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Genetics

Functional haplodiploidy: A mechanism for the spread of insecticide resistance in an important international insect pest

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ABSTRACT **The coffee berry borer, *Hypothenemus hampei*, is the most important insect pest of coffee worldwide and has an unusual life history that ensures a high degree of inbreeding. Individual females lay a predominantly female brood within individual coffee berries and because males are flightless there is almost entirely full sib mating. We investigated the genetics associated with this interesting life history after the important discovery of resistance to the cyclodiene type insecticide endosulfan. Both the inheritance of the resistance phenotype and the resistance-associated point mutation in the γ-aminobutyric acid receptor gene *Rdl* were examined. Consistent with haplodiploidy, males failed to express and transmit paternally derived resistance alleles. Furthermore, while cytological examination revealed that males are diploid, one set of chromosomes was condensed, and probably nonfunctional, in the somatic cells of all males examined. Moreover, although two sets of chromosomes were present in primary spermatocytes, the chromosomes failed to pair before the single meiotic division, and only one set was packaged in sperm. Thus, the coffee berry borer is "functionally" haplodiploid. Its genetics and life history may therefore represent an interesting intermediate step in the evolution of true haplodiploidy. The influence of this breeding system on the spread of insecticide resistance is discussed.**

The coffee berry borer (*Hypothenemus hampei*) is the major insect pest of coffee worldwide. This destructive beetle has an interesting and unusual life history similar to some other members of the bark beetle family (Scolytidae) (1). Single females enter individual coffee berries where they build galleries and lay their eggs (Fig. 1). The resulting brood are predominantly female (≈10:1), and the smaller males are flightless and never leave the berry. Thus, in addition to generating a highly female-biased sex ratio (spanandry) this life history promotes inbreeding between the large numbers of females and their few male sibs (inbreeding polygyny).

Spanandry is sometimes associated with haplodiploidy, in which haploid males develop from unfertilized eggs. Furthermore, haplodiploidy has been documented among scolytid beetles within the subfamily Xyleborini (1). However, two important lines of evidence suggest that *H. hampei* is not truly haplodiploid. First, unfertilized eggs are inviable (unpublished observations). Second, preliminary cytological investigation suggests that both males and females are diploid (2). Furthermore, haplodiploidy has not been associated with the subfamily Cryaphilini, to which *H. hampei* belongs (1).

Endosulfan (a cyclodiene type insecticide) is the most effective compound for control of *H. hampei* as its fumigant action penetrates the coffee berry and kills the resident brood. The spread of endosulfan resistance from the South Pacific island of New Caledonia, or its independent origin elsewhere, could therefore lead to loss of one of the major control agents for the borer and is thus a serious threat to the international coffee industry as a whole (4). Following the observation that resistance appeared to be sex linked (5), as would be consistent with haplodiploidy, we recognized that insecticide resistance was not only economically important but could also provide a useful marker for dissecting the genetics underlying the interesting life history of *H. hampei*.

Previous studies have shown that endosulfan resistance in *H. hampei* is associated with a single point mutation in the γ-aminobutyric acid receptor gene Resistance to dieldrin (*Rdl*) (6), which encodes the target site for cyclodiene insecticides (7). After this discovery, we developed a molecular diagnostic PCR amplification of specific alleles (PASA), capable of distinguishing between susceptible (Rdl^S or *S*) and resistant (Rdl^R or *R*) alleles at this locus. In this study, we use both the insecticide-resistance phenotype, as determined by bioassay, and PASA to study the genetics underlying the *H. hampei* life cycle and to correlate these findings with the unusual chromosome cycle.

