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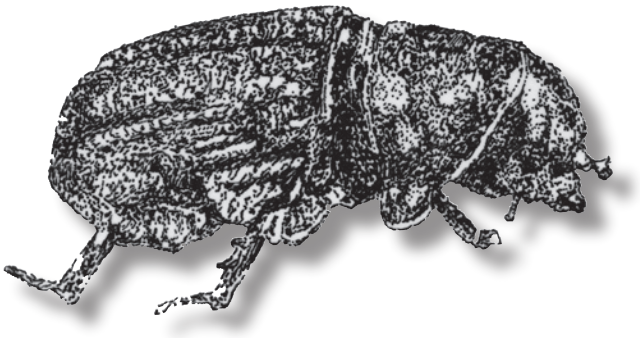
Genetics of Bark Beetles and Associated Microorganisms:

Third Workshop Proceedings

May 20 and 21, 2006
Asheville, North Carolina



In Association with IUFRO WP *7.03.05 - Integrated Control of Scolytid Bark Beetles*

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These proceedings provide a synopsis of the Third Workshop on Genetics of Bark Beetles and Association Microorganisms, which was held May 20-2, 2006 in Asheville, NC. Twenty- five participants from five countries attended the meeting. The proceedings are structured into four parts: Phylogenetics of Bark Beetles, Population Genetics of Bark Beetles, Bark Beetle Gene Structure and Function, and Genetics of Symbionts, Natural Enemies, and Hosts. The abstracts give a snapshot of our current understanding of the genetics of bark beetles and associated microorganisms.

In Association with IUFRO WP *7.03.05 - Integrated Control of Scolytid Bark Beetles*

Workshop Organizers: Barbara Bentz, Anthony Cognato, and Kenneth Raffa

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Proceedings from the Third Workshop on Genetics of Bark Beetles and Associated Microorganisms

In Association with IUFRO WP *7.03.05 - Integrated Control of Scolytid Bark Beetles*

Workshop Organizers: Barbara Bentz, Anthony Cognato, and Kenneth Raffa

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Preface

These proceedings provide a synopsis of the Third Workshop on Genetics of Bark Beetles and Associated Microorganisms held May 20 and 21, 2006, in Asheville, NC.

The first workshop was held at the University of California, Berkeley on May 17 and 18, 1992. It was organized by Jayne Hayes and Jacqueline Robertson to address the increasing realization that our understanding of bark beetle biology and phylogeny suffered from a fundamental lack of knowledge about their genetics. Further, research on bark beetles had made relatively minor use of recent and emerging technologies in addressing this gap. There were 23 participants. The workshop had a very informal structure, without assigned speaking orders, which promoted vibrant discussion. The final session was devoted to an open discussion of critical needs for future research. The proceedings are reported in USDA Forest Service, General Technical Report PSW-GTR-138, 31 p.

The second workshop was held at the University of Wisconsin, Madison, on July 17 and 18, 1998. It was organized by Jayne Hayes and Kenneth Raffa. The meeting had 27 participants. It was structured into three parts: Genetic Structure of Bark Beetle Populations, Variability in Ecologically Important Traits, and Systematics of Bark Beetles. Despite this structure, the meeting maintained an informal atmosphere for freewheeling discussion, which we consider a hallmark. The second meeting was distinguished from the first by including more "basic genetics" and "molecular approaches." However, most talks still clustered around Variability in Ecologically Important Traits. The proceedings are reported in USDA Forest Service General Technical Report PNW-GTR-466, 63 p.

The third workshop, encapsulated by these proceedings, was held immediately prior to, and in the same venue as the North American Forest Insect Work Conference. It was organized by Barbara Bentz, Anthony Cognato, and Kenneth Raffa. There were 25 participants from five countries. The meeting was structured into four parts: Phylogenetics of Bark Beetles, Population Genetics of Bark Beetles, Bark Beetle Gene Structure and Function, and Genetics of Symbionts, Natural Enemies, and Hosts. In response to participant feedback, the major goal of the third workshop organizers was to maintain the informal nature and open discussions of the prior meetings, despite its larger size. Based on closing session comments, we achieved this goal.

Three general features distinguished this workshop from the previous two: 1) There was a substantial increase in the amount of basic genetics, such that the meeting is now attractive to scientists who are not primarily focused on bark beetles *per se*; 2) Molecular methods were infused throughout most of the talks and have achieved the status of common operational tools within various thematic areas; and 3) Genetics of symbiotic fungi and bacteria, arthropods closely associated with bark beetles, and host trees are within the scope. The closing session yielded a group commitment to sponsor a bark beetle genomics project.

In contrast to their prior ad hoc status and timing, organizers and participants took three steps to contribute to the sustainability of these meetings:

- The third workshop was conducted within the auspices of IUFRO, as WP 7.03.05—Integrated Control of Scolytid Bark Beetles. This will be continued.
- There was agreement to routinely hold meetings the weekend before NAFIWC.
- The three organizers agreed to continue in this capacity. However, additional leadership is welcomed.

The enclosed abstracts give a snapshot of our current understanding of the Genetics of Bark Beetles and Associated Microorganisms. We look forward to a continuing rapid advancement and application of this knowledge.

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Phylogenetics of Bark Beetles

Reconstructing the Phylogeny of Scolytinae and Close Allies: Major Obstacles and Prospects for a Solution

Bjarte H. Jordal [1]

Abstract—To enable the resolution of deep phylogenetic divergence in Scolytinae and closely related weevils, several new molecular markers were screened for their phylogenetic potential. The nuclear protein encoding genes, CAD and Arginine Kinase, were particularly promising and will be added to future phylogenetic studies in combination with 28S, COI, and Elongation Factor 1α. The combined analysis of multiple molecular markers and wide taxon sampling is expected to resolve many previously unresolved nodes in scolytine phylogeny, but a completely resolved topology seems dependent on the inclusion of a large number of morphological characters. A well founded phylogeny will provide a powerful framework for testing evolutionary hypotheses on habitat selection and reproductive biology.

Introduction

Bark and timber beetles in the weevil subfamily Scolytinae (Kuschel and others 2000) comprise nearly 6,000 species worldwide and constitute a significant factor in forest ecosystems. These insects receive increased attention, not only from forest entomologists concerned with forest health, but from evolutionary biologists fascinated by an unprecedented wide range of different biological and ecological features. Host plants and diets include most woody substrates imaginable, from living to dead trees and shrubs, dry to soggy substrates, large logs to herbs and tiny seeds, to the cultivation of asexual "ambrosia" fungi as food for adults and larvae. Their reproductive biology varies even more, including various forms of monogyny, harem polygamy, inbreeding by regular sibling mating, and parthenogenesis.

As a key to understanding how these features may have influenced diversification in Scolytinae, the generation of a baseline phylogenetic hypothesis is of paramount importance. Particularly interesting evolutionary transitions that can be tested in a phylogenetic framework include the multiple transitions between gymnosperm and angiosperm host plants and the evolution of ambrosia fungus symbiosis, mating systems (including the origin of various pheromones used in mate attraction), and haplodiploidy and paternal genome elimination in regularly inbreeding species. However, a phylogenetic reclassification of the Scolytinae and close relatives is still in its infancy without yet a clear picture of the relationship between most groups. Current classification (Wood 1986, Wood 1993) is based on rather few morphological characters not yet subject to a full cladistic analysis, although a smaller sample of scolytine taxa was recently analyzed by Kuschel and others (2000). They found a monophyletic Platypodinae nested inside Scolytinae, with Cossoninae

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as the closest outgroup. Wider taxon sampling and addition of molecular data will provide a more rigorous test for this hypothesis.

This project thus aims at closing many of the gaps in our current understanding of scolytine phylogeny and continues our recent phylogenetic work using DNA sequence data (Farrell and others 2001). Using such data to resolve deep divergence (for example, Cretaceous) is not straight forward; however, most genes demonstrate substitution rates not suitable for phylogenetic reconstruction at this level. Thus, one necessary step towards a prospective phylogeny of Scolytinae and allies includes screening for new molecular markers and sampling from a broad range of taxa. This paper reports preliminary data on evolutionary rates and phylogenetic properties of five little used genes in beetle phylogenetics and compares these data to previously used genes with respect to resolving deep divergences. The role for morphological data in conjunction with DNA sequence data is briefly discussed.

Methods

Several criteria were used to select genes for future sequencing and phylogenetic analyses of Scolytinae: 1) the amplification rate must be high, at least 70 percent of the samples must amplify to reduce the negative effects from large amounts of missing data in phylogenetic analyses; 2) substitution rates must be low, measured by sequence divergence and compared to the slowly evolving Elongation Factor 1α (EF-1α); and 3) phylogenetic signal must be high, judged by the capability of resolving likely sister relationship (based on 28S + EF-1α or morphology).

