 1989) were sequenced using the Sequenase II kit (Bethesda Research Laboratories, Bethesda) as double-stranded templates. Primer T7 (Invitrogen), within the plasmid, was used for the sequencing reaction. Specific primers were then designed within the region framed by degenerate primers F5 and R8.

Genomic DNA from single individuals was extracted after grinding each insect in 50 μL grinding buffer (0.01 M Tris HCl, pH 7.8; 0.005 M EDTA; 1 per cent SDS), then heating at 70°C for 45 min, then precipitating the proteins in 1 M K acetate, cooling on ice and spinning. The supernatant was then phenol-chloroform extracted (Sambrook *et al.*, 1989). Thirty-five cycles of PCR were then performed (94°C for 1 min; 50°C for 1 min; 72°C for 1 min), following a 2 min start at 94°C, on a reaction mixture containing about 50 ng genomic DNA, 0.1 mM primers, 3 mM $MgCl_2$, 0.4 mM dNTPs and 2 units of Taq polymerase.

The products of PCR using specific primers were subjected to SSCP. Single-stranded DNA conformation variants were cloned into plasmid PCRII and sequenced using Sequenase II.

Degenerate and specific primers for the *H. hampei Rdl* gene were synthesized by the University of Wisconsin Biotechnology Centre, Madison, according to our specifications.

Methods for SSCP

PCR products were heat-denatured and subjected to SSCP according to the methods of Coustau & ffrench-Constant (1995) for *Drosophila*. Gels were silver-stained according to a protocol similar to that of Wray *et al.* (1981). Controls incorporated into each SSCP gel included the PCR products from the *Ser* and *Ala* clones and a no-DNA PCR control.

Results

Three clones of the 251 bp degenerate PCR product of exon 7 in the reference *H. hampei* resistant strain (PN106, Ponerihouen, New Caledonia) were sequenced. All three sequences were identical (Fig. 1). These showed the same serine amino acid at site 302 homologous to that found in cyclodiene resistant *D. melanogaster*.

Specific forward (5′-TCGATGGGTTACTACTTA-3′) and reverse (5′-GCAGGTACCCAAATAGACG-3′) primers were designed from the sequence. They framed a fragment of size 216 bp, suitable for SSCP analysis. The PCR products of two reference susceptible individuals were cloned and three clones of each were sequenced. The only consistent change from the sequence in Fig. 1 was that a guanine (G) was present instead of a thymine (T) at the triplet responsible for the change from Ser 302 (TCT) to Ala (GCT). The other changes consisted of single base-pair substitutions at a rate of about 0.5 per cent. They were not consistent among clones and thus could be ascribed to random copy-errors in PCR.

Three different phenotypes were revealed through SSCP on PCR products of *Rdl* exon no. 7 in *H. hampei* (Fig. 2). The allele encoding Ala 302 (*Ala*) was expressed as a two-band pattern and the allele encoding Ser 302 (*Ser*) as a distinctive two-band pattern. Female progeny of S × S and R × R crosses

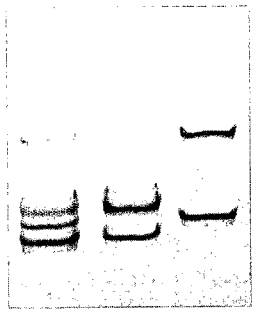

Fig. 2 Silver-stained SSCP gel showing the three phenotypes observed at the *Rdl* locus in *Hypothenemus hampei*. Ser, Ala, and *Ser/Ala*: SSCP phenotypes for PCR products from R individuals, S individuals and heterozygous R × S females, respectively.

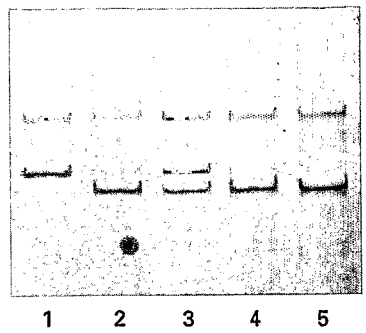

Fig. 3 Silver-stained SSCP gel showing artefactual variation in SSCP patterns for allele *Ala* at the *Rdl* locus in *Hypothenemus hampei*. SSCP phenotype in lane 5 was identical to the *Ala* phenotype of Fig. 2.

Table 1 SSCP phenotypes for F_1 male and female *Hypothenemus hampei* of different pedigrees

Mother	Father	SSCP phenotypes	*N*
F_1 females			
S	S	Ala	10
S	R	Ser/Ala	10
R	S	Ser/Ala	10
R	R	Ser	8
F_1 males			
S	S	Ala	7
S	R	Ala	17
R	S	Ser	17
		Ala	1
		Ser/Ala	3
R	R	Ser	7

S: La Foa reference susceptible strain; R: PN106 reference resistant strain; *N*: sample size.

exhibited the *Ala* and *Ser* phenotypes, respectively (Table 1). Female progeny of the mother R × father S or mother S × father R crosses exhibited a four band phenotype with bands migrating to the same position as a mixture of *Ala* and *Ser* bands (*Ser/Ala* heterozygous SSCP phenotype) (Fig. 2). Single-stranded DNA conformation phenotypes for males and females from all four types of crosses are reported in Table 1. Whereas the SSCP phenotypes exhibited by F_1 females were in accordance with Mendelian expectations, those for males somewhat departed from the Mendelian model.

