Genetic Linkage Analysis of the Lesser Grain Borer *Rhyzopertha dominica* Identifies Two Loci That Confer High-Level Resistance to the Fumigant Phosphine

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ABSTRACT

High levels of inheritable resistance to phosphine in *Rhyzopertha dominica* have recently been detected in Australia and in an effort to isolate the genes responsible for resistance we have used random amplified DNA fingerprinting (RAF) to produce a genetic linkage map of *R. dominica*. The map consists of 94 dominant DNA markers with an average distance between markers of 4.6 cM and defines nine linkage groups with a total recombination distance of 390.1 cM. We have identified two loci that are responsible for high-level resistance. One provides ~50× resistance to phosphine while the other provides 12.5× resistance and in combination, the two genes act synergistically to provide a resistance level 250× greater than that of fully susceptible beetles. The haploid genome size has been determined to be 4.76×10^8 bp, resulting in an average physical distance of 1.2 Mbp per map unit. No recombination has been observed between either of the two resistance loci and their adjacent DNA markers in a population of 44 fully resistant F_5 individuals, which indicates that the genes are likely to reside within 0.91 cM (1.1 Mbp) of the DNA markers.

PHOSPHINE has been used worldwide as an insecticidal fumigant for stored grains since the 1930s because it is relatively inexpensive, easy to apply, and essentially residue free (Chaudhry 1997). In recent years the importance of phosphine usage to protect stored grain products has increased due to international agreements to phase out use of the fumigant methyl bromide (United Nations Environment Programme 1996). Unfortunately, high-level resistance to phosphine in stored product pests, initially reported in Bangladesh (Tyler *et al.* 1983) and later in India and other tropical countries (Rajendran and Narasimhan 1994), threatens the useful life of this fumigant. In eastern Australia a "weak" resistance phenotype now seems to be common in field populations of *Rhyzopertha dominica* (White and Lambkin 1990). More recently, a higher-level resistance to phosphine was detected in *R. dominica* in Australia in 1997 (Collins 1998; Collins *et al.* 2002) and it is highly likely that a similar level of resistance will shortly arise in other countries with temperate climates. There is no equally suitable alternative to phosphine and this highlights the need for effective monitoring and management of resistance to allow for its continued use (Chaudhry 1997).

Despite several decades of research, the mode of action of phosphine (Bond 1963; Chefurka *et al.* 1976; Nakakita 1976; Chaudhry and Price 1992; Hsu *et al.* 1998) and the mechanisms of resistance to its effects (Price 1984; Nakakita and Kuroda 1986; Chaudhry and Price 1989, 1990) are still unclear (reviewed in Chaudhry 1997). Using classical genetic techniques, there is evidence that two or more genes may be responsible for high-level resistance in *Sitophilus oryzae* (Li *et al.* 1994), *Tribolium castaneum*, and *R. dominica* (Ansell *et al.* 1990; Collins *et al.* 2002). Furthermore, Ansell *et al.* (1990) and Collins *et al.* (2002) have shown that the resistance mechanism is complex, in that the trait is dominant at low doses of phosphine and recessive at high doses. No work has been published on the molecular genetics of phosphine resistance, but identification of molecular genetic markers linked to resistance would allow (1) an assay to be developed with which to accurately survey the occurrence of resistance, (2) the number of genes potentially responsible for resistance to be determined, and (3) the genes directly responsible for resistance to be found, thereby allowing the mechanisms of resistance to be elucidated.

In an effort to identify the mechanisms of resistance we have taken a DNA-based genetic mapping approach. This has allowed us to estimate the number of genes responsible for conferring high-level resistance and will eventually allow us to discover their identity by map-based cloning. This should allow us to determine resistance mechanisms without having to rely on a previous understanding of the specific mode of action of phosphine.

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Since there has been very little analysis of the genome of *R. dominica*, we have employed a very rapid, arbitrary DNA marker generating protocol, *r*andom *a*mplified DNA *f*ingerprinting (RAF; Schlipalius *et al.* 2001), to create a genetic linkage map from an unselected F_2 intercross from susceptible and resistant insect lines. We have used this map to identify two loci responsible for high-level resistance. To estimate physical genomic distances, we also estimated the genome size of *R. dominica* via flow cytometry of propidium iodide-stained cell nuclei.

MATERIALS AND METHODS

Flow cytometry for genome size determination: The protocol adopted for flow cytometric determination of genome size (Marescalchi *et al.* 1990) used cell nuclei isolated from *Drosophila melanogaster* adults as an internal standard. *R. dominica* and *D. melanogaster* were homogenized separately: five beetles in 3 ml and 10 flies in 3 ml of Hanks' solution (137 mm NaCl, 5.5 mm KCl, 5.6 mm glucose, 4.2 mm $NaHCO_3$, 33 μm Na_2HPO_4). The homogenates were then filtered through a 54-μm nylon mesh filter and diluted 10-fold in Hanks' solution. In addition to the two separate homogenates, a third sample was prepared by mixing 500 μl each of the original two extracts. DNA was fluorescently stained by addition of 50 μl of propidium iodide (1 mg/ml in PBS) stain containing 5 μl RNaseA (10 mg/ml) to 1 ml of homogenate. The homogenate preparations were left standing in the dark at room temperature for 30 min to 1 hr to allow the fluorescent dye to equilibrate with the DNA. Flow cytometry was performed on a Becton Dickinson (Franklin Lakes, NJ) FACSCalibur flow cytometry system utilizing a laser wavelength of 488 nm and an FL2-H (peak height) detector for propidium iodide staining. For each sample ~1300–2000 cells were usually measured. Data were processed on a Power Macintosh 7600/120 utilizing CELLQuest v.3.1f software, providing histograms across 1024 channels on the *x*-axis and number of analyzed cells on the *y*-axis.