EXPERIMENTAL PROCEDURES

Beetle Strains and Insecticide Bioassay. The origins of the susceptible (La Foa2) and resistant (Ponerihouen106) strains have been described elsewhere (5). Beetles were reared in the laboratory in New Caledonia on an artificial diet (8) compressed into the wells of 96-well microtiter plates. Pupae were isolated to collect virgin females and males. Crosses were established between homozygous susceptible adult females (*SS*) and F_1 males where either the *R* or the *S* allele was paternally derived [paternal allele is indicated in parentheses—i.e., *S*(*R*) or *R*(*S*)]. Individual crosses were established with a single male and 10 females in a single microtiter well. After 2 weeks males were removed, and the mated females were transferred to individual cells. Parent females were then also removed after the appearance of larvae.

The resulting adult progeny were bioassayed with endosulfan in New Caledonia as described (5, 9). Briefly, insects were exposed, via vapor action, to a fixed dose of insecticide (50 and 400 ppm for males and females, respectively) and mortality was scored over time. After 6 hr all *SS* insects were dead; all other genotypes survived. After 24 hr all *RS* insects were dead; only *RR* insects survived. These time points were therefore used to discriminate between *SS* and *RS* and between *RS* and *RR* beetles. After bioassay, insects were scored as alive or dead and then placed in the individual wells of microtiter plates and shipped by air mail to Madison, Wisconsin. DNA was successfully recovered from both alive and dead insects upon arrival (see below). For cytological analysis, a range of life stages were shipped to Madison.

PASA Analysis. After insecticide bioassays, performed at the Institut Francais de Recherché Scientifique pour le Dé-

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Abbreviation: PASA, PCR amplification of specific alleles.

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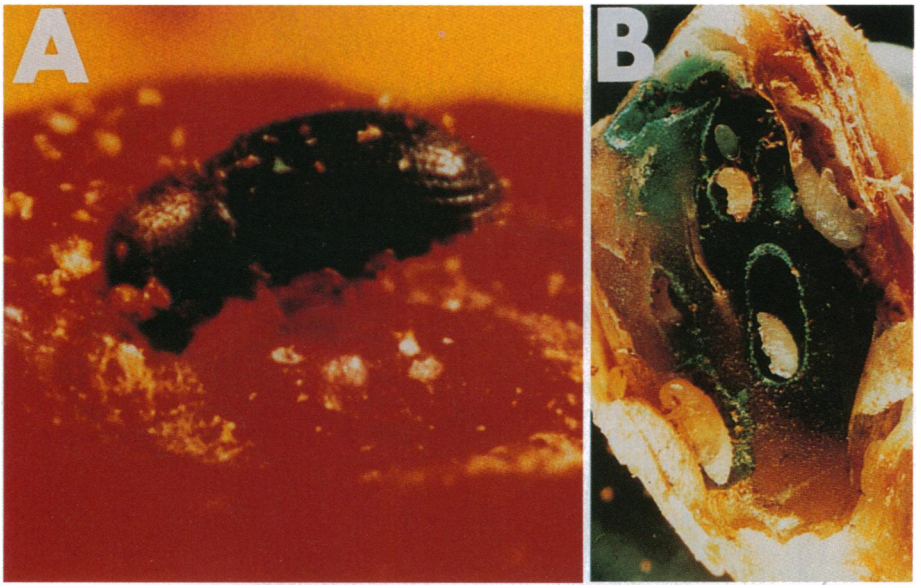

FIG. 1. (*A*) Female coffee berry borer entering coffee bean. (*B*) Larval brood of single female inside dissected coffee berry. The tunneling of individual broods can completely excavate the coffee bean, leading to severe economic damage.

velopment en Cooperation, New Caledonia, beetles scored as *SS*, *RS*, or *RR* were inserted individually into the wells of flat-bottomed microtiter plates and shipped at ambient temperature to Madison, Wisconsin, for PASA analysis. Upon arrival, plates were stored at −80°C before analysis. PASA was then performed as described with existing primers (6), except that a new forward primer specific for the susceptible allele (5′-GCC ACC CCG GCT CGT GTG-3′) was designed.

