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ORIGINAL ARTICLE

Phosphine resistance in India is characterised by a dihydrolipoamide dehydrogenase variant that is otherwise unobserved in eukaryotes

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Phosphine (PH_3) fumigation is the primary method worldwide for controlling insect pests of stored commodities. Over-reliance on phosphine, however, has led to the emergence of strong resistance. Detailed genetic studies previously identified two loci, *rph1* and *rph2*, that interact synergistically to create a strong resistance phenotype. We compared the genetics of phosphine resistance in strains of *Rhyzopertha dominica* and *Tribolium castaneum* from India and Australia, countries having similar pest species but widely differing in pest management practices. Sequencing analysis of the *rph2* locus, dihydrolipoamide dehydrogenase (*dld*), identified two structurally equivalent variants, Proline49 > Serine (P49S) in one *R. dominica* strain and P45S in three strains of *T. castaneum* from India. These variants of the DLD protein likely affect FAD cofactor interaction with the enzyme. A survey of insects from storage facilities across southern India revealed that the P45/49S variant is distributed throughout the region at very high frequencies, in up to 94% of *R. dominica* and 97% of *T. castaneum* in the state of Tamil Nadu. The abundance of the P45/49S variant in insect populations contrasted sharply with the evolutionary record in which the variant was absent from eukaryotic DLD sequences. This suggests that the variant is unlikely to provide a strong selective advantage in the absence of phosphine fumigation.
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INTRODUCTION

Phosphine (PH_3) fumigation has been used across the world for more than four decades as the primary method to disinfest stored grain from insect pests. Widespread and frequent use of PH_3 from the time of the United Nations Food and Agriculture Organisation (FAO) global survey in the 1970s (FAO, 1975), and which subsequently increased because of methyl bromide phase out led to the development of high levels of PH_3 resistance in several pest species including *Rhyzopertha dominica* (F.), *Tribolium castaneum* (Herbst) and *Cryptolestes ferrugineus* (Stephens) (Tyler *et al.*, 1983; Rajendran, 1992; Ren *et al.*, 1994; Acda *et al.*, 2000; Collins *et al.*, 2002; Benhalima *et al.*, 2004; Lorini *et al.*, 2007; Opit *et al.*, 2012; Nayak *et al.*, 2013). In Australia, strong resistance in *R. dominica* (600×) was first detected in 1997 (Collins *et al.*, 2002) and in *T. castaneum* strong resistance (431×) was found in 2000 (Jagadeesan *et al.*, 2012).

Detailed genetic analysis of these strains identified two loci conferring strong resistance in both *R. dominica* and *T. castaneum* (Jagadeesan *et al.*, 2012; Mau *et al.*, 2012a, b). The first, *rph1* (*tc-rph1* for *T. castaneum*), is responsible for weak resistance, whereas the second, *rph2*, acts synergistically with *rph1* to confer strong resistance (Schlipalius *et al.*, 2002). Complementation analysis of *R. dominica* strains collected from widely geographically separated locations in Australia (Queensland, New South Wales and South Australia) confirmed that the genes responsible for high-level resistance are highly conserved, in that *rph1* and *rph2* contribute to the resistance phenotype in all the outbreaks studied (Mau *et al.*, 2012a, b). This raises the question of whether this is the case for samples from different countries.

Following the above studies, Schlipalius *et al.* (2012) recently identified *rph2* as dihydrolipoamide dehydrogenase (DLD), a flavin-dependent oxidoreductase essential for energy metabolism (Patel and Roche, 1990) that contains a reactive disulfide and a FAD cofactor, which are directly involved in electron transfer (Williams, 1992). Resolution of the structure of DLD from human and yeast showed that the three-dimensional protein folding was highly conserved across taxa (Brautigam *et al.*, 2005). The protein structure also revealed that protein variants causing E3 deficiency diseases in humans occur at three general locations in the human enzyme: the dimer interface, the reactive disulfide and the FAD/NAD^+-binding sites. Schlipalius *et al.* (2012) also showed that the variants that cause resistance in *R. dominica*, *T. castaneum* and the nematode *Caenorhabditis elegans* (Maupas) also occurred at these general sites. This information now enables direct detection of resistance variants at the *rph2* locus in field populations (Kaur *et al.*, 2013).

Although high levels of PH_3 resistance have been reported in the key grain insect pests in India (Rajendran *et al.*, 2004), there has been no previous investigation into the genetics of resistance in that country. The observation that the same two genes mediate resistance

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in independently arising Australian strains (Mau *et al*., 2012a, b) indicated us that there may be a similar situation prevailing in Indian populations.

