

1. Genetic Structure of *Ips pini* (Say) Populations

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The pine engraver, *Ips pini* (Say) is an important pest of North American pine trees and has been the subject of considerable biological research (Wood and Bright 1992). Although these beetles feed mostly on dead or dying trees, epidemic populations can attack and kill living trees. Aggregation pheromones mediate the attraction of *I. pini* and other bark beetles to suitable food sources (Wood 1982).

Pheromones and their role in aggregation behavior have been investigated extensively in *I. pini* (for example, Miller and others 1996, 1997; Seybold and others 1992, 1995; Teale and others 1991). The pheromone components lanierone and ipsdienol are known to be active for *I. pini*; however beetle populations across North America vary in the production of and response to lanierone and to the enantiomeric blend of ipsdienol. Generally, populations in eastern North America produce and respond to <60 percent (-) - ipsdienol ("New York" phenotype) whereas in western North America they produce and respond to >90 percent (-) - ipsdienol ("California" phenotype) (Seybold and others 1995). Lanierone is produced in small quantities by male *I. pini* from New York (Teale and others 1991) and has recently been discovered in a California population as well (Quilici and others, in press). The synergistic effect of lanierone with ipsdienol is weaker for California *I. pini* when compared with New York *I. pini* (Seybold and others 1992). The attraction of California *I. pini* to >90 percent (-) - ipsdienol is hindered by the presence of high quantities of (+) - ipsdienol (Birch and others 1980). Therefore, the preference of female *I. pini* for the "conspecific" pheromone phenotype may preclude mating among eastern and western *I. pini*. (Hager and Teale 1996; Piston and Lanier 1974; Teale and others 1994)

Pheromone phenotypic variation also occurs within western North America. Populations from southeastern

British Columbia, Idaho, and Montana produce between 91 percent and 95 percent (-) - ipsdienol while other western populations produce > 94 percent (-) - ipsdienol (Seybold and others 1995). Based on pheromone and cuticular hydrocarbon data, Seybold and others (1995) hypothesize that two phenotypes exist in western North America south of the Canadian border.

A more extensive investigation of pheromone phenotypes in British Columbia showed the existence of "New York," "California," and intermediate pheromone phenotypes in different individuals within and among populations (Miller and others 1996). British Columbia may represent an area of intergradation of eastern and western populations, given the variation of pheromone production among individuals. These populations presumably interbreed (Miller and others 1996), although the rate of interbreeding will be affected by female pheromone phenotype preference.

Eastern and western populations of *I. pini* were not always considered a single species. They were described as separate species: *I. pini* in the east and *I. oregonis* in the west. Morphological differences, such as the density of punctures on the pronotum and the smoothness of the elytra, were used to distinguish the eastern and western species. However, these morphological characters intergrade in western Canada and the species were synonymized by Hopping (1964). Lanier (1972) added further evidence of conspecificity with laboratory breeding experiments showing that the eastern and western individuals produce viable offspring.

However, the population variation found in pheromone and morphological characters once again calls into question the species status of *I. pini*. Do two species exist and hybridize in British Columbia, or is *I. pini* one species whose populations were allopatrically separated in the past?

To address this hypothesis, a genetic marker independent of pheromone production is needed to assess the relationship among *I. pini* populations. DNA sequence from the mitochondrial cytochrome oxidase I gene was used because it is presumably independent of pheromone production, and provides enough nucleotide variation to resolve relationships among populations (see Simon and others 1994, for review of mitochondrial genome use in insect phylogenetics).

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A total of 97 individuals were PCR-amplified and directly sequenced over 354 base pairs of the mitochondrial cytochrome oxidase I gene. These sequences were used to reconstruct a single most parsimonious phylogeny (heuristic search with 10 random stepwise additions, Swofford 1993). Beetles were sampled from 10 localities, including 3 from California, 2 from New York and 1 each from Alberta, Colorado, Washington, Montana, and Arizona. Ten specimens were sampled from each locality except Montana, where only seven specimens were obtained.

Two measures of gene flow (Lynch and Crease 1990, Slatkin and Maddison 1989) indicate that the number of migrating individuals per generation (N_m) is 0.4 (by both measures) among all *I. pini* populations. An N_m of approximately 1 is theoretically sufficient to maintain genetic homogeneity among populations (Avice 1994). Therefore, estimated gene flow for the sampled *I. pini* populations is moderate.

Phylogenetic analysis revealed three monophyletic lineages: "eastern" (New York), "Rocky Mountain" (Alberta and Colorado), and "western" (all Californian populations and Arizona). One Arizona individual had a haplotype consistent with the "eastern" lineage. All three lineages were present in the Washington and Montana populations. This result shows genetic polymorphism in regions of pheromone phenotype variation.

The mitochondrial phylogeny is generally concordant with the geographic pattern of pheromone phenotypes (Seybold and others 1995). The "New York" and "California" pheromone phenotypes are associated, respectively, with the "eastern" and "western" lineages. The "Rocky Mountain" lineage may be associated with the second western pheromone phenotype (91 percent to 95 percent (-)- ipsdienol); however pheromone analysis from additional collection localities in this area are needed.

The estimated moderate gene flow among populations supports the hypothesis that *I. pini* is one species. However, the mitochondrial phylogeny of populations indicates that there are three distinct lineages. Since female pheromone phenotype preferences exist between eastern and western *I. pini*, it may be reasonable to formalize the trivial names of "New York" and "California" *I. pini* with recognition of the "eastern" and "western" lineages as subspecies. The "Rocky Mountain" lineage may also represent a subspecies; however few populations have been sampled and diagnostic morphological characters have not yet been demonstrated for

this lineage.

More specimens from all regions, especially British Columbia and northwest US localities, will test the robustness of these preliminary results. Mitochondrial markers only reflect maternal lineage, and the presence of individuals with "eastern" lineage in Arizona, Montana, and Washington may indicate introgression due to "eastern" lineage females breeding with "western" lineage males. The addition of a nuclear gene marker will assess the phylogeny and gene flow for both sexes and should help determine if "eastern" lineage males ever breed with "western" lineage females and conversely, determine if "western" lineage males breed with "eastern" lineage females. Concordance of the mitochondrial and nuclear data sets would allow a better assessment of the need for recognition of eastern and western *I. pini* as subspecies.

2. Insecticide Resistance and Nucleotide Variability in the Coffee Berry Borer

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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, whilst 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.

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 and others 1989, 1990), which is one of the main chemicals used in control of the borer. Whilst 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 (10 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 and others 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 and others 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 and others 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*). Whilst a mitochondrial locus, cytochrome oxidase I (COI) retains some nucleotide variation. Phylogenetic analysis of this mitochondrial DNA variation suggests that only a few inbreeding strains of the coffee berry borer have colonized the globe. 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

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sequenced all three loci in 17 strains collected globally. Sequencing of 800 bp from the insecticide resistance associated locus *Resistance to dieldrin* (French-Constant and others 1994) 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. 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 percent divergence, respectively, from *H. hampei*, was carried out to guard against unintended repeated PCR amplification of exactly the same product. We also sequenced 1,203 bp from the *H. hampei* mitochondrial locus COI and found 21 variable positions (1.8 percent 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 percent.

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 and Brookes 1989). Thus, in aphids average biochemical polymorphism (P) is reduced, as is average heterozygosity (H) with values of approximately 4.5 percent against an average of 7.3 percent in other insects (Loxdale and others 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 initial 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 percent loss at each subsequent generation (Wright 1921). Such a decline would dramatically reduce heterozygosity, 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 eliminate 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 16 allozymes (Breilid and 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 (French-Constant and others 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 and others, in press).

Global colonization by a few inbreeding lines

Examination of the most parsimonious tree of the mitochondrial data suggests that outside of Kenya, the putative ancestral origin of the species, the globe 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 maintained 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 global 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.

3. Genetic Variation in *Dendroctonus frontalis*, Within and Between Populations

Jane Leslie Hayes¹

Many species of *Dendroctonus*, particularly the so-called aggressive species, are notorious outbreak organisms with more or less predictable or characteristic, cyclic patterns of outbreak locally if not regionally. *D. frontalis*, for example, exhibits an approximately 7-10 year cycle with 2-3 year duration of outbreak. The last south-wide outbreak of *D. frontalis* began in 1992 and peaked in 1995, with an estimated timber resource loss of \$350 million in that year alone (Price and others 1998). The previous peak occurred in 1986. The presumption is that at epidemic levels, beetle populations move toward a maximum rate of increase; environmental resistance eventually imposes limits, and the populations begin to wane. Although numerous efforts have been made to determine the principle factor(s) responsible for contributing to this phenomenon, none have yet been identified. Turchin and others (1991) found that the pattern of *D. frontalis* population fluctuations in east Texas over a 30-year span most resembled a delayed density-dependent function. They suggested that this pattern is consistent with the notion that natural enemies exert the most significant influence and regulate bark beetle populations. There are numerous pieces of evidence to support this hypothesis, such as the ability to forecast beetle populations for the present year using the ratio of bark beetles to natural enemies (in particular, a clerid predator *Thanasimus dubius*) captured in traps (developed by Dr. R. F. Billings, Texas Forest Service), and the recent body of work by Reeve (for example, 1997) which may lead to a possible year-in-advance predictive model based on clerid and beetle numbers.

Because of the regularity of these outbreaks and the apparent population phase shift from endemic to epidemic, it is tempting to speculate about the potential role of genetics in the epidemiology of bark beetles. Such a possibility is not inconsistent with the mathematical description of outbreak cycles and has long been speculated about by early researchers attempting to

explain outbreaks in forest defoliators (for example, Chitty 1965, Wellington 1964) and bark beetles (Raffa and Berryman 1987). Whether cause or effect—that is, as a consequence of expansion to epidemic levels or as a factor contributing to expansion—the genetic structure of epidemic populations is presumably different from endemic populations (see discussion by Mitter and Schneider 1987). Indeed, the genetic changes in an explosive population over time may actually contribute to the decline (or “implosion”) of the population, and thus contribute to the pattern of outbreak. Relatively high levels of genetic variation in *D. frontalis* (Anderson and other 1979, Florence and Kulhavey 1981, Namkoon and others 1979) and other bark beetles (reviewed in Mitton and Sturgeon 1982, and in Hayes and Robertson 1992) were initially detected with enzyme electrophoretic studies. Florence and others (1982) were among the first, using a polymorphic esterase in *D. frontalis*, to document the connection between genetic structure and population behavior, finding that genetic diversity is maintained with dispersal. Similarly, isozyme variation in bark beetles has been related to differences in host species and/or the margin of a particular host species range (reviewed by Stock and other 1992 and Lakatos this proceedings). Stauffer (1997b) has found enzyme differences between first-attacking and later-arriving beetles. However, no studies to date have provided direct genetic evidence of differences between endemic and epidemic populations.

Current molecular techniques and sophisticated programs have opened up new opportunities for genetic studies of bark beetle populations (Hayes and Robertson 1992). A technique as simple as RAPD-PCR (random amplification of polymorphic DNA through polymerase chain reaction—see Carter, this proceedings for a detailed discussion of this technique) may permit the fingerprinting of a population and snapshots through time as an infestation progresses. Ultimately, a look at *D. frontalis* populations across the southeastern United States may provide a new predictive tool, if changes in infestations or population structure are consistent in epidemic vs. endemic populations. Differences between disparate populations, such as those found in the United

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States compared to those in Mexico or Central America, may be equally important. The identification of unique biotypes may have significant bearing on the development of different resource management programs.

Within-Population Variation--Differences in Paternity of Multiple Broods

Before the population structural dynamic can be understood, the mating pattern of bark beetles such as *D. frontalis* or *D. brevicornis*, which can produce multiple broods per generation, must be examined. Work by Gagne and others (1980) and Wagner and others (1981) uncovered the potentially important contribution of second and even third broods to the population dynamics of *D. frontalis*, but no studies have addressed the genetic consequences of multiple broods. Most significantly, the role of the male and his contribution with each successive mating are unknown. In their studies (Gagne and others 1980, Wagner and others 1981), new males were provided to females in production of second and subsequent broods. It is not known whether the sperm of the first male is expended or shunted prior to the next mating, whether there is sperm competition, mixing, or precedence, or whether a new male and/or additional sperm are required.

Paternity in insects has been assessed a number of ways using molecular genetic techniques (for example, Benken and others 1998; Fjerdingstad and others, in press; Hooper and Siva-Jothy 1996; Moritz and others 1995). I proposed to identify and use RAPD markers to examine the parents and offspring of multiple broods of *D. frontalis*. There are two major hurdles in this effort (the work described here involves multiple collaborations that are identified in Future Research Direction sections below and Hayes and others 1998). The first is finding suitably unique populations, that is, populations with unique polymorphisms or consistent proportions of polymorphisms. The second hurdle is arriving at a protocol that will permit the retrieval of parent adults as well as brood from two successive matings. This is a particular problem with *Dendroctonus* species because continuous rearing is problematic. A satisfactory solution has developed to the second problem, but not the first. However, while attempting to resolve the first problem, we (Hayes and others 1998) made some intriguing observations about two geographically isolated populations of *D. frontalis*, which will be described below.

To obtain brood from two successive matings, we employed a modification of methods developed by

Rhodes and others (1998). A protocol was developed using prepared sections of freshly cut loblolly pine (*Pinus taeda*, a preferred host of *D. frontalis*) bole. These bolts were approximately 15-20 cm in diameter and length, each was halved, and all exposed areas were coated with paraffin to reduce desiccation. A newly emerged female and male pair was introduced into half of a gelatin capsule inserted into a small hole that had been cut with a cork borer through the bark to the phloem. After 7 to 14 days, the bark was carefully peeled off of the bolt to expose the parent adults and developing brood. The female was then paired with a second newly emerged male and introduced into a fresh bolt in the manner described above. Again, in 7 to 14 days this bolt was stripped and the parent adults retrieved along with eggs or larvae from the second brood. A minimum of 20 offspring from each brood is needed for adequate sample size. Additionally, a minimum of 20 first-brood bolts must be established to achieve adequate replication because for unknown reasons, a proportion of attempted matings (about 25%) are not successful in both first and second pairings. The longer the bolts are held before peeling the bark, the further developed the offspring are. For practical reasons, larval stages are easier to work with than eggs; however, we have successfully isolated DNA from individual eggs.

Future Research Direction

To identify distinguishing markers, we screened nearly 50 primers using the robotic technology of the Genetic Institute of the South (USDA-FS, SRS, Saucier, MS). Taken altogether we found considerable variation within and among *D. frontalis* obtained from different infestation sources in central Louisiana; however, few distinctive polymorphisms were revealed. In a second screening effort, using about 20 primers, we identified distinctive markers for *D. frontalis* from Florida (supplied by Dr. J. Meeker, Florida Division of Forestry). Unfortunately, we were unable to obtain additional infested bark from Florida to correspond with the availability of newly-emerged individuals from central Louisiana or elsewhere. This effort will be continued.

Between-Population Variation – Differences Between Allopatric Populations

An opportunity to conduct mating trials with a *D. frontalis* population from southern Chiapas, Mexico, presented itself in April 1998. The presumption was that, compared to beetles from central Louisiana, these

beetles were likely to be genetically distinctive, as revealed through RAPD-PCR techniques, given the extreme geographic isolation of the two populations. Live specimens of these presumed *D. frontalis* were collected from a known host tree *P. oocarpa* (Tovar and others 1995) and were transported on ice to the Alexandria Forestry Laboratory (USDA-FS, Pineville, LA) for genetic analysis. Surviving individuals that were apparently healthy (missing no tarsi and able to respond to stimuli), were introduced into six loblolly bolts as described above with the hope that the females could then be mated to males from central Louisiana. One of the six bolts was used to cross a female from Mexico to a male from Louisiana; this combination produced approximately 10 cm of gallery but no brood. Of the five Mexico by Mexico crosses, one pair failed to initiate a nuptial chamber, but 50% of the others produced both gallery and a small number of apparently viable eggs. No second matings were possible. Although these results indicate that the *D. frontalis* from Mexico can use a novel host, the results are inconclusive relative to the mating ability of members of these two allopatric populations (Hayes and others 1998). Previous efforts to mate geographically distinct *D. frontalis* populations (Lanier and others 1988, Vite and others 1974) showed relatively high interfertility, suggesting the absence of post-mating isolating mechanisms.

The preliminary results of subsequent RAPD-PCR analyses confirmed the genetic differentiation between individuals from the Mexico and Louisiana populations. Although a relatively small number of primers were screened (about 12). Several showed distinctive population identifying markers, as well as markers that could be used to distinguish between the two observed morphs found among the individuals collected in Mexico (Hayes and others 1998).

We observed two external morphological features, overall size and corresponding mycangial conformation of females of both sizes, that differed consistently among individuals collected from the Chiapas site and from the same host tree. *D. frontalis* size is known to vary over the flight season within a region (for example, Hedden and Billings 1977) and among regions (Lanier and others 1988). Among the beetles collected in Mexico, we found they fell into two relatively distinct size classes; males and females of one morph were about 40% (n=20) larger in surface area on average than males and females of the other morph; there was no difference in size between the smaller morph from

Mexico and beetles from central Louisiana (Hayes and others 1998). Additionally, the mycangia, a characteristic cuticular collar-like structure containing symbiotic fungi, of the large females from Mexico was less pronounced and appears to be narrower when compared to females from the smaller morph or central Louisiana.

In their review of the geographic distribution of *Dendroctonus* throughout Mexico, Zúñiga and others (in press) indicate that both *D. frontalis* and *D. mexicanus*, a larger member of the *frontalis*-complex, use *P. oocarpa*. Additionally, *D. mexicanus* and *D. frontalis* are known to occur in the same host tree (Zúñiga and others 1995). Genitalia of males from the two Mexican morphs and from specimen from central Louisiana (n=6-12) were dissected, and seminal rods were isolated and mounted (following Lanier and others 1988). Seminal rod is one of several characteristics used by Lanier and others (1988) to distinguish members of the *frontalis*-complex, which includes *D. approximatus*, *D. frontalis*, *D. mexicanus*, and the little known *D. vitei* (compare Tovar and others 1995). Despite the distinctive external morphology differences, the male seminal rod does not appear to differ among the two morphs from Mexico, nor the samples from central Louisiana (Hayes and others 1998). Chromosome numbers also differ between *D. frontalis* (diploid no. = 16) and *D. mexicanus* (diploid no. = 12) (Lanier 1981, Salinas-Moreno and others 1994). Chromosomal preparations of approximately 20 individuals made by Cisneros and Zúñiga (IPN, Mexico City, Mexico) also suggested that these two morphs were *D. frontalis*. Whether the observed morphological differences are genetically based remains to be demonstrated.

Future Research Directions

Additional genetic work is planned in collaboration with G. Zúñiga, R. Cisneros, and J. Macías-Sámano (see Zúñiga and others this proceedings). In addition to these efforts, samples have been supplied to Dr. Kelley, for preliminary mitochondrial DNA analysis using techniques described in Kelley and Farrell (1998) and in these proceedings.