Cytological Analysis. Tissues were dissected and prepared for cytology as previously described (10). Briefly, to examine somatic cells, brains were dissected from both the male and the female prepupae, pupae, and adults. To examine spermatogenesis, testes were dissected from male pupae and adults. Chromosome preparations were strained with 2% Giemsa in phosphate buffer (pH 6.8) and observed by light microscopy with an Olympus Vanox microscope. Twenty-five somatic metaphase spreads from 10 females were examined. In addition, several hundred meiotic cells from the testes of both pupae and adult males (total of 50 insects) were also characterized. Photographs were taken with Kodak Technical Pan film.

RESULTS

Insecticide Bioassay and PASA. Previous investigations have shown that many cases of cyclodiene resistance are associated with a single point mutation (11) in *Rdl*, which is inherited as a semidominant resistance phenotype. We therefore predicted that we would observe three classes of progeny—homozygous susceptible beetles *SS*, homozygous resistant beetles *RR*, and insects heterozygous for resistance *RS* and therefore intermediate in response. Based on this hypothesis, males appeared to express only their maternally derived alleles; i.e., male progeny of both $SS \times S(R)$ and $SS \times R(S)$ matings (Fig. 2 *A* and *B*) were completely susceptible and male progeny of both $RR \times S(R)$ and $RR \times R(S)$ matings (Fig. 2 *C* and *D*) were completely resistant. Furthermore, females survived insecticide bioassay only when their father had a maternally derived resistance alleles (Fig. 2 *B* and *D*). Thus, as would be consistent with haplodiploidy, males apparently inherited and expressed only the maternally derived genome, but females inherited two, one from their mother and one from their father's mother.

To further examine our prediction, we tested the same beetles by PASA. Unfortunately, a substantial proportion (31.5%; $n = 19$) of the males and a small proportion of the females (1.4%; $n = 277$) analyzed by PASA failed to produce any PCR product with either *S* or *R* allele-specific primer. As males are much smaller than females, this suggests that there was difficulty in extracting enough intact DNA from males after prolonged shipping at ambient temperature. Nonetheless, results from the PASA analysis were again consistent with both the bioassay results and haplodiploid inheritance (Fig. 3). Thus, among the male progeny of each mating, paternally derived alleles were never amplified. Furthermore, heterozygous females were never observed among the $SS \times S(R)$ (Fig. 2*A*) and $RR \times R(S)$ (Fig. 2*D*) matings, whereas only heterozygous females were observed among the $SS \times R(S)$ (Fig. 2*B*) and $RR \times S(R)$ (Fig. 2*C*) matings.

Cytology. As reported (12), female metaphase nuclei appeared to contain $2n = 14$ chromosomes (Fig. 4*A*). Furthermore, in female somatic prophase cells, seven or eight small heteropycnotic spots, which presumably correspond to the paired centromeres of 14 chromosomes, are visible. Probably because of the smaller number of males available for examination, male somatic cells in metaphase were not observed. However, these also appeared to be diploid, although when compared to female cells in prophase, they have a distinctly different morphology. Among the small heteropycnotic spots that correspond to the centromeres of the prophase chromosomes was a large heteropycnotic mass (Fig. 4 *C–E*). Thus, it appears that male somatic cells are diploid, but while one set of chromosomes is decondensed in prophase, the other (presumably the paternally derived set) is condensed, in a fashion similar to that previously observed in coccids (13), into a darkly staining mass of chromatin.

A similar condition was observed as the germ line progressed through spermatogenesis (Fig. 4 *F–H*). Like male somatic prophase nuclei, primary spermatocytes were observed to contain two distinct sets of chromosomes as meiosis begins: one set of seven relatively decondensed chromosomes (Fig. 4*F*) and a second and presumably paternally derived highly condensed set (arrows in Fig. 4 *F* and *G*). As meiosis progressed, the former set condensed and underwent a mitotic-like division, while the latter degenerated and was eventually lost. A second meiotic division did not occur and the net result of meiosis is therefore a mitotic division of the maternally derived chromosomes.