The present study investigates the extent to which *rph2* mediates PH_3 resistance in two the key stored product pests, *R. dominica* and *T. castaneum*, from two different geographic regions of the world with radically different grain storage and pest management practices. The widespread distribution and abundance of a single resistance allele in India led us to search the evolutionary record for evidence of it being neutral or under positive or negative selection in species that had not been exposed to PH_3.

MATERIALS AND METHODS

Insect strains

Two strongly PH_3-resistant *R. dominica* strains (an Australian strain, QRD569 and an Indian strain IRD_{Mdu}) were used in this study. QRD569 was collected near Millmerran, Queensland, in 1997 (Collins *et al*., 2002) and IRD_{Mdu} generated from samples collected in 2009 from central and state grain storage reserves in Madurai, Tamil Nadu and southern India. Four strains of *T. castaneum* (an Australian strain QTC931 and three Indian strains, ITC_{Mdu}, ITC_{Cbe} and ITC_{ND}) were used in this study. QTC931 was collected from a central storage at Natcha, Queensland, in 2000 (Jagadeesan *et al*., 2012), whereas ITC_{Mdu}, ITC_{Cbe} and ITC_{ND} were established from samples collected in 2009 from central and state storage reserves in Madurai (Tamil Nadu, southern India), Coimbatore (Tamil Nadu, southern India) and New Delhi (Delhi, northern India), respectively. The Australian strains had been selected previously for homozygosity, whereas the Indian strains had undergone three successive selections, soon after their initial collection to promote homozygosity of the resistance genes within the populations. Following preliminary resistance bioassays (FAO, 1975), *R. dominica* strain IRD_{Mdu} had been diagnosed as strongly resistant; therefore, this strain was purified using a discriminating dose for strong resistance of $0.25\,mg\,l^{-1}$ of PH_3 for 48 h. Between selections, the survivors of PH_3 selection were allowed to mate freely and their progenies were collected for further selection. Similarly, discriminating dose testing for the *T. castaneum* strains showed that ITC_{Mdu} was diagnosed as strongly resistant, whereas ITC_{Cbe} and ITC_{ND} were diagnosed as weakly resistant. Therefore, ITC_{Mdu} was purified using a discriminating dose for strong resistance of $0.25\,mg\,l^{-1}$ of PH_3 for 20 h, and a discriminating dose for weak resistance of $0.03\,mg\,l^{-1}$ for 20 h was used for ITC_{Cbe} and ITC_{ND}. *R. dominica* strains were maintained on organically grown wheat and *T. castaneum* on whole-wheat flour and yeast 20:1 at controlled regimes of 30 °C and 55% relative humidity (RH).

Insect survey collection from India

R. dominica and *T. castaneum* insect samples were collected in 2011–2012 at 30 and 31 different locations, respectively, from the southern Indian states of Tamil Nadu, Karnataka and Andhra Pradesh (Supplementary Figure S1, Supplementary Table S1). Grain samples were sieved for insects, which were identified, counted and subsequently cultured on whole-wheat flour at 30 °C and 55% RH.

Fumigation

All fumigations were undertaken according to the published standard method (FAO, 1975) for 20 h (*T. castaneum*) and 48 h (*R. dominica*) at 25 °C and 70% RH to enable more accurate probit analysis (Daglish *et al*., 2002). PH_3 was generated in a collection tube containing aluminium phosphide introduced into a solution of sulphuric acid (5%). PH_3 concentration was determined with gas chromatography using a thermal conductivity detector with nitrogen (N_2) as the standard (Winks and Waterford, 1986).

Mortality responses to PH_3 of the resistant strains were measured against a range of PH_3 concentrations, that is, $0.025–5.0\,mg\,l^{-1}$ for *R. dominica* and $0.008–15\,mg\,l^{-1}$ for *T. castaneum*. Fumigation was undertaken by placing 50 adult beetles (1- to 2-week-old) in a 30-ml ventilated plastic cup containing 10 g whole grain as one replicate inside the gas-tight desiccators and injecting PH_3 through a rubber septum in the lid using a gas-tight syringe. Insects were exposed to PH_3 for 20 (*T. castaneum*) or 48 h (*R. dominica*) at 25 °C and 70% RH, and then removed from the desiccators and held at 25 °C and 55% RH until end point mortality was assessed after 7 days. All experiments were replicated three times.

