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SHORT NOTE

Lack of nucleotide variability in a beetle pest with extreme inbreeding

D. Andreev, H. Brellid,* L. Kirkendall,* L. O. Brun† and R. H. ffrench-Constant

*Department of Entomology, University of Wisconsin-Madison, Madison, Wisconsin, USA, *Zoology Museum, University of Bergen, Norway, and †ORSTOM, Noumea, New Caledonia*

Abstract

The coffee berry borer beetle *Hypothenemus hampei* (Ferrari) (Curculionidae: Scolytinae) is the major insect pest of coffee and has spread to most of the coffee-growing countries of the world. This beetle also displays an unusual life cycle, with regular sibling mating. This regular inbreeding and the population bottlenecks occurring on colonization of new regions should lead to low levels of genetic diversity. We were therefore interested in determining the level of nucleotide variation in nuclear and mitochondrial genomes of this beetle worldwide. Here we show that two nuclear loci (*Resistance to dieldrin* and ITS2) are completely invariant, whereas some variability is maintained at a mitochondrial locus (COI), probably corresponding to a higher mutation rate in the mitochondrial genome. Phylogenetic analysis of the mitochondrial data shows only two clades of beetle haplotypes outside of Kenya, the proposed origin of the species. These data confirm that inbreeding greatly reduces nucleotide variation and suggest the recent global spread of only two inbreeding lines of this bark beetle.

Keywords: inbreeding, nucleotide variability, *Resistance to dieldrin*, mitochondrial DNA, coffee berry borer, *Hypothenemus hampei*.

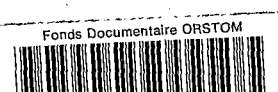
Introduction

The coffee berry borer, *Hypothenemus hampei*, is the major insect pest of coffee and has spread to most of the coffee-growing countries of the world (Le Pelley, 1968). This insect has also recently evolved resistance to the insecticide endosulfan (a cyclodiene type compound) (Brun *et al.*, 1989, 1990), which is one of the main chemicals used in control of the borer. As well as being a remarkably successful insect pest, *H. hampei* also displays an unusual life cycle with regular inbreeding. Simple population genetic theory suggests that this regular inbreeding and the founder effects occurring on the colonization of new regions should lead to low levels of genetic diversity. We were therefore interested in documenting the levels of nucleotide variability in this inbreeder and in trying to reconcile the expected reduction in variability with the ecological success of the insect and its ability to evolve insecticide resistance.

The coffee berry borer shows regular inbreeding. Single mated female *H. hampei* burrow into coffee berries and produce large single families with highly distorted sex ratios (ten females to one male). The dwarfed males are flightless and mate with their sisters in the natal berry. The beetle is also 'functionally haplodiploid', as paternal genes in males are neither expressed nor transmitted (Brun *et al.*, 1995a, b). Thus, both the nuclear and mitochondrial genome are strictly maternally inherited and should share an identical genealogy. Resistance to the cyclodiene type insecticide endosulfan has also recently been documented in *H. hampei* in the South Pacific island of New Caledonia (Brun *et al.*, 1989, 1990). Resistance is associated with replacement of a single amino acid, alanine302 > serine, in the γ-aminobutyric acid (GABA) receptor subunit coded for by the *Resistance to dieldrin* gene (ffrench-Constant *et al.*, 1994).

Here we report that both the cyclodiene resistance gene *Rdl* itself and a different nuclear locus, the Intergenic Spacer region (ITS2) of the 5.8S and 28S ribosomal RNA, show a complete absence of nucleotide variability across the globe (with the exception of the resistance associated point mutation in *Rdl*). Although a mitochondrial locus, cytochrome oxidase I (COI) retains some nucleotide variation. Phylogenetic

Received 11 August 1997; accepted 1 October 1997. Correspondence: Dr R. H. ffrench-Constant, 237 Russell Laboratories, 1630 Linden Drive, Madison, WI 53706, USA. e-mail: ffrench@vms2.macc.wisc.edu.



analysis of this mitochondrial DNA variation suggests that only a few inbreeding strains of the coffee berry borer have colonized the world. The factors likely to reduce nucleotide variation in this inbreeder and the contrast with the remarkable ecological success of this beetle are discussed.