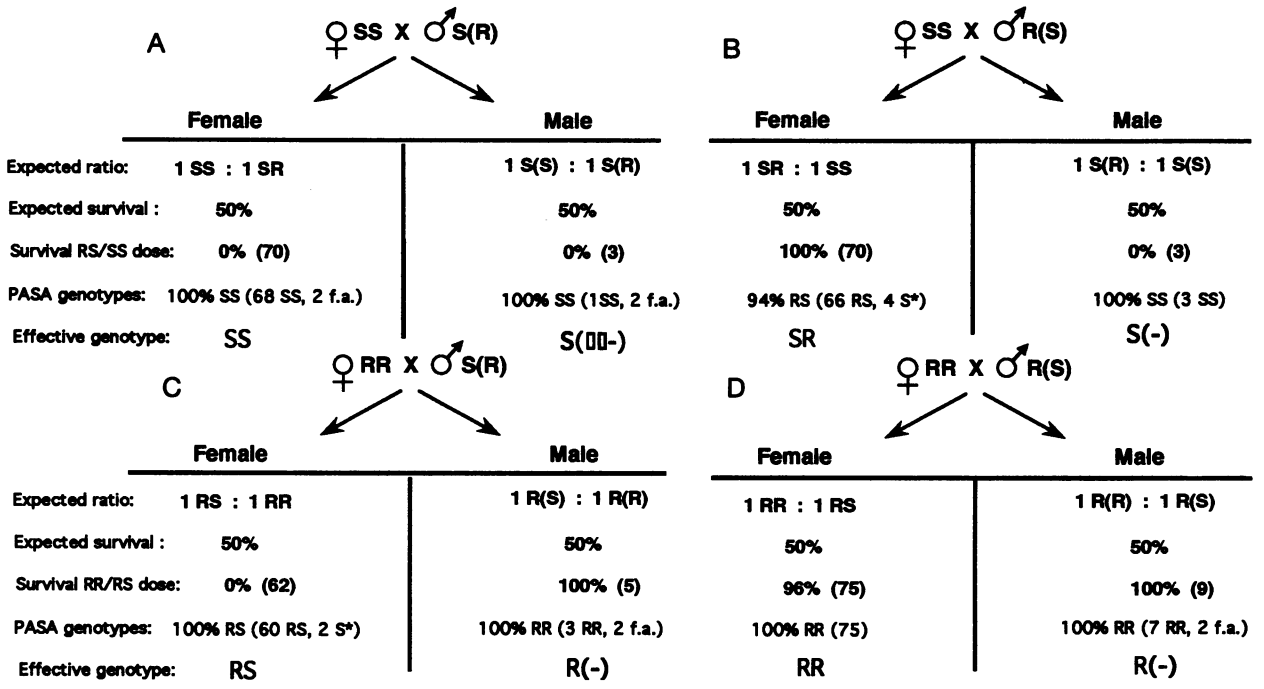

FIG. 2. Crossing schemes to show expected and observed insecticide-resistance phenotypes of male and female progeny from crosses of susceptible females *SS* to either *S(R)* (*A*) or *R(S)* (*B*) males and crosses of resistant females *RR* to either *S(R)* (*C*) or *R(S)* (*D*) males. In each case, the expected genotypic ratio from the cross and the expected and observed percentage survival at each discriminating dose of insecticide (doses discriminating either *RS* from *SS*, *RS/SS* or *RR* from *RS*, *RR/RS*) are shown. Genotypes determined by PASA are given as percentage *SS*, *RS*, or *RR* where numbers in parentheses indicate (*i*) the number of insects genotyped and (*ii*) either the number of insects that failed to give a PCR amplification product because of failed amplification of the susceptible allele-specific primer alone (S*) or failure of both S- and R-specific primers [failed amplification (f.a.), probably corresponding to insufficient target DNA in the PCR]. Finally, the effective genotype resulting from the cross is given, highlighting the fact that the paternally derived allele (−) is not inherited in the males.

