The Ecological Association between Ambrosia Beetles (*Monarthrum scutellare* and *M. dentiger*) and *Phytophthora ramorum*, the Pathogen that causes Sudden Oak Death

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Abstract A recently discovered fungus-like oomycete, *Phytophthora ramorum*, has been identified as the pathogen responsible for the epidemic levels of oak mortality throughout California and southern portions of Oregon. The means of pathogen dispersal is currently unknown. This study explored the possibility of bark beetles (*Pseudopityophthorus pubipennis*) and ambrosia beetles (*Monarthrum scutellare* and *M. dentiger*) as vectors of *P. ramorum,* which results in the disease complex termed Sudden Oak Death (SOD). To confirm beetles as vectors of the pathogen, logs of coast live oak (*Quercus agrifolia*) exhibiting SOD infection and evidence of beetle colonization were placed in isolation chambers and emergent beetles were collected and plated on a selective media designed to promote *P. ramorum* growth. Additionally, logs were cross-sectioned to determine if the pathogen could be isolated from tunnel walls. Of the 1,344 beetles sampled, none were shown to associate with the pathogen. Since 98% of the beetles sampled belonged to *M. scutellare*, no conclusions could be made concerning *M. dentiger* and *P. pubipennis* due to inadequate sample size. *P. ramorum* was not isolated from tunnel wall shavings. These results suggest that *M. scutellare* is not a vector of *P. ramorum* and therefore neither capable of spreading the pathogen within the vascular tissue of the host nor capable of transporting the pathogen to a new host.

Introduction

Since 1995, oak mortality has risen to epidemic levels from as far south as Monterey County, California, to as far north as southern portions of Oregon (Garbelotto et al. 2001). Evidence suggests that a previously unknown pathogen, *Phytophthora ramorum* (McPherson, 2001), is responsible for this rapid decline in oak health. Infection by the pathogen results in a disease complex termed Sudden Oak Death (SOD). Susceptible oak species include black oak (*Quercus kellogii*), coast live oak (*Quercus agrifolia*), Shreve oak (*Quercus parvula* var. *shrevei*), and tan oak (*Lithocarpus densiflorus*). However, the pathogen is not restricted to these four species in the family Fagaceae. The pathogen has also been confirmed to attack huckleberry (*Vaccinium ovatum*), *Rhododendron* spp., madrone (*Arbutus menziesii*), bay laurel (*Umbellularia californica*), buckeye (*Aesculus californica*), bigleaf maple (*Acer macrophyllum*), and manzanita (*Arctostaphylos manzanita*). The host list continues to lengthen as researchers discover new susceptible species. Recently, coastal redwoods (*Sequoia sempervirens*) were identified as potential hosts; research has yet to confirm these statements (California Oak Mortality Task Force, 2002). The number of species the pathogen is capable of infecting remains unknown.

Hardest hit counties include Marin, Santa Cruz, Monterey, Napa, San Mateo, Santa Clara, and Sonoma (Figure 1). Although the disease distribution is patchy, infected areas can experience 40-80% oak stand mortality (Garbelotto et al. 2001). As a result, within these hard-hit counties, thousands of dead and dying oaks have generated a wealth of social, economic, and environmental problems (Kan-Rice, 2001). At present, there is no cure for this affliction. The consequences of changes in forest structure as a result of thousands of dying and dead oaks are uncertain. Changes in fire ecology, wildlife habitat, and aesthetics are issues raising awareness of the disease among the scientific community and public (Kan-Rice, 2001). With so many lingering questions, research to combat this epidemic is urgent.

Sudden Oak Death has devastated many oak woodland areas. However, ecological devastation as a consequence of exotic pathogens is nothing extraordinary. Unlike native pathogens which have co-evolved with their hosts, a new or exotic pathogen finds its host plants unable to adequately defend themselves. For example, the exotic Asian fungus *Cryphonectria*