Beetle strains and genetic crosses: The map is based on a single-pair intercross population derived from susceptible and resistant lines of *R. dominica*. Samples of partially inbred strains of resistant (QRD569) and susceptible (QRD14; Daglish and Collins 1998) beetles were obtained from the Queensland Department of Primary Industries, Indooroopilly, Queensland, Australia. Classical genetics have shown that these lines appear to be homozygous for the respective major phosphine resistance and susceptibility alleles (Collins *et al.* 2002). The lines are highly heterogeneous throughout the rest of the genome (Schlipalius *et al.* 2001).

Nondestructive sex determination of virgin males and females was performed by visual inspection of pupae (Potter 1935) and a susceptible (QRD14) virgin female was mated with a resistant (QRD569) male to produce F_1 hybrids. A single virgin F_1 female was then crossed with a sibling male to produce an F_2 population of 104 individuals from which 92 were chosen as a mapping population. This mapping population was not subjected to selection for resistance to phosphine; rather, it was used to produce a framework genetic map that was later used to determine the genomic location of DNA markers linked to the resistance trait.

Identification of DNA markers tightly linked to resistance genes required selection of individuals that were homozygous for resistance at all resistance loci. The very low survival frequencies following selection by exposure to phosphine necessitated the generation of larger populations than could be obtained from single-pair crosses between sibling F_1 individuals. Two distinct strategies were employed to generate large populations of individuals for resistance selection, generation of a mass F_2 population and generation of an F_5 population descended directly from the F_2 population used for mapping.

First, at least 100 siblings of the F_1 pair used to generate the mapping population were allowed to mate freely. The resulting mass F_2 population contained all of the polymorphisms present in the 92 F_2 beetles used in the mapping population because they were all derived from the same parental beetles (P_0). They also contained additional markers that were not transmitted to the pair of F_1 sibs used to produce the mapping population, but these additional markers were not examined further. The mass F_2 beetles were subsequently fumigated at high doses and analyzed by bulk segregant analysis (Michelmore *et al.* 1991) to allow DNA markers linked to resistance to be identified.

Second, 12 siblings of the individuals that constituted the F_2 mapping population were used as founders of a population that was subsequently subjected to selection. The immature stages of the beetle exist within grains of wheat, making estimation of progeny sizes difficult. Because the total progeny size was only 104 individuals and we had already processed 92 beetles for mapping, 12 is simply the number of beetles that remained. Although a small founder population could skew allele frequency, we subsequently demonstrated that there was no loss of resistance alleles. The 12 founder F_2 beetles were allowed to mate randomly to produce a large F_3 population. The F_3 beetles were allowed to mate randomly to produce a large F_4 population, which produced an F_5 population that was subjected to resistance selection by phosphine fumigation. This provided a population with a high level of recombination for identification of markers tightly linked to the gene conferring resistance and for the fine-scale mapping of the locus. To prevent further founder effects, nearly 1000 individuals were transferred to establish each of the subsequent generations.

Fumigations: Fumigations were performed according to Daglish and Collins (1998) in desiccation chambers of fixed volume. Phosphine concentration was determined by gas chromatography [Varian (San Fernando, CA) aerograph model 90-P], utilizing dichlorofluoromethane as the carrier gas and a gas density balance detector. Fumigations lasted 48 hr, with the surviving beetles left to recover under normal atmosphere for 14 days at 25° to determine end point mortality. Fumigation of F_2 beetles to select fully resistant individuals for bulk segregant analysis was carried out at 0.2 mg/liter, 0.5 mg/liter, and 1.0 mg/liter phosphine. Fumigation of F_5 beetles for bulk segregant analysis was carried out at 1.0 mg/liter phosphine. F_5 progeny were also selected at a range of doses to allow the relative contribution of each locus to the resistance phenotype to be determined. These fumigations were carried out at 0.001 mg/liter, 0.003 mg/liter, 0.004 mg/liter, 0.005 mg/liter, 0.01 mg/liter, 0.05 mg/liter, 0.1 mg/liter, 0.3 mg/liter, 0.4 mg/liter, 0.5 mg/liter, and 1.0 mg/liter phosphine. In every case between 100 and 500 beetles were exposed to phosphine with 7500 being exposed at 1.0 mg/liter.

Survivor backcross: Survivors of the 1.0 mg/liter fumigation that were used for bulk segregant analysis were assumed to be homozygous for at least the major resistance genes. This assumption was tested by setting up a backcross between one of the presumed homozygous resistant individuals and a known homozygous resistant individual from line QRD569. Males were identified by putting each beetle that survived fumigation at 1.0 mg/liter phosphine into a container with whole wheat for 2 weeks. If no progeny were detected by visual inspection, the adult placed in the container was assumed to be male and

was then mated to a virgin female from the highly resistant strain (QRD569) to produce a backcross progeny that was expected to be homozygous for every major resistance locus and therefore fully resistant. The progeny were then fumigated at a discriminating dose of phosphine (0.1 mg/liter), which results in 100% mortality of F_1 hybrids between QRD569 and QRD14, but which results in >95% survival of the resistant line, QRD569 (Collins *et al.* 2002).