DISCUSSION

Functional Haplodiploidy. Inbreeding polygyny, whereby a few males fertilize all their sisters, has evolved a number of times in the Scolytid beetles—namely, in the Hyorrhynchini, Xyleborini, Drocoetini, and the Cryaphilini (1) (to which *Hypothenemus* belongs). Furthermore, spanandry has often been used to implicate the occurrence of haplodiploidy in insects and mites. This correlation between highly biased sex ratios and haplodiploidy is thought to occur because deviations from unit sex ratio are difficult to maintain in diplodiploid systems (14). However, haplodiploidy is not the only means by which a species can maintain deviations from unit sex ratio and other mechanisms such as the presence of microorganisms can also cause sex ratio distortion (14, 15). In fact, within the scolytids themselves, true haplodiploidy, whereby males develop from unfertilized eggs, has been demonstrated only in the Xyleborini (1).

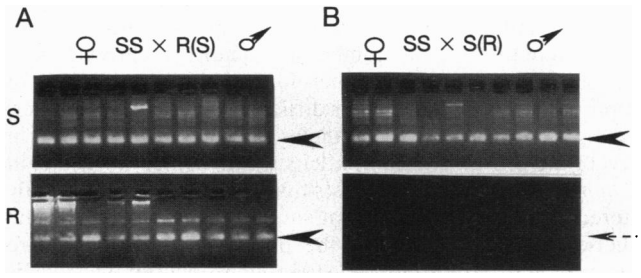

FIG. 3. PASA analysis of female progeny from crosses of homozygous susceptible *SS* females to heterozygous males where either the resistant allele *S(R)* (*A*) or the susceptible allele *S(R)* (*B*) is paternally derived (shown in parentheses). Note that in *A* both *S* and *R* alleles (arrowheads) are amplified in the progeny as the cross is effectively *SS* × *R*(−) and all the progeny are thus *RS*, whereas in *B* only the *S* allele is amplified (broken arrow indicates expected position of allele-specific PCR product; however, only very faint nonspecific products are observed). The latter cross is therefore effectively *SS* × *S*(−) [where (−) indicates failure of the paternally derived allele to be inherited] and all the progeny are thus *SS*.

In the case of the coffee berry borer, insecticide bioassays and PASA analysis of specific resistance alleles indicate that resistance is determined solely by a single point mutation in *Rdl* and that its inheritance is consistent with haplodiploidy. Interestingly, our cytological examination indicates that males are diploid but effectively shut off the expression of the paternally derived set of chromosomes by condensing those chromosomes in prophase. In addition, meiosis has apparently been modified in males so that a father's paternally derived chromosomes are not transmitted to his offspring. Fascinatingly then, the genetics associated with the life history of this important cosmopolitan pest are functionally haplodiploid even though males are diploid and females must mate before they can produce viable eggs. As polygyny has apparently evolved independently a number of times in the Scolytidae (1), it is interesting to speculate on the significance of our findings in *Hypothenemus*. Functional haplodiploidy in *H. hampei* may simply represent another mechanism whereby a species can benefit from polygyny (16). Alternatively, as previously postulated for other insects and mites (16), the destruction of paternally derived chromosomes in *H. hampei* may represent a possible intermediate step in the evolution of true haplodiploidy.