Data analysis

All mortality data were corrected using Abbott's correction for control mortality ($\leqslant$10%; Abbott, 1925) before the probit analysis (Finney, 1971). The analysis was performed using GenStat11 statistical package (Payne *et al*., 2008). The resistance ratio for the resistant strains was calculated by dividing the LC_{50} of resistant strain by the LC_{50} value of a reference Queensland strain, a susceptible *R. dominica*, QRD14 (Collins *et al*., 2002) and *T. castaneum* strain, QTC4 (Bengston *et al*., 1999). The 95% confidence interval (CI) for the resistance ratio at LC_{50} was calculated following the method established by Robertson and Preisler. (1992). The resulting CI was used to test the equality of the two LC_{50} values (that is, if the value 1 is contained in the CI for the resistance ratio, and then the LC_{50} are not significantly different).

Sequencing of the *rph2* resistance gene (DLD)

cDNA synthesis and sample preparation for sequencing. Total RNA was extracted from 20 mixed-sex adult beetles of the selected resistant IRD_{Mdu}, ITC_{Mdu}, ITC_{Cbe} and ITC_{ND} strains using the QIAGEN (Melbourne, VIC, Australia) RNA extraction kit as per the manufacturer's protocol. First-strand cDNA synthesis was generated from 1 μg of total RNA (Bioline) according to the manufacturer's protocol. The DLD fragment was amplified in a reaction volume of 20 μl containing 4 μl 5× PCR buffer, 1.5 μl 50 mM $MgCl_2$, 1.0 μl 10 mM dNTPs, 0.2 μl $1.25\,U\,\mu l^{-1}$ Taq DNA polymerase, 2.5 μl 10 μM of forward (5′-TCCCGGTCCAACATTTTAGTA-3′) and reverse (5′-CGGCCTAACCTTA AAAATAC-3′) primers and 2 μl of 1:10 diluted template cDNA stock. The PCR conditions were as follows: denaturation for 3 min at 95 °C, and then 35 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min and 30 s, with a final extension at 72 °C for 2 min. The PCR products were visualised using gel electrophoresis (1.5% agarose in 1× TAE, 100 V for 45 min). The resulting PCR product was purified and sequenced.

Genomic DNA extraction

Genomic DNA of *R. dominica* and *T. castaneum* samples collected from different storage sites in India was extracted using a CTAB DNA extraction method as described by Doyle and Doyle (1987). Briefly, the DNA extraction buffer contained 100 mM Tris-HCl (pH 8), 10 mM EDTA, 1.4 M NaCl, 2% CTAB and 5% β-mercaptoethanol. Individual insect samples were homogenised with 200 μl of DNA extraction buffer and incubated at 65 °C for 1 h. Chloroform: isoamyl alcohol (24:1) was added and mixed by inversion for 10 min, and subsequently centrifuged at 15 000 *g* at 4 °C for 10 min. The clear aqueous phase was then transferred into a new sterile microfuge tube and 200 μl of ice-cold isopropanol was added, mixed gently by inversion and kept at −20 °C for overnight incubation. The precipitate was then pelleted using centrifugation at 15 000 *g* at 4 °C for 10 min, after which the pellet was washed with 70% of ethanol and resuspended in 20 μl TE buffer. The DNA was left to dissolve overnight at 4 °C before use.

Resistance marker visualisation

A cleaved amplified polymorphic sequence marker assay was designed on the basis of the single-nucleotide polymorphism (SNP)that confers a substitution in the DLD gene found in the both species collected in India. In *R. dominica*, the DLD gene region was amplified using PCR in a reaction volume of 25 μl containing 2.5 μl of 10× PCR buffer, 1 μl each of 10 μM forward (5′-CTTAATTTCAGCGACAGACT-3′) and reverse (5′-GCCATGTGATAATAG TGTGAG-3′) primer, 0.5 μl of Taq polymerase (1.5 U) and 2 μl (25–50 ng) of template DNA. The PCR conditions were as follows: initial denaturation for 5 min at 95 °C, followed by 35 cycles of 95 °C for 1 min, 57 °C for 30 s and 72 °C for 1 min and a final extension at 72 °C for 7 min. The resistance polymorphism causes a C to T substitution at position 111 of the PCR fragments. The amplified 315-bp product was digested with *Mbo*I at 37 °C for 4 h and, the presence of the resistant variant results in two fragments of 108 and 207 bp, whereas the sensitive genotype results in no cleavage.