Results and Discussion

Lack of nuclear nucleotide variation

To test whether or not the level of nucleotide variation in *H. hampei* is reduced by inbreeding we examined two nuclear loci (*Rdl* and ITS2) and one mitochondrial locus (COI) via PCR amplification and direct nucleotide sequencing of the PCR products. We sequenced all three loci in seventeen strains collected world-wide. Sequencing of 800 bp from the insecticide resistance associated locus *Resistance to dieldrin* (ffrench-Constant *et al.*, 1994) (Fig. 1a) revealed no nucleotide variation, except for the presence or absence of the insecticide resistance associated nucleotide substitution. To guard against the possibility that this locus was under abnormally strong selection (although resistant strains have only been collected from the South Pacific island of New Caledonia) we also examined ITS2, a non-coding region of a second nuclear locus highly variable in other insects (Fig. 1b). Sequencing of 747 bp from ITS2 of all strains also revealed no variation.

Amplification and sequencing of both *Rdl* and ITS2 from the related inbreeding beetle *H. obscurus*, showing 1.9% and 4.0% divergence respectively from *H. hampei*, was carried out to guard against unintended repeated PCR amplification of exactly the same product. We also sequenced 1203 bp from the *H. hampei* mitochondrial locus COI (Fig. 1c) and found twenty-one variable positions (1.8% variation) within populations. This higher level of variation in the mitochondrial genome can be explained by an apparent rapid rate of evolution (higher mutation rate) in *H. hampei* mitochondrial DNA. Thus, the open reading frame of the mitochondrial COI locus of *H. hampei* and *H. obscurus* shows a very high level of divergence (Ks, 0.84), whereas intron sequence from the nuclear locus *Rdl* diverges by only 2%.

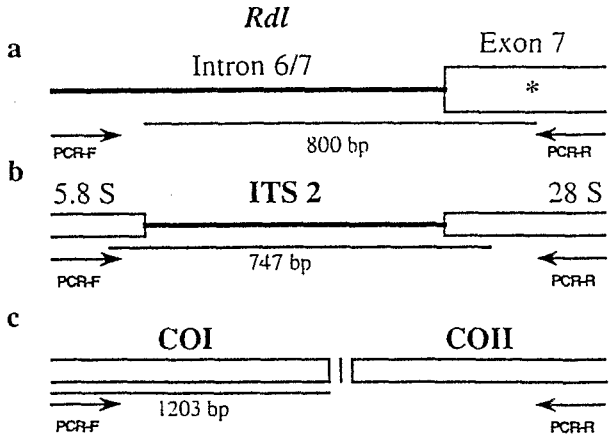


Figure 1. Diagram showing the relative location and lengths of the regions sequenced in the two nuclear loci (a) *Resistance to dieldrin* (*Rdl*, DDBL/EMBL/GenBank accession number AF037324) and (b) the second Intergenic Spacer (ITS2) of the two ribosomal RNA genes (accession number AF037326), and (c) the single mitochondrial locus cytochrome oxidase I (COI, accession number AF037325). A star indicates the location of the resistance associated mutation in *Rdl*.

Factors contributing to reduction in nucleotide variation

Several factors associated with different insect life cycles might be expected to reduce genetic polymorphism. In aphids, for example, these include parthenogenicity (anholocycly), annual population bottlenecks and founder effects (Loxdale & Brookes, 1989). Thus in aphids average biochemical polymorphism (P) is reduced, as is average heterozygosity (H) with values of approximately 4.5% against an average of 7.3% in other insects (Loxdale *et al.*, 1985). However, two factors relating to these studies are relevant to the current discussion. Firstly, biochemical polymorphism is maintained in aphids even in the presence of anholocycly. Secondly, many studies of variability in insects have been restricted to allozyme analysis and the level of underlying nucleotide variability has not been assessed.