4. Using RAPD-PCR to Study Intraspecific Variation Among Bark Beetles

M. Carol Carter¹

Detection of differences between genetically similar individuals, or identification of clonal populations, requires methods with high resolution. Characterization of samples using methods based on nucleotide sequence variations have distinct advantages over phenotype-based analyses, because nucleotide variation is free of confounding effects from environmental and developmental influences. Currently, the most widely used molecular methods for characterizing variability between operational taxonomic units (OTUs) use the polymerase chain reaction (PCR, Mullis and others 1986) at some stage in the characterization process (Newton and Graham 1997). The PCR amplifies targeted DNA by repeated rounds of thermal denaturation, cooling, and polymerase-driven replication. Target sequences are amplified exponentially as: $a = (2p)^n$, where a is the number of double-stranded targeted sites after amplification, p is the number of double-stranded targeted sites prior to the initial denaturation, and n is the number of cycles (M.C. Carter, unpublished calculation). While less than optimal conditions, including declining efficiency of the polymerase, will always result in less product than $(2p)^n$, the PCR can routinely produce an increase of 10^5 after 25 cycles (Newton and Graham 1997).

Random amplification of polymorphic DNA (RAPD) is a PCR method that utilizes single oligonucleotide primers to target multiple, anonymous sites in a DNA sample. Upon electrophoretic separation and staining of the amplification products, a fingerprint of DNA fragments is produced (Williams and others 1993). A series of RAPD reactions, each with a different primer, produces a set of fingerprints for each DNA sample.

RAPD analysis can provide a more robust estimation of genotypic diversity than is possible with standard PCR. Robustness is related to the number and distribution of genotypic polymorphisms. Whereas standard PCR typically targets a single gene locus, RAPD-PCR

typically targets many genomic sites simultaneously. The sites targeted by RAPD primers are likely to be within non-coding regions throughout genomes and therefore are more likely to be neutral for fitness than are variations within functional genes (Li and Graur 1991). Because these non-coding sites can be considered relatively equal (evolutionarily), all polymorphisms in all fingerprints can be analyzed together to give a single, broadly based picture of variation among samples.

The versatility of the RAPD-PCR approach to studies of insect population was reviewed by Hoy (1994). More recent application of the RAPD method include determination of multiple founder populations of exotic insects (Carter and others 1996); paternity analysis (Hooper and Siva-Jothy 1996), and development of a molecular identification key to the species of a genus (Frey and Frey 1997).

While RAPD technology is now an accepted approach for generating data for population studies, there is less agreement on the most appropriate method for scoring and analyzing the data. In RAPD analysis, the presence of a band (+) is considered by many investigators to be the dominant expression of a locus, while the absence of the band (-), due to primer mismatch, is considered recessive (for example, Williams and others 1993, Hoy 1994). Theoretically, then, the homozygous dominant (++) with two fold target templates prior to amplification (p in the equation above), should produce a stronger band than the heterozygous (+ -) condition. However, experience has shown that such discrimination is unreliable and inaccurate under normal amplification and detection conditions (Williams and others 1993). Therefore, most investigators score RAPD patterns from individual insects strictly in relationship to presence/absence of fragments (Hoy 1994) with no adjustment in score based on band intensity, except, perhaps to count very weak bands as absent.

There are two schools of thought on tactics to employ for relatedness determinations—the cladistic school and the phenetic school. The cladistic method attempts to determine relatedness within an evolutionary

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context. The phenetic method requires no such assumptions about past events, and sorts out similarities and differences among OTUs strictly in an extant context. Most investigators using RAPDs take a modified phenetic approach, since assumptions concerning the evolutionary significance of the anonymous band polymorphisms that are generated by the RAPD are not possible. Additionally, RAPD analysis is most suited for closely related OTUs (reference Clark and Lanigan 1993, Weising and others 1995, p.151-152), which is also compatible with the phenetic approach.

In RAPD studies, dendrograms showing phenetic relatedness among samples are often constructed by unweighted pair group mean analysis (UPGMA, Sneath and Sokal 1973), an algorithm that develops clusters from a distance matrix derived from comparisons of band positions among all pairwise combinations of OTUs.

There are different ways to score fingerprints in order to produce a data set for distance calculation. For pairwise distance, some investigators calculate distance from the occurrence of shared fragments (presence only) (for example, Parani and others 1997). However, I agree with other investigators (Puterka and others 1993, Hooper and Siva-Jothy 1996) that variability is more accurately estimated (in closely related OTUs) by calculating distance from both alleles (+ and -). The proportion of matches, M (the shared presence and the shared absence of a band), is estimated from: $M = N_{ab} / N_T$, where N_{ab} is the number of matches between individuals a and b , and N_T is the total number of bands scored in the study (Puterka and others 1993). Individuals are said to be identical when $M = 1$, and share nothing in common (no matching bands, no matching absence of bands) when $M = 0$. For distance calculations with these relationships use: $D = 1 - M$ (Puterka and others 1993).

The equations given above are most commonly used for measures of distance between individual insects. However measures of distances between *populations* can also be attained by bulking individuals from single populations. (Here, populations may be defined loosely, according to, for example, collection site, host, or temporal factors.) In the bulking strategy, several individuals from a population are pooled for DNA extraction, and a multiple genome DNA sample is produced (for example, Michelmore and others 1991, Carter and others 1996). PCR reactions are set up in the typical way for RAPD analysis, and banding patterns similar to that which would have been obtained

from individual insects are produced. Bulked DNA produces consensus fingerprints that emphasize common characters in the population and minimize individual variation (Carter and others 1996). When the bulking strategy is used, reproducible band intensity differences are indicative of allele dosage differences, so that as in the first equation above [$a = (2p)^n$], p = dosage.

Dosage is likely to be a discriminating feature of populations, and distance calculations should provide for a measure of dosage by utilizing intensity of fragment bands as a basis for multistate scoring for each band position (Carter and others 1996). For multistate scoring of s bulked samples the equation

$$d_{ij} = \frac{1}{C_{ij}} \sum_{k=1}^{C_{ij}} |x_{ik} - x_{jk}| \quad \text{can be used, where } d_{ij} \text{ is the}$$

distance between two bulked samples ($i, j = 1, 2, \dots, s$), C_{ij} is the number of characters (fragment positions) that are scored in both populations, and x is character state (for example, one of the linearly related values 0, 1, 2, 3) representing dosage value (as estimated by band intensity) for the k th character (Carter and others 1996).

Since most people are accustomed to distance values (D) ranging from 0 to 1.0, distance between any two bulked samples (for example, i and j) may be expressed as $D_{ij} = d_{ij}/n-1$, where n is the number of character states.

Computerized imaging systems allow quantification of band intensity (representing dosage) by densitometry, giving the possibility of generating hundreds of character states, depending on the resolution of the instrument. The continuum of intensity values should probably be transformed into only a few character states, however, (for example, 4 states representing very low, low, medium, and high densitometry values) to de-emphasize variations due to methodology and instrument noise.

Whatever one's selection for distance calculation, the distance values obtained should be viewed as approximations that may underestimate distance. Erroneous similarities will be scored when amplicons arising from different loci occupy a single band position or when shared absence of band is unrelated to shared loci characteristics.

Inaccuracies in distance calculations will be exacerbated when there are differences in DNA quality in the sample set. Larger amplicons will be severely underrepresented in samples with significantly degraded DNA. Therefore, samples that lack higher molecular weight bands relative to the other samples, should be viewed with suspicion and perhaps should be excluded

from the study. Even when the samples that are to be compared all seem to have DNA of comparable quality, not all bands should be scored. Scoring should be limited to bands between about 1800 to 200 bp for ordinary protocols using Taq polymerase, since outside this range, artifacts of amplification become more pronounced.

Avoid contamination of samples by non-target organisms. Contamination becomes more likely when two morphologically similar species are sympatric, or when some of the target organisms are heavily parasitized. However, gut and hemocoel organisms may not be a problem (Cognato and others 1995). Second, when bulking, avoid confounding the analysis with sex-linked genetic variation.

Can the RAPD method be improved? Recently an approach termed amplified fragment length polymorphism (AFLP) has been developed (and patented!) that combines RFLP analysis and PCR to produce highly informative fingerprints. AFLP can be fine-tuned, like RAPDs, to define relationships within species, subspecies, populations, or kinships and can produce robust, broadly-based data sets (Hill and others 1996). In addition, AFLP amplifications can be conducted under higher stringency conditions than RAPDs, so that only perfect matches are allowed and scoring is less problematic. However, AFLP requires substantially more involved and expensive procedures including additional enzymatic reactions, custom made primers, and high resolution polyacrylamide gel electrophoresis.

In my opinion, neither AFLP nor hypervariable repetitive sequences (Post and others 1992) will supplant the RAPD approach as long as they require significantly more time and expense to perform than the latter, yet do not produce substantially improved results. However, the RAPD approach would benefit from innovations that allow more stable primer/template/polymerase interactions under higher-stringency conditions. With such improvements in methodology, scoring accuracy may be improved and problems of nonreproducibility between labs due to instrumentation and protocol differences may be alleviated.

5. Genetic Variation of *Ips typographus* L. Populations from Within and Outside of the Native Range of Its Host *Picea abies* (L.) Karsten

Ferenc Lakatos¹

The Norway spruce (*Picea abies* (L.) Karsten) has a wide autochthonous range in Europe. These stands are of economic importance and trees are planted far into the allochthonous range too. The most important pest in Norway spruce stands is the eight-spined spruce bark beetle, *Ips typographus* L., which destroys millions of cubic meters of spruce during years of mass outbreaks. There are many suggestions, that in the allochthonous stands the frequency and intensity of mass infestations are higher than in the autochthonous regions.

This polygamous beetle species has up to three generations per year in lower altitudes, and many sister breedings are possible too (Postner 1974). The infestation of the tree through pioneer beetles is directed by monoterpene released by the host and the mass attack by the pheromone release of the pioneer beetles (Führer and others 1991). The population density of *I. typographus* is influenced mainly by two factors: climate and suitable trees for breeding. Outside of the native range of *P. abies*, the climate conditions are advantageous for the beetle: quicker ontogeny and more generations in lower altitudes (Wegensteiner and Führer 1991), but disadvantageous for the host: dryer and hotter vegetation period.

Isozyme and morphological studies detected high genetic variation within and genetic differentiation among *P. abies* populations in Europe (Lagercrantz and Ryman 1990).

Geographic patterns of different genetic and physiological characteristics of bark beetles were the aims of many studies (Lanier and others 1972, Stauffer and others 1997). Physiological investigation of several European bark beetle species detected probably genetically determined characteristics. Bakke (1968) detected the parthenogenesis of *Ips acuminatus*. Führer (1977) described the incompatibility among *Pityogenes chalcographus* L. populations from several parts of Europe. Klipstein (1986) showed there is an increased potential to attack trees by progeny of crossed inbred

populations. All these arguments can be taken as a part of a population development process. The explanation may be the genetic structure of the bark beetles. The *I. typographus* populations in the allochthonous regions of Hungary cause different epidemiological behaviour (for example, more generations/year), and the genetic composition of these populations might be different than in those populations in the autochthonous regions.

Methods

I. typographus adults were collected from trap trees (minimum number of trees was three, to avoid the founder effect) in the spring of 1993 and further on in 1994-96. Altogether 8 populations from autochthonous and 10 from allochthonous stands were analyzed.

Electrophoresis: Thirteen enzymes were stained and 24 loci could be found. Five loci of three enzymes (*Aat-2*, *Amy-1*, *Est-2*, -3, -4) were polymorphic and screened in all populations.

Statistics: From allele frequencies, the observed (H_{obs}) and the expected (H_{exp}) heterozygosity (Nei 1973) were calculated and deviations from the Hardy-Weinberg equilibrium were tested by the χ^2 test. Population structure was analyzed using F-statistics (Wright 1978). The significance of the deviations of F_{IS} from zero were tested using χ^2 test. Gene flow calculations were also made. A graphic method (Slatkin 1987) was used for the gene flow comparison among different beetle populations.

Current Questions

Early (Sturgeon and Mitton 1986) and recent (Stauffer and others 1997) studies of bark beetles pointed out connection between the genetic structure and ecological and/or physiological features of a given species. In our study we wanted to obtain more information about the influence of the wide commercial use of *P. abies* in the allochthonous areas.

The high polymorphism of bark beetles has been well known since the early 80s, after the first electrophoretic studies on bark beetles (Anderson and others 1979, Florence and Kulhavy 1981). In our study the

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number of alleles seems to be higher in the autochthonous stands than in the allochthonous. The results varied from significantly higher to slight, but there were no significant differences among populations' polymorphism in and outside of the native range. On the basis of unique or special alleles, not one of the investigated populations could be identified.

Heterozygosity values were one of the first genetic characteristics used in population genetics. It is easy to handle, and the test of deviation from Hardy-Weinberg expectation is a good value to describe the population structure. Populations near the margins of the species are expected to have different heterozygosity (suboptimal conditions), than populations from the native range. Langor and Spence (1991) and Stock and others (1992) attributed the changing level of heterozygosity of *Dendroctonus ponderosae* with the species distribution. Pavlicek and others (1997) suggest that in the stressed *I. typographus* populations, the proportion of heterozygotes increased. In our study, the testing of homozygous and heterozygous genotypes as by the H_{obs} & H_{exp} comparison as by the F Statistics (F_{IS} and F_{IT}) resulted in homozygote excess. All of the investigated loci show heterozygote deficiencies, and no differences could be detected among the autochthonous and the allochthonous stands.

The rare presence of the host *P. abies* outside of its native range can lead to the isolation of bark beetle populations if the necessary migration is not possible. The best way to "measure" this is the calculation of inbreeding coefficients. The inbreeding coefficients from populations collected in 1993 gave results contradictory to the values from populations collected in 1994-96. The inbreeding coefficients from 1993 are significantly higher in the allochthonous stands compared to the autochthonous stands. On the other hand, the coefficients from the years 1994-96 are significantly higher in the autochthonous stands. Under the influences of inbreeding alone, however, values of F_{IS} and F_{IT} would be expected to be identical across all loci (Wright 1978), but these values are different. Is this opposition the result of the genetic structure of the chosen populations or may there be any other reason? Former studies (Sturgeon and Mitton 1986) on Scolytidae have reported a great variance of non-random mating (F_{IS} values) ($0.05 < F_{IS} < 0.22$), nearly so high as in one of the investigated populations, K2.

Also from the Wright F_{ST} values, calculated migration rate is different. This depends on the effective population size (N_e) and the migration rate (m): $F_{ST} = 1 /$

$(4Nm+1)$ (Wright 1943). Taken an equivalent population density on both stands, the migration rate on allochthonous stands is about 1/3 of the autochthonous stands.

Direct and indirect gene flow calculations gave the same conflict. The results of the two different data sets are in some cases contradictory. On the allochthonous stands it might be restricted in comparison to the autochthonous stands (data from 1993), but no differences could be found in the years 1994-96. The possible evidence is the high frequency of rare alleles, which are present in few populations. The gene flow comparison supports our opinion; although in both stands there is a high value of gene flow, this may be restricted in the allochthonous stands.

Theory

A model was presented of how natural and artificial barriers or natural and human activities can influence the genetic structure of *I. typographus*:

Norway spruce has a wide native range in Eurasia. Most of its associated insects have wide distribution area as well. The gene flow of *I. typographus* is high and unlimited in the overwhelming part of autochthonous *P. abies* regions (Stauffer and others 1997). But if the migration is limited, genetic and physiological changes can be detected. Mating incompatibility between populations of *P. chalcographus* L. from a wide geographic range in Norway spruce was detected by Führer (1977). This can be taken as the first step in the development of a new species. Here the migration and the gene flow can be considered as impossible.

However between the two extremes—the continuous gene flow within small distances of autochthonous stands and the excess of gene flow in great distances—there must be other gene flow stages as well. The isolation and inbreeding occur in the "long-time breeding" populations under artificial conditions as well. After the controlled cross breeding of these *P. chalcographus* populations, the resulting F1 generation had a higher attacking potential (Klipstein 1986). These populations have, as expected, a high bottleneck and inbreeding effect before crossing.

The outbreeding with the rapidly increased gene flow can occur under natural conditions in the island-like host stands of the allochthonous Norway spruce regions or perhaps in the deep valleys of higher mountains. A unique opportunity for natural gene flow restriction is the border zone of *P. abies* with the human-created spruce cultures outside of it.

Human activity, like transporting wood with the bark still on it, can be the major gene flow reason as well.

Conclusion

The allochthonous regions of *P. abies* seem to be geographically restricted for *I. typographus* because of the diffuse presence of the host plant. The different environmental factors in the allochthonous stands may appear also in the genetic structure of the beetle populations. The internal factors, for example, selection and the different genetic processes, like the founder effect, drift, and Wahlund effect (Wahlund 1928) could partially influence the genetic characteristics as well.

The high variability within *I. typographus* populations and the high gene flow make the populations homogenous. The homozygote excess, the great genetic differences within single populations, and the high gene flow make the *I. typographus* populations diverse. Overall genetic differentiation levels (mean of F_{ST} over all polymorphic loci) showed that in the overall populations only 1.1 - 1.4 percent of the variance of allele frequencies occurred in the genetic differentiation among populations; the remaining 98.6-98.9 percent being accounted for by variations among beetles within the locality.

The differences between *I. typographus* from autochthonous and allochthonous stands appear in the lower number of alleles, the restricted migration, and gene flow in allochthonous stands. On the basis of heterozygosity and the inbreeding coefficients (F_{IS} and F_{IT}) no differences could be found.

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6. Ecological Genetics of European *Ips* Species

Christian Stauffer¹

Ecological Genetics

Ecological genetics addresses adaptations and adjustments in the genetics of wild populations in relation to their environment, and allows us to investigate actual past and present evolutionary processes (Real 1994). The research field is characterized by the study—through a combination of field and laboratory work—of evolution in natural populations. *Ecological genetics* represents the Modern Synthesis, which recognizes that the roots of evolution can only be studied by looking at changes in populations (Mayr and Provine 1980). The Modern Synthesis represents the relationship between Darwin's hypothesis and Mendel's rules and was described by Dobzhansky (1936) in *Drosophila pseudoobscura*. Since then, the population genetics of many different organisms has been studied by investigating morphological and behavioral characteristics.

Behavioural and eco-physiological characteristics like aggressivity, dormancy reaction, and pheromone response vary geographically and are often based on the genetic structure of a population or individual. The understanding of such characteristics is important for long-term biological pest control programs. Physiological and genetic research techniques can help to understand such relevant characteristics, such as the dormancy status of populations. Koomanschop and others (1995) isolated the partial gene encoding the diapause protein of *Leptinotarsa decemlineata* (Coleoptera, Chrysomelidae). Genetic techniques have also helped in the analysis of population structure, efficient population size, and gene flow in insect species. This kind of information is important for understanding epidemiological differences among populations, or for establishing conservation programs for endangered insect species. For example, two morphologically identical *Cicindela puritana* (Coleoptera, Cicindelidae) populations were found to be genetically different. They were considered as two independent populations for the purposes of conservation (Vogler and others 1993). In a population of *Melitaea cinxia* (Lepidoptera,

Nymphalidae). Sacceri and others (1998) showed that, in addition to demographic and environmental factors, inbreeding is an important element contributing to extinction.