The DLD gene fragment of *T. castaneum* was amplified using PCR in a reaction volume of 25 µl containing 2.5 µl of 10× PCR buffer, 1 µl of 10 µM forward (5′-GCCCTGACTGTCTTCCACCA-3′) and reverse (5′-AGCCTTGAC AGCATTTTCCT-3′) primer, 0.5 µl (1.5 U) of Taq polymerase and 2 µl (25–50 ng) of template DNA. The PCR conditions consisted of 5 min at 95°C, followed by 35 cycles of 95 °C for 1 min, 55 °C for 30 s and 72 °C for 1 min and a final extension at 72 °C for 7 min. The amplified 368-bp product was digested with 1 U of *Mbo*I at 37 °C for 4 h in a reaction volume of 15 µl containing 5 µl of PCR product, 1 µl of 10× buffer and 9 µl of nuclease-free water. The digested product was visualised as above. The polymorphism causes a G to A substitution at position 296 of PCR fragment, which causes the formation of an *Mbo*I restriction enzyme recognition site, similar to that found in *R. dominica*. Thus, presence of the resistant variant results in cleavage of the PCR product into 296- and 72-bp fragments, whereas the wild-type sequence does not digest.

Evolutionary analysis of the DLD Proline > Serine variant by BLAST

Natural variants corresponding to *T. castaneum* DLD proline 45 (P45) were identified through BLAST search against the nonredundant database at NCBI (http://www.ncbi.nlm.nih.gov) using CLC Genomics Workbench 6.5 (http://www.clcbio.com). An *E*-value of 1×10^{-58} was observed to be the threshold between DLD orthologues and the closest paralogue of DLD, glutathione reductase. Sequences near the threshold were tested by BLAST homology search to confirm their identity. The resulting 669 sequences were aligned and scanned visually to identify variants of P45/49.

RESULTS

Comparative analysis of Australian and Indian *R. dominica* and *T. castaneum* strains

Two genes have previously been identified, *rph1* and *rph2*, that explain the PH_3 resistance phenotype of a strongly resistant strain of *R. dominica* (QRD569) and *T. castaneum* (QTC931) from Queensland, Australia. The response to PH_3 of each strain of *R. dominica* and *T. castaneum* from Australia and India were analysed using the probit mortality model.

The observed response curve of *R. dominica* strains shows that IRD_{Mdu} was more resistant to PH_3 than QRD569 (Figure 1). The resistance ratio of QRD569 is 435× that of QRD14 parent, whereas, for IRD_{Mdu} the resistance ratio is 1283× greater than that of QRD14 (LC_{50} = 0.00174 mg l^{-1}), which is ~3x greater than that of QRD569 (Supplementary Table S2). We hypothesised that the additional resistance in IRD_{Mdu} could be due to stronger resistance effect from different alleles of *rph1* and/or *rph2* genes.

Likewise, the observed response curve of *T. castaneum* strains shows that the resistant phenotype of ITC_{Mdu} was double, whereas the ITC_{Cbe} strain was half that of the strong resistant reference strain from Queensland, QTC931 (Figure 1 and Supplementary Table S2). The resistance ratio of ITC_{Mdu} is 1081× that of the QTC4 parent, whereas for ITC_{Cbe} the resistance ratio is 280× greater than that of QTC4 (LC_{50} = 0.007 mg l^{-1}).

Sequencing, protein prediction and alignment

DNA sequence of the dihydrolipoamide dehydrogenase gene in the IRD_{Mdu} (GenBank accession KP843188) strain revealed a P49S polymorphism, which corresponds to a previously reported allele present in a strongly resistant Australian strain, NNRD2864 (Table 1; Schlipalius *et al.*, 2012). This polymorphism is near the active site of the protein and is equivalent to amino acid 15 of the yeast DLD protein (1JEH) and participates in FAD cofactor-binding (Figure 2; Brautigam *et al.*, 2005). The previously described K142E polymorphism (Schlipalius *et al.*, 2012) of QRD569 is present in a different region of the protein that affects the accessibility of the active site to the lipoamide cofactor and is not involved in FAD cofactor binding. There were several other polymorphisms detected in the strain; however, none of these were characterised for association with resistance (see Table 1).

Sequencing of the two southern Indian *T. castaneum* strains (ITC_{Mdu} and ITC_{Cbe}, GenBank accession KP843190 and KP843189) revealed a shared polymorphism that causes a missense amino-acid substitution P45S (Table 1), which corresponds to the P49S substitution reported for the Indian *R. dominica* strain and NNRD2864 from