The relationship of our results to the only other published study on the cytology of *H. hampei* (12) is unclear. According to this previous study, females are $2n = 14$ and males are $2n = 15$. The odd Y chromosome in males was argued to be male determining; its frequent loss during spermatogenesis, via nondisjunction, was thought to lead to the disproportionate number of females in each brood. Our results agree with the previous study with respect to the following: females have $2n$

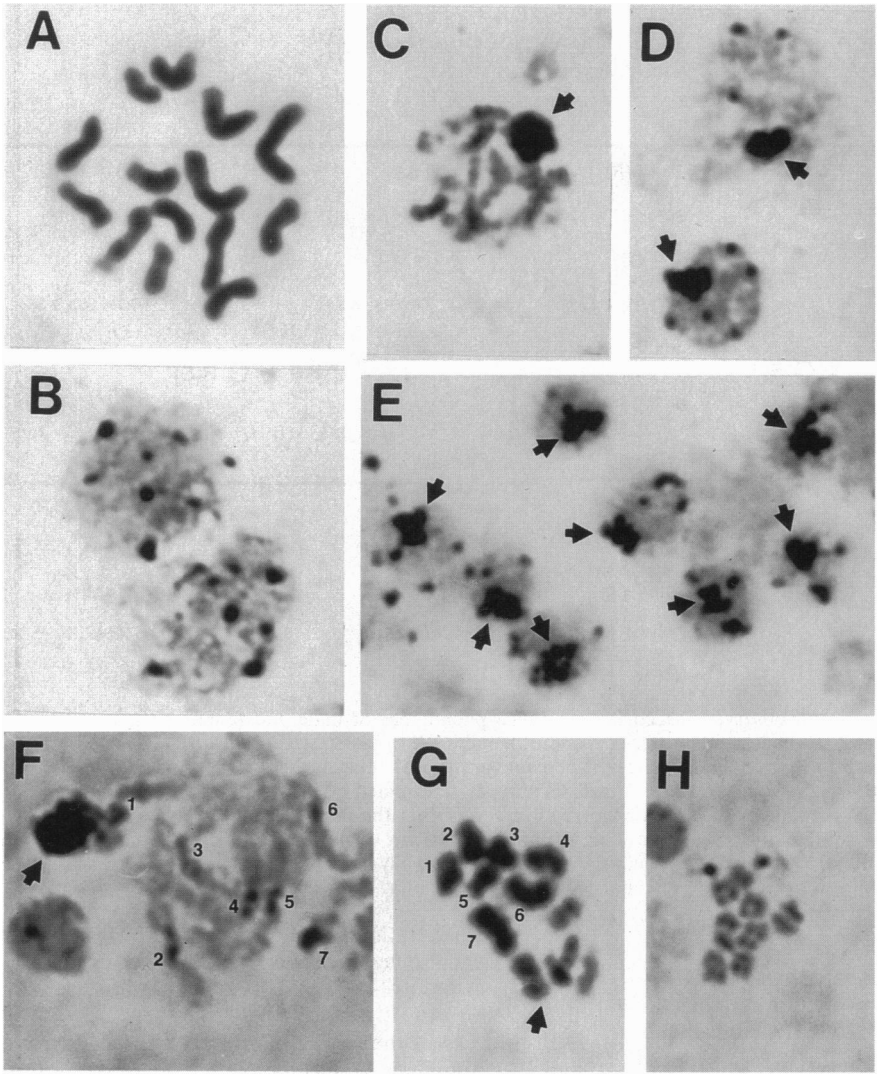

FIG. 4. Cytology of female (*A* and *B*) and male (*C–E*) somatic tissues and male germ line (*F–H*). (*A*) Female neuroblast showing 14 chromosomes in metaphase. (*B*) Female neuroblast in interphase showing paired homologous centromeres corresponding to the 14 chromosomes. (*C*) Male neuroblast in prophase. (*D* and *E*) Male neuroblasts in interphase. Note the presence of a dark heterochromatic mass (arrows) found only in the male cells, corresponding to condensation of the putative paternally derived chromosomes. (*F–H*) Spermatocytes in prophase I (*F*), metaphase I (*G*), and early anaphase I (*H*). Condensed paternally derived chromosomes seen in *F* (arrow) degenerate during meiosis I in *G* (arrow) and are eventually lost (*H*). Second meiotic division does not occur and spermatogenesis is therefore essentially mitotic.