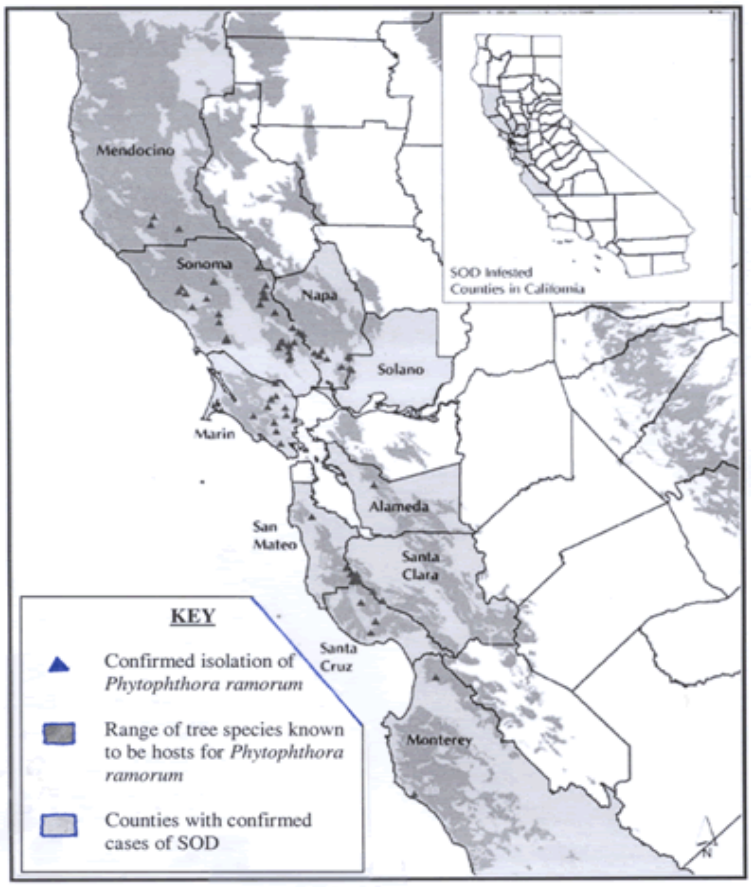

Figure 1. Areas of Confirmed SOD Infection as of April 18, 2002
(Monitoring SOD in California, 2002)

parasitica, from the time of its introduction to United States in 1900 to 1940, killed 3.5 billion American chestnut trees (*Castanea dentata*). The blight successfully downgraded the most important tree species of the eastern forest to insignificance (American Chestnut Cooperator's Foundation, 2002). Similar notable cases of ecological devastation as a result of exotic pathogen introduction include Dutch elm disease, White Pine Blister Rust, and Pine Pitch Canker. Today, researchers struggle to prevent similar catastrophic losses and species extinction from Sudden Oak Death.

The pathogen causing Sudden Oak Death belongs to the large genus of plant-pathogenic fungi called *Phytophthora* ("plant destroyer"). Although *Phytophthora ramorum* is a recently discovered species, members of the genus *Phytophthora* have caused economic and social problems for more than a century. Many species of the genus are known to cause diseases on an increasing number of host plants, including major food crops, tropical fruit trees, nut trees,

berries, and ornamental plants (Zentmeyer, 1983). In 1845 *P. infestans* caused a potato blight devastating potato crops throughout Western Europe. Ireland was hardest hit, suffering widespread famine and massive emigration (Gregory, 1983).

The remarkable success of *Phytophthora* species can be attributed to their plasticity and tolerance of environmental change. The lifecycle of *Phytophthora* species is complex, involving a variety of structures, which can transition back and forth depending on external conditions (Gregory, 1983). At present, knowledge of *P. ramorum*'s lifecycle remains incomplete. Furthermore, the dispersal mechanism of *Phytophthora* spores vary among species, including wind (*P. infestans* and *P. phaseoli*), water (*P. coloasiae*), ants (*P. magekarya*), and even snails (Gregory, 1983; El-Hamalawi et. al., 1996). This research project addresses the mechanism of *Phytophthora ramorum* dispersal. Two Ambrosia beetles (*Monarthrum scutellare* and *M. dentiger*) and the Western Oak bark beetle (*Pseudopityophthorus pubipennis*) are commonly found in infected oak species and are hypothesized to serve as *P. ramorum* vectors. Here, I will explore the possibility of an oak/pathogen/beetle association. I will investigate the beetle tunneling and gallery establishment within infected trees to determine whether the pathogen can be isolated from larvae and gallery walls. Such an inquiry may help determine whether beetles and larvae are capable of transporting the pathogen further into the sapwood of the host, perhaps hastening host death by facilitating the spread of the *Phytophthora* and other beetle-associated fungi.