Based on promising preliminary results from recent phylogenetic studies of bees, primer information for five different genes were downloaded from the web: Abdominal-A (Abd-a), RNA polymerase II (POL), Sodium-Potassium ATPase (NaK), CAD (rudimentary), and Arginine Kinase (ArgK) (see: http://www.entomology.cornell.edu/BeePhylogeny). PCR cocktails included Qiagen Hotstar taq and conditions were optimized on a MJ-200 gradient cycler with the following specifications: 95 °F for 15 min, then 40 cycles of 46 to 58 °F (60s), 72 °F (90s), final extension cycle (72 °F) of 10 min. Final optimal amplification temperatures were 50 to 52 °F (60s), except Abd-A, which did not amplify the correct gene products.

Results and Discussion

Screening for New Molecular Markers

PCR products of putative Abd-A, POL, NaK, CAD, and ArgK were sequenced and submitted to blast searches in Gen Bank. Correct gene products were confirmed in all cases except Abd-A. Characteristics of each verified gene fragment are listed below (see also table 1).

POL—the primers polfor2a and polrev2a amplified 822 base pairs from 43 percent of the samples (n=72). None of the sequences contained introns. Several sequences of Platypodinae had up to 33 amino acid substitutions compared to other Platypodinae and Scolytinae, suggesting paralogous copies. After excluding these sequences, the substitution rate was nevertheless surprisingly high (fig. 1) given that it is supposed to be one of the more conservatively evolving protein coding genes in insects (Danforth and others 2006). The high substitution rate was furthermore consistent through the full

Table 1—Characteristics of molecular markers used in phylogenetic studies of Scolytinae, including the new markers screened in this study.

Gene	Amp. rate[a]	Length[b]	Intron[c]	Signal nuc[d]	Signal aa[e]	Paralogs
12S	99	400	-	low	-	no
16S	98	500	-	low	-	no
COI	95	1200	0	low	high	yes[f]
18S	98	1900	-	low	-	no
28S	98	800	-	high	-	no
EF-1α, C1	90	927	0-1	high	low	yes
Enolase, ni	20	687	0	low	moderate	yes
Histone H3	50	328	0	high	low	yes
NaK	50	713	1	low	low	no
POL	43	822	0	low	low	yes
CAD	73	900	1	moderate	high	no
ArgK	80	1120	1	high	moderate	no

[a] Average proportion of samples that amplify.
[b] The longest fragment sequenced of any Scolytinae (bp).
[c] Uncorrected maximum divergence level in Scolytinae.
[d] Phylogenetic signal from nucleotide sequences.
[e] Phylogenetic signal from amino acid coded sequences.
[f] Mitochondrial copies inserted into the nuclear genome (numts, pseudogenes).

length of the sequence, which impedes ready design of more specific primers. Taken together with the incongruent topology and limited support for clades

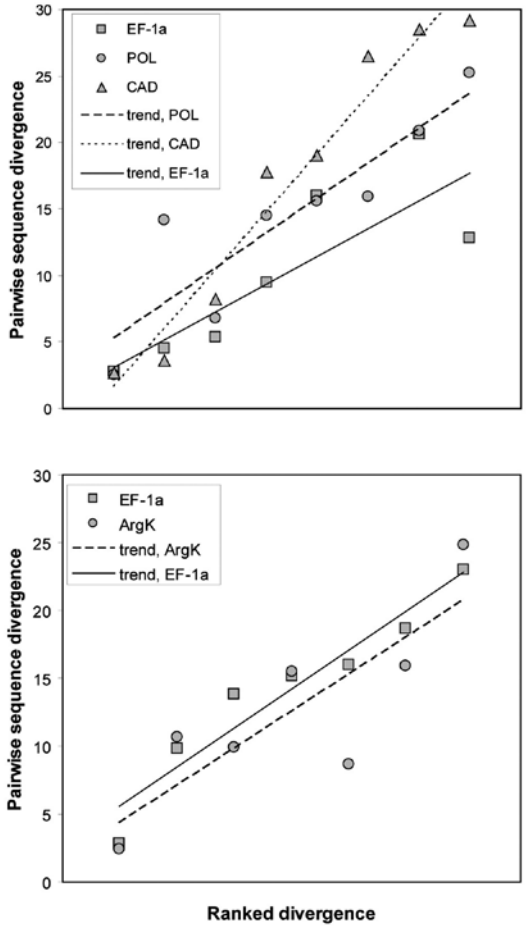

Figure 1. Sequence divergence (HKY corrected) between eight (above) or seven (below) species pairs of Scolytinae and Platypodinae, ranked by increasing divergence levels. Linear trend lines are added for illustrative purposes only.

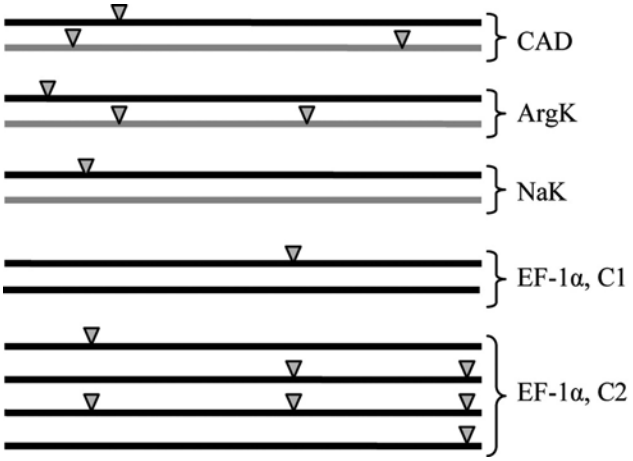

Figure 2. Intron maps for CAD, ArgK, NaK, and two copies of EF-1α in Scolytinae. Gray lines indicate intron maps in bees (Halictidae). Triangles indicate the relative position of introns. Maps of different genes are not to scale.

that are otherwise strongly supported by EF-1α and 28S data, this marker appears to have a limited phylogenetic potential in Scolytinae.

NaK—each of the primer pairs NaKfor1-NaKrev1, Nakfor1-NaKrev1a, and NaKfor2-NaKrev2 amplified multiple bands of various lengths in the majority of samples. The first primer pair amplified the correct product more consistently, amplifying 50 percent of the samples (n=16). The amplified fragment consisted of one intron close to the 5' annealing site (112 base pairs downstream from NaKfor1), ranging from 56 to 71 base pairs. There is no intron known from bees or from *Tribolium* (fig. 2). The substitution rate was high and ranged between 21 and 28 percent (p-distance), but a maximum of only five amino acid substitutions were found in the 642 base pair coding region. A phylogenetic analysis of six scolytine sequences was not at all consistent with previously well established clades. Taken together with the relatively low amplification rate and the high synonymous substitution rate, this gene seems less productive in higher level studies. It could, on the other hand, be an excellent marker for phylogenetic studies of genera and species complexes.

CAD—the primers 581F, apCADfor1, and apCADfor4, in conjunction with apCADrev1mod, produced band lengths of about 900, 700, and 455 base pairs. The correct product was amplified in 38, 56, and 73 percent of the screened samples (n=40), respectively, with very few multiple band patterns. One intron occurred in the two longest amplicons, close to the apCADfor1 annealing site. The intron ranged between 51 and 127 base pairs, but was universally missing in all Platypodinae in addition to one sequence of Scolytinae. Introns 5 and 6 in bees (Danforth and others 2006) were missing in these sequences. Several conserved primer sites provided ample opportunities to design more specific primers for consistent amplification of more than 800 base pairs (in progress). The substitution rate was higher than in POL and NaK (up to 32 percent, see also fig. 1), but even so the phylogenetic placement of scolytine and platypodine taxa was more consistent with the current classification. The Platypodinae was nevertheless slightly paraphyletic, but amino acid coding of these data resulted in a strongly supported, long branch of monophyletic Platypodinae. The amino acid data furthermore grouped distantly related genera in the tribes Corthylini and Polygraphini. The strong signal at the amino acid level may suggest that this gene holds a strong potential in separating tribes and subfamilies of various weevil groups. Dense sampling of genera and species within tribes will also probably increase phylogenetic accuracy for nucleotide analyses.

ArgK—the primers ArgKfor2 and ArgKLTfor3, in combination with the reverse primer ArgKLTrev2, produced band lengths of 1,120 and 490 base pairs, respectively. Very few samples amplified the longer fragment, while 80 percent of the samples amplified the shorter fragment (n=16). One intron was found close to the 5' end of the longest fragment, 93 base pairs downstream from the ArgKfor2 annealing site. Intron 1 and 2 in bees were not present in these sequences (fig. 2). The substitution rate was relatively low, not exceeding 22.2 percent for the taxa included (25.5 percent HKY corrected), which is about the same rate or lower than for EF-1α (fig. 2). A phylogeny of 14 nucleotide sequences demonstrated high congruence with previously hypothesized clades (Ipini + [Dryocoetini + Xyleborini]; Platypodinae; *Cryphalus* + *Trypophloeus*; *Dendroctonus* + Hylastini).

Building a Complementary Data Matrix

Several of the screened markers demonstrated sufficient amplification, evolutionary conservatism, and phylogenetic potential, to be included in the forthcoming higher level analysis of Scolytinae and close relatives (table 1). Particularly promising in this respect were CAD and ArgK. Each demonstrated consistent orthology and ample regions for primer design. These two gene regions will add some 1,500 to 1,700 base pairs to a matrix consisting of a mixture of conserved nucleotide sequences, rapidly evolving amino acid sequences, and morphological characters.