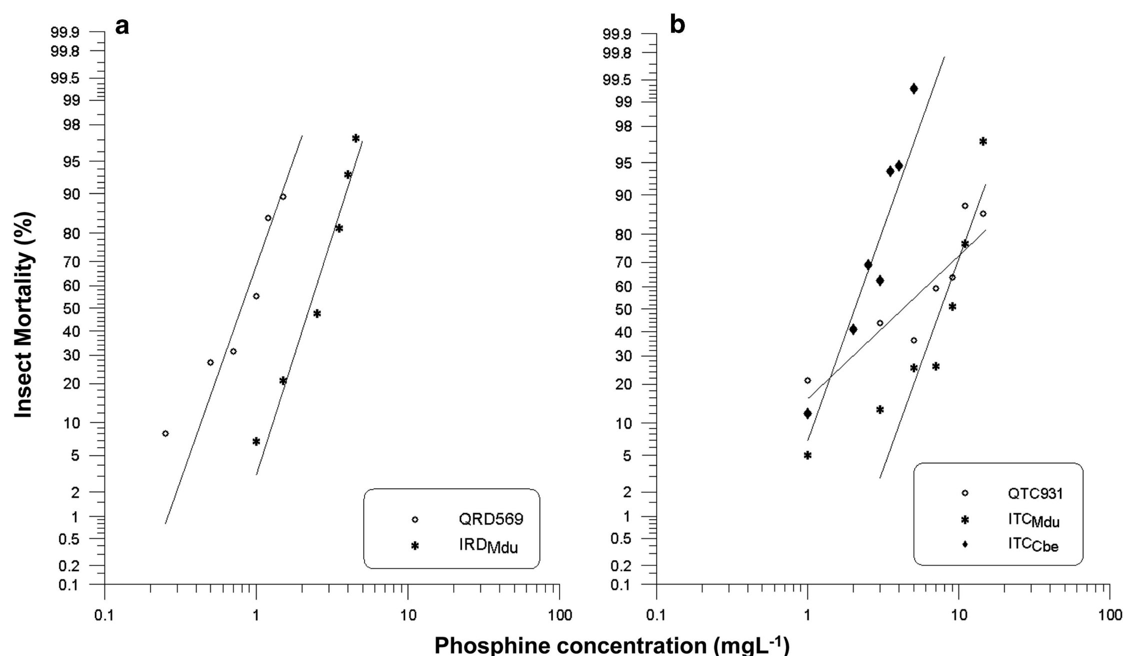

Figure 1 Response to phosphine strongly resistant insect strains from India and Australia. Results are presented as log-dose mortality of the resistant strains; (**a**) QRD569 and IRD_{Mdu}, (**b**) QTC931, ITC_{Mdu} and ITC_{Cbe}.

Table 1 Table of amino-acid variants sequenced from resistant and susceptible strains of *R. dominica* and *T. castaneum*

Species	*Strain*	*Phenotype*	*rph2*	*AA position*[a]										
				3 (3)	*49 (45)*	*81 (77)*	*135 (131)*	*142 (138)*	*334 (330)*	*361 (357)*	*376 (372)*	*411 (407)*	*416 (412)*	*476 (472)*
R. dominica	QRD14	Susceptible	−	Y	P	V	G	K	R	A	K	S	V	E
R. dominica	QRD369	Weak	−	Y	P	V	G	K	C	A	K	G	V	E
R. dominica	QRD569	Strong	+	Y	P	V	G	E	C	A	K	G	V	E
R. dominica	NNRD2864	Strong	+	C	S	V	G	K	R	A	K	G	V	E
R. dominica	IRD_{Mdu}	Strong	+	Y	S(het)	D(het)	G	K	R	G	E	G	V	G
T. castaneum	QTC4	Susceptible	−	S	P	V	G	K	R	A	T	G	I	E
T. castaneum	QTC931	Strong	+	S	P	V	S	K	R	A	T	G	T	E
T. castaneum	ITC_{Cbe}	Strong	+	S	S	V	G	K	R	A	T	G	I	E
T. castaneum	ITC_{Mdu}	Strong	+	S	S	V	G	K	R	A	T	G	I	E
T. castaneum	ITC_{ND}	Strong	+	S	S	V	G	K	R	A	T	G	I	E

Candidate resistance mutations are highlighted in grey.
[a]Position numbers are given in native sequence position with the *R. dominica* position and the aligned *T. castaneum* position in brackets.