Marker generation: DNA extraction methods, PCR conditions, and electrophoretic separation were as described in Schlipalius *et al.* (2001). Briefly, beetles were snap frozen in liquid nitrogen and DNA was extracted from individuals by homogenization with a pestle in a microcentrifuge tube followed by boiling in a 5% Chelex-100 (Bio-Rad, Richmond, CA) suspension in TE buffer (100 mm Tris HCl pH 8.0, 1 mm EDTA, 2.5×10^{-7} mg/liter Rnase A) for 15 min. DNA markers were generated using arbitrary 10–11 mer primers by RAF analysis performed on individuals and pooled DNA samples. Each reaction volume of 10 μl contained 1 μl 10× PCR buffer (100 mm Tris HCl pH 8.0, 100 mm KCl, 50 mm $MgCl_2$), 0.2 μl of 1 mm dNTPs, 0.15 μl of 10 units/μl AmpliTaq Stoffel fragment DNA polymerase (Perkin-Elmer, Norwalk, CT), 2.5 μCi/10 μl α-labeled [^{33}P]dATP, 5 μl of 10 μm primer, and 1 μl of diluted template DNA stock. The PCR reaction was preincubated at 94° for 5 min, and then subjected to 30 cycles of 94° 30 sec, 57° 1 min, 56° 1 min, 55° 1 min, 54° 1 min, 53° 1 min, with a final extension of 72° for 5 min. In reactions in which fingerprints were generated from pooled templates, the equivalent of only 0.2 μl of each template was included in the reaction. Resulting radiolabeled PCR products were separated on 4% polyacrylamide manual sequencing gels and visualized by overnight exposure of Biomax MR (Eastman Kodak, Rochester, NY) autoradiography film to the dried gels.

Linkage analysis: DNA fingerprints from the resistant and sensitive parents, as well as from the F_1 hybrids, were compared with the fingerprints of 92 unselected F_2 progeny. Analysis of genetic linkage of markers was performed using Map Manager QTXb06 software for the Macintosh computer (Manly 1993), created by Jane Meer, Robert Cudmore, and Kenneth F. Manly and available at the website http://mcbio.med.buffalo.edu/mmQTX.html/. In cases where a PCR reaction failed (<4%), an entry was made to indicate that the data were missing. To compensate for the heterozygosity of the parents (P_0), a strategy was employed that allowed us to treat the parents as "virtual" (even if not actual) homozygotes. Only bands originating from the susceptible parent and that were absent from the resistant parent and present in both F_1 hybrids (*i.e.*, heterozygous in the F_1 hybrids) were used in the linkage analysis. All other polymorphisms were excluded from the analysis. This resulted in a robust linkage map that allowed unambiguous integration of markers subsequently determined to be linked to resistance loci. Markers transmitted to only one of the F_1 hybrids were ignored for the purposes of this analysis to eliminate ambiguities in determining inheritance. The critical value for linkage detection was set at $P = 0.0001$ (>99.99% probability that a predicted linkage was not a false positive). Recombination frequencies were converted to map distances using the MapManager's Morgan function, which assumes complete interference, *i.e.*, no double crossover events between markers. The "hide locus" function was used to test whether any markers caused map expansion of >3.0 cM. Such markers were found, but upon rechecking the scoring was deemed to be accurate.

Bulk segregant analysis: Bulk segregant analysis of resistant F_2 beetles was used to identify DNA markers putatively linked to the resistance loci and the linkage of these markers was subsequently confirmed on individual F_2 and F_5 beetles. Specifically, beetles that were maximally resistant to phosphine were selected from the mass cross F_2 population by fumigation at 0.2, 0.5, and 1.0 mg/liter phosphine. RAF fingerprints were then produced from the susceptible and resistant parents, both F_1 hybrids, and four bulked DNA samples, which had been prepared by combining the DNA from five beetles that survived phosphine fumigation. Two pooled DNA preparations were produced from survivors of 0.2 mg/liter phosphine treatment and one each from survivors of 0.5 and 1.0 mg/liter treatments. Because phosphine resistance had previously been shown to be an almost completely recessive trait, homozygosity for a resistance allele was expected to be required for maximal expression of the resistance phenotype. When using dominant markers the absence of the marker is the unambiguous way to determine homozygosity. Thus, we scored fingerprints from the bulked samples (and subsequently the individual members of the bulks) for the loss of a marker originating from the susceptible line. As with the mapping population, markers were used only if they were present in the susceptible parent, absent in the resistant parent, and present in both F_1 individuals. Markers that fit these criteria but were not present in the fingerprints of the resistant F_2 beetles were regarded as putatively linked to the susceptible alleles of resistance genes. In all cases, fingerprints of the unselected F_2 mapping population were used as a control for marker frequency. DNA markers exhibiting potentially tight linkage to resistance genes were subsequently confirmed in a population of 44 F_5 individuals that had survived fumigation at a dose of 1.0 mg/liter. The F_5 population was derived from F_2 siblings of the mapping population and (in all instances that were tested) retained all of the polymorphisms mapped originally in the unchallenged F_2 population. These DNA markers were subsequently scored in the F_2 mapping progeny and incorporated into the linkage map. The F_5 analysis allowed fine-scale mapping of resistance loci to a resolution of 0.6 cM.