The aim of this study was therefore to quantitate the reduction in nucleotide variation associated with the unusual life cycle of the coffee berry borer. Several factors associated with the life cycle of *H. hampei* would be expected to reduce nucleotide variation. (1) Only single families are usually found in each coffee berry. Full-sib mating would therefore be expected to reduce heterozygosity at a rapid rate. Calculations show a rapid initial decline in heterozygosity over intial generations (values reducing as follows for each generation; 1.0, 1.0, 0.75, 0.62, 0.50, 0.40, 0.32, 0.14, 0.04, 0.01 and 0.002) followed by an average of a 19% loss at each subsequent generation (Wright, 1921). Such a decline would dramatically reduce heterozgosity and full-sib mating may therefore be the dominant factor in reducing nucleotide variation in *H. hampei*. (2) Bottlenecks occurring upon colonization of new regions. As the spread of *H. hampei* to many countries has been relatively recent, little divergence within these founder populations would be expected. This factor would be expected to affect both mitochondrial and nuclear genomes. Further, as a result of both the level of inbreeding and population bottlenecks, as a particular mutation fixes in a population, variants present in the genome in which it arose will also be fixed, as there is no outcrossing. (3) Finally, the appearance of deleterious mutations will also elimi-

nate the genomes in which they are found via natural selection, also reducing diversity. Our results on *H. hampei* thus confirm the predicted reduction in the nucleotide variability of this beetle, data also supported by the apparent lack of variation in a global survey of sixteen allozymes (Breilid & Kirkendall, unpublished).

Interestingly, despite the apparent absence of nucleotide variation in the two nuclear loci sequenced, the resistance associated point mutation in *Rdl* was present and has been selected for at least in the South Pacific island of New Caledonia (ffrench-Constant *et al.*, 1994). If resistance is absent from the rest of the world (a systematic survey has not been completed), this may represent an independent origin of resistance in a single inbreeding line. However, regardless of the uniqueness of resistance in New Caledonia, the level of inbreeding in *H. hampei* will constrain independent resistance associated mutations within a limited number of colonizing lines. In contrast, examination of a global *Rdl* phylogeny in an outbreeding flour beetle, *Tribolium castaneum*, shows clear evidence for multiple independent origins of cyclodiene resistance associated mutations on different continents (Andreev *et al.*, 1997).

Global colonization by a few inbreeding lines

Examination of the most parsimonious tree of the mitochondrial data (Fig. 2) suggests that outside of Kenya, the putative ancestral origin of the species, the world has been colonized by only two inbreeding lines of *H. hampei*, one encompassing central and southern America and the other all strains from south east Asia, the south Pacific, Jamaica and the Ivory Coast. Two further factors are of interest in relation to the mitochondrial variability. Firstly, on a geographical level, the particular strain sequenced from Kenya does not correspond to either of the two colonizing clades. This may reflect a greater mitochondrial haplotype diversity in the source country. However, this assumption would have to be supported by analysis of a larger number of strains from Kenya. Secondly, given the unusual chromosome/life cycle of *H. hampei*, in which both the nuclear and mitochondrial genomes are effectively maternally inherited, it is interesting that variation is present in the mitochondrial genome but virtually absent from the nuclear loci examined. This higher level of mitochondrial variation is probably due to a higher mutation rate versus the nuclear genome, as documented for other organisms, or could be main-