When Darwin (1859) presented his theory in the lecture "*The Origin of Species by Means of Natural Selection, or the Preservation of Favored Races in the Struggle for Life*," and when Mendel (1865) wrote the book "*Versuche über Pflanzenhybride*," the biological basis of inheritance was unknown. At the beginning of the 20th century, cytogenetic, behavioral, and morphological studies were used to investigate the evolution of species and populations, in particular of *Drosophila* species. At the beginning of the 60s, allozyme electrophoresis allowed the separation of enzymes and histochemical detection of the specific protein products of single loci (Harris 1966, Lewontin and Hubby 1966). This technique has revolutionized many research fields, including those of systematics, sociobiology, genomic organization, and population genetics, and the technique has since been used to study evolution. It offers a relatively inexpensive and fast method for analyzing single locus variation in populations of any life form, from bacteria to man (May 1994). In the mid-90s, the discovery that DNA can be polymerized by applying repeated thermal steps—called polymerase chain reactions (PCR)—using a thermostable DNA polymerase (Mullis and Faloona 1987) led to the development of other techniques for studying evolution, such as random amplified polymorphism (RAPD) using unspecific primers and DNA sequence analysis using specific primers. Since the development of the PCR, sequence analysis has frequently been used in population genetics. Today, specific primers are available for the genomic and mitochondrial DNA (mtDNA) of many organisms. Most population genetic studies have been carried out using mtDNA markers since the mtDNA has properties that make it an ideal molecular marker. The mt genome is ubiquitously distributed, so that reliable homologous comparisons can be made between a wide variety of organisms. The mt genome also has a simple genetic structure (it lacks introns), exhibits a straightforward mode of genetic inheritance (recombination does not occur), is maternally inherited and evolves at a rapid

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pace (Lunt and others 1996).

Although more than 6000 species of bark beetles have been described from throughout the world (Wood 1982) and several species are important pests, our knowledge in *Ecological Genetics* of scolytids is fairly rudimentary. Enzyme electrophoresis has been established for phylogeographic studies on several species (for review see Hayes and Robertson 1992), and RAPD has been used to analyse the *Ips grandicollis* group (Cognato and others 1995) and *Tomicus piniperda* populations (Carter and others 1996). These two methods are not destined to answer phylogenetic relevant questions, however they can help to estimate the genetic variation among and within the populations (May 1994).

The preliminary aim of our work was to establish genetic markers to define eco-physiological reaction types in European scolytid species. Scolytids have an efficient infestation behavior that is directed by pheromones. In biological pest management programs, synthetic odors are often used. But scolytid populations vary often in their reaction towards such odors, and thus the use of pheromone traps is often limited to monitoring outbreaks. The reasons for these differences are unknown. Further, first-attacking beetles (pioneer beetles) have a different reaction behavior than later-attacking ones (Borden 1985). The definition of reaction types (pheromone beetles, pioneer beetles, or diapause beetles) in scolytids is important to understand epidemiological differences among populations. For evaluating genetic data, it is necessary to know both intra- and the interspecific variation. Thus during our work, first the phylogenetic relationships of the *Ips* species were studied to understand the ancestry within this genus and speciation processes like host adaptation. *Ips typographus* and *Ips cembrae*, two economically important *Ips* species were studied by analyzing populations over a wider geographic range. This was done to provide information on evolutionary questions like: what is the genetic structure of bark beetle populations; how strong is the gene flow among the populations; are there correlations between the genetic structure and epidemiological findings? During the investigations, phenomena like parasites and pathogens and *null alleles* were detected, which were important regarding the interpretation of the data.

Methods

For our work, *Ips typographus* populations were

analyzed using enzyme electrophoresis, sequence analysis, and microsatellites in order to study phylogenetic and phylogeographic issues.

Enzyme Electrophoresis

The *Ips* species were screened for 13 enzymes, and 9 of 24 loci were found to be polymorphic. Five enzyme-loci, *Aspartate Amino Transferase-2* (*Aat-2*), *Amylase-1* (*Amy-1*), *Esterase-2* (*Est-2*), *Est-3*, and *Est-4* showed between 8 and 18 alleles and stained consistently well over all the assays. The allozyme pattern and the mode of inheritance of the alleles was tested using pedigree analysis (Stauffer and others 1992). The *Est* isozymes were the most polymorphic ones. In contrast to *Amy-1* and *Aat-2*, little is known about the physiological function of the *Esterases*, other than that they are involved in catabolising toxic substances. The *Est* loci were analyzed using biochemical and molecular techniques. *Est-2* is involved in catabolism of the juvenile hormone, and *Est-3* and *Est-4* are *General Esterases* (Stauffer and others 1997a). An understanding of the physiological function of loci was important for phylogeographic analysis and for investigation of an *Ips typographus* population. Quantitative enzyme assays indicated that first-attacking beetles are different from the later-attacking beetles (Stauffer 1997b).

Sequence Analysis

Primers developed for *Drosophila yakuba* (Clary and Wolstenholme 1985) were used for the sequence analyses in most of the papers presented here. Primers polymerizing a region between the 5' end of COI and the t-RNA_{leu}, and primers for the 3' end of the COII gene were chosen (Stauffer and others 1997b).

Microsatellites

A method that has appeared during the last decade is the use of microsatellites; short, tandem repeated nucleotide stretches that are of variable length and that are found abundantly on eukaryotic genomes. Microsatellites have become the markers of choice in population genetic studies and are available for hymenopteran and dipteran species. A lot of effort has been made to isolate microsatellites from lepidopteran, coleopteran, and other insect orders, but only a few polymorphic loci have been isolated compared to the above-mentioned insect orders. Bogdanowicz and others (1997) isolated four polymorphic loci from *Lymantria dispar* (Lepidoptera, Lymantriidae), Saccheri and others

(1998) isolated one polymorphic locus from *Melitaea cinxia* (Lepidoptera, Nymphalidae), Sunnucks and others (1996) isolated four polymorphic loci from *Sitobion miscanthi* (Hemiptera, Aphididae), and Batley and others (1998) isolated 14 loci for *Phyllodecta vulgatissima* (Coleoptera Chrysomelidae). In the work presented here, a genomic DNA library of *I. typographus* was established and 13 di- and trinucleotide microsatellites were isolated. Two of these primer pairs polymerized a product not showing polymorphism in the 14 populations analyzed. These microsatellite primers also amplified DNA from *I. cembrae*, the phylogenetic sister taxon of *I. typographus*, but not the DNA of other *Ips* species. These results support the conclusion derived from phylogenetic data—that these two species are sister taxa. The other microsatellite loci could not be polymerised (Stauffer and Schlötterer, unpublished data), and thus the isolation of polymorphic microsatellites in this scolytid genus has failed so far.

Others

In Stauffer and Zuber (1998) morphological measurements were used, together with breeding, behavioural (olfactometer), and biochemical (cuticular hydrocarbons) studies, to produce data that was then compared with genetic findings (allozyme and sequence data) to test whether *I. amitinus* var. *montana* differs from *I. amitinus*.

Species and Subspecies

In Europe, 154 scolytid species have been described wherever woody plants grow (Pfeffer 1995). The seven species of the genus *Ips* found in Europe are distinguished by morphology, by their host tree, and by their frass system. However, until recently, nothing was known about their phylogenetic relationships. The *Ips* species infest either spruce (*Picea* sp.), larch (*Larix* sp.), or pine (*Pinus* sp.) trees—all of which are economically important conifers. The phylogenetic relationships of the genus *Ips* is presented in Stauffer and others (1997b) and in Stauffer (1997a), which reveals that the genus *Ips* is a paraphyletic group. The eight spined bark beetles and *I. acuminatus* form a monophyletic group. This is confirmed by Cognato and others (unpublished data), who investigated the American genus *Ips* and the American genera *Orthotomicus* and *Pityokteines*. In comparison with other coleopteran species, the genus *Ips* had a relatively high sequence divergence. Using the calculation devised by Boyce and others (1994), the

time back to common ancestry for the two most closely related haplotypes of *I. typographus* and *Ips cembrae* is estimated at 50-100 million years.

I. amitinus var. *montana* infesting *Pinus cembrae* or *Pinus montana* was originally described in Switzerland and the southern Alps. A re-investigation of this race (Stauffer and Zuber 1998) using ecological and genetic methods shows that no differentiation between *I. amitinus* and *I. amitinus* var. *montana* can be found.

In Japan, *I. japonicus* infests *Picea jezoensis*. This insect species was first described by Nijima in 1909 and is morphologically closely related to *I. typographus*. The mtDNA of *I. japonicus* (AF036157) and the European *I. typographus* (U82589) was found to differ by 1.5 percent. As the European populations had a sequence divergence of about 0.6 percent, the status of *I. japonicus* as a species or subspecies is doubtful (Stauffer, unpublished data). *Ips japonicus* might be a local population of *I. typographus*, as suggested by Kono and Tamanuki (1939).

Phylogeography

The population structure of insects in Europe is characterized by events since the last ice ages. Phytophagous species have spread parallel with their hosts. Since the discovery that populations differ genetically and that the population structure may influence the epidemiological reaction of insects, many individual insect pest populations have been investigated. In natural forests, mass infestations rarely occur, as plants have a natural defense system. In allochthonous stands, where insect populations may benefit from a more convenient climate, trees are often more stressed and thus less resistant against infestations. For economic reasons, larch stands have been planted in northern regions in recent decades and *Ips cembrae* causes lots of damage in these areas. Many scientists (for example, Escherich 1923) warned that *Ips* species could cause tremendous forest health problems through mass infestations in allochthonous zones. Different populations of an insect species can behave differently in newly colonized areas, dependent on prevailing environmental and genetic factors. This could be also demonstrated in genetic studies of European populations of *I. typographus* (Stauffer and others, in press). Sequence and allozyme analysis showed that the northern populations had less genetic variation than the Central European populations. The populations were selected for screening because behavioral and epidemiological

differences among the populations had been reported; the Scandinavian populations, for example, emerge from their winter site in May/June and have longer development times (due to the climate) than their more southern counterparts. Thus, the Scandinavian populations must be under a different selection pressure regarding voltinism.

One to three generations are reported in the south (Postner 1974). In Stauffer and others (in press), it is suggested that the different types of voltinism might be correlated to the haplotypes, an idea that is currently being tested in ecological-physiological and genetic studies.

Another reason for the original interest in the genetics of the European populations was the fact that the Scandinavian populations could be controlled more efficiently using synthetic pheromone traps than could the more southern populations (for example, Bakke 1989). This might be due to the lower genetic variability in the north. One haplotype was found in the northern countries, and between two and six in the southern countries. A correlation between haplotype and pheromone reaction could also explain the different capture rates using synthetic odors. The validity of this hypothesis is currently being tested by studying the response of beetles to the odor (using an olfactometer). The olfactometer assay is being followed by a genetic analysis of the beetles.

Parasites and Pathogens

Extracting beetles for genetic analysis, one has to consider the presence of parasites and pathogens as they can influence the electrophoretic banding pattern; for example, nematodes and fungi can weaken the expression of the esterase system and make it difficult to interpret. The hymenopteran braconid *Tomicobia seitneri* and other braconid species may cause additional bands between *Est-1* and *Est-2*, making the interpretation of *Est-2* difficult. As some populations had a large number of hymenopteran parasites, it was important to dissect at least a portion of the individual beetles investigated (Stauffer and others 1992).

In contrast to the mtDNA sequence divergence, isozyme markers revealed high polymorphism. This could be caused by the presence of *Wolbachia*, an endosymbiotic proteobacteria that is known to cause a reduction of mtDNA polymorphism (Moran and Baumann 1994). The detection of *Wolbachia* in four *I. typographus* populations was carried out using specific

primers (Stauffer and others 1997c). Sequencing revealed a *Wolbachia* strain that was the same as the *Wolbachia* found in *I. sexdentatus* and *I. duplicatus*. The sequence was also similar to the *Wolbachia* found in *Trichogramma deion*. However, further screening of populations failed to detect this proteobacteria (Stauffer and Riegler, unpublished data).

Null Alleles

Another genetic data factor that influences the interpretation are *null alleles*. In all scolytid species studied, *null alleles* were found for the enzyme locus *Aat-2*. *Null alleles* are defined as alleles with reduced or no expression of a protein product. They are identified through observation of reduced staining intensity or a complete absence of staining activity (Hillis and others 1996). This interpretation is often ambiguous and requires breeding studies of monomeric enzyme loci. In scolytids, *Aat-2* is a dimeric isozyme—the active enzyme consists of two molecules. The homozygous beetle has one homodimeric band. The heterozygous beetle has two homodimeric and one heterodimeric band, the latter migrating between the two homodimeric bands. These three bands appear in a relative concentration of 1:2:1, since random combination of subunits follows the binomial theorem. In dimeric enzyme systems, *null alleles* can be detected in heterozygote individuals through the presence of only two bands or by the presence of two bands and one band with residual activity. There are two hypotheses used to explain *null alleles* (Burkhart and others 1984); either the homodimeric form is affected by mutation in the active site, or the heterodimeric band is affected by mutation in the dimerization region. The different forms of *null alleles* found in scolytid species indicate that the heterodimeric band is affected. Lane 7 shows one normal homodimeric band (AA), one heterodimeric band (AB^N), and one homodimeric band (B^NB^N) with residual or no activity. The residual activity of B^N also affects the staining intensity of the heterodimeric band. About 15 percent of an *I. amitinus* population had *null alleles* in *Aat-2*. However, no homozygous *null allele* individuals were found. The *Hardy-Weinberg* law would predict 2.3 percent homozygote *null allele* individuals for this population. These were never found, as it seems that *Aat-2* is an essential enzyme—so a non-presence of this enzyme is lethal (Stauffer, unpublished data).

Conclusions

In our work, molecular markers were applied to investigate inter- and intraspecific relationships of European scolytid species. Besides enzyme electrophoresis, sequence analysis was used for the first time to analyze bark beetle species. *Ips* species were screened to determine the sequence divergence among closely related taxa and among populations of two species studied. Primers were applied that proved to have high sequence divergence among the species. The sequence divergence within the species, however, was very low. Due to the distribution of the haplotypes, eco-physiological reaction types could be genetically characterized. The low variation of the Scandinavian populations indicate that the haplotype found might correlate with beetles reacting towards the synthetic pheromone traps and beetles with obligate diapause. Currently genetic markers are isolated that we hope will determine eco-physiological reaction types. The esterase that catabolizes juvenile hormone is currently studied by molecular means in order to clone this protein. It is more likely that mutations in such genes could define, for example, pioneer beetles. If this is the case, a screening of populations could give an evaluation of the epidemic potential of the population.

7. Karyology, Geographic Distribution and Origin of the Genus *Dendroctonus* Erichson (Coleoptera: Scolytidae)

Gerardo Zúñiga ¹, Ramón Cisneros ¹, and J. Macías-Sámano ²

The worldwide importance of genus *Dendroctonus* is due to its ecological and economical impact on coniferous forests (Fam: Pinaceae). The geographic range of the genus is located in America, from Alaska to Honduras, and in Eurasia, from France to Siberia and China.

Wood (1982) recognized 19 species in the last taxonomic revision of the genus, 17 of which inhabit North and Central America, and the last two live in Eurasia. Previously, *Dendroctonus* species had been ordered in five groups of closely related species based on anatomical, biological, and behavioral characteristics, and host species (Wood 1963).

Considering the high concentration of species and diversity in Mexico, Wood (1982, 1985) suggests that *Dendroctonus* is a biogeographical relict derived from species infesting ancient *Araucaria* forests, and whose origin was Mexico with a comparatively recent invasion of North and Central America.

To date, the karyology of 16 out of 19 *Dendroctonus* species are known. Such information derives from studies of 120 populations distributed from Canada to Guatemala (Lanier and Wood 1968, Lanier 1981, Lanier and others 1988, Pajares and Lanier 1990, Salinas-Moreno and others 1994, Zúñiga and others 1998). Data from these investigations show that karyotype is a characteristic feature of the species, which does not show variation throughout geographic ranges. Therefore, the available chromosomal evidence of the genus can be used to test the hypothesis of the Mexican origin of *Dendroctonus*.

The diploid chromosomal number species ranges from $2n = 30$ to $2n = 12$, with a modal value of $2n = 30$ and an average number of 23.375. Two karyological groups can be distinguished within the genus: the first is formed by *Dendroctonus rufipennis*, *D. punctatus*, *D. murrayanae*, *D. simplex*, *D. pseudotsugae*, *D.*

parallelocollis, *D. terebrans*, *D. valens*, *D. rhizophagus*, *D. ponderosae*, and *D. jeffreyi* with numbers above mean value. The second include to *frontalis* complex species: *D. adjunctus*, *D. brevicomis*, *D. approximatus*, *D. mexicanus*, and *D. frontalis* with values below it.

Regarding sex determining mechanism, the majority of the species show an Xyp sexual system, which is considered primitive for Coleoptera by most authors (Smith 1949, 1962; Smith and Virkki 1978; Virkki 1980). Only *D. ponderosa*, *D. jeffreyi*, *D. adjunctus*, *D. brevicomis* and *D. approximatus* have a neo-XY sexual system, originated from an Xyp sexual system (Smith 1949, 1962).

Thus, the analysis of the chromosomal number and geographic distribution allows establishing some general tendencies related with the origin and direction of dispersion of the group. Moreover, Lanier (1981) based on karyotype stability and on behavior of chromosome dynamics during meiosis, proposed that the reduction of the chromosome number is an advanced condition in *Dendroctonus*.

In such context, species inhabiting from the southern United States to Honduras exhibit low chromosomal numbers, and species with higher numbers are located in United States and Canada. Exceptions to this pattern are *D. valens*, distributed throughout of North and Central America, *D. rhizophagus*, endemic of northwest Mexico, and *D. ponderosae*, *D. jeffreyi* and *D. pseudotsugae*, mainly present along western United States and Canada, but with small populations in northwest Mexico.

This evidence permits us to ask if the genus really originated in Mexico (Wood 1982, 1985) or as karyological data suggest, the *Dendroctonus* origin occurred in the North with a subsequent southward dispersion.

The increase of the chromosomal number in *Dendroctonus* must be associated with the polyploidy or chromosomal rearrangements, such as centric fission, which have not been documented previously in *Dendroctonus*. On the other hand, the origin of the neo-XY sexual system, as result of a centric fusion of an autosome with a sex chromosome, is linked with a

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process of decreasing more than increasing of the chromosomal number.