RESULTS

Genome size determination: Flow cytometry was used to determine the genome size of *R. dominica* using the *D. melanogaster* genome as a standard (Figure 1). We plotted the relative fluorescence of Drosophila diploid (2C) and tetraploid (4C) nuclei (Ulrich 1990; see Figure 1) to a standard curve to determine the DNA content *vs.* fluorescence relationship of the staining procedure used. From this curve the relative fluorescence of the *R. dominica* genome was determined to be 2.8 times the size of the *D. melanogaster* genome. Using the estimated haploid genome size of *D. melanogaster* of 175 Mbp (Laird 1973), the haploid genome size of *R. dominica* was deduced to be ~476 Mbp. This falls within the expected range for a coleopteran genome (Juan and Petitpierre 1991).

Linkage analysis: A total of >300 RAF markers were generated by 18 arbitrary oligonucleotide primers, 94 of which were selected for construction of the genetic map (Figure 2). Of the 94 markers used for map construction, 81 markers appear on the map and 13 markers colocalized and do not appear separately on the map (Table 1). The selected markers consisted of DNA fragments that did not amplify from the resistant parent but did amplify from the sensitive parent as well as the sibling F_1 hybrids that were used to establish the F_2

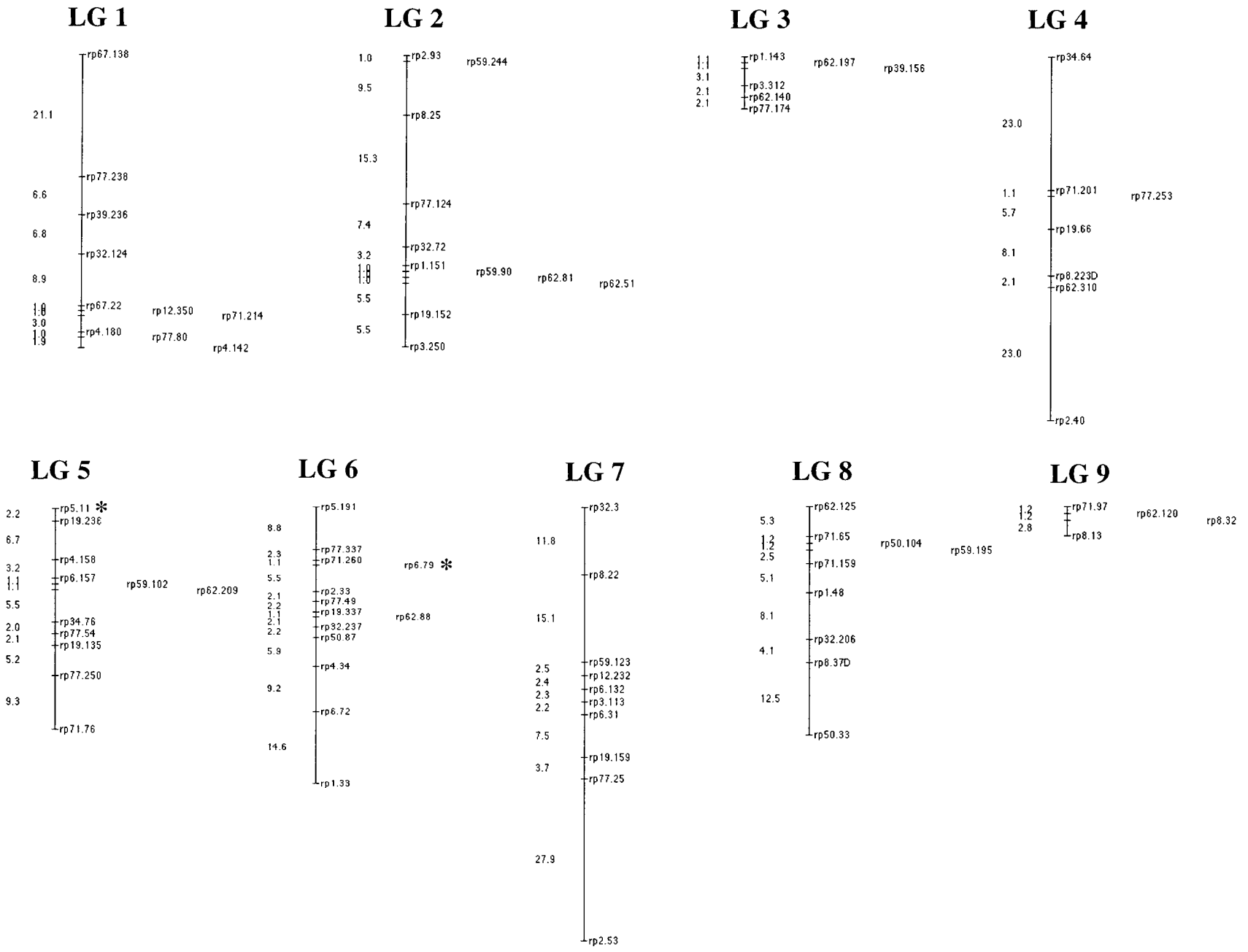

FIGURE 1.—The genetic linkage map of *R. dominica* consisting of 112 RAF markers in nine linkage groups with a total length of 398 cM. The critical value for linkage detection was set at $P = 0.0001$ (LOD ≈ 3.0). The loci that are tightly linked to resistance in linkage groups 5 and 6 are highlighted with an asterisk.