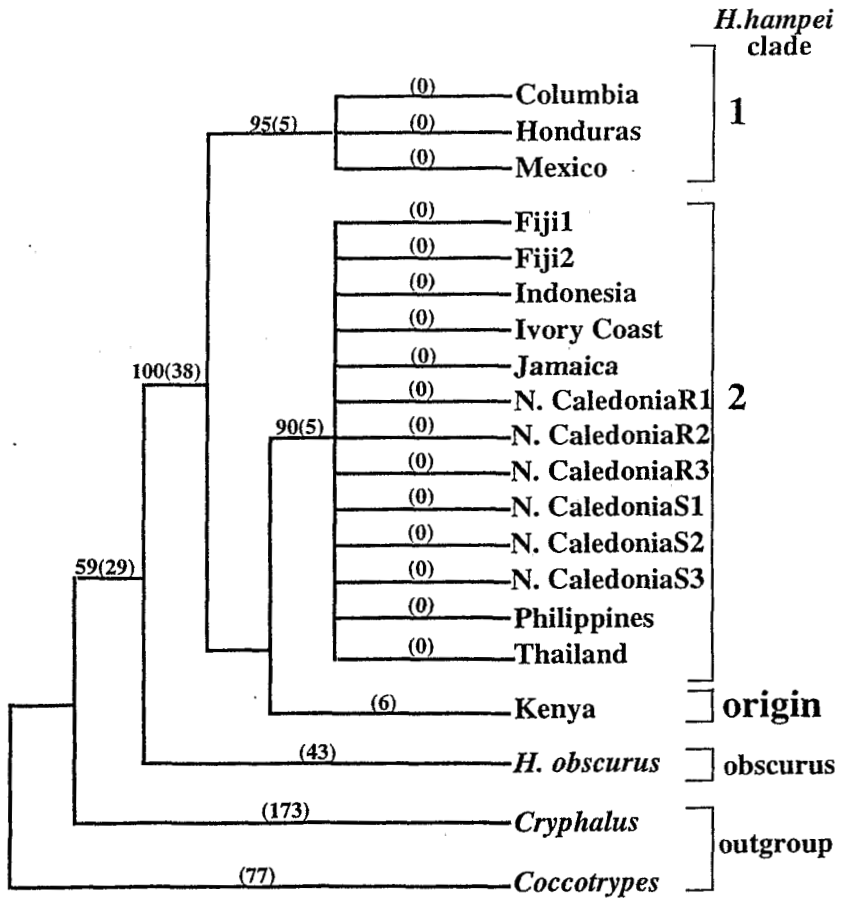


Figure 2. Strict consensus tree of COI data derived by phylogenetic analysis using parsimony (PAUP version 3.0). Bootstrap values greater than 50% are given alongside the number of nucleotide changes in parentheses. The tree is rooted to the two outgroup species *Coccotrypes dactyliperda* (F.) and *Cryphalus aff. indicus* (Eichoff). Outside of Kenya (the putative ancestral origin of *H. hampei*) note the two invariant clades corresponding to East Africa Central/Southern America and South-East Asia/South Pacific (including Jamaica and Ivory Coast). Strains are named according to their country of origin. The suffixes R1–R3 and S1–S3 indicate three cyclodiene resistant and three susceptible strains collected from New Caledonia.

tained by independent selection of the two different mitochondrial haplotypes. The possibility of the mitochondria being heteroplasmic (more than one haplotype per individual) can be excluded as no heterozygosity was apparent in the sequencing ladders (direct sequencing of PCR products). Formal estimates of the expected rate of decline in mitochondrial nucleotide variability are difficult to estimate in the absence of data relating to the number of mitochondrial particles transmitted per generation (J. Crow, pers. comm.).

Taken together, these data support a hypothesis of recent world-wide spread of a few inbreeding coffee berry borer lines and confirm the predicted theoretical depression in nucleotide variation associated with inbreeding and population bottlenecks. Interestingly, inbreeding has evolved repeatedly in bark beetles: if other species are similarly genetically depauperate, then their considerable ecological (Kirkendall, 1993) success poses an interesting challenge to evolutionary biologists.

Experimental procedures

Strain collection, PCR amplification and sequencing

H. hampei strains were collected in coffee berries from the field. Beetles were dissected from the berries in the laboratory and raised on artificial diet (Brun *et al.*, 1993). Progeny were bioassayed with endosulfan (Brun *et al.*, 1991) to select for resistant heterozygotes and strains showing resistance were then made homozygous by repeated insecticide selection and crossing of survivors *inter se*. Genomic DNA was prepared (Andreev *et al.*, 1994) from each homozygous strain (strains consistently surviving a dose of insecticide discriminating homozygous from heterozygous insects). For PCR, approximately 100 ng of genomic DNA was added to a 50 μl reaction containing 0.2 μM of each primer, 0.2 mM dNTPs and 1.5 units of *Taq* polymerase. Samples were denatured at 94°C for 2 min and then PCR was carried out for 35 cycles of 1 min denaturation at 94°C, 2 min annealing at 50°C and 3 min extension at 72°C. Regions of the three loci were amplified by the PCR and reaction products sequenced directly without cloning (to avoid polymerase errors often associated with individual cloned products). PCR primers used for *Rdl* were: forward TGTTGACTCTAAATATGACGTCGTCACCTCACCG, reverse GACCCCGGGTAAAATGTTACGAAAATAAAC, for ITS2: forward GTGGATCCTGTGAACTGCAGGACACATG, reverse GTGAATTCATGCTTAAATTTAGGGGGTA and for COI: forward GGATCACCTGATATAGCATTCCC, reverse GTTTAAGAGACCAGTACTTG. PCR products were sequenced using an ABI 373 automated sequencer (Applied Biosystems).