Among the numerous genes previously screened for scolytine phylogenetics, only a handful have proven useful in resolving deeper divergences (Farrell and others 2001, Sequeira and others 2000). Adding to the problem of rapidly evolving genes, the most extensively surveyed and slowly evolving gene, 18S ribosomal DNA, does not provide sufficient information to resolve relationships between most scolytines and other weevil groups (Marvaldi and others 2002). 28S seems more promising in this respect, with considerable contribution to combined data analyses (Sequeira and others 2000). A stronger phylogenetic signal is furthermore extractable from this gene if alignments are guided by secondary structure, potentially resolving the majority of scolytine tribes (Sequeira, pers. Com; Cognato and others, in progress).

Mitochondrial genes (12S, 16S, COI) are generally highly saturated by multiple substitutions in higher level studies. However, when COI nucleotides are translated into amino acids, there is apparently sufficient signal to resolve several important groups of Scolytinae (Sequeira and others 2001). Preliminary analyses of 223 COI amino acids (76 parsimony informative characters) from a wider range of taxa furthermore demonstrated a strongly supported Platypodinae nested within Scolytinae. Taken together with the benefits of DNA barcoding using COI nucleotides, the phylogenetic information in COI amino acids makes this gene a logical part of any large data set.

Among the nuclear protein encoding genes, *Enolase* has been used with some success (Farrell and others 2001, Jordal and Hewitt 2004). However, amplification of this gene is difficult, and the substitution rate is more suitable for generic level and below. *Histone* genes are more promising with respect to substitution rate, and the H3 copy is used frequently in metazoan phylogenetics. Scolytines nevertheless amplify paralogous copies, and the short length of the H3 copy (328 base pairs) increases the relative cost per base pair sequenced for this gene. The most promising marker for scolytine phylogenetics so far has been *Elongation Factor 1*α, although not without problems. Sometimes, two copies of different length can co-amplify (fig. 1), and are

usually distinguished by different intron structures (Jordal 2002). However, adding EF-1α sequences from a wider range of taxa raised some doubts about the integrity of intron structure in orthology assessment. Phylogenetic analyses nevertheless sort these copies into two distinct clades with intron variation, as shown in figure 2. These minor problems aside, EF-1α alone can provide considerable signal and support for a wide range of tribes and genera (Farrell and others 2001, Jordal 2002, Sequeira and others 2001).

Based on the preliminary results presented here, and the brief review of markers used in scolytine phylogenetics, five gene fragments are selected for further phylogenetic analyses. The combination of EF-1α, ArgK, CAD, 28S, and COI (amino acid) sequences will possibly enable resolution of currently unresolved nodes. However, support for some groups will most likely remain weak and the addition of morphological characters is needed to increase resolution and support. A data matrix consisting of 240 taxa and more than 150 morphological characters is currently in progress. Preliminary results based on 47 of these characters indicate a strongly supported Platypodinae, with *Schedlarius*, *Mecopelmus*, *Carphodicticus,* and *Coptonotus* as successive outgroups, and Scolytinae as the sister group to these taxa combined. These results are quite similar to Kuschel and others (2000) (see also Marvaldi and others 2002), but quite different from analyses of larval characters that relate Platypodinae to Dryophtorinae (Marvaldi 1997). The addition of molecular data and the remaining morphological characters will probably enable a definite test of these contrasting hypotheses.

Acknowledgments

The preliminary results presented here are part of a joint venture with A. Cognato, A. Sequeira, and B. Farrell. Larry Kirkendall collected some particularly valuable samples and reviewed the manuscript. Funding was provided by the Museum of Natural History and Archaeology, Trondheim.

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A Standard DNA Taxonomy for Insects?

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Identification of insect species is often problematic because of limited morphological and/or biological characters. DNA data have been used in many phylogenetic studies to help identify and revise species boundaries (Savolainen and others 2005). For many studies, percent similarity DNA compared between species was summarized and intra- and interspecific variation patterns were observed (for example, Brower and Boyce 1991). These patterns suggested that animal species, at most, exhibit 2.0 percent difference among conspecifics (Hebert and others 2004). A group of individuals that exhibited DNA difference greater than the 2.0 percent boundary would potentially represent a new species. Thus, the difference between intra- and interspecific percent DNA variation has been used as a "genetic yardstick" to recognize new species (for example, Hung and others 1999).

The establishment of a standardized percent nucleotide divergence to predict species boundaries would aid in cases where species status is suspect. However, given variation in nucleotide mutation rates and species concepts, association between a standard percent sequence divergence and species is questionable. Cognato (2006) reviewed the percent DNA sequence difference found between insect sister-species in order to assess whether a standard divergence was associated with named species. Comparisons of intra- and interspecific pairwise DNA differences were made for mitochondrial and nuclear loci spanning many insect families. Intra- and interspecific sequence divergences varied widely, 0.04 to 26.0 percent and 1.0 to 30.7 percent, respectively. The ranges of intra- and interspecific sequence divergences overlapped in 28 of 62 comparisons. This implied that a standardized percent sequence divergence would fail to correctly diagnose species for 45 percent of the cases. Common occurrence of non-monophyly among closely related species likely explains this observation. Non-monophyly and overlap of intra- and interspecific divergences were significantly associated. The reviewed studies suggested that a standard percent sequence divergence does not predict species boundaries.

However, a taxon specific genetic yardstick may better predict species boundaries. The application of a standard percent DNA difference to predict species boundaries for an important forest beetle pest, *Ips* was investigated. A phylogeny was reconstructed using nuclear and mitochondrial genes for all species including individuals collected in China whose species identity was suspect. One most parsimonious tree was recovered with a similar topology to previous studies (Cognato and Vogler 2001). Individuals of the suspect species were monophyletic and sister-clade to *Ips amitinus*. The amount and type of nucleotide difference that characterized this clade were similar to the changes observed with currently valid species. Compelling evidence of monophyly, shared nucleotide substitutions from multiple genes, and unique and diagnostic morphological characters suggested that the sister clade to *I. amitinus* is a new species and will be described.

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Mitochondrial cytochrome oxidase (c) I (COI) nucleotide data is currently the most popular gene for delimiting species boundaries. The *Ips* COI data was analyzed independently with an expanded taxon data set, including pairwise nucleotide differences between recognized sister species. The wide range of average intraspecific pairwise nucleotide difference (0.8 to 13.0 percent) was in concordance with the results found in Cognato (2006). This suggests a limited application of taxon specific standard percent nucleotide difference as a means to identify species boundaries. At most, the overall average COI nucleotide intraspecific difference (1.0 percent) provides an informal guide to identify potential clades that may warrant further systematic investigation. DNA data can help best to predict species boundaries via its inclusion in non-phenetic phylogenetic analysis and subsequent systematic expert scrutiny.

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Population Genetics of Bark Beetles

Pitfalls in Applying Mitochondrial Markers Onto the Scolytid Species *Pityogenes chalcographus*

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Markus Riegler[2]
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Abstract—*Pityogenes chalcographus* is one of the major pests in Eurasian spruce stands. Crossing experiments performed in the mid-1970s suggested race differentiation, and mtDNA analysis of *P. chalcographus* gave evidence that today's populations are divided into several clades. The genetic distance between clades favours a model of allopatric origin with a separation about one million years ago while today haplotypes of the major clades exist sympatrically all over Europe. Within the last few years, the use of mtDNA as a sole genetic marker became a matter of critical discussion. It was shown that nuclear copies of mtDNA (*numts*) led to artefacts in some of the derived genealogies. A long PCR based approach for elimination of potential *numt* sequences was developed to validate the dataset of *P. chalcographus*. This method showed that the beetle's genome does not contain *numts*. Another factor that may influence mitogenomes is the presence of endosymbiotic *Wolbachia*, which causes alterations in insect reproduction and thus influences the population's mtDNA patterns. While *Wolbachia* was not found in *P. chalcographus* in past studies, the use of long and nested PCR, cloning and sequencing of PCR products, and *in situ* hybridization techniques gave evidence that at least a certain percentage of European populations harbour this intracellular endosymbiont. An influence on the mitochondrial dataset can not be excluded and further research is proposed to estimate the prevalence of *Wolbachia* in *P. chalcographus*.

In the mid-1970s, intraspecific variation and unidirectional incompatibility were detected in the Eurasian scolytid species *Pityogenes chalcographus* (Coleoptera, Scolytinae) when males from northern European regions were crossed with females from Central Europe (Führer 1976). This differentiation was further verified by morphological data and the existence of two races among the European populations of *P. chalcographus* was suggested (Führer 1978). Recently, mitochondrial markers were applied and a phylogenetic reconstruction assigned 58 haplotypes to six clades (Avtzis 2006). The two major clades exhibited a sympatric distribution in most of the European terrain, with clade I dominating in northern and clade IIIa in central Europe. The results supported the hypothesis of allopatric divergence of the mtDNA lineages, which postglacially came into sympatric existence in Europe. However, due to partial crossing incompatibility, the diverged lineages retained their genetic identity through the Ice Ages. During the re-colonization of Europe after the last Ice Age, diverged lineages of *P. chalcographus* perhaps also confronted differentiated geographic lineages of *P. abies*. Differential adaptation to diverse

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spruce biochemistry potentially played a role in shaping the current distribution of various *P. chalcographus* genotypes in Europe. The general pattern of geographic separation was in congruence with the findings of Führer (1976); however, it is not as strict as previously described.