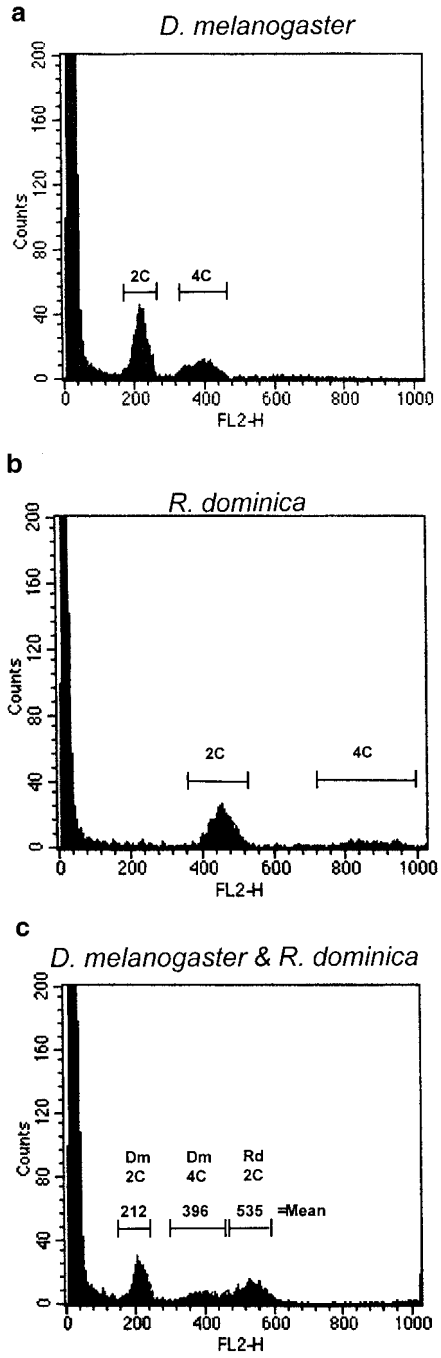

FIGURE 2.—Flow histograms showing the relative fluorescence of the DNA content in (a) *D. melanogaster*, (b) *R. dominica*, and (c) *R. dominica* with *D. melanogaster* combined. The channel number is shown on the *x*-axis and the total number of counts analyzed is shown on the *y*-axis.

progeny. These markers were selected to reflect the fact that only absence of a band can be scored in bulk segregant analysis. Because absence of a band is recessive to presence of a band, absence of a band will show the same inheritance as the recessive resistance allele. Insects that are fully resistant to phosphine are expected to be homozygous for the recessive resistance alleles. Therefore, the 94 selected DNA markers and the resistance loci can be integrated into a single unambiguous genetic map. All adjacent markers were joined at LOD > 3.0. The 94 markers are distributed over nine linkage groups (Figure 2). These linkage groups may plausibly be considered to correspond to the 8 + XY karyotype of *R. dominica* (SMITH and BROWER 1974). The total recombination distance over the nine linkage groups is 390 cM with an average genetic distance of 4.6 cM between markers. Given the estimated genome size of *R. dominica* of 476 Mbp, the average physical distance per recombination unit is calculated to be 476 Mbp/390 cM or 1.22 Mbp/cM.

TABLE 1

Colocalizing markers not appearing on final map and their associated mapped markers

Linkage group	Mapped marker	Colocalizing markers
Group 1	rp71.214	rp12.61
		rp39.23
		rp12.164
		rp8.116
Group 2	rp62.81	rp6.188
		rp1.151
Group 3	rp62.197	rp59.63
		rp32.89
	rp62.140	rp1.77
Group 4	rp77.283	rp32.283
	rp62.310	rp1.183
Group 5	rp59.102	rp12.68
Group 8	rp71.65	rp50.49

Survivor backcross: We wished to determine whether the F_2 individuals used for bulk segregant analysis actually contained all resistance alleles present in the line QRD569. To do this, we carried out a progeny analysis to determine the genotype of one of the F_2 males that survived selection at 1.0 mg/liter phosphine. This involved determining the sex of the survivors, backcrossing a male to a virgin female from the high-level resistance line, and determining the resistance phenotype of the offspring. Because sex determination of adult *R. dominica* is often lethal, we chose to determine the sex indirectly by assessing the ability of surviving beetles to produce offspring. Individual beetles were left on whole wheat for 4 weeks at which time the grain was inspected for evidence of larvae. The extended incubation period was required due to a phosphine-induced interruption to oviposition by fertile females. Only one beetle out of the five survivors assessed in this manner did not produce offspring. This beetle was mated with a virgin phosphine-resistant female from the high-level resistance line (QRD569) and 100 offspring were exposed to a discriminating dose of 0.1 mg/liter, all of which survived. At this dose of 0.1 mg/liter, >95% of the QRD569 resistant line but 0% of the F_1 hybrids and 0% of the sensitive QRD14 beetles survive (Figure 3). If the F_2

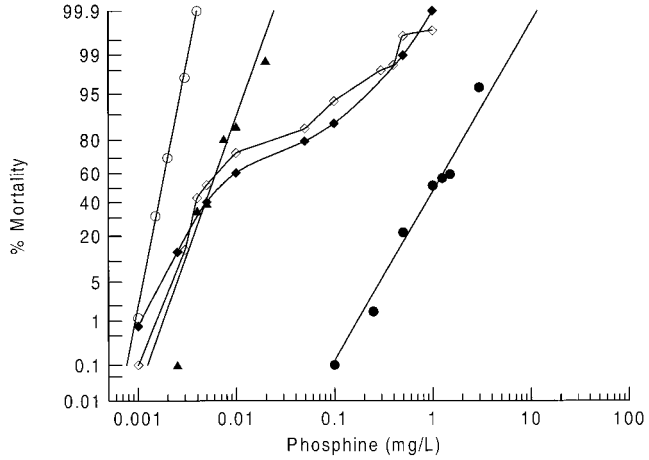

FIGURE 3.—A probit dose *vs.* mortality response curve of the (◇) F_5 progeny of a single-parent cross between a (●) strong resistance strain (QRD569) and a (○) susceptible strain (QRD369). Original strain and (▲) F_1 and (◆) F_2 progeny response data are reproduced with permission from COLLINS *et al.* (2002).

male identified above was homozygous for all resistant alleles present in line QRD569, then we expected the mortality of the offspring to be <5%. Our result shows the selected male was homozygous for all resistance alleles, confirming our expectation that all beetles selected at a dose of 1.0 mg/liter contain all the resistance alleles present in line QRD569.