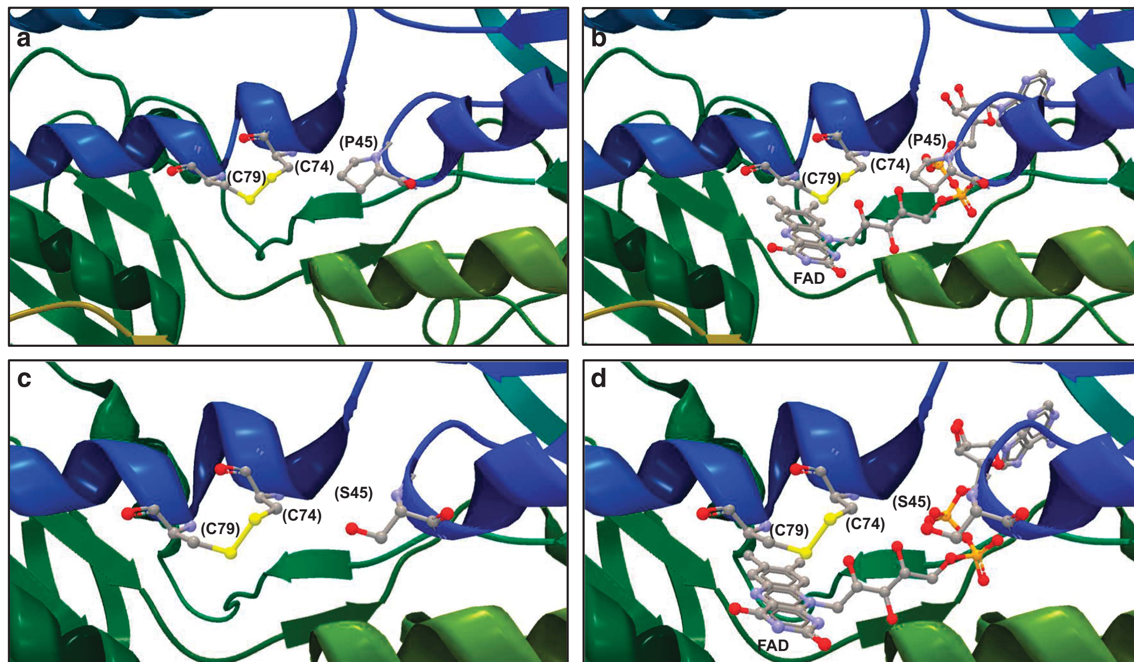

Figure 2 Structure of the DLD and glutathione reductase protein-active sites. The protein structures have not been determined for either *T. castaneum* or *R. dominica*; therefore, the highly conserved orthologues of the DLD and glutathione reductase proteins from *Saccharomyces cerevisiae* (1JEH and 2HQM; Brautigam *et al.*, 2005; Yu and Zhou, 2007) are shown to illustrate the location of the P>S variant amino acids relative to the active sites of the respective proteins. The extreme degree of sequence conservation between the paralogous enzymes is reflected in easily recognisable structural similarity between the DLD protein (**a**, **b**) and glutathione reductase (**c**, **d**). The placement of the FAD cofactor shown in **b**, **d** indicates that the P>S change is unlikely to affect the electron transfer reaction that occurs between the cysteine residues and the three-ring structure of the FAD. The cysteine (as the disulfide) and proline residues are highlighted and numbered according to the equivalent *T. castaneum* coordinates.

Australia (Schlipalius *et al.*, 2012). To determine whether the observed allele was present in northern India, we selected the ITC_{ND} strain from New Delhi for strong resistance with a discriminating dose of $0.25\,mg\,l^{-1}$ (20 h), and sequenced a pool of the survivors. We found the same P45S polymorphism homozygous in the DLD sequence (KP843191). No other polymorphisms that may be related to resistance were detected in the Indian *T. castaneum* strains. To test the possibility whether this mutation arose once and spread throughout India, we compared the synonymous SNP variants that reside within the coding sequence of the gene (Table 2). This revealed unique polymorphisms for each strain within the gene, suggesting that the resistant strains are likely to be independently arising.

Resistant allelic frequency for *R. dominica* and *T. castaneum* populations in India

The results of the *rph2*-resistant marker allele frequency survey are given in Table 3. In *R. dominica*, the highest allele frequency was found in the state of Tamil Nadu, in south India, where more than 94% of individuals carried a resistance allele at *rph2*, followed by those recorded for the other two south Indian states, Karnataka (78% of

individuals) and Andhra Pradesh (76% of individuals). In *T. castaneum*, the resistance allele frequency was quite high in all three states investigated, with >95% of individuals observed carrying the resistance allele.