High mutation rates established mtDNA as a popular genetic marker for inferring the demography of populations and speciation processes. The availability of conserved universal primers increased its extensive use and PCR amplification made mtDNA easily accessible for direct sequencing as well as for PCR-RFLP and SSCP techniques. After more than a decade of strong reliance on mtDNA, the last years brought emerging awareness that phylogenies derived solely from mtDNA may be biased by several influencing factors (Ballard and Whitlock 2004). Besides the fact that the comparatively small mtDNA molecule represents only one single locus and upcoming doubts if the evolution of the mitochondrial genome is strictly neutral, two main limitations for the reliability of mtDNA sequences must be considered:

First, transferred nuclear copies of mtDNA (*numts*) might be co-amplified using universal mitochondrial primers and grouped together into one distinct clade. Strategies to avoid *numt* based errors include *in silico* analysis of sequences (Bensasson and others 2001a) and PCR of long DNA regions following nested PCR (Thalman and others 2004).

Second, mtDNA transmission can be influenced by any selection for maternally selective traits. Several maternally transmitted endosymbionts are well known in invertebrates, with *Wolbachia* being the most common besides *Cardinium* and Rickettsia. *Wolbachia* infections are widespread among insects (Stouthamer and others 1999). While studies using standard PCR methods estimated a prevalence of the endosymbiont in about 20 percent of insect species (Werren and Windsor 2000), the application of long PCR (Jeyaprakash and Hoy 2000) indicated infection rates up to 70 percent. Due to manipulation of the hosts, reproduction by male killing, cytoplasmic incompatibility (CI), and parthenogenesis and feminization (Stouthamer and others 1999), *Wolbachia* influences mtDNA variation in infected populations (Shoemaker and others 2004). In a population newly infected with a reproductive parasite, the mtDNA associated with the initial infectious individuals will hitchhike along with the expanding reproductive parasite and replace the uninfected haplotypes (Hurst and Jiggins 2005). From a phylogenetic point of view, such a selective sweep may easily be mistaken for a population bottleneck.

The aim of our work was to analyze the mtDNA based phylogeny of *P. chalcographus* (Avtzis 2006) and to rule out any possible influence of *numt*- or *Wolbachia*-caused bias on its authenticity.

Numt Problem

As amplification of mtDNA of *P. chalcographus* was initially performed with universal primers, an erratic co-amplification of *numts* had to be excluded. *In silico* analysis of the *P. chalcographus* sequences was performed to identify non-synonymous base substitutions, additional stop codons, insertions and deletions, frameshifts, and the transition:transversion ratio. Observed patterns in the *P. chalcographus* dataset were all within a 5 percent confidence interval of the expected values. The fact that the GC is often methylated in nuclear DNA and that 5-methylcytosine mutates often to T (Bird 1980) was used as additional indicator to distinguish between mitochondrial and nuclear sequences. Only 12 percent of all observed C→T mutations were of

the GC→GT type indicating a non-methylated, and therefore, most probably non-nuclear molecular origin. Since in most cases *numts* are no longer than 1 kbp (Bensasson and others 2001b), we performed a long PCR amplifying a 3.5 kbp fragment that covered the whole ND2 and CO1, as well as parts of the CO2 gene. To achieve primers highly specific for coleopteran mitochondria, an alignment of currently known coleopteran mitogenomes was performed and conserved regions were selected as primer loci. Products of the long PCR were highly diluted to remove any amplifyable traces of original insect DNA and used as a template for nested PCR with CO1 universal primers. A comparison of the phylogenetic trees from 14 haplotypes of *P. chalcographus* derived from direct and nested PCR showed identical topologies. As *numts* co-amplified erroneously by universal primers tend to group together into a distinct clade (Bensasson and others 2001a), complete removal of ncDNA from the template and re-PCR with identical primers will lead to changes in tree topology. The absence of such changes allows exclusion of *numt* presence in the analyzed populations of *P. chalcographus*.

Wolbachia Problem

A sensitive detection system for *Wolbachia* is based on PCR amplification of the endosymbionts *wsp* gene using proofreading polymerases and high cycle numbers (Jeyaprakash and Hoy 2000, Zhou and others 1998). While Riegler (1999) did not detect *Wolbachia* infections in *P. chalcographus* screening only a limited number of Austrian individuals, a long PCR approach on 189 European individuals resulted in 14.3 percent positive reactions. In contrast to control experiments with *Wolbachia* in *Rhagoletis cerasi* (Riegler and Stauffer 2002), signals were often weaker. The PCR product was cloned and the sequence (GenBank DQ993183) revealed a high homology to a B-strain *Wolbachia pipientis* isolated from *Tipula aino* by Kittayapong and others (2003). Distribution of *Wolbachia* infection was compared with the distribution of mtDNA haplotypes and also haplotypes used in crossing experiments (Avtzis 2006) and no correlation of clade affiliation and *Wolbachia* infection was detected.

Possible sources of false positive results from PCR detection of *Wolbachia* are infected parasitoids harboured in *P. chalcographus*. This error source can be circumvented by *in situ* hybridization, which offers a possibility to detect *Wolbachia* directly in infected tissues (Chen and others 2005, Gómez-Valero and others 2005). Therefore, beetles were dissected and ovarial tissue recovered, split, and used for PCR and for *in situ* hybridization with *wsp* specific DIG-labelled probes (Chen and others 2005). Hybridization and PCR results were compared. As a control, ovarial tissues of *Drosophila simulans* with known infectious state were also objected to hybridization and showed accumulation of dark colour in ovarioles of infected samples, while uninfected tissue remained unstained. Hybridization of four specimens of *P. chalcographus* resulted in three cases of an accumulation of purplish brown color at different intensities. Although low sample numbers do not allow general conclusions, the binding of the *wsp* probe at ovarial tissue supports the hypothesis that positive PCR detections are not obtained due to amplification of contaminants.

It was shown that sympatric European lineages of *P. chalcographus* exhibit strong genetic divergence on mtDNA level. We have proven that these results are not biased by erratic co-amplification of *numts* but represent the authentic state of the beetle's mitochondrial genealogy. Furthermore, we have detected

the presence of the reproductive endosymbiont *Wolbachia* in *P. chalcographus* for the first time. Presently, little information is available on its abundance, geographic distribution, and phenotype. Further research is necessary to elucidate whether the current haplotype distribution of the beetle is solely an effect of differentiation driven by quaternary climate changes or if endosymbionts co-shaped its molecular history.

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Invasion Genetics of Emerald Ash Borer (*Agrilus planipennis* FAIRMAIRE) in North America

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Emerald ash borer (EAB) was first detected in Michigan and Canada in 2002. Efforts by federal and state regulatory agencies to control this destructive pest have been challenged by the biology of the pest and the speed in which it has spread. Invasion dynamics of the beetle and identifying source populations from Asia may help identify geographic localities of potential biocontrol agents. Genetic techniques, such as mtDNA gene sequencing, amplified fragment length polymorphisms (AFLP), nuclear gene sequencing, and microsatellite analysis will help determine the geographic origin of the native east Asian population(s) of EAB that have given rise to the EAB that is invasive in North America.

In North America, EAB has been detected in much of the Lower Peninsula of Michigan and Ontario, Canada, as well as northern Indiana and Ohio. To represent the diversity of the introduced population, we have obtained approximately 2,100 EAB individuals, mainly from 32 localities in Michigan, but also from three localities in Ohio and one locality each in Indiana and Ontario. DNA was isolated from EAB individuals from seven localities in China, four localities in South Korea (kindly loaned to us by Dr. Dave Williams, USDA-APHIS), and one adult from Shiroishi, Japan (kindly loaned to us by Dr. Paul Schaefer, USDA-ARS) to represent the native range of EAB in Asia. Identical mitochondrial cytochrome oxidase I (COI) sequences (485 bp) were obtained from all North American EAB analyzed (Michigan [76], Ohio [2], Indiana [4], and Canada [6]), all EAB from China (Dagong [5], Hangu [1], Heilongjiang Province [4], Hebei [2], Jilin [2], and Liaonging [2]), and six EAB individuals from three localities in South Korea. However, mitochondrial COI sequences from five individuals in two populations in South Korea differed from this common haplotype by two to four nucleotides, and the Japanese sample differed from the common EAB mtDNA haplotype by 3.7 percent. Therefore, the mtDNA COI sequence of the Japanese sample is very different from any other individual sampled and there is COI haplotype variation in two of the three localities in South Korea. We have obtained AFLP profiles from EAB individuals from Michigan (46), Ohio (2), Ontario(6), South Korea (4), Dagong (4), Heilongjiang Province (3), Liaonging (2), Hebei (2), Jilin (2) and 1 individual from Japan (four selective AFLP primer pairs; 139 scoreable loci). We have observed differences in AFLP profiles, both within populations from the same location, as well as between all

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populations. Neighbor-joining analysis of the 139-band AFLP data set indicates that individuals from MI cluster more often with individuals from China, while the Japan individual fell into a separate, more distantly related group. However, we cannot rule out South Korea as the geographic origin of North American EAB since the common mtDNA haplotype that is shared by all Chinese and North American EAB individuals exists in each of the Korean populations sampled. Thus, it will be necessary to increase our sampling of Asian populations if we hope to make valid inferences about the geographic location(s) of source populations that gave rise to the North American EAB infestations. To this end, collections will be conducted over the summer of 2006 in South Korea, China, and Japan to expand our EAB sample from its native range. We are also working to obtain DNA sequences from the nuclear genes wingless (*Wg*), phosphoenolpyruvate carboxykinase (*Pep*CK), cytochrome c (*Cytc*), and elongation factor-1α (EF-1α). Finally, microsatellite markers are being developed for EAB to incorporate these highly polymorphic markers into the data set. We expect that analysis of the expanded data set will improve resolution of the genetic structure of EAB populations and allow us to determine which populations are most closely related to each other and if there was a single introduction or multiple introductions of this pest.