From above, it is difficult to explain the karyotype increase without chromosomal evidence that support this. In contrast, the reverse process, decrease in the chromosomal number, is an event largely documented in Coleoptera (Smith 1949, 1962; Smith and Virkki 1978; Virkki 1980), which reinforces the hypothesis about *Dendroctonus* northern origin.

Nevertheless, it is not possible to discard another scenario raised from the presence of two karyological groups observed in *Dendroctonus*, which suggest a possible continental dispersion of the genus toward south and northward in recent times. These groups might be originated from a basic karyotype number between $2n = 12$ and $2n = 9$, from which through centric fusion and fission, the increase and decrease of the chromosomal number was attained. The existence of such hypothetical karyotype is supported by the general tendency shown by karyological data from other scolytids (for example, *Conophorus*, *Ips*, *Pityogenes*, *Tomicus* (= *Blastophagus*), *Hylurgops*, *Pissodes* and *Orthotomicus*), which are genera with a chromosomal number equal to or higher than the formula $9II + Xyp$; considered primitive in Coleoptera (Smith 1952, Smith and Virkki 1978, Virkki 1984).

To prove these hypotheses, we need to know the chromosome number of *D. micans*, *D. armandi*, and *D. vitei*, because they are species located in the limits of the geographical range. Furthermore, it is of vital importance for revealing chromosomal changes to study mitotic chromosomes and to apply other cytogenetics techniques for karyotypes comparison (for example, banding techniques and DNA-amount of nucleus).

8. Behavioral and Genetic Basis of Pheromone Evolution in *Ips pini*

S.A. Teale¹

Most insect pheromone systems exhibit very limited intrapopulational variation in component blend ratios. An exception is the pine engraver, *Ips pini* (Say), where there is considerable intrapopulational variation in the ratio of ipsdienol enantiomers (Miller and others 1989, Teale and Lanier 1991, Teale and others 1994). Because the pheromone system is the primary means of mate location, uncommon variants should experience reduced mating success and become extinct over time. Such selection would deplete the additive genetic variance in the component blend ratio, and we should see low heritability estimates and limited phenotypic variance. Frequency dependent selection is one mechanism that can maintain high additive genetic variance in traits highly correlated with fitness. The selective forces acting on the pheromone system of *I. pini* are not well understood, but there are predators and parasitoids that exploit the pheromone system in prey and host location (Herms and others 1991, Raffa and Klepzig 1989, Rice 1969, Senger and Roitberg 1992).

If a pheromone system responds to directional selection, there must be a genetic mechanism by which signal preference and production remain correlated if the system is to function. Two possibilities are pleiotropy and the maintenance of linkage disequilibrium through assortative mating. The purpose of this work was to determine which of these mechanisms is operating in the pheromone system of *I. pini*, to estimate the phenotypic and genetic components of pheromone preference and production, and to determine if assortative mating maintains a genetic correlation of these two traits.

Assortative mating was investigated by measuring the phenotypic correlation of response preference with ipsdienol enantiomeric blend production. Traps were baited with 11 blends of ipsdienol enantiomers in 10 percent increments, live beetles were removed, and the ipsdienol enantiomeric blends produced by individual males was measured in the laboratory. There was a significant relationship of the two traits ($R^2=0.78$, $P<0.001$) (Teale and others 1994). In the next experi-

ment, male-female pairs were sampled in the field, the response preference of individual females was measured in a laboratory olfactometer, and the enantiomeric blend production of individual males was also measured. Again, the relationship between the two traits was significant ($r=0.45$, $P<0.0001$) confirming the presence of a phenotypic correlation of blend production with response preference (Teale and others 1994).

The pleiotropy hypothesis was tested by measuring the genetic correlation of signal and response in wild beetles under assortative mating (control) and after five generations of random mating (treatment). The genetic correlation in the control group was 0.207 ± 0.216 ($n=226$), which was significantly different ($P<0.001$) from that of the treatment group ($r_G=-0.272\pm 0.367$; $n=189$). Heritabilities ranged from 0.269 to 0.627 for response preference and from 0.823 to 0.951 for blend production (Hager and Teale 1996).

While the selective pressures that maintain high intrapopulational variation in this pheromone system remain largely unknown, the mechanisms by which variation in pheromone blend composition remain coordinated with variation in response preference are now clear. The variation in female preference observed in field studies (for example, Teale and Lanier 1991) is composed of comparatively narrow individual preferences (Hager and Teale 1994) that, when combined, form a broader population profile. Thus, if selection acts disproportionately on certain component blends relative to others, the population should quickly adapt through assortative mating, which maintains the genetic correlation. The magnitude of directional shifts in blend ratios is probably constrained by interspecific interactions with congeneric species (Cognato and others 1995). The high heritabilities suggest that there are either interacting or fluctuating (frequency-dependent) selective forces acting on this system.

The role of the third component of the pheromone, lanierone (Teale and others 1991) in intrapopulational behavioral genetics is largely unknown. However, lanierone appears to function in a qualitative manner at naturally occurring levels. Only at release rates that are orders of magnitude above natural levels, is there a progressive decrease in the proportion of males attracted

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(Teale and others 1991). While individual variation in lanierone production or response preference have not been studied, it seems unlikely that these traits would be invariant. Teale and Lanier (1991) indicated that lanierone is required for response to ipsdienol in the spring but less so in the summer. This suggests that some individuals respond to ipsdienol in the absence of lanierone while others do not. The important question is: Does variation in lanierone production and response affect the genetic and behavioral interactions concerning ipsdienol? The answer to this question depends on whether or not the genes controlling ipsdienol and lanierone production and response are linked. If they are unlinked, then lanierone simply represents a third, but independent variable worthy of study in its own right, but would not affect the general conclusions regarding the evolution of communication based on ipsdienol alone. If there is linkage, then the interactions between ipsdienol and lanierone variation would be crucial.

There are no direct studies addressing this linkage question, but we can infer from indirect evidence that there are no ipsdienol-lanierone interactions. In the two experiments conducted by Teale and others (1994), the first utilized synthetic ipsdienol enantiomeric blends without lanierone and the second involved the measurement of enantiomeric ratios produced by male beetles. In both experiments, a significant correlation of production and response was observed, which indicates that assortative mating based on ipsdienol enantiomeric composition occurs both in the presence and absence of lanierone. When the correlation was measured in summer it was positive and significant, but in the spring it was not significantly different from zero. As discussed by Hager and Teale (1996), the basis for this seasonal difference was related to overwintering physiology and its effect on ipsdienol enantiomeric composition; there is no reason to infer that lanierone played any role.

9. Allozyme Diversity, Gene Flow, and Evidence for Non-Random Mating in the Jeffrey Pine Beetle, *Dendroctonus jeffreyi*

Diana L. Six¹, T. D. Paine², and J. Daniel Hare²

Background and Objectives

Unlike other *Dendroctonus*, the Jeffrey pine beetle, *Dendroctonus jeffreyi* Hopkins, is monophagous colonizing only *Pinus jeffreyi* Grev. & Balf (Wood 1982). Partly because of its limited host range and geographic distribution (southern Oregon to northern Baja California, Mexico), *D. jeffreyi* has not been the focus of as intensive study. The sibling species of *D. jeffreyi*, the mountain pine beetle, *D. ponderosae* Hopkins, possesses many similarities with *D. jeffreyi* including almost identical morphologies (Wood 1982), the same mycangial type (T.D. Paine personal observation, Whitney and Farris 1970), and a mycangial fungal associate in common (Six and Paine 1997, in press); however, the mountain pine beetle is polyphagous and has a much greater geographic distribution (Wood 1982).

Substantial variation has been found to occur among populations of *D. ponderosae* morphology (Stock and others 1984), host species preference (Baker and others 1971, Hopkins 1916, Wood 1982), tree diameter preference (Amman 1977, Cole and Amman 1969, McCambridge 1967), allozymes (Langor and Spence 1991; Stock and Amman 1980, 1985; Stock and Guenther 1979; Stock and others 1984; Sturgeon and Mitton 1986), and response to pheromones and pheromone synergists (Borden and others 1983, Pitman 1971); however, variation in *D. jeffreyi*, had not been assessed.

In this study, we assessed genetic variation within and among populations of *D. jeffreyi* across the majority of its geographic range. Because *D. jeffreyi* periodically develops outbreaks and thus undergoes great fluctuations in population size, we also assessed possible effects of fluctuating and small population size on beetle genetic variation. Some consequences of small population size include a reduction of the effective population size, an increase in inbreeding, and a decrease in genetic

variation. Fluctuations in population size may exacerbate these effects (Woodworth and others 1994).

Methods

Dendroctonus jeffreyi adults were collected from under bark of *P. jeffreyi* at 10 sites across California. Using horizontal starch gel electrophoresis we assayed 16 enzymes exhibiting 20 putative gene loci to assess genetic variation in this beetle.

Results and Discussion

Polymorphism—Polymorphism was relatively low in *D. jeffreyi*. Of the 20 loci assayed, 6 were polymorphic in at least 1 of the 10 populations surveyed. Only two loci were polymorphic in all populations assayed.

Population differentiation

At two loci, alleles were present in the two most southern populations that were not present in any of the more northern populations. These southern populations were also missing an allele at one locus that was present in all northern populations, indicating that there is no gene flow between these two population groups. These results were not surprising because the southern populations are geographically isolated from the northern populations by an expanse of the Mojave Desert across which migration is unlikely.

Nei's genetic distances (Nei 1972) among the beetle populations indicated that high levels of overall genetic similarity occurred among all populations but that the southern populations were the most divergent. Cavalli-Sforza and Edwards' (1967) cord distances showed the same general relationships among the populations.

The isolation-by-distance principle predicts that pairs of populations that are geographically more distant would be expected to show greater genetic distance, assuming roughly equal rates of migration (Hartl and Clarke 1989). Nei's genetic distance and Cavalli-Sforza and Edwards' cord distance were positively and significantly correlated with geographic distance when all populations were included in the analyses ($P=0.0001$ for both distance measures); however, the correlation was no longer significant (Nei, $P = 0.316$) or only slightly

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significant (Cavalli-Sforza and Edwards, $P = 0.0418$) when the two southern populations were removed from the analysis and the northern population group was analyzed separately. These results indicate that geographic distance and genetic distance may be related only when geographic distances are great, or more likely, that the isolation and subsequent divergence of the southern populations from northern populations may be solely, or mostly, responsible for the significant correlation when all populations were included in the analysis.

Heterozygosity

Heterozygosity varied considerably within and among the 10 populations. Mean heterozygosity over all loci and all populations was 4 percent, a relatively low value when compared with values observed in other *Dendroctonus*. Estimates for *Dendroctonus* species from other studies range from 10-31 percent (Anderson and others 1983; Bentz and Stock 1986; Higby and Stock 1982; Langor and Spence 1991; Roberds and others 1987; Stock and Amman 1980, 1985; Stock and others 1984) except for *D. micans*, a highly inbred species, for which heterozygosity has been estimated at 5 percent (Stock and others 1987).

Conformance to Hardy-Weinberg expectations

Genotype frequencies at each locus were compared for conformance to Hardy-Weinberg expectations for random mating using variations on Wright's F statistics: F_{IS} (the inbreeding coefficient), F_{ST} (the genetic index of fixation), and F_{IT} (the overall inbreeding coefficient) (Nei 1977).

The F-statistics indicated a strong departure from random mating in the *D. jeffreyi* populations. F_{IS} , F_{ST} , and F_{IT} values were positive and differed significantly from zero at all polymorphic loci indicating a deficiency of heterozygotes. The mean F_{IS} value across all loci indicated that 52 percent of the reduction in heterozygosity observed within populations was due to non-random mating, while the mean F_{ST} value revealed that 15 percent of the observed genetic variability was due to geographic subdivision and random genetic drift. The mean F_{IT} value indicated that 61 percent of the total observed variation across all populations was attributable to the combined effects of non-random mating and genetic drift.

A high degree of inbreeding may be occurring in these beetles as indicated by the significant departure of the F_{IS} statistics from zero; however, inbreeding is not

predicted to occur in *Dendroctonus* species that utilize long-distance attractant pheromones (such as *D. jeffreyi*). It does apparently occur in species that do not utilize long-distance pheromones and do not develop gregariously under bark such as *D. micans* (Kugelann) and *D. punctatus* (LeConte) (Kirkendall 1993). Siblings of *D. micans* and *D. punctatus* apparently mate under the bark prior to emergence from the natal host. However, pre-emergence sib-mating is unlikely for *D. jeffreyi*, which apparently emerges directly out of bark from individual pupal chambers with little opportunity of under-bark mating. Likewise, mating on the outer bark of the natal host is unlikely because *D. jeffreyi* utilizes a complex pheromone system that combines the attack of a new host tree with the attraction of mates. Therefore, if inbreeding does occur in *D. jeffreyi*, it should occur after emergence and off the natal host.

Inbreeding in *D. jeffreyi* may be a direct consequence of population size. The populations sampled in this study were in various stages of expansion or contraction, yet, in all cases, at all polymorphic loci, significant deviations from random mating were indicated. Different factors may contribute to the likelihood of inbreeding in outbreak and non-outbreak populations. During non-outbreak periods the beetles are often maintained in small single-tree populations scattered widely throughout a forest. Many or most beetles emerging from these trees may be related, and the probability of mating with a close relative on a newly attacked tree may be very high.

Other factors may account for a high degree of non-random mating in larger populations. In expanded populations, the apparent high degree of inbreeding may indicate that during outbreaks the dispersal distance of these beetles is very short, and may often extend no farther than an adjacent tree when susceptible trees are present. In this case, if brood emergence is synchronized, the probability of mating among sibs would be increased.

Future work

Future work should include research on the factors responsible for the occurrence of inbreeding in *D. jeffreyi*. While under-bark pre-emergence mating is believed to be unlikely, experiments to confirm or deny its existence should be done. Studies on the synchronicity of brood emergence and dispersal of emerging adults may also shed light on this question. We must also begin to address the question of what effects inbreeding may have on this species.

10. Semiochemical Disparities Among Bark Beetles and Natural Enemies Responding to Bark Beetle Pheromones

Kenneth F. Raffa and Kenneth Hobson¹

It has long been known that natural enemies of bark beetles use beetle pheromones to orient towards their prey (Gregoire and others 1991, Silverstein and others 1966, Wood and others 1968, Wood and Silverstein 1970, Wood 1982,). There is also strong evidence that natural enemies can greatly impact bark beetle population dynamics (Amman 1984, Dahlsten and Stephen 1974, Miller and others 1987, Raffa 1995, Reeve 1997, Riley and Goyer 1986, Turchin and others 1991, Weslien 1992), and thus could likely impose selective pressures on their life history characters (Raffa and Klepzig 1989, Raffa and Dahlsten 1995). We have been investigating mechanisms by which herbivores might avoid natural enemies that exploit their pheromones as kairomones in host finding. Specifically, we have been testing the underlying assumptions and putative mechanisms of a coevolutionary model, in which alterations in ratios of stereoisomers and synergists may allow for partial, and presumably temporary, escape yet maintain intraspecific functionality. Recent information on the population genetics, heritability, and molecular biology of bark beetles (Bentz and Stock 1986; Cane and others 1990b; Gast and others 1993; Gast and Stock 1994; Hager and Teale 1994, 1996; Stauffer 1997a; Teale and others 1994) have made such studies possible. We are also applying this model to improve the biological control of bark beetle pests. We are using the pine engraver, *Ips pini*, as a model.

Aspects Being Evaluated

Behavioral Responses to Variable Chemically Defined Cues

Ips pini is a transcontinentally distributed insect, which is known to vary geographically in its relative portions of (+) to (-) ipsdienol (Lanier and Wood 1975; Mustaparta and others 1980, 1985; Seybold 1993; Seybold and others 1995), and synergistic component, lanierone (Seybold and others 1992, Teale and others 1991).

In Wisconsin, *I. pini* males produce predominantly (+) ipsdienol (ratios vary throughout different portions of the state, 50-75 percent (+). A large and diverse guild of predators, competitors, and parasites are also attracted to ipsdienol (Raffa 1991, 1995). Some of these major predators, however, prefer predominantly (-) ipsdienol (Herms and others 1991, Raffa and Klepzig 1989). Attraction to ipsdienol by Wisconsin beetles is strongly synergized by addition of lanierone (Miller and others 1997). In California, *I. pini* males produce almost entirely (-) ipsdienol (Lanier and Wood 1975). As elsewhere, a large and diverse guild of predators, competitors, and parasites are attracted to ipsdienol. Some of these major predators, however, prefer predominantly (+) ipsdienol. Attraction to ipsdienol by California *I. pini* is not synergized by addition of lanierone (Miller and others 1997).

In a collaborative study, we conducted a reciprocal exchange experiment with Donald Dahlsten at the University of California at Berkeley. After obtaining permits, we mailed each other local *I. pini* males, established them on caged logs, transported them to the field, and exposed them to natural populations of insects. In California, *I. pini* were attracted almost exclusively to caged California *I. pini*, but the major predator preferred caged Wisconsin *I. pini*. In Wisconsin, *I. pini* were attracted to caged Wisconsin *I. pini*, but the most abundant predator preferred caged California *I. pini* (Raffa and Dahlsten 1995).

We have completed a 4-year analysis of the chemical ecology of *I. pini* and its associates in Wisconsin. Spatial and temporal variation interact strongly with chemical variation (Herms and others 1991, Raffa 1991, Teale and Lanier 1991). Sites as close as 50 km can show significantly different patterns.

Predator Impact on Beetle Reproduction

Predator impact studies have been conducted in the laboratory and the field. Strong overall effects were observed by predators in the field. In laboratory assays, *Thanasimus dubius* (Coleoptera: Cleridae) and *Platysoma cylindrica* (Coleoptera: Histeridae) exerted

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strong impacts on breeding *I. pini*. *Thanasimus dubius* also exerted strong impacts on *I. pini* adults.

Independent *Ips pini* Breeding Lines: Predator Responses

We developed independent breeding lines of *I. pini* through selection on males. Colonies diverged on the basis of both ipsdienol chirality and lanierone content. Individual beetles were analyzed for pheromone signatures by gas-liquid chromatography. Males from rearing lines were established on logs and taken to the field, and they experienced differential reproductive success. There were several sources of variation in differential replacement rates, of which predation was a major effect.

Effects of Host Trees & Host Tree Phytochemicals

Host tree species and chemistry can influence the response of bark beetles to their pheromones (Miller and Borden 1990, Piston and Lanier 1974, Wood 1982). We are conducting studies with red pine (*Pinus resinosa*), jack pine (*Pinus banksiana*), and white pine (*Pinus strobus*) on the relative attraction of host tree species, selected monoterpenes, and background forest types on responses by *I. pini* and its associates. Host chemistry provides an additional source of disparity between herbivore prey and its predators.