Evolutionary analysis of the DLD Proline>Serine variant

The abundance and widespread distribution of the P45/49S variant suggested to us that it may have been a common pre-existing variant in India before the use of PH_3 that gained a selective advantage once PH_3 fumigation became common practice. We reasoned that if it was indeed a common variant, we would find it in DLD sequences of other insects in the NCBI database. When we carried out BLASTp analysis, however, we found that not only was the P>S variant not found in any of 64 arthropod DLD sequences, other than reported PH_3-resistant pest insects of stored grain, but neither was the variant found in any of 605 non-insect eukaryotic sequences (Supplementary Table S3). The only variants seen in eukaryotes of the proline equivalent to P45 of *T. castaneum* was a valine substitution in some plants and fungi and a single example of a tyrosine substitution in an amoeboid with a cyanobacterium endosymbiont. We do not show the numbers, but a fraction of eubacterial and archaeal DLD proteins varied at the equivalent proline, a small proportion of which carried the P>S substitution. The scarcity of the P45/49S variant across all eukaryotic taxa provides no evidence to support the hypothesis that it was either neutral or under positive selection in insects or other species that had not been exposed to PH_3 fumigation. The complete absence of the variant in eukaryotes other than PH_3-resistant grain pests suggests that it was more likely to have been under strong negative selection under natural conditions. Despite the paucity of this variant in the DLD protein, it is present in two close paralogues, glutathione reductase and thioredoxin reductase. The three-dimensional structure of the three related sequences are extremely similar, two of which, DLD and glutathione reductase, are shown in Figure 2 (Rice *et al*., 1984; Brautigam *et al*., 2005; Yu and Zhou, 2007). As can be seen, the active site disulfide, the FAD cofactor and the proline or serine residues are similarly aligned between the two proteins. This similarity suggests that the P>S substitution is unlikely to eliminate enzyme activity, but may well change the activity of the variant DLD.

Table 2 Table of SNP variants in the coding region of DLD sequenced from Indian *T. castaneum* compared with Australian reference strain QTC4

Strain	Position[a]										
	133	219	222	339	390	741	810	966	987	1278	1344
QTC4	C	C	C	C	G	C	A	G	A	C	C
ITC_{Cbe}	T	T	T	T	A	C	G	A	G	T	T
ITC_{Mdu}	T	C	T	T	G	C	G	A	G	C	T
ITC_{ND}	T	T	T	T	A	T	G	A	G	C	T

Abbreviations: DLD, dihydrolipoamide dehydrogenase; SNP, single-nucleotide polymorphism.
[a]Denotes position referenced to the start of the coding region for QTC4 DLD.
Nucleotide variations that are different to the QTC4 reference are highlighted in grey.

DISCUSSION

In each of eight previously tested strains of *R. dominica* and *T. castaneum* from Australia, strong resistance to PH_3 was because of alleles of *rph2/dld* together with resistance alleles at a second locus alternatively named *rph1* or *tc-rph1* (Jagadeesan *et al*., 2012; Schlipalius *et al*., 2012; Mau *et al*., 2012a, b). This indicated that only a limited number of genes could be modified to generate resistance, but the analysis was limited to one country with well-defined national guidelines for the management of post-harvest grain pests. The current study extends the analysis of PH_3 resistance factors to India, a country with quite different post-harvest grain storage system and pest management practices, different climate conditions and a different emphasis on the types of grain produced. In addition, factors such as type of storages (for example, bag stacks), typical length of storage and domestic mobility of grains within India are significantly different to Australia. These differences alter the spectrum of insect pests of primary and secondary importance between the two countries, although *R. dominica* and *T. castaneum* are significant pests in both countries. Interestingly, the currently studied Indian strains that we tested had a resistance phenotype nearly twice of that observed in Australia, with *R. dominica* 1283× and *T. castaneum* 1081× more resistant than the fully susceptible reference strains from Australia, QRD14 and QTC4, respectively. Despite these differences and the potentially large number of targets of PH_3 action, our analysis revealed that the previously identified resistance locus *rph2* is also responsible for the strong resistance phenotype in India.

The identity of *rph1* is unknown, but *rph2* encodes DLD (2012), a key enzyme of energy metabolism (Patel and Roche, 1990) that contains a reactive disulfide and a FAD cofactor that are directly involved in electron transfer (Williams, 1992). Sequencing of the *dld* gene showed a P45S variant in all three of the Indian *T. castaneum* strains. This serine substitution for the original proline occurs in the FAD cofactor-binding site in the enzyme. Interestingly, a P49S variant exists at the equivalent position in the protein of an Australian *R. dominica* strain that is the most strongly resistant strain of this species in Australia (Mau *et al*., 2012a).