Bulk segregant analysis and resistance locus detection: Fine-scale mapping was achieved using a total of 77 arbitrary primers for bulk segregant analysis of 20 F_2 individuals that survived a high level of phosphine exposure to identify markers linked to resistance loci. This number of primers enabled fine-scale mapping of resistance loci by virtue of greater coverage of the genome than was achieved by the 18 primers used to construct the map. We found markers that mapped to two distinct loci, one each in linkage groups 5 and 6. The resolution of the map was then enhanced by scoring DNA markers linked to resistance in 44 F_5 individuals that had survived an extremely high dose of phosphine (1.0 mg/liter for 48 hr at 25°). We calculated map distance in the F_2 generation as cM = number of observable recombinations × 100/number of F_2 progeny. For subsequent generations, we used the formula cM = (number of recombinations × 100)/number of progeny × (number of generations/2), to calculate map distances from the accumulated recombination events over multiple generations. There was no recombination between either marker rp5.11 (in linkage group 5) or rp6.79 (in linkage group 6) and their respective linked resistance genes in any of the 44 fully resistant F_5 beetles. This corresponds to a genetic distance of 0.91 cM between each of the two resistance loci and their closest respective DNA markers.

Relative level of resistance conferred by each resistance allele: To determine the relative contribution of each genetic locus to the resistance phenotype, we selected resistant F_5 individuals at a range of phosphine concentrations. The genotype of the survivors at each dose, with respect to markers rp5.11 and rp6.79, was used to determine the degree to which each resistance allele contributed to survival.

Because the resistance alleles are nearly completely recessive, individuals expressing the resistance phenotype must be homozygous. Furthermore, because presence of the DNA fragments rp5.11 and rp6.79 are both associated with sensitivity to phosphine, homozygosity for the resistance allele is indicated by the lack of DNA fragment amplification in both cases. Analysis of 98 arbitrarily chosen individuals from the F_5 population revealed a frequency of homozygosity of the rp5.11-associated resistance allele of 13% in individuals that have not been exposed to phosphine. The frequency of homozygosity of the rp6.79-associated resistance allele was 31% in these same individuals (Table 2). This deviation from the expected 25% is probably due to founder population effects (only 12 beetles from the 104 segregating F_2 population were used to found the F_3 population). Analysis of 84 surviving beetles from the same 98 F_5 individuals following exposure to a phosphine concentration of 0.003 mg/liter revealed no significantly divergent rates of homozygosity of these resistance alleles ($\chi^2 = 1.225$, $\alpha > 0.10$). However, selection at 0.01 mg/liter resulted in 96% of the surviving beetles being homozygous for one or the other or both of the two resistance-associated markers as compared to a combined basal rate of 40%, a highly significant result ($\chi^2 = 78.4$, $\alpha \ll 0.001$). Similarly, 95% of the survivors at selection doses >0.05 mg/liter were homozygous for rp6.79 ($\chi^2 = 75.6$, $\alpha \ll 0.001$) and 100% of survivors at doses >0.3 mg/liter were homozygous for both alleles ($\chi^2 = 90$, $\alpha \ll 0.001$). The presence of markers rp5.11 and rp6.79 in the F_5 survivors at the various doses between 0.001 and 1.0 mg/liter is summarized in Table 2.

In the F_5 population four individuals survived at 0.01 mg/liter or above, despite having a genotype exhibiting susceptibility at both loci. Of these, two exhibit recombination between the markers that flank rp6.79. It is reasonable to hypothesize that this recombination event separates the marker rp6.79 from the resistance gene. The remaining two survivors exhibiting a susceptible genotype may have been due to two recombination events within the 5.5-cM interval over four generations, giving the appearance of no recombination. Alternatively, some of these individuals may have unexpectedly survived selection due to recombination near rp5.11; this could be the case particularly for the survivor of the lower dose (0.01 mg/liter) since this dose represents the threshold at which either resistance gene was required. However, this possibility remains unexplored at this time due to lack of flanking markers on either side of rp5.11.

TABLE 2

Genotype analysis of F_5 survivors of phosphine fumigation

Genotype		Dose of phosphine (mg/liter)										
rp6.79	rp5.11	0.0	0.003	0.004	0.005	0.01	0.05	0.1	0.2[a]	0.3	0.4	1.0
		% of beetles analyzed										
−	−	60	53	44	23	4	6	5	0	0	0	0
−	+	9	11	9	32	39	0	0	0	0	0	0
+	−	27	31	34	41	57	78	65	0	0	0	0
+	+	4	5	13	4	0	16	30	100	100	100	100
		No. of beetles analyzed (survivors)										
		98	84	32	22	23	31	22	10	10	8	44

Genotypes of surviving F_5 beetles at various doses are expressed as a percentage of the total survivors analyzed. +, homozygous for the resistant allele; −, heterozygous or homozygous for the susceptible allele. Underlined percentages indicate genotypes of four individuals discussed in further detail in the text.