Host Range (Kairomonal Attraction) of Predators

The extent and rate to which natural enemies could respond (genetic and phenotypic plasticity) to variation in bark beetle pheromones depends in part on the extent to which they are host specific. Natural enemies of bark beetles do not readily fall into conventional definitions. They are specialists in the sense that they feed and develop almost entirely within trees colonized by bark beetles. However, once in such trees, some species are generalists in the sense that they will feed on most inhabitants within this community. Some biologists have employed the term ecological specialists in such situations, which seems to apply here. We are evaluating the host ranges of the major predators and parasites of *I. pini* in Wisconsin from a chemical ecology standpoint. Assays include a variety of potential herbivore prey. The most specialized species, *Tomicobia tibialis* (Hymenoptera: Pteromalidae) shows tightly defined chemical responses in Wisconsin. Likewise, in the reciprocal exchange experiment, *T. tibialis* in California were almost exclusively attracted to California *I. pini* (Raffa and Dahlsten 1995).

Population Trends

We are conducting long-term population monitoring of *I. pini* and its major associates in 17 stands of red pine, *Pinus resinosa*, throughout Wisconsin.

Implications for Biological Control

We are developing these results to improve the biological control of bark beetle pests. In particular, we are conducting experiments to: 1) improve sampling efficiency and accuracy in estimating the relative numbers of pests and natural enemies; 2) reduce negative impacts of pheromonally based "trap-out" tactics on beneficial insects (DeMars and others 1986); 3) increase the arrival rate of beneficial insects to breeding sites, and 4) guide introduction strategies.

Future Work

Future work will emphasize:

1. Molecular analyses of insects from chemically defined breeding lines and traps
2. Responses of natural enemies to volatiles from microbial symbionts of bark beetles, and from altered host chemistry.
3. Continued studies on the chemical ecology, population dynamics, and evolution of *I. pini* - natural enemy interactions.

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11. Variation in Two Life History Traits of *Dendroctonus ponderosae* from Lodgepole and Ponderosa Pines in Idaho and Utah

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The polyphagous nature of the mountain pine beetle, *Dendroctonus ponderosae* Hopkins (Coleoptera: Scolytidae), is well documented. Adult mountain pine beetle can successfully reproduce in at least 13 species in the genus *Pinus* (Cerezke 1995, Furniss and Schenk 1969, Wood 1963), although populations are most often found in lodgepole pine, ponderosa pine, limber pine, whitebark pine, and western white pine. In mixed species stands, mountain pine beetle brood may infest a different host species than the one they were reared on, suggesting a preference in host selection (Baker and others 1971, Wood 1963). In a laboratory study in which adult beetles from lodgepole pine were allowed to infest ponderosa, whitebark, western white and lodgepole pine, Amman (1982) observed that brood did best, overall, in ponderosa pine, and poorest in lodgepole pine, despite the fact that parent beetles came from lodgepole pine. Additionally, mountain pine beetle development, survival, and reproduction was greater in limber than in lodgepole pine in an area where both hosts occurred (Langor 1989). Differences in fitness parameters such as larvae size (Logan and others 1998), emergence time (Bentz, unpublished data), and cold-hardiness (Bentz and Mullins, in press) in mountain pine beetles from lodgepole and ponderosa pine in Idaho and Utah have also been documented.

Differential host use has been associated with genetic differentiation in several phytophagous insect species, including the mountain pine beetle (Anderson and others 1979, Edmunds and Alstad 1978, Mitter and Futuyma 1979, Stock and Amman 1980, Sturgeon and Mitton 1986). Stock and Amman (1980) and Sturgeon and Mitton (1986) attributed observed differences in allele frequencies in mountain pine beetles from lodgepole and ponderosa pine to be more associated with host tree species than with geographic distances among the sites. Langor and Spence (1991), however, made the argument that genetic differentiation found among beetles from the two hosts may be due to differential survival of beetle genotypes in response to selection

pressures within each host. In this same study they also observed relatively high levels of genetic differentiation among beetles from different sites in Alberta and British Columbia (Langor and Spence 1991). Although past research has shed light on the matter, the question still remains whether the differences in mountain pine beetle fitness and behavioral parameters are due to host factors, environmental factors (for example, temperature), or whether they are based on genetic differences among beetles from the different host species or geographic locations.

We chose to explore this puzzle by rearing mountain pine beetles from different hosts in the laboratory, maintaining a constant environment (temperature and humidity) so that the host the brood was reared in was the only factor varied. Our assumption was that if environment was held constant, any differences observed in fitness parameters between beetles reared in the host they came from or an alternative host could be due to some host-related inherited influence.

Adult beetles from naturally infested lodgepole pine were manually infested into half of a lodgepole pine bolt and half of a ponderosa pine bolt. Likewise, beetles from a naturally infested ponderosa pine bolt were manually infested into the other half of both the lodgepole and ponderosa pine bolts. This resulted in replicates consisting of a brood reared in the host its parents came from and in the alternate host: 1) parents reared in lodgepole pine and brood reared in ponderosa pine {LP@PP}, 2) parents reared in lodgepole pine and brood reared in lodgepole pine {LP@LP}, 3) parents reared in ponderosa pine and brood reared in lodgepole pine {PP@LP}, and 4) parents reared in ponderosa pine and brood reared in ponderosa pine {PP@PP}. Because of the available distribution of naturally infested hosts in the field, beetles came from two geographic regions. Infested lodgepole pine billets were collected from central Idaho, and infested ponderosa pine billets were collected from southern Utah. A male and female pair was initiated into pre-drilled holes in the phloem of each bolt. A consistent spacing between parent galleries was maintained among bolts. Equal replicates of the bolts

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were kept at 17°C and 21°C until brood emergence. Total time for development and pronotum width of emerged adults were measured.

An interaction between temperature and development time was observed, although not consistently across the hosts. The PP@PP brood remained relatively the same size and required, on average, a similar amount of time to develop at both 17°C and 21°C. In contrast, the LP@LP brood required significantly less time to develop at 21°C than at 17°C, and individuals were slightly smaller at 17°C. At 17°C the LP@LP brood individuals were smaller and took longer to develop than the PP@PP brood at the same temperature. At 21°C the LP@LP brood was still smaller than the PP@PP brood, although they developed significantly faster. At both 17°C and 21°C, the LP@PP brood developed faster than did the PP@LP brood. At 21°C, broods from LP@PP and PP@LP were similar in size, although at 17°C the LP@PP brood was smaller than the PP@LP brood.

In summary, individuals with parents from lodgepole pine were always smaller than individuals with ponderosa pine parents, no matter the host they were reared in. Individuals with lodgepole pine parents developed faster when reared in ponderosa pine at 17°C, and were slightly larger when reared at 21°C (as compared to being reared in lodgepole pine at both temperatures). In contrast, individuals with ponderosa pine parents developed slower and were smaller when reared in lodgepole pine at both 17°C and 21°C (as compared to being reared in ponderosa pine). These results indicate that ponderosa pine is a better host for mountain pine beetle in terms of faster development time and larger size. At 21°C, individuals reared in ponderosa pine that came from parents reared in lodgepole pine developed almost 20 days faster than individuals that were reared in ponderosa pine that had parents also from ponderosa pine. Those individuals with ponderosa pine parents were always larger however.

The minimum development time required to complete a generation was approximately 60 days, accomplished by beetles with lodgepole pine parents, reared at 21°C in either lodgepole or ponderosa pine. Beetles with parents from lodgepole pine made more fitness-tradeoffs, reducing adult size for a faster development time when given warmer temperatures or the better food source of ponderosa pine. A trade-off between development time and size at maturity is a common component of life-history models, with current theory involving a trade-off in fitness advantages between obtaining a large

size and taking less time to develop through a single generation (Nylin and Gotthard 1998). Although in many situations a faster development time is advantageous, the importance of such fast development time varies, such as in cold vs. warm climates.

Because parent beetles came from different geographic regions, as well as different hosts, it is difficult to separate host differences from regional population differences. Parent beetles collected from lodgepole pine in central Idaho had experienced significantly colder winter temperatures than had those beetles collected from ponderosa pine in southern Utah (Bentz and Mullins, in press). The capacity of a brood from lodgepole pine parents to compromise size for development time could be due to a selection pressure in the colder environment for putting all resources into making sure development occurs in a single generation. In the warmer climate, where parents from ponderosa pine were collected, the developmental season is longer and selection pressure may be greater on the size fitness component. Inheritance of body size has been shown with several insect genera (see Roff 1980), while it is unclear if development time is also an inheritable trait. Although host and regional effects are confounded in this preliminary data, under constant environmental conditions, the source of parent beetles had a significant effect on size and developmental fitness characteristics of first-generation brood adults regardless of the brood host. This suggests a genetic basis to observed differences. These observed differences and tradeoffs in mountain pine beetle fitness components most likely help to maintain the polyphagous nature and widespread distribution of this important bark beetle species.

12. Selection on Pheromone Production and Preference in *Ips pini*

Alice M. Shumate and Matthew P. Ayres¹

Background

A central problem in evolutionary ecology is explaining the maintenance of genetic variation in traits linked to fitness. Variation can facilitate population persistence in a changing environment, but tends to be minimized by stabilizing natural selection. Although high genetic variation in important ecological traits has recently been documented in a number of natural systems (for example, Bossart and Scriber 1995, Hairston and Dillon 1990), we still lack a complete understanding of the circumstances under which this variation may persist.

Pheromone use in the pine engraver beetle, *Ips pini*, is an example of a trait for which there is large unexplained genetic variation. The main component of the *Ips pini* pheromone mixture, ipsdienol, is a chiral compound with two enantiomers (optical isomers); the isomeric blend used by *I. pini* populations varies geographically (Birch and others 1980, Lanier and others 1980, Miller and others 1989, Seybold and others 1995). Studies have also shown extensive variation in pheromone blend within populations, particularly in eastern populations where the blend preference is intermediate (lying between 40 and 80 percent (+) ipsdienol) (Lanier and others 1980, Miller and others 1989).

Both pheromone blend production and pheromone blend preference are likely to be under strong selective pressures because they are involved in mate attraction, can influence intraspecific competition through effects on colonization density, and impact susceptibility to specialist predators that use ipsdienol as a kairomone (Herms and others 1991, Raffa and Klepzig 1989). These characters could evolve quite quickly, because they have high heritability (Hager and Teale 1996), and are phenotypically correlated due to assortative mating (Teale and others 1994).

How, then, might phenotypic variation in these characters be maintained within populations of *Ips pini*? One hypothesis invokes frequency-dependent selection

acting through predator preference (Herms and others 1991, Raffa and Klepzig 1989). While a direct test of this and other hypotheses (see Raffa, this proceedings) is necessary to establish a mechanistic understanding of the maintenance of variation, to form a complete picture of natural selection it is necessary to quantify fitness components in the wild throughout the entire life cycle of the organism (Arnold and Wade 1984). Our approach has been to break down the life cycle of *Ips pini* into biologically distinct stages, and then to estimate and compare fitness components from all stages. The result should be a framework of natural selection, within which results of mechanistic tests can be placed.

Selection Model

Our life cycle model is male-based. It breaks down the *Ips pini* reproductive cycle into five discrete steps: eggs, larvae, flying adults in search of host material, adults boring into the bark, and adults in the nuptial chamber attempting to attract females and mate with them.

This conceptualization of the life cycle can be parameterized as a stage-based model and analyzed as a Lefkovich matrix (Caswell 1989) to identify the processes that most strongly influence population growth rate. Based upon these analyses, Wisconsin *Ips pini* seem to be most sensitive to survival in the larval stage, and secondarily to survival and mate procurement during the adult reproductive stage. While our population dynamic analysis points to these stages as most critical in determining overall population growth rate, it is important to note that these stages might not be the most important for selection. For example, the larval stage is critical to population dynamics because mortality is so high during this period; however, if the probability of survival is not related at all to an individual's phenotype, selection will not be acting at all during this population bottleneck. Fitness analyses should combine information regarding strength of selection at each stage with the relative importance of survival or reproductive rate at that stage.

We are parameterizing our model for a number of different phenotypes, and will ultimately model selec-

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tion in a population of *Ips pini* with an array of matrices, one for each phenotype. This will allow us to explicitly model assortative mating, and follow evolutionary dynamics through multiple phenotypes.

Methods

Fitness components for reproduction and larval survival were parameterized from field data collected during the 1996 and 1997 field seasons on naturally-colonized host material in Dunn County, Wisconsin. Red pine trees (*Pinus resinosa*) were felled in plantations, which comprise the majority of *Ips pini* habitat in this area of west-central Wisconsin. Log sections (bolts) were cut from the boles of the trees, placed in five arrays of five bolts each at four sites, and monitored for colonization. Entrance holes of colonizing beetles were numbered and mapped. A pheromone sample was collected from each entrance hole for GC/MS analysis. In 1996, the logs were peeled after 2-4 weeks, and each numbered male entrance hole was surveyed for the total number of female egg galleries, and the number of eggs within each of those egg galleries. In 1997, bolts were placed in emergence traps once colonization was complete, and offspring were counted and identified to species as they emerged from the bolts. Once emergence was complete, the exit holes were counted. In both years the number of *Ips*, weevil, and borer galleries were tallied, and bolt dimensions and surface area were measured.

Reproductive Fitness Components

Mating success of males varied as a function of their pheromone production, with a U-shaped response surface. Males averaging the lowest number of mates in this analysis lay near the peak of the phenotypic distribution, at 69 percent (+) ipsdienol. Males averaging the highest numbers of mates (approximately two times as many) were found at the lower and higher tails of the main portion of the phenotypic distribution, 63 percent (+) and 73 percent (+) ipsdienol, respectively. (Small sample sizes precluded calculation of mating success for the extremely rare portions of the distribution.)

This disruptive, frequency-dependent selection surface would tend to maintain variation in a character. As opposed to directional or stabilizing selection, where rare individuals at one or both ends of the phenotypic distribution have lower fitness and are rapidly eliminated from the population, the selective surface we have found indicates that rare males obtain a greater number

of mates. This mechanism, allowing rare males to persist on both ends of the phenotypic distribution, tends to broaden the phenotypic distribution of the population.

Although there was high variation in female oviposition, we found no systematic relation between the number of eggs oviposited by a female and the pheromone blend of her mate.

Larval Fitness Components

Larval survival is likely to be affected by host quality, predation, and competition. Host quality will operate on the spatial scale of an individual bolt. An entire bolt is probably of similar nutritional quality; therefore host quality will only be an important component of fitness if different bolts are colonized by different arrays of phenotypes, with some phenotypes colonizing higher-quality bolts than others. We have found, however, that bolts do not vary in their average pheromone blend or phenotypic distribution. Thus, variation in food quality among trees presumably exerts very little selection on pheromone production.

Predation and competition are both likely to vary with colonization density. Densely colonized host material will have less space per larval gallery, and send a stronger kairomonal signal to predators that roam the entire surface of the bolt. Competition could be from conspecifics, other *Ips* species, or other phloem-feeding insects (mainly weevils, and Cerambycid and Buprestid borers). In our studies, *Ips* density is usually inversely related to larval emergence, with a decreasing number of exit holes per gallery as the colonization density increases. This relation could be due to direct competition and/or increased predator presence.

Surprisingly, survival to emergence tended to increase with increasing densities of both weevils and borers. Both weevils and borers appear to compete intensely for phloem with *Ips* larvae, which would suggest the opposite pattern. It could be that interspecific competition is somehow less than it appears or that weevils and borers preferentially oviposit in host material that is of the highest quality for *Ips*.

Although there are patterns between larval survival and colonization density at the scale of logs, these trends are not clearly related to pheromone phenotype and thus will not obviously affect selection on pheromone production or preference. However, on a smaller spatial scale, pheromone phenotype could affect spacing amongst neighbors and/or the pheromone phenotype of those neighbors, thereby influencing colonization or

predation density in small patches on bolts and imposing selection on pheromone phenotypes. There was no relation between nearest neighbor distance and male pheromone production, indicating that pheromone phenotype does not affect resource availability for larval development. We also analyzed the distance between randomly chosen pairs of males, and found that males are no more likely to be closer to individuals of similar phenotype. This argues against the hypothesis that there are phenotypic patches within bolts that predators might find more or less attractive. To date, we have found no indication of selection acting on pheromone use during larval development on any spatial scale.

Future Research Plans

We plan to continue parameterization for the remaining *Ips pini* life stages and complete the phenotypically-structured selection model. This will allow us to investigate the strength and importance of selection at different life stages, and evaluate the effects of potential future shifts in relative importance of selective agents (for example, a relatively large population of predators, or an unusually hot and dry summer that decreases larval survival).

Another current experiment is aimed at separating the causal agents in larval survival, to determine the relative importance of competition (both intra- and inter-generic), predation, and host quality at this stage of high mortality. As suggested by K. Raffa and others during the workshop, we are also pursuing analyses of temporal colonization patterns in our existing data sets. These analyses will determine if there exist temporal patterns in colonization of different phenotypes that might affect larval survival yet not be revealed by the spatial analyses we conducted after all colonization was complete.

13. Application of Chemical Ecology to Conservation and Augmentation of Bark Beetle Predators, 1997 Results for Northern California and Wisconsin

D.L. Dahlsten¹, K.F. Raffa², D.L. Six³, B.H. Aukema², and D.L. Rowney¹

Introduction

A common control strategy for bark beetles is to trap flying adults in pheromone traps. Unfortunately, beneficial insect predators of the beetles are also attracted and trapped (Dahlsten and Stephen 1974, DeMars and others 1986, Wood 1982). The goal of this research is to develop means to reduce the removal of predators during bark beetle trap-out programs and to increase predator-to-prey ratios in bark beetle-attractive timber harvest sites (Herms and others 1991, Raffa 1991, Raffa and Dahlsten 1995, Raffa and Klepzig 1989). Specific objectives are: 1) to determine which synthetic lures attract actual ratios of predator-to-bark beetles (*Ips pini*) that arrive at host trees; 2) to determine which combinations of synthetic attractants generate the highest bark beetle-to-predator ratios during simulated trap out; 3) to determine the pheromonal mixtures that would most augment predator arrival rates.

Parallel experiments were done in California and Wisconsin. These studies are part of a joint 3-year USDA-CSRS grant to the University of California and the University of Wisconsin.

Procedures

In the first experiment, we deployed nine treatments in a behavioral choice test twice a season and with several replicates. Six treatment lures are made of ipsdienol plus lanierone in different configurations of + or - stereoisomers (Herms and others 1991; Lanier and Wood 1975; Miller and others 1997; Seybold and others 1992, 1995; Teale and Lanier 1991; Teale and others 1991). There are two control type treatments and one infested bolt treatment. The second experiment simu-

lates trap-out treatments, as they would be used in an actual control project, but with six different attractants. It was deployed as a series of no-choice tests in a Latin square design, repeated twice a season. Six plots were used, each with nine traps deployed in a 3x3 grid and baited with one lure type (using the same six treatments of synthetic lures as in objective 1). The traps were sampled every 3 days and treatments randomized among plots for a total of six sampling intervals.