Phylogenetic analysis of COI data

A strict consensus tree of COI data was derived by phylogenetic analysis using parsimony using the PAUP software package version 3.0. Bootstrap values were calculated and those greater than 50% are given alongside the number of nucleotide changes in parentheses on the tree (Fig. 2). The tree is rooted to the two outgroup beetle species *Coccotrypes dactyliperda* (F.) and *Cryphalus aff. indicus* (Eichoff).

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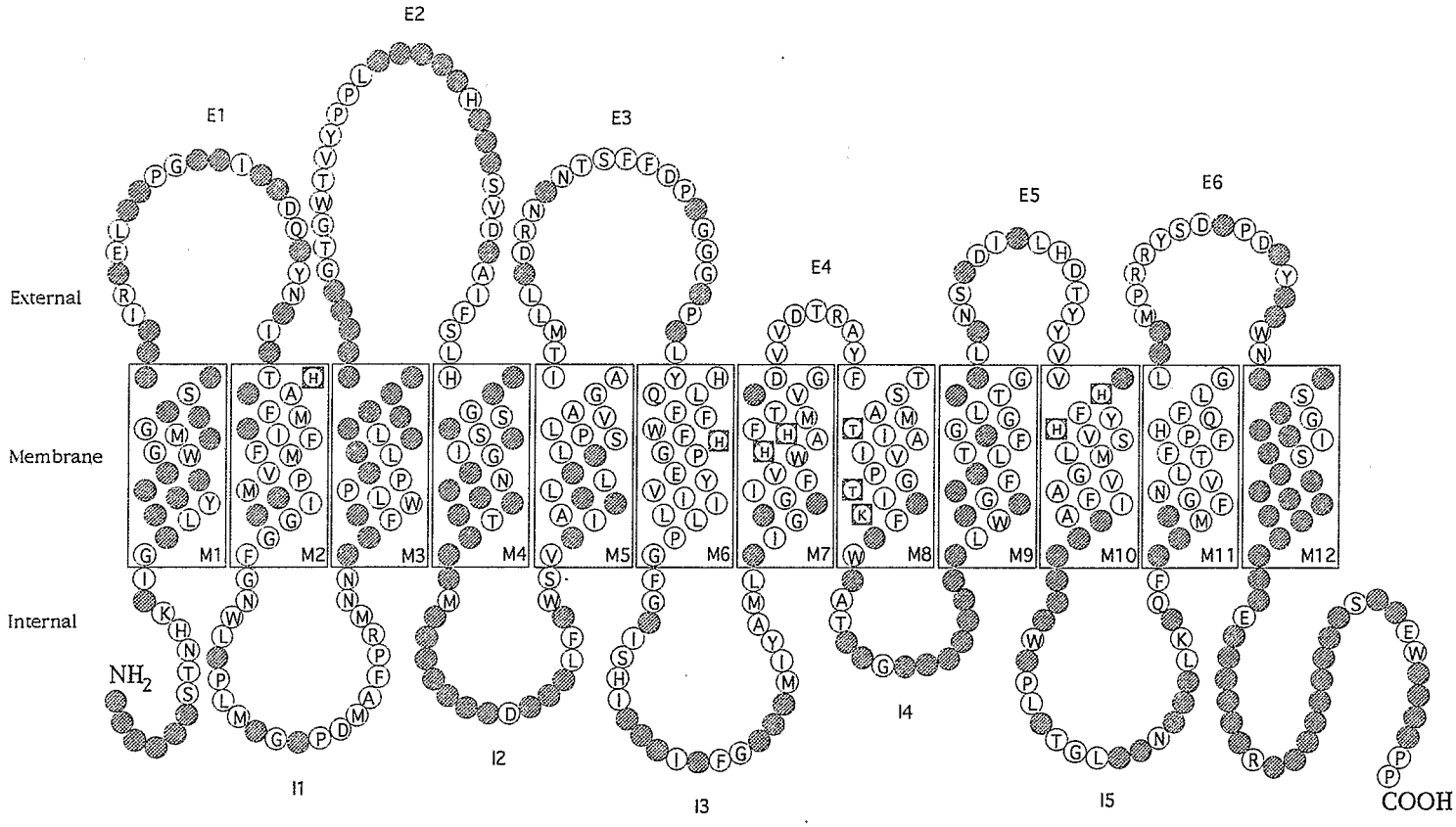

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