Genetic and Phenotypic Resistance in Lodgepole Pine to Attack by Mountain Pine Beetle

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The recent outbreak of mountain pine beetle (MPB) (*Dendroctonus ponderosae*) in British Columbia provided an opportunity to examine genetic variation of differential attack and resistance in a 20-year old lodgepole pine open-pollinated (OP) family trial. Approximately 2,500 individuals from 180 OP parent-tree collections (~14 trees per parent), from several geographic collection zones in B.C., were scored for several traits relating to attack, survival, gallery formation, and infection from the fungi associated with MPB, *Ophiostoma* sp. Successful initial attack was assumed by the presence of pitch tubes, and the mean number of pitch tubes per tree (PT#05) (in an area of 10 x 15 cm at breast height) was 1.5 with a range from 0 to 14. Family mean differences for the presence of "green crowns" (in other words, survival) in 2005 (GC05) ranged from 46 to 100 percent, and for pitch tubes present or absent (PTPA05), family means ranged from 7 to 100 percent. Significant levels of genetic variation were found for these two attributes, with the heritability for GC05 being 0.59 (s.e. = 0.28) and 0.43 for PTPA05 (s.e. = 0.26). The correlation between breeding values of 10-year height growth (20-year height or diameter has not been measured) and PTPA05 was 0.22, indicating that faster growing families may be slightly more subject to attack.

An intensive survey of a sub-sample of 442 trees in the test (arbitrarily selected from 50 families with a range of attack levels, and of adequate size for beetle attack) was conducted, and trees were re-assessed for the presence of attack, blue stain in the wood (due to *Ophiostoma* sp.), galleries, and egg chambers/eggs in the gallery. Thirty-two percent of the 442 trees had pitch tubes present, 19 percent had blue stain, 34 percent had galleries, and 18 percent had egg chambers/eggs. Family means ranged from 0 to 57 percent for blue stain, 11 percent to 63 percent for galleries and 0 to 57 percent for egg chambers/eggs. Due to the sample of families not being truly random (in other words, estimates of heritability are expected to be inflated) and the small family sizes in the intensive survey, estimates of heritability for blue stain, galleries, and egg chambers/eggs were all greater than 1.0, indicating the sample was not large enough for meaningful quantitative genetic parameter estimation. Provenance or stand differences were also significant, indicating that some population structure is present for these "resistance" attributes. Further research is underway to determine the actual mechanisms at work, which are contributing to the differential levels of attack and resistance to MPB and its associated fungi.

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Genetic Architecture of Differences in Fitness Traits Among Geographically Separated *Dendroctonus ponderosae* Populations

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Introduction

The mountain pine beetle, *Dendroctonus ponderosae* Hopkins (Coleoptera: Curculionidae, Scolytinae) (MPB), is widely distributed across western North America spanning 25 degrees latitude and more than 2,500 m elevation. In a common garden experiment, Bentz and others (2001) observed that MPB populations from a southern location required significantly more time to develop than individuals from a population in the northern part of the range, although both populations exhibited univoltinism. Adults from the southern population were also significantly larger, even when reared in a common host and temperature. These results suggest that local selection plays a role in MPB adaptation to temperature, which can vary dramatically across the broad range of MPB. In a recent phylogeographic analyses of MPB, Mock and others (2007) found evidence of genetic structuring among populations that followed a broad isolation-by-distance pattern, confirming that genetic differences exist among geographically isolated populations. Little is known, however, about the underlying genetic architecture of important MPB life history traits, such as development time. To adequately forecast the effects of climate change on MPB population success and adaptability, a better understanding of the underlying environmental and genetic control of these traits, and variability across geographically separated populations, is needed. The main objective of our study was to examine the genetic architecture of differences among three geographically separated populations of MPB that differ in total development time and adult size. We used line cross experiments to analyze the relative influences of additive and nonadditive genetic effects on population differences in these traits.

General Approach

Adult MPB from three geographically isolated populations and infesting three host tree species were used in the study: 1) MPB from southern California infesting pinyon pine (*Pinus monophylla*), 2) MPB from central South Dakota infesting ponderosa pine (*P. ponderosa*), and 3) MPB from central Idaho infesting lodgepole pine (*P. contorta*). We quantified total development time

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and adult size of beetles from each population reared in the lab in a common host, lodgepole pine, at a range of temperatures (12.5 °C, 17.5 °C, 22.5 °C, 27.5 °C). Using beetles reared at 22.5 °C, reciprocal crosses were also made among the populations by mating males and females, respectively, from each population. Offspring from each population cross were also back-crossed to the original parent population. Fitness characteristics, total time to complete development, and adult size were measured and sex determined for all offspring by mating group and generation. Line-cross or generation means analysis was used to compare observed means with expected means derived from genetic models accounting for additive, dominance, epistatic, and maternal effects (Mather and Jinks 1982).

Results

In general, mean development time for all populations was slower and adults were larger at cold rearing temperatures when compared to warm temperatures. However, population response to temperature was not consistent across latitude, and a greater proportion of beetles from the southern population completed development at 17.5 °C compared to the northern populations. At this temperature, a large proportion of individuals from the northern populations did not pupate. These results suggest adaptation to local environments that could lead to geographically specific voltinism patterns under a global warming scenario. Results from line-cross analyses indicate that epistatic interactions are responsible for many of the genetic differences in adult size and total development time among the MPB populations sampled. We also observed sterility in F_2 males from the southern California population when mated with adults from the northern populations. Although infestation by differing strains of *Wolbachia* may cause F_2 breakdown, F_2 sterility may also be an indication of epistatically linked loci and is the first trait to evolve in incipient speciation. The shallow mtDNA lineage found by Mock and others (2007) suggests that if *Wolbachia* spp. are not the cause, it may be a very recent incipient speciation event for MPB.

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Phylogeography of *Dendroctonus rufipennis* Based on mtDNA and Microsatellites

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Spruce beetle, *Dendroctonus rufipennis* (Kirby), is one of the most broadly distributed bark beetles in North America, extending from Alaska to Newfoundland, south to Arizona. It colonizes most species of spruce within its range. Usually it is associated with highly stressed or killed trees, but under certain conditions undergoes landscape level eruptions that kill millions of trees (Werner and others 1977), regardless of their physiological condition (Wallin and Raffa 2004). Populations may be semivoltine or univoltine, depending on temperature (Hansen and others 2001). Spruce beetles show relatively close associations with several species of fungi (Six and Bentz 2003), some of which vary in frequency with beetle population phase (Aukema and others 2005).

Adult beetles were collected from sixteen sites extending across the full range of *D. rufipennis*. DNA was extracted, and approximately 100 beetles were sequenced for mtDNA and approximately 550 beetles were genotyped for microsatellites (Maroja and others subm). Phylogenies were reconstructed by several means, including Maximum Parsimony and Bayesian Posterior approaches. We also computed isolation by distance, neutral evolution, migration and divergence time between populations, linkage disequilibrium, and deviation from Hardy-Weinberg equilibrium (Maroja and others 2007).

Three distinct clades were apparent (Maroja and others 2007). Two are northern, extending west to east from Alaska to Newfoundland, and the third extends north to south throughout the Rocky Mountains. The Rocky Mountain clade further subdivides into northern and southern clades. A zone of overlap is located in southern British Columbia, which appears to represent secondary contact between beetles that moved north along the Rocky Mountains and beetles occupying the boreal forests of Canada. There is high among-clade divergence.

Across all populations, there was a strong relationship between genetic distance and physical distance. This relationship was also significant within one northern clade. However, there was no isolation by distance effect within the other two clades, or within the pooled northern clades. There was no evidence for migration between the northern and southern Rocky Mountain clades. These same patterns segregate according to spruce species, and there is little evidence for migration between white and Engelmann spruce. However, host plant relationships cannot be teased apart from underlying geographic distribution patterns of tree species.

Based on historical records of spruce and the estimated divergence times from this study, the most parsimonious explanation is that spruce beetle

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populations were isolated during recent glaciations and have since become reconnected (Maroja and others 2007). However, initial divergence of these lineages must have occurred much earlier.