[a] Data obtained from F_2 mass cross (bulk segregant) analysis.

The two genes for which we have identified markers confer different levels of resistance and are synergistic in their action rather than additive. In this analysis it is useful to refer to a resistance threshold, which is defined as the minimum dose of phosphine at which a particular resistance allele must be homozygous for an individual to survive. The threshold concentration for the resistance allele linked to rp5.11 is between 0.005 and 0.01 mg/liter for a 48-hr exposure at 25°. The threshold concentration for the resistance allele linked to rp6.79 is between 0.01 and 0.05 mg/liter and for both alleles together the threshold concentration is between 0.1 and 0.2 mg/liter (Table 2). Given that the lethal dose $(LD)_{99.9}$ for the fully susceptible phenotype is 0.004 mg/liter, we estimated that the resistance alleles linked to rp5.11 and rp6.79 have individual contributions toward resistance of ~12.5× and ~50× (at $LD_{99.9}$), respectively, calculated using the upper limits of these threshold values. For both resistance alleles together the resistance factor is >250× (at $LD_{99.9}$).

DISCUSSION

The rapid generation of markers by RAF (Schlipalius *et al.* 2001) has allowed us to efficiently map the genes for phosphine resistance in an insect that had not previously been analyzed at the molecular level. The efficacy of the RAF technique is demonstrated by the fact that little or no primer screening is needed to generate abundant, reproducible markers. We used only 18 primers to generate a linkage map of 94 markers with an additional 59 primers used in bulk segregant analysis to identify markers that were very tightly linked to resistance loci. To construct a map of 122 randomly amplified polymorphic DNA (RAPD) markers, Beeman and Brown (1999) screened ~1000 DNA primers against *T. castaneum* to find 79 of them that produced acceptable fingerprints. For a linkage map of the honeybee *Apis mellifera*, Hunt and Page (1995) relied on screening 1000 primers to find 132 that produced 365 RAPD markers. The efficiency of the RAF technique allowed us to produce our map from an F_2 intercross from lines that were highly heterozygous rather than rely on haplodiploidy or inbreeding of the parental lines as was done in the other studies (Hunt and Page 1995; Beeman and Brown 1999).

For some previous studies on insects, backcross populations from inbred parental lines have been utilized for analysis of markers linked to a particular trait of interest, usually insecticide resistance (Heckel *et al.* 1997; Hawthorne 2001). The nature of our system limits the suitability of this approach to analyzing phosphine resistance on *R. dominica*. *R. dominica* has a limited reproductive capacity and because it is highly genetically heterozygous, bulk breeding to produce large numbers of progeny is of limited value when the desired outcomes include construction of a reliable genetic map. Furthermore, the assay that we employed, lethality at a given dose of phosphine, does not allow quantitative determination of resistance levels in individuals. We decided on a three-step procedure to allow precise mapping of resistance loci and reliable quantification of the resistance phenotype. First, we created a genetic map using arbitrarily chosen untreated individuals to provide a supporting framework for subsequent identification of the genomic location of markers linked to resistance loci. Second, we identified markers associated with inheritance of the maximal level of phosphine resistance. Third, we determined the relative levels of resistance conferred by each of the two identified resistance loci. This approach allowed each of the steps to be carried out without ambiguity, but it did require that the markers be useful in not only the F_2 but also subsequent generations as well as in bulk segregant analysis. One final requirement was that markers mimic the inheritance pattern of resistance. While these requirements

resulted in most of the markers produced being excluded from analysis, the ease and abundance with which markers were generated facilitated our approach.

Noninvasive determination of the gender of *R. dominica* adults is difficult and only pupae could be sexed nondestructively. Without knowing the sex of individuals in the mapping population, maternally derived markers segregate in a manner indistinguishable from autosomal markers, hence the X linkage group has not been identified. However, recombination rates along the X linkage group should be approximately one-half that observed in the autosomes because X chromosome recombination cannot occur in males. Using this criterion, either linkage group 3 or 9 may represent the X chromosome. For similar reasons, we have not identified Y-linked genetic markers. Not knowing the sex chromosome does not interfere with our objective of identifying resistance loci because classical genetic studies of the lines used in this study have shown that the phosphine resistance phenotypes are not sex linked (Collins *et al.* 2002). Our finding that resistance loci mapped to linkage groups 5 and 6 is consistent with linkage groups 3 or 9 representing the X chromosome.

The presence of two loci that account for most, if not all, of the phosphine resistance phenotype in highly resistant beetles is in agreement with previous nonmolecular genetic analyses (Ansell *et al.* 1990; Li *et al.* 1994; Collins *et al.* 2002). Collins *et al.* (2002) concluded that two or more genes were responsible for high-level resistance to phosphine in the highly resistant strain used in this study. A more definitive determination was not possible by classical genetic analysis, mainly due to the heterozygous genetic background of the strains. Ansell *et al.* (1990) also concluded that at least two genes contributed to high-level resistance to phosphine in a strain collected from Brazil. However, it remains to be determined whether the same genes are involved in separate outbreaks of resistance to phosphine.