Results

Choice Tests

California—Run one had relatively low numbers of *Ips pini* attracted to all treatments compared to run two, however there were significant differences in treatments. The infested bolt treatment was highest in both run one (6.33 m ± 2.11 se) and run two (15.56 m ± 5.04 se). This peak was not significantly different (p<0.05) in run one compared to the 3 percent + ipsdienol and 3 percent + ipsdienol/lanierone treatments, but was significantly higher than all other treatments. In run two, the infested bolt treatment was significantly different from both of these pheromone treatments, but these two pheromone treatments were higher than all other pheromone combinations and controls.

In both run one and run two the predator *Enoclerus lecontei* was significantly more attracted to treatments that had Lanierone as a component, especially in combination with the +50 percent and +75 percent ipsdienol. This difference was highly significant for run two, when this predator was much more common.

In run one the predator *Enoclerus sphegeus* was somewhat common and was significantly attracted to the infested bolt, but not to any pheromone treatments or controls. Not enough *E. sphegeus* were present in run two to determine trends.

Wisconsin—The relative responses of *Ips pini*, other *Ips*, and predators to a range of pheromones (including various enantiomers and synergist combinations), and

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also to natural sources of pheromone, were compared. This experiment was conducted twice during 1997, during early season and late season. The experiment was conducted as planned, insect populations were adequate for analysis, and all of the collected insects have been identified, tabulated, and stored into computer files.

The major predators obtained were clerid and histerid beetles. The overwhelming majority of the clerid beetles were *Thanasimus dubius*. The predominant histerid beetle was *Platysoma cylindrica*.

There was strong variation in insect responses to the various plumes. Depending on the synthetic mixture chosen, a forest manager's estimate of the relative abundance of pest bark beetles to beneficial insects could range from 24 times to 0.2 times as much. Responses to infested logs were 1:1. These results support our working hypothesis, that an improved knowledge of how various synthetic lures relate to actual colonization patterns could greatly improve our ability to gauge beetle populations, and thus be directly employed in treatment decisions.

No Choice Tests

California—For both tests, *Ips pini* was attracted to the treatment of 3 percent + ipsdienol in significantly higher numbers than all other treatments except for the 3 percent + ipsdienol/lanierone combination.

For run two, the predator *Enoclerus lecontei* was significantly more attracted to treatments that had lanierone as a component, especially in combination with the +50 percent and +75 percent ipsdienol. Insufficient numbers of this and other predators were caught in run one for analysis.

Several other scolytid species and other insect associates were trapped in both the choice and no choice tests but attraction to any of the test materials showed no distinct patterns. We are still awaiting verification of identifications for a number of the insects that were trapped in both tests.

Wisconsin—Bark beetle and predator responses to various pheromone mixtures were evaluated under no-choice conditions. This experiment was designed to emulate a trap-out operation, to see if manipulating chemical signals could reduce deleterious effects on predator populations. The experiment was conducted as planned, insect populations were adequate for analysis,

and all of the collected insects have been identified, tabulated, and stored into computer files.

As in objective one, the major predators obtained were clerid and histerid beetles. The overwhelming majority of the clerid beetles were *Thanasimus dubius*. The predominant histerid beetle in this experiment was *Platysoma parallelum*.

The high variation in insect responses, as well as the differences between bark beetles and predators to bark beetle pheromones, persisted under these no-choice conditions. The ratio of pest to predator insects collected in funnel traps varied from 7.5 to 0.2. Stated another way, a forest manager's choice of plumes could result in anywhere from 83 percent of the insects removed from the forest being predators, to as low as 3 percent of the insects removed being beneficial. These results support our working hypothesis that deployment of judiciously selected plumes can conserve predator insects during pheromonally based trap-out operations.

Tentative Conclusions Based on First Year of Study

The differential in attraction provides us with the opportunity to manipulate predator-prey ratios, which we will test under field conditions in 1999. The results for the 1998 field season are still being counted. Our preliminary findings based on predator response are that predator augmentation should be done early in the season and trap-out for *Ips pini* should be done late in the season.

14. Environmental and Genetic Influences on Host Selection Behavior of Bark Beetles: Roles of Population Phase and Geographic Origin

Kimberly F. Wallin and Kenneth F. Raffa¹

This research is aimed at linking shifts in population phase with underlying causes (Chandra and Williams 1983, Lance and others 1986, Mitter and Schneider 1987, Price 1991, Redfern and Pimm 1988, Rossiter 1987). It places particular emphasis on the genetic basis of host plant selection by bark beetles. Our approach builds on previous studies on the mechanisms of tree resistance against bark beetles, the role of aggregation pheromones in mediating rapid concentrations that collectively exhaust these defenses, individual behavioral decisions by beetles, individual replacement rates, and population dynamics (Klepzig and others 1996; Raffa and Berryman 1983, 1987). In particular, we are testing whether beetle populations show genetic shifts that reflect differences in selection pressures on individual choices to colonize relatively healthy vs. stressed trees (Raffa and Berryman 1983, 1987). We are testing genetic and alternate, complementary hypotheses, such as effects of age, lipid content, repeated rejections, and maternal effects on individual host selection decisions (Kinn and others 1994, Langor and others 1990, Rossiter 1994, Thomson and Sahota 1981).

Our experiments involve two models: Spruce beetle, *Dendroctonus rufipennis*, in white spruce in western and midwestern North America, and the pine engraver *Ips pini* in red pine in Wisconsin. The first system involves an outbreak species (Reynolds and Hard 1991, Werner and others 1977), which facilitates comparisons between endemic and epidemic populations. Study sites are located in the Kenai Peninsula and Fairbanks regions of Alaska, the Canadian Yukon, northern British Columbia, Utah, and portions of the Great Lakes region (Upper Peninsula MI, northern WI, northeastern MI). *Ips pini* is more easily cultured than *D. rufipennis*, and lends itself more readily to mutigenerational and classic heritability studies.

This research is considering whether factors associated with herbivore population density affect host plant acceptance. In particular, we are evaluating whether

bark beetle aversion from high concentrations of host allelochemicals (monoterpenes) can be reduced by 1) two environmental factors, an extended inability to locate stressed trees (containing low monoterpene concentrations) and the presence of aggregation pheromones, and 2) whether such aversion might have a genetic component that is subject to controlled selection, and varies with eruptive population phase. Each experiment involves variations on a common laboratory bioassay and biochemical analysis of total lipids. Parallel experiments are being conducted with *D. rufipennis* and *I. pini* for addressing the above questions.

Heritability of host acceptance behavior and multiple-generation selection experiments (Via 1984, 1990) are being analyzed for both *D. rufipennis* and *I. pini*. Within- and between-population and within- and between-geographical location comparisons are being conducted by assaying field collected beetles from high- and low-density populations of *D. rufipennis*. Independent breeding lines based on field population phase and location (*D. rufipennis*) and performance in bioassays (*I. pini*) have been established.

Future Directions

The following studies are planned for 1999:

1. Field caging assays using field collected adult beetles and breeding line adult beetles to verify results from laboratory assays.
2. Employ SEM techniques to qualitatively and quantitatively assess differences between and among beetle antennae setae from the four breeding lines.
3. Using quantitative genetics to determine heritability, parental effects, and relative strengths of environmental and genetic influences on insect vigor, insect morphology, and how they relate to host selection behavior.
4. Molecular analyses of *I. pini* from independent breeding lines based on performance in host selection assays.
5. Molecular analyses of *D. rufipennis* based on geographic origin and population phase.
6. Mathematical modeling of interactions among

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population density, host selection behavior, and genetic and environmental inputs.

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15. Bark Beetle Genetics Research in the Bark Beetle/Pitch Canker/Pine System

Andrew J. Storer¹, Pierluigi Bonello², David L. Wood¹ and Thomas R. Gordon²

Introduction

Pitch canker disease, caused by *Fusarium subglutinans* f. sp. *pini*, has affected Monterey pines in California since at least 1986 (McCain and others 1987). The geographic and host range of the pathogen has increased rapidly in the last 12 years (Gordon and others 1996, Storer and others 1994). The pathogen is now considered to have a continuous distribution in coastal areas of central and southern California wherever suitable hosts are found. All native pines in these areas are susceptible to pitch canker disease. However, some of the exotic species planted in central and southern California show lower levels of damage from the disease than the native species (Gordon and others 1998).

The pitch canker pathogen is vectored to pines by a number of insect species including *Ips* spp., *Pityophthorus* spp., *Conophthorus radiatae* (Coleoptera: Scolytidae), and *Ernobius punctulatus* (Coleoptera: Anobiidae) (Fox and others 1991, Hoover and others 1996). The pathogen is also associated with feeding sites of the spittlebug *Aphrophora canadensis* (Homoptera: Cercopidae), and transmission of *F. s. pini* by this insect to Monterey pines in the greenhouse has been demonstrated (Storer and others 1998). It is possible that other insects feeding on Monterey pine are also capable of vectoring the pathogen. While the *Ips* spp. are well studied, and *Conophthorus* spp. have recently received some attention, relatively little is known about the *Pityophthorus* spp. Further study of *Pityophthorus* spp. is merited due to the association of these beetles with *F. s. pini* infected branch tips, and a recent demonstration of vectoring of *F. s. pini* to healthy branch tips in the field (Storer and others, unpublished).

Our knowledge of the genetics of some groups of bark beetles has increased greatly over the years, and a

number of molecular techniques have been used in addition to studies of cross-mating, pheromone and cuticular hydrocarbon chemistry, and morphology. We hope to utilize some of these techniques in order to gain a greater understanding of the interactions among species in the bark beetle/pitch canker/pine system.

Previous Uses of Genetic Techniques in the Pitch Canker Disease System

Designation of the causal agent as forma specialis *pini*.

Correll and others (1991) proposed that strains of *F. subglutinans* from diseased pine tissue and from pine feeding insects be designated as a forma specialis since non-pine strains of *F. subglutinans* were not pathogenic to pines in greenhouse pathogenicity tests. This initial designation was based solely on greenhouse inoculation studies, but was later supported by studies of mitochondrial DNA (mtDNA) restriction fragment length polymorphisms (RFLPs) (Correll and others 1992). In the latter study, isolates of *F. s. pini* from different geographical locations and different pine hosts shared identical mtDNA restriction patterns. These mtDNA restriction patterns were quite different from those of non-pine isolates of *F. subglutinans*. Work in South Africa has subsequently supported the designation of the causal agent of pitch canker disease as *F. s. pini* (Viljoen and others 1994).

Determination of the population structure of the pathogen.

Isolates of *F. s. pini* were characterized by vegetative compatibility using nitrate non-utilizing mutants (Correll and others 1992). Five distinct vegetative compatibility groups (VCGs) were identified among 209 isolates collected in California between 1986 and 1989. In contrast, 45 VCGs were identified from a collection of 116 isolates of *F. s. pini* from Florida (Correll and others 1992). The limited genetic diversity of the pathogen in California, reflected in the low number of VCGs is indicative of a recently introduced, non-

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sexually reproducing population.

In an additional collection of 170 isolates made in California between 1993 and 1995, 152 isolates were vegetatively compatible with the five previously characterized VCGs, and 18 isolates were determined to be associated with three new VCGs (Gordon and others 1996). The distribution of these VCGs indicates how movement of infected trees may have contributed to the spread of the disease. For example, one VCG from the 1986-9 collection was found only in nursery stock in Los Angeles Co. and in an isolated infestation in Santa Barbara. In the 1993-5 collection, this VCG was found to predominate a large infestation in San Luis Obispo Co. (Gordon and others 1996).

Studies in South Africa revealed 23 VCGs from 69 isolates of the pathogen, and this higher diversity is thought to reflect some level of sexual reproduction in the population (Viljoen and others 1997a). Cluster patterns constructed from random amplified polymorphic DNA (RAPD) indicated that South African isolates from pine were related to pitch canker isolates from other areas, but were distinct from non-pine isolates (Viljoen and others 1997b). Evidence from virulence tests, genetic analyses and composite RAPD data suggest that pine isolates probably represent a distinct new species rather than a forma specialis of *F. subglutinans* (Viljoen and others 1997b), and the name, *Fusarium circinatum* has been proposed as the name for this species (Nirenberg and O'Donnell 1998).

The use of mutant forms of the pathogen to study epidemiology

A benomyl-resistant mutant of the pitch canker pathogen was developed using VCG C3 (from Christmas trees in southern California), which does not otherwise occur at New Brighton State Beach, the field site named in the permit for the release. *Ips paraconfusus* were inoculated with the marked strain of the pathogen, and reared for one generation in fresh Monterey pine logs. Progeny were allowed to emerge from these logs at the release site in July 1993. Insect and plant material from the release sites and from sites 1 km north, south, and east of the release site was collected to screen for the presence of the mutant form of *F. s. pini*. This sampling consisted of rearing insects from infected branch tips, infected trees, and uninfected branches cut and hung in the canopy of trees, and collected from pheromone-baited insect traps. The mutant was not recovered during a 6-month sampling period from April to October 1994. *F. s. pini* was isolated from 328 of 3451 (9.5

percent) twig and engraver beetles collected at this site (Storer and others, unpublished), and none of the fungal colonies grew on a *Fusarium* selective medium (Correll and others 1992) containing benomyl.

A second release was carried out at Monterey Bay Academy (following established regulatory procedures), using a new benomyl-resistant strain of *F. s. pini*. The second benomyl-resistant mutant of *F. s. pini* was produced using VCG C8, which is not found in the release area, and a different VCG from that of the previous released isolate. This unique marker ensured that recovery of the mutant in the future could be related to the first or second release. The twig beetle, *Pityophthorus setosus*, was contaminated with this new strain. This species is common at this location, and is found throughout central, coastal California (Dallara 1997). It is known to carry *F. s. pini* and has recently been demonstrated to vector the pitch canker pathogen to native Monterey pines (Storer and others, unpublished). A species-specific pheromone, pityol (Dallara and others 1995), was used to trap this insect, enabling the collection of a large number of insects in a short period of time.

To prepare for the release, 2200 *P. setosus*, trapped at Monterey Bay Academy from 10-23 August 1995, were mixed with 1.5 ml of a spore suspension (7.5×10^7 spores/ml) of the second mutant of *F. s. pini*, and dried on filter paper. These inoculated insects were allowed to fly from a release point in a mature Monterey pine stand. A sample of 30 of the unreleased beetles showed all of them were carrying *F. s. pini*, and estimates of over 100 propagules per insect were obtained by sequentially vortexing a small sample of the beetles in water, plating the water containing the removed propagules onto selective medium, and counting the colonies that grew after 3 days. We feel that the chances of establishment of this mutant are much higher than from the previous release because this release was not dependent on transmission of the pathogen from parents to progeny, as was the case with the first release. Beginning in November 1995, isolates of *F. s. pini* were collected in the vicinity of the release site from insects reared from infected Monterey pine branch tips, and stem cankers. To date, screening of over 400 isolates of *F. s. pini* has not yielded any benomyl-resistant colonies. However, details of the genetic structure of the *F. s. pini* population within this localized area is being determined using VCGs as described previously (Gordon and others, unpublished).

Future Uses of Genetic Techniques in the Pitch Canker Disease System

Further use of a genetically marked strain of the pathogen to study interactions among insects, the pathogen, and the host tree

The potential knowledge that could be gained from making successful releases and recoveries of genetically marked strains of the pitch canker pathogen include: 1) the relative importance of the insect vectors within a stand, 2) the relative importance of the insect vectors in transporting the pathogen to new stands, and 3) the ability of the pathogen to be transmitted from one vector species to another. We hope to maximize the possibility of establishing one or more genetically marked strains of the pathogen by releasing a number of different strains, each on different insect or plant material. Marked strains will utilize the benomyl-resistant character that we have already produced for VCGs 3 and 8. In the future we hope to produce similarly marked strains of the other VCGs. Each marked VCG will be released at the same site, but on different plant or insect material. We are also working on the transformation of *F. s. pini* with the gene for Green Fluorescent Protein (GFP), a visual molecular marker. The GFP tag should considerably simplify identification of marked strains released in the field and allow determination of where the fungus is carried on the insects. The release techniques will include inoculating *F. s. pini* into branch tips (with and without cones), into stems, and on *Ips* spp., and *Pityophthorus* spp. prior to release into the forest.

Biosystematics of *Pityophthorus* spp. that vector the pathogen.

Based on the published host and geographic ranges of the insects involved in the transmission of the pitch canker pathogen, it is possible to hypothesize how pitch canker disease may spread to other pine species and regions of California (Storer and others 1997). This prediction is dependent on the assumption that insects all currently classified as one species infesting two or more hosts are one species. There is a precedent in other scolytids for insects that appear morphologically identical but are, in fact, different species. For example, *Ips confusus*, *I. hoppingi* and *I. paraconfusus* are virtually identical based on morphology, and electrophoretic analyses (Cane and others 1990b). Species status for these *Ips* spp. is based upon their host association, geographic distribution, failure to hybridize, karyology, and striation density on the parastriations of the stridulatory organ (Cane and others, 1990c; Lanier 1966, 1970, 1971, 1972; Merrill 1991). Studies of pheromone chemistry did not reveal differences among these species (Cane and others 1990a). However, some

divergence between *I. paraconfusus* and *I. confusus* was indicated in behavioral studies (Cane and others 1990c).

Three species of twig beetle appear to have an important role in the epidemiology of pitch canker disease. *Pityophthorus setosus* is only reported to attack Monterey and Bishop pine; *P. carmeli* has a host range that is limited to Monterey, Bishop, Torrey and Coulter pine; and *P. nitidulus* has a broad host range that apparently includes all *Pinaceae* (Bright and Stark 1973). Since none of these insects was of economic importance prior to the establishment of associations with the pitch canker pathogen, they have been studied little, and no genetic work has been undertaken to determine whether the classification based on morphological characteristics accurately identifies these species from their various hosts.

In the scolytidae, isozyme electrophoresis has been used to determine relatedness of species of *Dendroctonus* (Bentz and Stock 1986, Stock and others 1987), *Ips* (Cane and others 1990b) and *Conophthorus* (DeGroot and others 1992). Analysis of cuticular hydrocarbons has focused on *Dendroctonus* (Page and others 1990b), *Conophthorus* (Page and others 1990a) and *Ips* (Page and others 1997). RAPDs have been used to determine the relatedness of *Tomicus piniperda* populations (Carter and others 1996) and *Ips* spp. (Cognato and others 1997). RAPDs have also been used to study the genetics of species in other beetle families such as Curculionidae (Taberner and others 1997) and Silvanidae (Brown and others 1997)

We intend to use molecular approaches to define species or species complexes in the genus *Pityophthorus*. We propose to use a hierarchical design in which polymorphisms would be sought along a low-to-high resolution gradient. The first step would be to sequence variable regions of the nuclear genome, specifically the internally transcribed spacers (ITS) and intergenic spacer (IGS) of the nuclear rRNA gene tandem repeat. Phylogenetic analysis using parsimony (PAUP) would be employed to define clades against close outgroups, such as closely related species or geographically distant populations of the same species. A useful byproduct of this approach would be the creation of a molecular database that could be continuously expanded and used for the identification of unknown samples/groups in a manner similar to that used for other taxa, for instance, fungi (for example, Bruns and others 1998). If polymorphisms are not found at this level of variability, more sensitive molecular analyses would be carried out. Although RAPDs are very sensitive and have been used repeatedly by other authors for similar purposes, we believe that a better approach for highly sensitive analyses would be to look for arbitrary fragment length polymorphisms (AFLP)

(Chalhoub and others 1997). This technique is conceptually a derivatization of RAPDs analysis, but it is much more robust, and offers the possibility of selecting species- or population-specific alleles that could be used for the development of diagnostic PCR tests, if necessary or desirable. Definition of population structure at this level of resolution would be best achieved by using analysis of molecular variance (AMOVA) (Stewart and Excoffier 1996). Whatever the test that will ultimately be used, members of different clades, indicative of significantly distinctive molecular groupings, would then be tested in other confirmatory ways, for example, by conducting mating studies and morphological examinations. This should give us rather definitive answers regarding the genetic structuring of any given group of bark beetles in the insect/pathosystem under study here.