DNA sequencing of the *dld* gene from eight strongly PH_3-resistant strains of *R. dominica* and *T. castaneum* from Australia resulted in

Table 3 Frequency and distribution of *rph2* P45/49S resistance variant in Indian populations of *R. dominica* and *T. castaneum*

Species	Location by state	No. of insect tested	Total of each genotype			R alleles (%)	S alleles (%)	Individuals with R allele (%)
			R	S	H			
R. dominica	Tamil Nadu	35	8	2	25	58.6	41.4	94.3
	Andhra Pradesh	21	3	5	13	45.2	54.8	76.2
	Karnataka	32	7	7	18	50.0	50.0	78.1
T. castaneum	Tamil Nadu	39	25	1	13	80.8	19.2	97.4
	Andhra Pradesh	45	13	2	30	62.2	37.8	95.6
	Karnataka	40	19	1	20	72.5	27.5	97.5

Abbreviations: H, heterozygous; R, homozygous resistant; S, homozygous susceptible.

identification of five different resistance-causing variants (Mau *et al.*, 2012a; Schlipalius *et al.*, 2012), whereas in this study all four Indian strains (one *R. dominica* and three *T. castaneum*) carried the same resistance-causing amino-acid variant. The *T. castaneum* strains, in particular ITC_{Mdu} and ITC_{ND}, came from widely geographically separated regions (2500 km apart), making it likely that the resistance variants were independently derived, a hypothesis that we confirmed by DNA sequencing of the *dld* gene from each strain. The *dld* gene sequences of each strain contained unique nucleotide variants in addition to the common variant responsible for resistance. This supports the notion that the resistance mutations occurred independently in pre-existing unique gene sequence contexts. The actual mutation in the proline codon was the same in each of the *T. castaneum* strains as well as the *R. dominica* strain from India and two previously published *R. dominica* strains from Australia (CCT to TCT); however, that is the only single-nucleotide change that can result in a proline to serine change.

The observation that the P45/49S variant was responsible for resistance in all four strains from India motivated us to survey the distribution and abundance of this allele in southern India more broadly. We found that at grain storage sites across three states of southern India (Table 3, Supplementary Table S1), the P45/49S allele is present in $\geqslant$95% of *T. castaneum* and 76–94% of *R. dominica* that were tested. The most obvious conclusion is that the high frequency of this resistance variant is the result of pest management practice in India that differs from practices in Australia where strong resistance in general and this variant in particular are rare.

While PH_3 exposure clearly selects for DLD variants that result in strong resistance, it was not possible to determine *a priori* whether DLD variants that confer PH_3 resistance existed in populations of pest insects before PH_3 exposure because of being neutral or under positive selection or whether they were unlikely to persist in the population because of negative selection. It is quite possible that variants in such a key enzyme of energy metabolism as DLD could significantly affect the fitness of the animal carrying the variant. If metabolism was altered in a way that provided a selective advantage under natural environmental conditions, it could favour the persistence of the variant in wild populations. If this were the case, the low frequency of the P45/49S variant in Australia as opposed to its abundance across southern India could reflect climatic differences between the two countries. This is an important consideration as it could not only influence the development of resistance as a pest management problem, but may also provide information for the effective management of resistance. This is a difficult problem to address, as any pre-existing variants still could be quite rare before PH_3 use and therefore difficult to detect.

To resolve this issue, we pursued a bioinformatic analysis of DLD sequences present in GenBank to scan for variants across the span of evolutionary time. This analysis revealed that the P45/49S variant was not found in any insect, other than reported PH_3-resistant pest insects of stored grain. Neither was the variant found in any other eukaryotic gene sequence (Supplementary Table S3). The complete absence of the variant across the span of eukaryotic evolution, throughout diverse environments and diverse species contexts, makes it highly unlikely that the variant is either neutral or under positive selection in natural insect populations. The absence of the variant in eukaryotes that have not been exposed to PH_3 makes it much more likely that, under natural conditions, the variant is under strong negative selection. The only other variants at P45/49 observed in multicellular eukaryotes were a change to valine (V) in fungi and plants (Supplementary Table S3) and to tyrosine (Y) in an amoeboid with a cyanobacterium endosymbiont. Thus, it appears that there are extreme constraints on changes to the proline at position 45/49, probably due to a disruption to the normal function of DLD. The appearance and seemingly rapid proliferation of the P45/49S variant in insects exposed to PH_3 suggest that fumigation is providing extreme positive selective pressure for change of the DLD protein. It is interesting to note that two paralogues of DLD, glutathione reductase and thioredoxin reductase, not only have structures that are extremely similar to that of DLD, but they each have a serine amino acid at the position corresponding to P45/49 from DLD. This may explain why DLD, but not glutathione reductase or thioredoxin reductase, is uniquely sensitive to PH_3 as indicated by the absence of resistance variants in the other two proteins. A more detailed understanding of the modified enzyme function that gives rise to PH_3 resistance may help to identify an as yet undiscovered vulnerability of PH_3-resistant insects.

DATA ARCHIVING

DLD sequences have been deposited in GenBank, accession numbers KP843188–KP843191.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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