Collins *et al.* (2002) observed in a cross between QRD569 (highly resistant) and QRD14 (susceptible) that F_1 hybrids were slightly more resistant than susceptible beetles (2.5×). This means that at least one of the resistance genes in QRD569 is incompletely recessive. These authors also observed that at least one of the genes found in the highly resistant QRD569 strain is shared in a weakly resistant strain (QRD369) and additional genes confer a resistance of 2.5× in the heterozygous phase (*i.e.*, in the F_1 generation). When we compare the phosphine resistance phenotype of the F_5 beetles and the corresponding genotypes in Table 2 to the data of Collins *et al.* (2002), the evidence suggests that the resistance allele linked to rp6.79 is shared between these strains and that the resistance allele linked to rp5.11 is incompletely recessive, conferring a resistance factor of 2.5 in the heterozygous phase. Standard treatments with phosphine are designed to kill beetles that are homozygous resistant for the rp6.79 locus, which is currently fixed in field populations in eastern Australia. This type of treatment would allow the survival of individuals that are heterozygous at the rp5.11 locus that are present in low frequency in field populations. Some progeny from these survivors would be homozygous resistant at both loci, indicating that this type of selection pressure could rapidly fix a locus of this type (rp5.11) in a population. The implications for resistance management are self-evident; treatment should ensure killing of individuals that are heterozygous at the rp5.11 locus.

In combination, the two loci we have observed are synergistic in action, which suggests that they contribute to two separate mechanisms of resistance (Raymond *et al.* 1989). Price (1984) and Chaudhry and Price (1990) first noted in *R. dominica* that there were apparently two distinct mechanisms of resistance to phosphine, the first being reduced uptake of phosphine in resistant insects and the second being a coping mechanism, whereby resistant insects that had been forced to take up as much phosphine as susceptible individuals could nevertheless successfully evade phosphine toxicity. Whether the two resistance loci identified by our study correspond to these two types of mechanisms will be the subject of further study.

The nature of phosphine action is different from that of other chemical insecticides. In a comparative study of chemical fumigants Bond (1963) determined that oxygen was essential for phosphine toxicity, unlike six other fumigants that exhibited toxicity under anaerobic conditions. In keeping with this is the observation that phosphine affects the activity of complex IV (cytochrome C oxidase), a component of the mitochondrial electron transport chain that requires oxygen as an electron acceptor (Chefurka *et al.* 1976; Kashi and Chefurka 1976; Nakakita 1976). Evidence suggests that phosphine kills via the production of reactive oxygen species within the cell (Bolter and Chefurka 1990; Hsu *et al.* 1998), probably via the disruption of mitochondrial electron transport. Phosphine is a strong reducing agent with a high affinity for metal ions, notably copper and iron, and the fact that complex IV contains essential copper and iron atoms at its active site suggests a mechanism for phosphine action. Complex IV delivers electrons to oxygen (the final electron acceptor in oxidative phosphorylation), indicating that disruption of complex IV could lead to an altered production of reduced oxygen molecules, *e.g.*, O_2^-, the superoxide anion. The superoxide anion could then initiate a chain of reactions leading to oxidative stress, causing widespread disruption of many cell macromolecules. However, there may be other modes of phosphine action and alternative theories have been presented in the literature, *e.g.*, Quistad *et al.* (2000). However, target site modification is not necessarily likely to be the main contributor of resistance to phosphine (Chaudhry

1997) as resistance mechanisms coping with oxidative stress do not have to be functionally related to the toxicity mechanism responsible for generation of reactive oxygen species.

There are several mechanisms whereby phosphine resistance might be acquired: target site modification, detoxification of deleterious compounds, and active exclusion or reduced uptake of phosphine. For many insecticidal compounds, target site mutation is established to be responsible for resistance (ffrench-Constant *et al.* 1998). However, the core subunits of cytochrome C oxidase (I, II, and III), which contain the iron and copper ions required for electron translocation, are encoded by the mitochondrial genome. The fact that the resistance alleles we have observed are not maternally inherited, as is the mitochondrial genome, leads us to conclude that modification of the active site of complex IV is not a resistance mechanism. Complex IV does contain 10 additional nuclear-encoded subunits that could possibly be altered to confer resistance to phosphine.

The oxidative stress response protects aerobic organisms from the oxidative by-products of respiration and also provides protection against environmentally encountered inducers of oxidative stress. The same oxidative stress response provides the basis of potential resistance mechanisms. A straightforward proposal may be that one mechanism of resistance to phosphine is a simple enhancement of a preexisting oxidative stress response. This may occur through mutation of a regulatory gene leading to a higher constitutive or induced level of response gene expression. Although to the best of our knowledge this mechanism has never been demonstrated in insects, in yeast it has been demonstrated that mutations in the transcription factor genes Yap1 and Skn7 alter resistance to a variety of oxidative stressors (Lee *et al.* 1999). Orthologs of Yap1 and Skn7 are not obvious in the genome of the insect *D. melanogaster*. However, it is plausible that a mutation in a functionally analogous transcription factor in insects could lead rapidly to high-level resistance under field selection conditions.

The role of active exclusion in phosphine resistance as described by Price (1984) remains obscure. At present there are no candidate genes or models for active exclusion of a molecule like phosphine. In this case, we believe genetic mapping will be the most productive way of finding the actual genes responsible for resistance.

In the future, we will clone and sequence the marker DNA fragments linked to the resistance loci for use in positional cloning of the resistance genes. With a genome size of 476 Mbp, the approximate average physical distance is 1.2 Mbp/cM. Since both loci lie within 0.91 cM (or 1.1 Mbp) of their respective resistance alleles, positional cloning is feasible. The markers linked to the resistance loci will also be used in genetic surveys of insects collected from other resistance outbreaks. This will allow us to determine whether the same genes are mutated to create a resistance allele in each case. The linkage map will also be used to map candidate genes, which may aid the rapid isolation of resistance genes and provide evidence for their involvement, or lack thereof, in the mechanisms of resistance.

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