Our short-term goals include clarifying potential relationships with host tree species, including whether there may be incipient host races. Long-term goals are to develop genetic markers that could help distinguish between localized population buildup vs. eruptions due to migration, and help distinguish between eruptive and noneruptive populations.

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Phylogeographic Analysis of the Douglas-fir Beetle *Dendroctonus pseudotsugae* Hopkins (Coleoptera: Curculionidae: Scolytinae)

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Introduction

Population genetic structure studies made in genus *Dendroctonus* have been conducted from the perspectives of allopatric and sympatric models. In the first case, host effect and historical contingency were not recognized as a source of variation, while the later considered the host itself as a source of reproductive isolation. Nevertheless, both models show that genus *Dendroctonus* has the highest values of genetic variation among Scolytidae (Anderson and others 1979; Anderson and others 1983; Higby and Stock 1982; Langor and Spence 1991; Namkoong and others 1979; Roberds and others 1987; Stock and Guenther 1979; Stock and others 1979; Sturgeon and Mitton 1986; Zuñiga and others, in press). In this sense, few studies have been performed dealing with genetic population differences in *D. pseudotsugae*. For instance, through the use of Isozymes produced by 13 gene loci, Stock and others (1979) found a pattern characteristic of populations differentiated to, or beyond, the race or subspecies level between Idaho and Oregon populations. This was a consistent result in later works (Bentz and Stock 1986). However, none of these works performed a population genetic structure analysis.

In a wide sense, classic theory of population genetics was used to describe how mutation, migration, genetic drift, and natural selection affect the distribution of genetic variation (Avise 2004). However, genetic population structure analyses provide us with limited information about historical processes involved in population differentiation. Consequently, a different approach that takes into account these population phenomena is needed to accurately estimate gene genealogies at the population level. The method of Templeton and others (1992) has been used with restriction site and nucleotide sequence data to infer population genealogies when divergence is low. In addition, this method has also been used, coupled with a nested clade procedure, to separate population structure from population history, and in this way, explore the phylogeography of many organisms (Templeton 1995). In this context, there are still few works using nested clade methods to understand phylogeography of bark beetles. Among these are those applied to *Ips typographus* and *Tomicus piniperda* (Ritzerow and others 2004; Stauffer and others 1999). Both species were demonstrated to be highly polymorphic when compared with other european scolytids, and last glaciations had a profound impact on

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their actual distribution ranges (as a result of more restricted host refugees). Another study with the bark beetle *Ips confusus* (Cognato and others 2003) revealed that past glaciation events better explain genetic population structure than isolation by neither the host type nor its past fragmentation. The purpose of this work is to perform a phylogeographic analysis to infer if mitochondrial haplotypes of *D. pseudotsugae* allow us to recognize which historical or demographical factors have been responsible for the actual genetic organization. Taxonomic status of *D. pseudotsugae* subspecies (*D. p. pseudotsugae* and *D. p. barragani*) *sensu* Furniss (2001) will be tested as well.

Methods

Until now, 38 different populations of *D. pseudotsugae* have been sampled from its distribution range in Mexico, United States, and Canada. mtDNA cytochrome oxidase I gene (COI) has been used to resolve relationships among populations due to its extensive intraspecific polymorphism and adequate nucleotide variation (Simon and others 1994). DNA extractions were carried out using DNeasy® Tissue Kit (QIAGEN Gmbh, Hilden, Germany), using whole, ground insect thorax (approximately 10 mg), following manufacturer's tissue protocol. To amplify a fragment of approximately 600bp of COI gene, primers C1-J-2441 and T12-N-3014 (Simon and others 1994) were used via polymerase chain reaction (PCR). Unincorporated dNTP's and oligonucleotides were removed using GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Buckinghamshire, UK) and were directly sequenced using the Dye Terminator Cycle® Sequencing Reaction Kit (Perkin Elmer).

To test statements about two different subspecies in *D. pseudotsugae* (Furniss 2001), cladistic analyses of sequences were performed with program PAUP* (Swofford 1998). The trees were generated under a parsimony optimality criterion. A heuristic search of most parsimonious trees with the stepwise addition and tree bisection-reconnection algorithms were performed. All other settings were default. Bootstrap proportions were determined with 500 replicates and all other PAUP* settings were default. To test the null hypothesis of random association between haplotypes and geographical distribution, frequency and spatial distribution were used to create a statistical parsimony network (Templeton and others 1992) using the program TCS (v. 1.21, Clement and others 2000). Haplotypes in network were nested in a hierarchical series of 0-step, 1-step, 2-step, etc., clades until the entire network was nested into a single clade. Resulting nesting network was used to perform statistical test of geographic association of the nested clades with the program GeoDis (v. 2.5, Posada and others 2000). Then, inference keys outlined in Templeton and others (1995) were used.

Results

No nucleotide insertion or deletions were observed. Twenty-seven populations were analyzed (DNA extraction, PCR, sequencing), resulting in a total of 276 sequences, 550bp long. These sequences yielded 73 different haplotypes. It was found that nucleotide divergence (0.039 ± 0.019) and haplotype diversity ($\bar{h}$ 0.945) were higher when compared with those reported for other related species (Cognato and others 2003; Ritzerow and others 2004; Stauffer and others 1999), with the solely exception of *D. valens* ($\bar{h}$ 0.99, Cognato

and others 2005). Only 73 of 550 sites were parsimony informative. In one of the most parsimonious trees, it is clear separation was observed between northern (USA, CAN) and southern (MEX) populations, thus supporting statements about two different subspecies in *D. pseudotsugae* (Furniss 2001) (even though significant homoplasy was found, RC = 0.2146). Nested clade analysis is ongoing.

Future Work

Additional genetic work is planned because of the bias introduced in this study due to a lack of an appropriate sample size (inadequate collection effort). Although a statistical parsimony network was already constructed, it has not yet been used to make inferences about historical or demographic processes. In collaboration with Javier Victor, a total evidence approach (COI sequences + morphological traits) will be implemented in order to test subspecies level among *D. pseudotsugae* populations.

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Evolutionary Ecology of Pheromone Signaling in *Dendroctonus frontalis*

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Although studies of pheromone production in the southern pine beetle (*Dendroctonus frontalis*) extend back to the dawn of chemical ecology, it is only recently that instrumentation has become sufficiently sensitive to measure pheromone production of individual beetles. Now, recent studies have revealed surprisingly high variation among individuals in their pheromone production. This seems paradoxical because pheromone signals in tree-killing bark beetles are apparently linked to fitness and have high heritability. We tested whether variation has been overestimated by hindgut extractions, which can only measure static pools of pheromones; but variation among individuals was similarly high for life time production of pheromones via individual aerations (CV = 60 to 182 percent). An alternative hypothesis is that natural selection is constrained by the aggregation behavior of *D. frontalis*. In fact, the phenotypic trait visible to selection is the pheromone plume emanating from a tree, which is the collective property of all the beetles in the group. We evaluated the effect of individual beetles on the pheromone plume by using the empirical frequency distributions of pheromone production to analyze simulated aggregations with variable group sizes. For realistic aggregation sizes of 300 to1,300 females per meter of host tree, the average effect of a beetle on the plume was very low (generally < 10 percent). By application of Fisher's Fundamental Theorem of Natural Selection, this represents the maximum opportunity for selection on individual pheromone production (assuming that heritability is perfect and the pheromone plume is the sole determinant of fitness). We conclude that pheromone production is a nearly neutral trait in *D. frontalis* because individuals have only very minor effects on the phenotypic trait under selection (the pheromone plume). Therefore, genetic variation accumulates via mutation and recombination, unchecked by natural selection, even though pheromone production is highly heritable and properties of the pheromone plume have generally strong effects on the fitness of individuals within the aggregation.

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Bark Beetle Gene Structure and Function

Red Turpentine *Dendroctonus valens* P450s: Diversity, Midgut Location, and Response to Alfa-pinene Enantiomers

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Introduction

Bark beetle colonization action is a biotic stress to the host, which responds to this action by secreting oleoresin. This mixture of several compounds can be highly toxic for the insects. We hypothesized that the first physiological reaction of the insects to these toxic compounds is to metabolize and subsequently use some of them as pheromone precursors. Epithelial cells from the bark beetle's midgut have been demonstrated to be involved in pheromone production (Nardi and others 2002); however, the site(s) and mechanism(s) where the detoxification process occurs are unknown.

The cytochrome P450-dependent monooxigenases are a metabolic system involved in (1) regulating the titers of endogenous compounds such as hormones, fatty acids, and steroids, and (2) the catabolism and anabolism of xenobiotics such as drugs, pesticides and plant toxins. Monooxygenases are found in virtually all aerobic organisms, including insects. P450's are a multigenic family with a common origin and are found in the endoplasmic reticulum and mitochondria. There are four main gene families in insects: CYP4, CYP6, CYP9, and CYP-mitochondrial (Fereyeisen 1999; Scott 1999; Scott and Wen 2001). Other mechanisms involved in the detoxification process, such as glutathion transferases enzyme (GTS) (Hemingway 2000) and non-specific-esterases, are being widely documented in insects. GTS enzymes are a multigenic family integrated in seven classes, whose function is to metabolize xenobiotic compounds and to protect cells against damage by oxidative stress. Non-specific-esterases (carboxylesterases) also participate in the detoxification process, and only two genes are known.