For the species with the broadest host and geographic ranges, we expect to identify sibling species or sub-species. By determining the host and geographic range of these species or sub-species, we will be able to better predict the potential for further host and geographic range expansion of the pitch canker fungus.

Sub-structuring of *Pityophthorus* spp. populations according to host of origin.

Pityophthorus nitidulus is listed as attacking all Pinaceae (Bright and Stark 1973). We propose to investigate the genetic diversity of *P. nitidulus* from diseased Monterey pine and apparently disease-free ponderosa pine at the same location in Santa Cruz County, as well as from other locations. This will enable us to determine if ponderosa pine escapes infection due to population sub-structuring of *P. nitidulus*, which results in contaminated individuals emerging from Monterey pine and selecting Monterey pine over ponderosa pine during host selection. There is some precedent for this in the scolytidae, as *Dendroctonus ponderosae* in mixed stands of lodgepole and ponderosa pines may select their larval host for mating and oviposition as adults (Sturgeon and Mitton 1982). We also propose to compare the genetic diversity of *P. setosus* and *P. carmeli* from Monterey pine and Bishop pine where they grow at the same site, compared with populations from other locations. Substructuring is unlikely in at least one of these two species, since Bishop pine growing in the vicinity of infected Monterey pine does not escape infection by the pitch canker fungus. This work would then be extended to include examination of the genetic diversity of

sympatric populations of *P. nitidulus* in Monterey pine, knobcone pine, and Douglas-fir at Point Año Nuevo in San Mateo Co. to determine whether Douglas-fir escapes infection at this location due to substructuring of populations of this species according to their host of origin.

An incipient sympatric speciation event may be uncovered during this work if sympatric population substructuring according to host of origin is revealed. In the apple maggot fly, *Rhagoletis pomonella* (Diptera: Tephritidae), a host shift from hawthorn (*Crataegus* spp.) to apple (*Malus pumila*) has occurred and is frequently cited as an example of an incipient sympatric speciation event. Variation in the fruiting phenologies of apple and hawthorn exert different selection pressures on the diapause and eclosion time characteristics of the host races of *R. pomonella* (Feder and others 1997). Hence reproductive isolation can arise as a consequence of host-associated adaptation in insects, and provides a mechanism for sympatric speciation via host shifts. Outside the insecta, the spider mite *Panonychus citri* populations on citrus and *Osmanthus* in Japan appear to be reproductively isolated and there is host range segregation among them. F₁ females of crosses between populations from each host were sterile (Osakabe and Komazaki 1997).

Conclusion

Most of the genetics techniques applied to the bark beetle/pitch canker/pine system have focused on the pathogen, or manipulation of the pathogen. There are clearly opportunities to utilize molecular techniques to learn further details about this new association between a plant pathogen and its insect vectors. Further use of genetically marked strains of the pathogen will help elucidate mechanisms of spread of the pathogen. Studies of the genetic diversity of twig beetle populations will enable more accurate predictions to be made about future spread of the pathogen into new host species. Molecular techniques can be added to previously used techniques to uncover sibling species relationships with allopatric (Cane and others 1990a) and sympatric (Seybold and others 1995) distributions. Molecular techniques will provide the opportunity to look for potential sympatric population substructuring of the insect vectors of the pitch canker fungus.

16. Genetic Perspectives on the Ecology and Management of *Tomicus piniperda* in North America

Therese M. Poland and Robert A. Haack¹

The pine shoot beetle (PSB), *Tomicus piniperda* L., is a serious pest of pine trees in its native range in Europe and Asia. PSB was first detected in North America in 1992, and as of August 1998 it is established in over 243 counties in 9 U.S. states and 18 counties in Ontario, Canada. Genetic analyses suggest that there were at least two separate introductions; one in northern Ohio along the shores of Lake Erie, and a later one in Illinois along the shores of Lake Michigan (Carter and others 1996). However, the origin of the introduced beetles is unknown. In general, European insects that invade North America may be very successful owing to the wealth of ecological opportunities in North America, reduced natural enemy pressure, and competitive superiority resulting from selection under disturbance and fragmented conditions in Europe (Niemela and Mattson 1996). The impact of PSB in North America will depend on numerous genetic and environmental factors and their interactions in different ecosystems. We present an overview of various studies we have recently conducted on the biology, ecology, and management of PSB in North America and discuss them from a genetic perspective, considering evolutionary adaptations of PSB with respect to North American and native European and Asian ecosystems.

Host Selection and Responses of the Pine Shoot Beetle to Non-Host Volatiles

Host tree species can influence survival and, hence, the genetic makeup of invading bark beetles (Sturgeon and Mitton 1982). Monoterpene vapors from tree oleoresin can be toxic to attacking bark beetles, but beetles tend to be most tolerant of vapors of their natural hosts (Smith 1965). Selection of suitable host trees is critical for bark beetle survival and reproduction. Long-range primary attraction to host volatiles has been well documented for PSB. Spring-flying PSB are able to discriminate between bolts of Scotch pine and Norway spruce (Tunset and others 1993) and are attracted to the

host volatiles α -pinene and ethanol (Byers and others 1985, Byers 1992; Vité and others 1986). Ethanol synergized attraction to α -pinene at low release rates of α -pinene; however no synergism was seen at high release rates of α -pinene. Ethanol was attractive to PSB but to a much lesser extent than α -pinene (Klimetzek and others 1986, Schroeder 1988). The proportion of α -pinene (prevalent in recently injured or felled pine trees) to ethanol (found in dead or dying trees) varies greatly among different types of breeding substrate.

The PSB generally breeds in relatively fresh material from which the release of ethanol is low while high amounts of terpenes may be released from exuding resin. Thus PSB is well adapted to recognize not only acceptable host species but also the most suitable condition of its hosts. It would also be adaptive for conifer-attacking bark beetles to avoid unsuitable or non-host trees. Green leaf volatiles (GLVs) are common 6-carbon alcohols or aldehydes that are prevalent in herbaceous plants and deciduous trees. There is increasing evidence that conifer-attacking bark beetles are disrupted by GLVs (Borden and others 1997, Dickens and others 1992, Wilson and others 1996). In Europe, responses to ethanol by PSB parent adults were disrupted by bolts of aspen (*Populus tremula*) and birch (*Betula pendula*) (Schroeder 1992). Therefore, PSB can detect and avoid particular non-host trees in its native Europe.

We have found that a blend of common GLV alcohols (hexanol, *E*-2-hexenol, *E*-3-hexenol, and *Z*-3-hexenol) disrupts attraction of PSB to α -pinene baited traps. In addition, we found that a blend of several antennally active volatiles from the bark and foliage of aspen (*Populus tremuloides*) and hybrid poplar was also disruptive to PSB parent adults (Poland and others unpublished data).

In North American forest ecosystems the number of potential host and non-host trees encountered is greater than that which PSB co-evolved with in Europe and Asia. Although tree species in North America differ from those in Eurasia, several tree genera are the same and thus their volatiles may be similar. The capacity to recognize non-host angiosperm species through general

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or common volatiles would be highly adaptive to coniferophagous bark beetles. In addition, beetles may recognize particular specific compounds or blends from certain non-host species with which they co-evolved.

Geographic Variation in Initiation of Spring Flight and Fall Shoot Departure

The current distribution of PSB in North America ranges from approximately 39°50'N to 47°10'N. Its native distribution ranges from Portugal east to Japan and from Scandinavia (70°N) south to southern China (<25°N) (Bakke 1968, Hui and Lieutier 1997). Therefore, this insect is remarkably adapted to a wide range of geographic and climatic conditions. In general, synchronization with new environments is likely to be more certain for insects transferring from high to lower latitudes (Niemela and Mattson 1996). Although we do not know the origin of the PSB populations in North America, there is probably considerable potential for PSB to spread in all directions from its current range in North America.

We investigated two temperature-dependent aspects of the pine shoot beetle's life history that are relevant to federal regulations on the timing of moving potentially-infested material out of the North American quarantine zone, that is, the initiation of adult flight in spring and the initiation of shoot departure in fall (Haack and others 1998). We used historical weather records (1950-1993) for the 22-state northeast quadrant of the United States to develop isopleth maps for average dates for when spring temperatures will first support strong adult flight (55° and 60° F), and for the first occurrence of hard frosts in autumn (24° and 28° F). Temperature data were averaged for each recording station in the 22-state region and then isopleth maps were developed with Kriging software using ARCINFO (ESRI 1994). The isopleth maps were presented to the Animal Plant Health Inspection Service (APHIS) for their potential use in adjusting the quarantine in a fashion that is biologically relevant as well as practical to both managers and regulators.

In addition, we tested the accuracy of these maps with actual dates of initial spring flight and fall shoot departure at four sites along a 275-km north-south gradient in Michigan and Indiana in 1998 (Poland and others unpublished data). Moreover, we recorded air, duff, and inside-bark temperatures in spring as well as air and inside-twigs temperatures in fall to test whether air temperatures accurately reflect the environment

where the beetles reside. Weekly averages of daily maximum temperature in spring and daily minimum temperatures in fall were approximately 4°F cooler at the northern site compared to the southern site. This gradient corresponded to a 4-week difference in initiation of spring flight and a 2-week difference in initiation of fall shoot departure across the gradient. Temperatures inside the shoots were similar to ambient air temperatures in the fall; however, the duff and bark provided insulation for over-wintering beetles and resulted in temperatures that were more moderate than ambient air temperatures in the spring.

The timing of spring emergence and duration of shoot feeding appear to be largely determined by prevailing weather conditions; however, temperature responses are likely genetically based. In general, bark beetles are well adapted to variable weather conditions and can adjust emergence accordingly (Stark 1982). Therefore, PSB will likely continue to spread in North America and adjust its period of spring flight and shoot feeding to local weather conditions.

Host Feeding Requirements and Reproductive Maturation

An unusual aspect of the PSB life cycle is the requirement for intermediate maturation feeding in the shoots of living pine trees before adults attack and reproduce in brood material. Although the development time of PSB is relatively short and F₁ brood adults emerge in late spring or early summer, PSB adults do not breed until the next year, and thus this species remains univoltine. Upon emergence, the reproductive organs of teneral adults are rudimentary; their development progresses throughout the maturation feeding period (Långström 1983). The duration of shoot feeding varies greatly over the geographic range of PSB. The amount, if any, of shoot feeding that is required for successful reproduction to occur is unknown. Therefore, in 1997, we investigated if shoot feeding is required by PSB prior to introduction into fresh brood material in order for viable egg production to occur (Poland and others, unpublished data). Unfed callow adults removed from host material, as well as F₁ adults excised every 2 weeks from shoots where they were feeding naturally, were introduced into Scotch pine bolts in the laboratory. Galleries were destructively sampled at weekly intervals until progeny were noted, then any remaining galleries were allowed to complete development. Successful reproduction eventually occurred in

all cases, that is, even for F_1 adults who had not shoot-fed. Thus it is theoretically possible for PSB to be multivoltine. The possible adaptive significance of shoot feeding vs. attempting a multivoltine live cycle may be (1) to avoid extended pre-emergence feeding under the bark and thus escape intense competition, both inter- and intra-specific, during maturation feeding; (2) to weaken trees and thereby provide additional future breeding material; or (3) to avoid interactions during the summer months with other multivoltine bark beetles with well-developed long range pheromone systems.

Reproduction in non-host conifer trap logs

Founding of a new population by a restricted group of individuals provides ideal conditions for genetic transilience through the founder-flush model, which may result in speciation or adaptive radiations in a new environment (Sturgeon and Mitton 1982). North American forest ecosystems offer a tremendous range of potential new host trees to PSB compared to the number of temperate tree species in Europe and Asia. Therefore, it may be possible for PSB to adopt new hosts in North America. We tested this hypothesis by setting out trap logs of five non-host conifers (black, white, Norway, and blue spruce, and Douglas-fir) in a heavily infested Scotch pine Christmas tree plantation in Michigan in 1998 (Haack and others, unpublished data). After natural colonization the logs were placed in sealed rearing tubes and held at room temperature. All emergent beetles were collected and then the logs were dissected. Successful reproduction and progeny adult emergence was found for all species of non-pine conifers tested except for black spruce. This indicates that PSB is capable of reproducing in a variety of non-hosts and under stringent selection pressures could possibly adopt additional host species in North America.

Overall, the above studies indicate the wide number of cases in which PSB life history traits have a strong genetic component. Adaptations of PSB to local environmental conditions will influence its distribution, impact, and management in North America.

17. *Tomicus* and *Anoplophora* Genetics: Important Research Needs

Robert A. Haack¹, Therese M. Poland¹, Jian Wu², and Hui Ye³

Tomicus piniperda (L.) (Scolytidae) and *Anoplophora glabripennis* (Motschulsky) (Cerambycidae) are two of the more recent exotic forest insects to become established in the United States. The native range of *T. piniperda*, the pine shoot beetle, includes much of the pine (*Pinus*) growing regions of Europe, Asia, and North Africa (Wood and Bright 1992). *Tomicus piniperda* was discovered in the United States near Cleveland, Ohio, in 1992 (Haack and Kucera 1993). It is primarily a pest of pine, but on occasion *T. piniperda* can reproduce and shoot-feed in other conifers such as trees in the genera *Abies*, *Larix*, *Picea*, and *Pseudotsuga* (Haack and others unpublished data, Lawrence and Haack 1995, Wood and Bright 1992). As for *A. glabripennis*, established populations were first discovered on Long Island, New York, in August 1996 (Haack and others 1997a) and then in Chicago, Illinois, in July 1998. *Anoplophora glabripennis* attacks hardwood trees, primarily maple (*Acer*), poplar (*Populus*), and willow (*Salix*). In this paper, we will briefly describe studies that address the genetics of *T. piniperda* and *A. glabripennis*, as well as other *Tomicus* and *Anoplophora* species.

Genetic Relatedness of North American Populations of *Tomicus piniperda*

In less than two months after the 1992 discovery of *T. piniperda* in Ohio, additional infestations were found in five nearby states: Illinois, Indiana, Michigan, New York, and Pennsylvania (Haack 1997, Haack and others 1997b). By the end of 1992, *T. piniperda* had been found in 43 counties in the above six US states. In spring 1993, Carter and others (1996) collected *T. piniperda* at eight locations among the original 43 infested counties (fig. 1). Subsequent tests of genetic relatedness, using random amplified polymorphic DNA

(RAPD) methods for polymerase chain reaction (PCR) amplification, suggested that there had been two separate introductions, one in Illinois or Indiana at the southern tip of Lake Michigan, and a second in Ohio along southern Lake Erie.

Since 1992, *T. piniperda* has been found in only three additional U.S. states: Maryland and West Virginia in 1995 and Wisconsin in 1997. In Canada, *T. piniperda* was first discovered in Ontario in 1993, at which time seven infested counties were found. As of January 1999⁴, *T. piniperda* was known to occur in 243 counties in 9 US states and in 22 counties in Ontario, Canada (fig. 1).

Given that the geographic range of *T. piniperda* has increased substantially from 1992 to 1998, it would be of interest to revisit the question of genetic relatedness. That is, would RAPD-PCR analysis of *T. piniperda* populations conducted in the near future provide evidence of additional new introductions? Or during the past several years could the North American *T. piniperda* populations have been sufficiently mixed (owing to natural spread and movement of infested pine

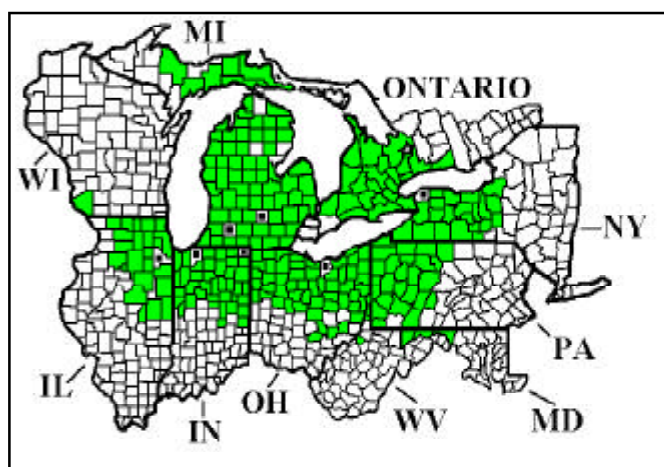


Figure 1—North American range of *Tomicus piniperda* as of January 1999. The seven infested counties with black rectangles represent the locations where *T. piniperda* was collected in 1993 for the *T. piniperda* genetics study reported in Carter and others (1996). There was one collection made in Illinois (IL), 2 in Indiana (IN), 2 in Michigan (MI), 1 in Ohio (OH), and 2 in New York (NY).

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⁴ A few additional occurrences have been added between presentation and publication of this proceedings.

products) so that any differences between the founding populations will now be obscured?

Origin of the North American Populations of *Tomicus piniperda*

We do not know the country or countries of origin for the North American populations of *T. piniperda*. Identifying the origin(s) of the North American founder populations would be useful to help (1) predict the future limits of *T. piniperda*'s geographic range in North America, and (2) target foreign exploration for biological control agents (Carter and others 1996). Considering the native range of *T. piniperda*, there are certainly many candidate countries that could have served as the source of the North American populations (Haack and Cavey 1997). If recent interception records are a good indication of the potential origin of *T. piniperda*, then the most likely sources include France, United Kingdom, Spain, and Italy (table 1). A worldwide collection of *T. piniperda* populations with subsequent genetic testing, using molecular methods such as RAPD-PCR, could aid in pinpointing the likely origin(s) of the *T. piniperda* populations now present in North America.

Table 1. Number of *Tomicus piniperda* interceptions on wood products at US ports of entry during 1985-1998 by country of origin. (Source: USDA APHIS)

Number of interceptions	Country
28	France
19	United Kingdom
15	Spain
15	Italy
8	Belgium
8	Europe*
8	Germany
3	Netherlands
3	Russia
2	Japan
2	Unknown
1	China
1	Finland
1	Greece
1	Hong Kong
1	Portugal
1	Sweden
1	Switzerland

*Cargo originated in Europe, but no individual country was listed.