= 14 chromosomes and males are diploid. However, we saw no evidence of a Y chromosome. We speculate that the difficulty of obtaining suitable somatic tissues led the earlier investigators to interpret the clustering of paternally derived chromosomes in males as an extra Y chromosome. The mechanism of sex determination is, therefore, still unresolved as males apparently develop from fertilized eggs and have the same chromosome complement as females.

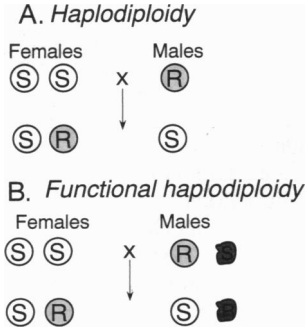

FIG. 5. Diagram of expected inheritance of resistance from either haplodiploidy (*A*) or functional haplodiploidy (*B*). Each haploid genome is represented by a circle; *R*, resistant; *S*, susceptible. In true haplodiploidy (*A*), haploid males develop from unfertilized eggs, receiving only one chromosome complement from their mother. In functional haplodiploidy (*B*), males arise from fertilized eggs but the paternally derived complement is condensed (darkly shaded circle) and only the maternally derived complement is transmitted and expressed. Therefore, functionally the transmission of resistance is the same in both modes of inheritance.

Our observations of heterochromatized paternally derived chromosomes in *H. hampei* are strikingly similar to the paternal genome loss described in coccids (13). However, unexpectedly, PASA failed to amplify paternally inherited resistance alleles from this condensed chromatin. Unfortunately, we were unable to find male somatic cells in metaphase; thus, we were unable to determine the number and quality of chromosomes present in the male soma. Therefore, the failure of PCR to amplify the resistance alleles is difficult to interpret. However, if the genes coding for these alleles are indeed present and in view of the proven allele specificity of the PASA diagnostic in *H. hampei* (6), then this suggests that their DNA is sufficiently altered or degraded to prevent successful amplification. The precise mechanism whereby the paternal chromosomes are clustered and fail to undergo subsequent meiotic divisions is therefore unclear. Furthermore, the genetic mechanism whereby paternal chromosomes are recognized by male cells also remains obscure. A likely possibility is imprinting of one set of chromosomes by either the male or the female.

Implications for the Spread of Resistance. Similar mechanisms for elimination of paternally inherited genetic material have been observed in other insects and mites (17, 18), and insecticide resistance has also been documented in putatively haplodiploid insects such as whiteflies (19). However, our findings represent a demonstration of the inheritance of

insecticide resistance via functional haplodiploidy. Thus, although the net product of this system is effectively the same as true haplodiploidy, the mechanism is very different (Fig. 5). Like haplodiploidy this system of sib mating will rapidly decrease the heterozygosity of females in the absence of selection [$H_t = (1/2)H_{t-1} + (1/4)H_{t-2}$, where H_t is the heterozygosity at generation t (20)].

In the presence of insecticide selection, the unique combination of this functional haplodiploidy and interesting mating system may therefore explain the rapid spread of resistance in New Caledonia (3, 21). As resistance is semidominant (or partially recessive), a single maternally derived resistance mutation may have been exposed directly to selection in a functionally hemizygous male [$R/-$ ($-$, failure to express a paternal allele)]. That male, mating with his sisters, would then perpetuate and amplify the resistance allele within all of his female progeny. Thus, in the presence of insecticide selection, within only a few generations a large number of homozygous resistant females can be produced. When mated to a resistant brother, those females would have dispersed to begin purely homozygous resistant inbreeding lines. The unique combination of the coffee berry borer's life history and chromosome cycle may thus promote the rapid spread of a single resistance-associated mutation through a number of individual inbreeding lines. In view of the past global dispersal of *H. hampei*, presumably in unprocessed coffee berries, the appearance of endosulfan-resistant lines in New Caledonia may thus represent a serious threat to the international coffee industry.

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