All *H. hampei* individuals that were successfully amplified and subsequently analysed through SSCP, from natural populations from Sumatera (21), Sumbawa (12), Jawa (11), Mindanao (12), Chumphon (14), La Foa (4), Canala (10), Tahiti-Nui (6), Tahiti-Iti (2), Man (3), Toubou (8) (sample size in parentheses) as well as all two individuals from Chiapas and ten females from the La Foa S strain, showed the *Ala* SSCP phenotype. All individuals from Ponerihouen (6) as well as all eight females from the PN106 R strain showed the *Ser* phenotype. The *Ala* phenotype encompassed three minor single-stranded DNA conformation variants as shown in Fig. 3 (lanes 1, 3 and 5), whereby silver-stained bands varied in their relative intensities. All three SSCP variants of Fig. 3 were subsequently cloned and three to six clones of each were sequenced to test their relationship to different *Rdl* alleles. All sequences were identical to the *Ala* allele present in the S strain (except nonconsistent, minor variation among clones attributable to random PCR copy-errors), ruling out that the three-banded individuals were heterozygotes and leading to the conclusion that this type of variation among SSCP phenotypes was artefactual.

Discussion

The difference between cyclodiene resistant and susceptible *H. hampei* consisted of one point mutation homologous to that found in two *Drosophila* species, *D. melanogaster* and *D. simulans*, the mosquito *Aedes aegypti*, the house fly *Musca domestica*, the red flour beetle *Tribolium castaneum* and the cockroach *Periplaneta americana* (ffrench-Constant *et al.*, 1993b; Thompson *et al.*, 1993). These findings support the idea of considerable functional constraints at the amino acid site 302 in the GABA receptor/ion channel in insects. The Ala 302 to Ser mutation, having been detected in several insect species from unrelated groups, provides a good

example of convergence which is likely to have arisen solely from selection pressure. Neither cyclodiene resistance (Kern *et al.*, 1990; P. Borsa, L.-O. Brun, V. Gaudichon and C. Marcillaud, unpublished data) nor the Ala 302 to Ser mutation have been detected in populations of *H. hampei* other than that from the Ponerihouen region on New Caledonia's east coast, despite the use of cyclodiene insecticides worldwide.

This observation enforces the presumption of Raymond *et al.* (1992) that mutation rate is a limiting factor in the advent of resistance to insecticides. Further support for the hypothesis of functional constraints stems from the observation of no polymorphism at the *Rdl* locus (identical SSCP patterns) in various *H. hampei* populations worldwide.

Genetic variation at five presumed Mendelian allozyme loci was investigated by Borsa & Gingerich (1995) in *H. hampei* samples from Côte d'Ivoire, Mexico and New Caledonia. Three loci (*Idh-1*, *Idh-2* and *Gpi*) exhibited sample monomorphism and one locus (*Mpi*) was slightly polymorphic whereas another locus (*Mdh-2*) was highly polymorphic. No significant differences in allelic frequencies were detected between populations from different countries. Moreover, no differences were detected between resistant and susceptible populations from New Caledonia (Borsa & Gingerich, 1995). Apart from the presumably highly selected *Ser* allele in New Caledonia, worldwide sample monomorphism at the *Rdl* locus does not contradict these results, although no further conclusion can be drawn on population structure when addressing a monomorphic marker.

Hypothenemus hampei has some of the features associated with local mate competition in other insects (Hamilton, 1967; Kirkendall, 1993): (i) reproduction occurs between sibs within the coffee bean colonized by their mother and females disperse after mating; (ii) males are about half the size of females, are flightless, and undergo one fewer moult than females; and (iii) the sex ratio is strongly female-biased with about one male for ten females. This last feature has been considered to imply haplo-diploidy in insects (arrhenotoky: Kirkendall, 1993), where males are produced parthenogenetically by the development of unfertilized eggs and females arise from fertilized eggs. However, karyological studies in *H. hampei* revealed that the chromosome number was $2n$ in males, as in females (Bergamin & Kerr, 1951). Also, eggs laid by unfertilized females do not hatch (Bergamin, 1943; Giordanengo, 1992; Barrera Gaytan, 1994; but see Muñoz, 1989). Yet half the chromosomes of somatic cells in males appear as an intensely stained pack of heterochromatin (Bergamin & Kerr, 1951; J. Stuart, unpublished data) suggesting that they are transcriptionally inactive. Furthermore, whereas F_1 females from $R \times S$ crosses invariably exhibit the [I] phenotype, F_1 males almost exclusively appeared to express the maternal phenotype (Brun *et al.*, 1995b; P. Borsa, L.-O. Brun and V. Gaudichon, unpublished data).

In the present work, some of the hybrid $R \times S$ males analysed by SSCP exhibited the allele that was presumably transmitted by their mother, and one, that of his father. However, three males exhibited the heterozygous SSCP phenotype as expected in a diplo-diploid organism. Although these results cannot be interpreted unambiguously at the moment (but see Bull, 1983), an interesting point is that both females and males can be heterozygous at the *Rdl* locus. Therefore, diplo-diploidy was confirmed in *H. hampei*. As the paternal resistance gene(s) usually appear not to be expressed in males, we conclude that the paternal set of chromosomes is, in the majority of cases, somehow inactivated. Hence, the mechanism of sex determination is pseudoarrhenotoky, similar to that described in Phytoseiid mites (Schulten, 1985).

It would be interesting to test whether the set of paternal chromosomes apparently inactivated in somatic cells in F_1 males is also not transmitted to the F_2, for example by its elimination at meiosis. Such results would constitute a further test of the evolution towards cytological mechanisms for the control of biased sex ratios in local mate competing species.

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