Sturgeon and Robertson (1985) proposed that cytochrome P45O monoxigenases could be involved in the detoxification process of some toxic monoterpenoids in bark beetles as α-pinene. White and others (1978) demonstrated that cytochrome P450 of gut epithelial cells participate in the transformation of α-pinene to *cis* and *trans*-verbenol in *Dendroctonus terebrans*. Aguilar (2002) showed by inmuno-histochemical techniques that cytochrome P450 and non-specific-esterases of midgut epithelial cells in *D.valens* and *D. mexicanus* are over-expressed when the insects are exposed to

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α-pinene vapors. With respect to GTS enzymes, there are no reports in bark beetles. However, in *Ips typographus*, four esterase isozymes were detected that participate in detoxification processes and/or in the hydrolysis of juvenile hormone (JH) (Stauffer and others 1997). Previous histochemical studies of *D. valens* alimentary canal have shown that non-specific-esterases of midgut increase their activity when insects are exposed to α-pinene (Aguilar 2002).

We are using *D. valens* as a model because it has been demonstrated that the monoterpenoids α -pinene, R-(+)-α-pinene, S-(-)-β-pinene, and S-(+)-α-pinene are highly toxic compounds. They are also very important for their chemical communication system (Hobson and others 1992; White and Hobson 1993). In this species, we are studying the ultra-structural changes in the midgut epithelial cells of *D. valens* after exposure of the insects to α-pinene. We are also studying the localization, diversity, and expression of CYP, non-specific-esterase and glutation–s-transferase genes in this region.

Methods

Ultrastructure

Samples of *D. valens* were collected directly from infested trees and transported alive to our lab. The insects were exposed to α-pinene during 24 hours at 4 °C. The insects were then dissected to obtain the alimentary canal and to extract the midgut. The anterior and posterior midgut were sectionaed and they were fixed in Eppendorf tubes with glutaraldehide 2.5 percent and cacodilate buffer 1M. The samples were then processed with conventional transmission electron microscope techniques.

P450 Complex

The insects were exposed to α-pinene and its enantiomers: R-(+)-α-pinene, S-(-)-β-pinene, and S-(-)-α-pinene during 24 hours at 4 °C. Subsequently, total RNA of midgut was extracted with Trizol method and single-stranded cDNAs for RT-PCR were synthetized from total RNA with MMLV reverse transcriptase. The CYP4, CYP6, and CYP9 (P45O) genes were amplified with degenerated primers (Rose and others 1997; Snyder and others 1996). The expected bands were cut off from the gel and cDNA was isolated using the QIAEX II Gel Extraction Kit. The isolated products were reamplified and sequenced. The nucleotide sequences were compared with sequences deposited in GeneBank.

Results and Discussion

The Midgut Ultra-structure of Insects Without Alpha-pinene

The midgut epithelium is composed of cylindrical cells with microvilli in the apical zone. The cells are limited by a basal membrane named basal lamina that forms invaginations in folds. The cytoplasm is granular and spongy. The nucleus is large-ovoid with one or several electron-dense nucleoli located in the center or mid part of the cell. The primary lysosomes are scarce and mitochondria are found in all the cytoplasm. Their crests have a normal structure and the rough endoplasmic reticulum is widely distributed. Ribosomes are found both free and adhered to the rough endoplasmic reticulum. It is very common to observe empty vacuoles and secretion vesicles irregularly scattered in all cytoplasm.

The Midgut Ultrastructure of Insects With Alpha-pinene

The microvilli, nucleus, and vesicles maintain their original structure; however, it is common to observe a significant increase in the number of mitochondria on the basal and apical zone of the cells. The mitochondria crests are modified, the primary and secondary lysosomes are abundant, the rough endoplasmic reticulum is observed near to the nucleus arranged in parallel packages, and the Golgi complex is evident and maintains its structure with crests in block piles and it is found near of nucleus and the apical zone. The smooth endoplasmic reticulum is very conspicuous. It has a tubular shape with vesicles associated in its extreme with indefinite arrange.

The differences observed in both experiments showed that α-pinene affects the midgut epithelial cells, changing and increasing the number of secondary lysosomes and mitochondria in the apical zone. The rough endoplasmic reticulum shows an overexpression. The Golgi complex and smooth endoplasmic reticulum are clearly evident. These ultrastructural changes suggest that these organelles participate actively in the detoxification process.

P450 Gene Complex

The midgut cDNA of stimulated insects amplify for a CYP4 gene (CYP4 family). The amplification product of this gene exhibited one band of the expected size of ~500bp. These products were sequenced, and Blast analysis showed that amino acid sequences of CYP4 of *D. valens* has a high identity (90, 91 percent) with CYP4G29 from *Leptinotarsa decemliniata*, and CYP4G15 from *Tribolium castaneum*. CYP6 and CYP9 genes have been amplified from total DNA of *D. valens*, and at this moment, we are amplifying these genes from midgut cDNA to obtain their sequences. These results show that three CYP genes are present in the midgut region.

Future Work

- The genes obtained (CYP4, CYP6, CYP9) will be cloned and sequenced to obtain subsequently complete genes using 5' RACE method. We are testing the presence of CYP-mitochondrial (CYP12).
- We are looking at where these CYP genes are located at ultrastructural level in *D. valens* midgut cells.
- We are quantifying the expression levels of CYP genes using Real-Time PCR.
- We are determining the midgut location and expression levels of other enzymes involved in the detoxification process, as esterases and Glutathion-S-Tranferases by histochemical and immunological methods, as well as by molecular techniques.

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Expression Patterns of Bark Beetle Cytochromes P450 During Host Colonization: Likely Physiological Functions and Potential Targets for Pest Management

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Introduction

Cytochromes P450 family genes (P450s) are found in a diverse array of organisms ranging from bacteria to mammals to plants to arthropods. Although there are exceptions to this rule, organisms generally contain a fairly large number of P450 genes and pseudogenes in their genomes. For instance, among arthropods whose genomes are well characterized, the mosquito, *Anopheles gambiae*, has about 100; the fruit fly, *Drosophila melanogaster*, has about 89; and the honeybee, *Apis mellifera*, has about 46.

While there are a very large number of P450s in nature, the general biochemistry of most P450s is similar. That is, P450s are typically involved in NADPH-mediated oxidative attack on various substrates. In practice, however, because specific P450s metabolize only certain substrates, they are involved in a diverse array of biologically important metabolic pathways. In insects, the functions of P450s include, but are not limited to, detoxification of plant secondary metabolites, detoxification of pesticides, biosynthesis of pheromone components and hormones, degradation of pheromone components and hormones, and omega oxidation of fatty acids. In tree-killing bark beetles, it is possible that P450s function in degradation of pheromone and kairomone components in antennae during host searching (Maïbèche-Coisne and others 2004), rapid detoxification of constitutive and induced plant secondary metabolites during host colonization, production of aggregation pheromone components, hormonal and other metabolic changes related to reproduction, and larval survival while feeding in host tissues.

Because of the importance of this group of genes and their associated proteins to insect survival, they are ideal targets for research into the development of novel pest management tools. For instance, because certain substances are capable of altering the activity of specific P450 enzymes (for example, Pelkonen and others 1998), an understanding of the biochemical and ecological functions of P450s in bark beetles may allow for the development of species-specific pesticides that disrupt important aspects of bark beetle host location, colonization, and development.

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More complete information pertaining to cytochromes P450 in insects can be found in reviews by Feyereisen (1999) and Omura (1999).

General Approach

Using degenerate PCR, and other PCR-based methods, we were able to isolate fragments of 13 P450s from the California fivespined ips, *Ips paraconfusus*. We separately fed newly emerged males and females fresh ponderosa pine, *Pinus ponderosa*, tissue for 0h, 8h, and 12h. We then assessed transcript levels in the beetles in a 12-replicate quantitative PCR experiment using TaqMan® primers and probes unique for each of the 13 novel P450s.

We found increased levels of transcripts of some P450s in males only, or in both sexes, following feeding. We also found decreased levels of transcripts of some other P450s in males only, or in both sexes, following feeding. We found that the transcript levels of one gene increased in females following feeding but decreased in males. The transcript levels of one gene remained constant in both sexes no matter their feeding state. These observations allowed us to make predictions as to putative biochemical and ecological functions for these P450s, and they will also guide further functional characterization and gene expression studies.

Further Research

Current and future work includes:

- Discovery of more P450 genes in *I. paraconfusus*, *Dendroctonus ponderosae*, and other bark beetles.
- Further exploration of expression patterns of P450s in *I. paraconfusus*, *D. ponderosae*, and other bark beetles under ecologically relevant conditions.
- Functional characterization of the protein products of P450 genes in bark beetles.
- Similar work with lepidopteran defoliators and seed orchard pests.