Genetic Variation Between Outbreak and Non-Outbreak Populations of *Tomicus piniperda* in China

In China, *T. piniperda* occurs throughout almost the entire country (fig. 2). However, based on the 1980 to 1998 annual forest pest reports from each province, *T. piniperda* was ranked as a major pest in only four provinces: Jiangsu, Jilin, Yunnan, and Zhejiang. In Yunnan, in southwestern China, *T. piniperda* outbreaks have occurred each year since the 1970s. Outbreaks are so severe in Yunnan that *T. piniperda* attacks and reproduces in live pine trees, resulting in significant tree mortality (Ye 1991, 1997). In Yunnan, the tree species that suffers the greatest mortality is Yunnan pine (*Pinus yunnanensis* Franch.). Yunnan pine is found primarily in Yunnan Province and in small portions of three neighboring provinces (Guangxi, Guizhou, and Sichuan) (Critchfield and Little 1966). Why has *T. piniperda* been so destructive in Yunnan? Are the *T. piniperda* populations in Yunnan more aggressive than in other parts of China? Or is the host tree, Yunnan pine, much more susceptible to stem-attack by *T. piniperda* than are other Chinese pines? One study that would address the

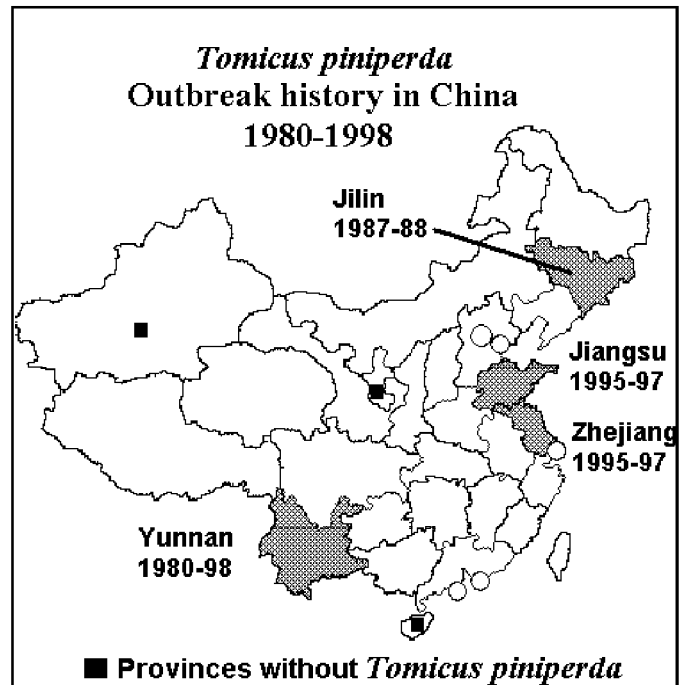


Figure 2—Map of China indicating the four provinces that reported *T. piniperda* as a major pest sometime during the period 1980 to 1998. *Tomicus piniperda* is not known to occur in the three “provinces” with black squares.

issue of *T. piniperda* aggressiveness would be to collect and compare the genetic relatedness of outbreak and non-outbreak populations within Yunnan as well as among *T. piniperda* populations from throughout China.

Genetic Relatedness Within the Genera *Tomicus* and *Anoplophora*

According to Wood and Bright (1992), six species of *Tomicus* occur worldwide. *Tomicus brevipilosus* (Eggers) occurs in China, India, Japan, and Korea. *Tomicus destruens* (Wollaston) occurs primarily in France, Italy, Portugal, Spain, Cyprus, Israel, and Turkey. *Tomicus minor* (Hartig) occurs throughout much of Eurasia; from Spain to Japan, and from Finland to southern China. *Tomicus pilifer* (Spessivtsev) occurs in China and eastern Russia. *Tomicus piniperda* has a range similar to that of *T. minor* but also includes parts of northern Africa such as Algeria and now North America. *Tomicus puellus* (Reitter) occurs mostly in eastern Russia. Each of these six *Tomicus* species uses *Pinus* as a host tree; however, on occasion some *Tomicus* species will use *Abies*, *Larix*, and *Picea*. It would be of interest to compare the genetic relatedness of these six *Tomicus* species and thereby reveal their evolutionary relatedness as well as provide evidence for the validity of each species.

In addition to *Tomicus*, the genetic relatedness within the Asian cerambycid genus *Anoplophora* should be examined. About 44 species of *Anoplophora* are currently recognized in Asia. Molecular techniques could be employed to determine the genetic relatedness of the *A. glabripennis* populations now established in New York and Chicago, as well as to compare them with *A. glabripennis* populations from throughout China. In addition, molecular techniques could be used to aid in the revision of the genus *Anoplophora*.

18. Phylogenetic Analysis of Resource Use and Specialization in *Dendroctonus* (Coleoptera: Scolytidae)

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In this paper, we provide a molecular phylogeny of the 19 species in the bark beetle genus *Dendroctonus*. These beetles collectively attack plant species in four

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different genera of the conifer family Pinaceae. Given substantial variation in diet breadth, in both the types and numbers of plant species utilized, we asked two general questions concerning the evolution of resource use in this group: 1) “How conservative is the evolution of host use in these insects?” and 2) “Does specialization tend to be derived (that is, a “dead-end”)?”

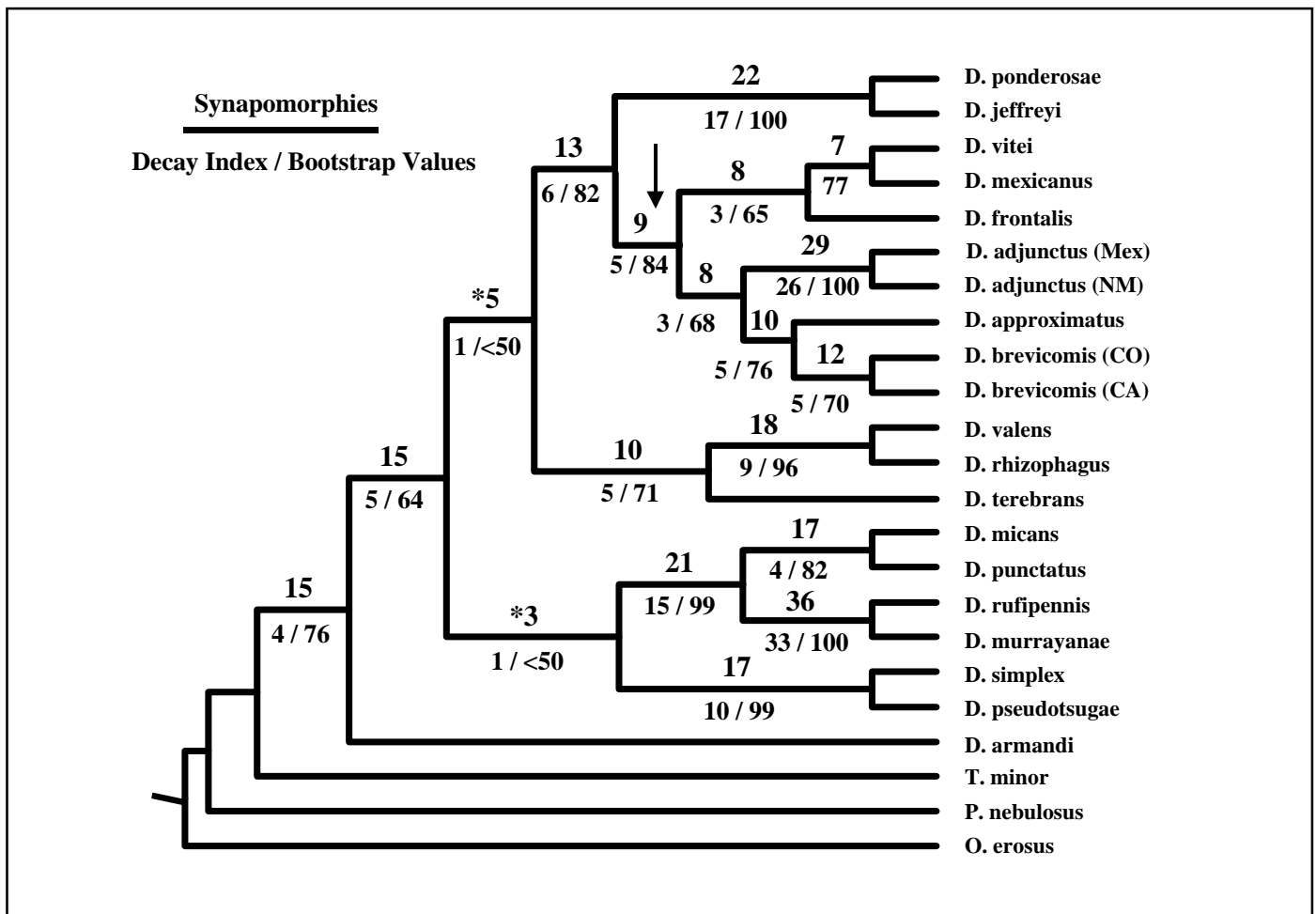


Figure 1—Single-most parsimonious tree for the relationships among 18/19 species of *Dendroctonus* based on information from the Cytochrome Oxidase I mitochondrial gene. Numbers of synapomorphies and autapomorphies are given above the branches. Decay indices and bootstrap values (calculated without *D. vitei*, which had much missing data) are presented below the branches. Asterisks denote clades with relatively weak support (bootstrap proportions < 0.50). The arrow indicates the alternative placement of *D. frontalis* when *D. vitei* was included in the analysis (Kelley and Farrell 1998; with permission from Evolution).

To answer these questions, we estimated the phylogeny of *Dendroctonus* using mitochondrial DNA sequences (fig. 1) and mapped transitions in resource use on the resulting phylogeny estimate. We found the evolution of affiliations with *Pinus* and *Picea* hosts in *Dendroctonus* to be conservative among beetle species ($P < 0.012$; fig. 2). However, in a comparison of *Dendroctonus* phylogeny to the phylogenies of Pinaceae genera (Price and others 1987) and *Pinus* species

(Krupkin and others 1996), we found no significant correspondence between the phylogeny of these beetles and the phylogeny among their Pinaceae hosts (among genera, $P = 0.28$, N.S.; among *Pinus* species, $P = 0.82$, N.S.). Thus, although *Dendroctonus* species tend to feed on the same host genera as their near relatives, there is no evidence that these beetles have co-specified with their hosts.

To determine whether specialization tends to be

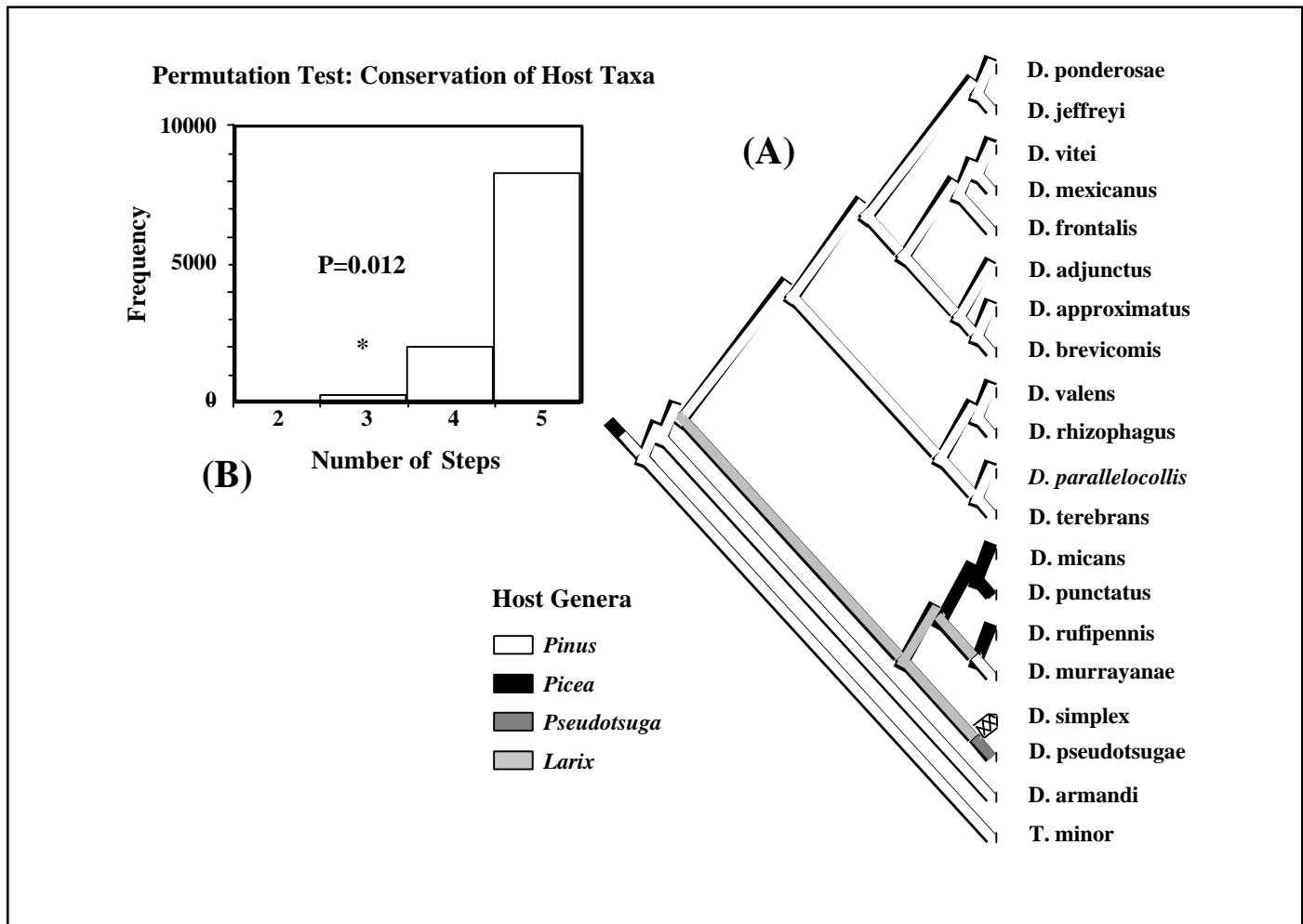


Figure 2—(A) Phylogeny estimate of all 19 *Dendroctonus* species mapped with most-parsimonious transitions between host genera of Pinaceae. The name of the one beetle species, *D. parallelocollis* for which we do not have sequence data, is in italics. Because the phylogeny estimate in Figure 1 corresponds very closely to Wood's (1982) groupings of *Dendroctonus* species, we place this taxon according to Wood (1982) for completeness. Its inclusion does not change the significance of any of the tests. The arrow indicates the alternative position of *D. frontalis* when *D. vitei* (which has missing data) is included in the analysis. Again, the alternative placement of *D. frontalis* does not affect any of the test results or interpretations. (B) Histogram detailing the results of a permutation tail probability test randomizing the character host genera across beetle species, holding the tree topology constant ($P = 0.012$) (Kelley and Farrell 1998; with permission from Evolution).

derived in *Dendroctonus*, we first defined the degree of specialization as the proportion of host species used over potential host species available in the range of the beetle species. We restricted the potential and observed host species to those within the plant genus the beetle species fed upon. For instance, *Dendroctonus jeffreyi*, a *Pinus*-feeder, encounters nine *Pinus* species in its range and has been recorded feeding on only one of them (1/9 of the available species). (Host records and beetle distributions were taken from Wood 1982 and Wood and Bright 1992, while host plant distributions came from Critchfield and Little 1966.)

Degree of specialization in *Dendroctonus* was bimodally distributed with “generalist” species using ≥ 60 percent of the congeneric hosts within their range and six specialist species using ≤ 40 percent of the

available hosts. Among the generalists, we found a strong correlation between the number of hosts encountered and the number of hosts used ($R = 0.97$; $P < 0.0001$), while there was no significant correlation among the specialists ($R = 0.27$; $P = 0.59$). The evolution of specialization in *Dendroctonus* proved highly labile: specialists arose from generalists at least six separate times (without reversal) all in derived positions (fig. 3). However, evidence from the ecological literature also suggests that several *Dendroctonus* generalists may have increased their range of host genera within the Pinaceae (Gregoire 1988, Wainhouse and Beech-Garwood 1994, Wood 1982).

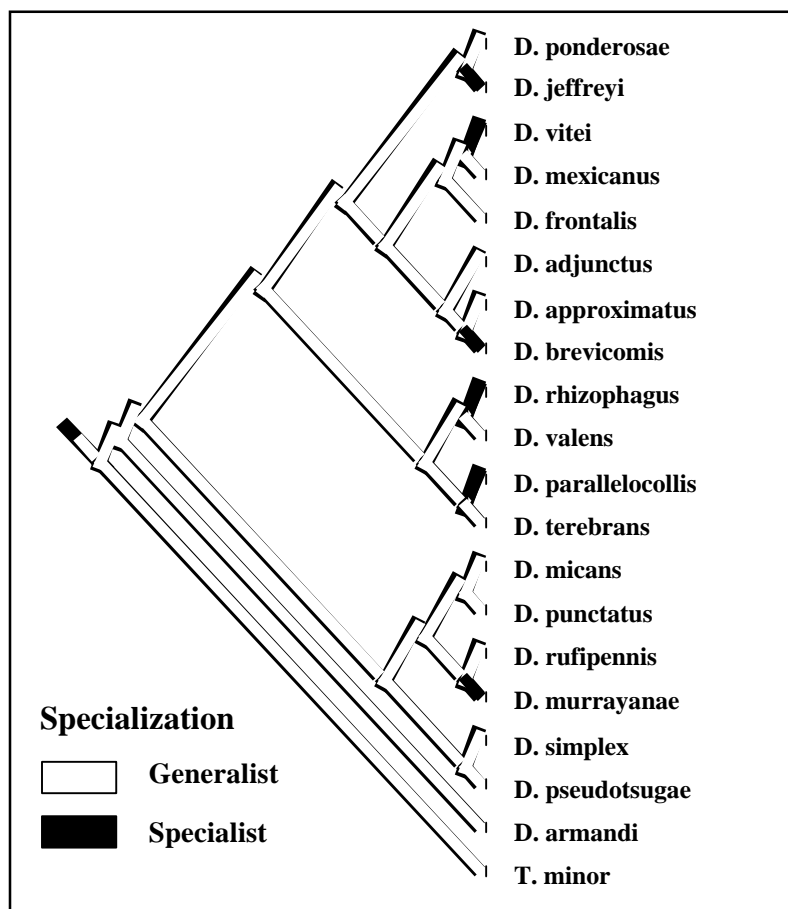


Figure 3—Most-parsimonious reconstruction of the transitions between (dichotomously defined) generalists and specialists in *Dendroctonus*. Specialized habits arose six different times in the genus and are not reversed (Kelley and Farrell 1998; with permission from Evolution).

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