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Genetics of Symbionts, Natural Enemies, and Hosts

Yeasts Associated with Bark Beetles of the Genus *Dendroctonus* Erichson (Coleoptera: Curculionidae: Scolytinae): Molecular Identification and Biochemical Characterization

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Introduction

The bark beetles (Scolytinae) increase their potential to colonize their hosts by associating symbiotically with microorganisms, particularly fungi, which are carried on specialized structures called mycangium (Harrington 1993; Klepzig 2001; Paine and others 1997; Six 2003; Whitney 1982). Additionally, there is experimental evidence that some yeasts and bacteria from the alimentary canal of these insects might be involved in digestion and detoxification processes and pheromone production. The latter are compounds fundamental to the chemical communication of these insects (Borden 1982; Harrington 1993; Paine and others 1997). The role of bark beetles-associated yeast cannot be studied if we do not know their diversity and location. Yeasts taxonomic identification have been commonly performed on morphological characters of vegetative and sexual stages as well as by physiological tests; however, the variable responses of yeasts to the physiological tests and their phenotypic plasticity have hindered their taxonomic identification. Recent investigations have demonstrated that the 18S rDNA and D1/D2 domain of the large subunit 26S rDNA are reliable attributes for yeasts identification. Thus, the purpose of this study is to elucidate yeasts diversity associated to different species of *Dendroctonus*. In order to asses how many biological entities (putative species) are present in our samples, we used species concept based on phylogenetic analysis of D1/D2 26S, 18S, and ITS1 rDNA data. In this context, we assume that the categories "species" or "groups" are not ranges, but different biological entities; the reference species or taxa were used to specify the name to which the group applies.

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Methods

Four hundred and fifty beetles of nine *Dendroctonus* species from 36 geographical sites were dissected under sterile conditions. The yeasts were isolated from gut and ovaries using RPMI 1640 medium for a 48 h period. Inocula were taken from the RPMI culture, placed in Sabouraud dextrose broth, and incubated for 24 to 48 h at 28 °C. The yeasts were isolated in Sabouraud dextrose agar and restreaked until pure cultures were obtained. Bark beetle frass and eggs were directly inoculated in Sabouraud broth and incubated under the same conditions above mentioned. Total genomic DNA was isolated from each yeast strain according to the protocol described in Lehmann and others (1992). The gene regions of D1/D2 domain 26S, 18S, and ITS1 of rDNA were amplified and sequenced. The sequences obtained were compared with yeast sequences from GenBank. The phylogenetic relations among the strains were established by parsimony methods using PAUP 4.0b10. The number of nucleotide differences per site among D1/D2 domain 26S sequences was estimated according to Nei and Li (1979). *Schizosaccharomyces pombe* was used as outgroup in all analyses. Finally, yeast groups were characterized by 29 assimilations and seven fermentation tests of carbohydrates, four additional assimilation tests, and morphological features following Yarrow (1998).

Results and Discussion

A total of 430 yeast isolates were obtained: 331 (gut), 33 (ovaries), 50 (frass), and 16 (eggs). Thirteen consistent groups were found throughout the phylogenetic trees of D1/D2 26S and ITS1 rDNA, however, some of them were not consistent on 18S topology. Eleven groups were associated to *Candida arabinofermentans, C. ernobii, C. oregonensis, C. picea, C. terebra, Pichia americana, P. canadensis, P. capsulata, P. glucozyma, P. guilliermondii, P. scolyti*, and two with no equivalent within the GenBank database. The great majority of yeast isolates, within groups, showed less that 1 percent nucleotide substitution respect to reference species, and among groups, more that 1 percent.

Phylogenetic analysis show, independent of the analyzed gene, the yeast community associated with *Dendroctonus* species only belong to *Candida* and *Pichia* genera. This observation agrees with previous studies carried out on bark beetles using conventional tests (Brand and others 1977; Bridges and others 1984; Leufvén and others 1984; Moore 1972; Shifrine and Phaff 1956), however, the species or groups are different. Also, this analysis shows that bark beetle-associated yeast groups do not present correlation with the isolate sites, geographic location, or *Dendroctonus* species. The only exception was *Pichia scolyti* group, which showed an association with its host, *Dendroctonus pseudotsugae*. Additionally, this analysis suggests that *C. arabinofermentans, C. ernobii*, and *P. capsulata* groups from *D. pseudotsugae* could have a vertical transmission from parents to progeny since they were isolated from eggs, ovaries, larvae, and adults in the same geographic site. However, it is necessary to carry out specific investigations to test if the transmission of the yeasts is really vertical.

Bark beetle-associated yeasts exhibit a physiological response highly variable within and among groups (data not shown, but obtainable upon request). Therefore, there is not correlation between groups and the fermentation or

assimilation of some carbohydrates. Nevertheless, a greater part of the isolates assimilated cellobiose, salicin, and D-xylose. Some studies reported that these carbohydrates are indicator substrates of enzymes involved in metabolic routes associated with digestive and detoxification processes (Vega and Dowd 2005). Unfortunately, we know little or nothing about the symbiotic association between *Dendroctonus species* and its associated yeast. Our findings of no association of yeasts with *Dendroctonus* species or geographic localities suggested no significant commensal or symbiotic relationship. However, we assume that this relationship can be very important in a nutritional context, as has been shown in other insect groups.

Future Research Plans

The following studies are planned for the future:

- Determine the role of yeast in the digestion process of bark beetles.
- Perform molecular analysis of multigenic family P450, esterases, and glutathione S-transferase of yeasts to learn about their role in the detoxification process in bark beetles.
- Determine if the transmission of the yeasts is vertical or horizontal.

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Gut Bacteria of Bark and Wood Boring Beetles

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Bark beetles are known to have complex associations with a variety of microorganisms (Paine and others 1987; Ayres and others 2000; Six and Klepzig 2004). However, most of our knowledge involves fungi, particularly external species. In contrast, we know very little about their associations with bacterial gut symbionts (Bridges 1981). Similarly, work with wood colonizing insects such as termites reveals a diverse and functionally important community of gut bacteria (Hongoh and others 2005). Yet our knowledge of the gut microbiota of wood boring beetles is rudimentary. Our work is aimed at addressing these gaps, as bark and wood boring beetles include important forest pests, natural disturbance agents, and invasive species.

This report describes the composition, variation among life stages, and ecological roles of beetle gut communities. The study systems include three bark beetles—the southern pine beetle, pine engraver, and spruce beetle—and three wood borers—the Asian longhorned beetle, linden borer, and emerald ash borer. Insects were evaluated by a combination of culture-independent and culture-dependent approaches. Culture-independent analyses involved extraction of total DNA from entire guts, amplification of bacterial 16S rDNA, and sequencing (Broderick and others 2004). Culture-dependent approaches involved plating dilutions of gut extracts, periodic counts of colonies, and molecular identification by the above processes. Sampling efficiency in culture independent studies was estimated by rarefaction curves.

The predominant bacteria in these six beetle species were members of the alpha-proteobacteria, beta-proteobactaeria, gamma-proteobactaeria, Firmicutes, and Actinobacteria. The diversities of gut communities varied dramatically. At one extreme, the gut bacteria of linden borer consisted entirely of gamma-proteobactaeria (Schloss and others 2006). At the other extreme, only half of Asian longhorned beetle's most common bacteria were in one group, Firmicutes. Overall, the gut communities of wood borers tended to be more diverse than those of bark beetles at the level of bacterial genera. The emerald ash borer contained 26 bacterial genera in larvae and 27 in adults (Vasanthakumar, in prep). Of these, 15 morphotypes were present in both stages. Bacterial densities were higher in adults than larvae. Asian longhorned beetle larvae contained 24 bacterial genera (Schloss and others 2006). The southern pine beetle contained 13 genera in larvae and nine genera in adults, with only three species in common (Vasanthakumar and others 2006). The

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pine engraver had six genera in larvae, three genera in pupae, and three genera in adults, with two genera common to all life stages (Delalibera and others, subm.). Densities of bacteria were higher in pine engraver larvae than in adults, and higher in adults than pupae. Although linden borer guts have a low diversity of gut bacteria, the densities of these bacteria are much higher than in pine engraver (Delalibera and others 2005). Variation in bacterial members varied widely among these six insects. No genera were detected in all six, only four were detected in three, and only seven genera were detected in even two beetle species.

Bacteria have been shown to confer a broad array of benefits and detriments to their host insects (Cazemier and others 1997). To date, we have evaluated two roles in bark- and wood-boring beetles. We observed cellulolytic activity in gut bacteria of both wood borers tested—linden borer and emerald ash borer, but in neither of the two bark beetles tested—southern pine beetle and pine engraver (Delalibera and others 2005; Vasanthakumar and others, in prep.). Secondly, bacteria in oral egestions from spruce beetle can defend against gallery invading fungi that otherwise reduce oviposition and increase adult mortality (Cardoza and others 2005). These bacteria appear almost entirely responsible for the highly fungistatic activities of the spruce beetle's oral egestions.

Ongoing work involves potential roles of gut bacteria in detoxification of tree defense compounds and analyses of the stability and resilience of the gut community. Understanding the functioning of gut microbiota can offer promising insights into novel approaches to pest management (Broderick and others 2006).

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