Although *Phytophthora* dispersal via beetles has never been observed, the possibility of an association exists. For example, *P. magekarya*, a pathogen found in the soils of cocoa plantations in Nigeria and Cameroon, relies on a species of ants that builds soil tents to carry spores up into host tree species (Gregory, 1983). The hypothesized pathogen transport by ambrosia and bark beetles may be more inadvertent. Active collection and transport of contaminated materials, as seen with *P. magekarya* and ants, is unlikely. However, the attachment of the pathogen's sticky zoospores and cysts to passing beetles and larvae is plausible. Beetles of coniferous forests have been known to vector pathogens. For instance, bark beetles (two species of curculionids and one scolytid) have been identified as vectors of the pathogen responsible for black stain root disease (*Leptographium wageneri* var. *pseudotsugae)*, which affects Douglas-Fir (*Pseudotsuga menziesii)* dominated forests of western North America (Goheen and Hansen, 1993). The fungus sporulates within the beetle galleries and conidiospores

(asexual spores), which are contained in a sticky droplet, attach to emerging beetles dispersing in the spring (Goheen and Hansen, 1993). Whether similar interactions between *M. scutellare, M. dentiger, P. pubipennis* and the pathogen *P. ramorum* exist is the subject of this investigation.

The ecological association between the exotic pathogen and the oaks which have co-evolved with the native bark beetle and ambrosia beetle is of particular interest to this study. Hosts exhibit a series of symptoms (Table 1). The ambrosia beetles, *M. scutellare* and *M. dentiger*, as well as the western oak bark beetle, *P. pubipennis*, have been observed to attack and colonize oaks that show seeping, which is an early indicator of *P. ramorum* infection (McPherson, 2000). Seeping is evidence of the host's attempts to defend itself from the invasive organism. The number of seeps exhibited in the host can be used as an indicator of severity of the infection. Seeping indicates the presence of pathogen cankers, which are presumed to be the primary cause of host death. These cankers basically "strangle" their host to death, killing the phloem and xylem tissue, that are responsible for nutrient and water transport, respectively. Removal of the outer bark of infected oak species reveals cankers delineated by necrotic tissue separated from healthy tissue by a dark and resinous line (Garbelotto et al. 2001). Initial beetle colonization occurs in the areas directly beneath these seeping zones on the lower trunks of oaks (McPherson, 2000). A common symptom is the presence of *Hypoxylon thouarsianum*, a fungal endophyte usually found on dead/downed trees. Like *H. thouarsianum*, beetles appear to attack

TABLE 1. Common Sequence of SOD Symptoms Among Infected Oak Species (Storer et. Al., 2001)

1) SEEPAGE APPEARS ON LOWER TRUNK (3 weeks after infection)

2) AMBROSIA / BARK BEETLES COLONIZE TREE WITHIN SEEPING AREAS (9 months or less)

3) *HYPOXYLON THOUARSIANUM* FRUITING BODIES APPEAR ON HOST TRUNK

4) BEETLES HEAVILY COLONIZE TRUNK (13 months)

5) TREE DEATH (occurring two or more years after initial infection, some may die sooner.*)

*Above observations remain insufficient to fully address the issue of time between the infection, symptoms and death of a mature tree. The above numbers are estimates based on one year of field data collected by Brice McPherson et. al. (unpublished, 2001) in the Marin Municipal Water District and China Camp State Park and based on studies by Rizzo et. al. (2002). Sapling death was reported to occur in a matter of weeks after inoculation with the pathogen in controlled nursery studies (Storer et. al., 2001).

the bark overlying dead host tissue. The *Phytophthora* pathogen has been noted to spread from the phloem into the sapwood toward the cambium and finally to the xylem (McPherson, 2000). Whether beetles and larvae, via tunnels and galleries, facilitate the spread of the pathogen inward is unknown and is of primary interest to this research project and will be addressed.

In a parallel project to this one, an undergraduate at UC Berkeley, Tamara Rich (2001), completed her senior thesis addressing Sudden Oak Death and the possibility of ambrosia beetles acting as pathogen vectors. She found no evidence of ambrosia beetles acting as vectors for the pathogen. However, Rich cited several problems associated with her methodology concerning beetle plating techniques, timing of plating, culture media preparation, and artificial laboratory conditions used to rear the beetles from cut logs. These complications may have prevented Rich from finding evidence of beetle–pathogen vectoring.

The research project addresses the following questions:

(1) Are *M. scutellare*, *M. dentiger*, and *P. pubipennis* vectors for the pathogen *P. ramorum*?

(2) If beetles do not vector the pathogen, can they transport the pathogen via larval offspring into the sapwood by way of galleries, possible hastening the death of the host? Can the pathogen be detected on beetle larvae?

(3) Can the pathogen be isolated from beetle galleries?

In addition to local environmental concerns about the inevitable change in oak woodland forest structure (COMTF, 2002 and Monitoring Sudden Oak Death in California. 2002), anxiety over the disease has spread to the Eastern United States. The spread of the pathogen to eastern hardwood forests would prove exceptionally devastating since many eastern oak species have been identified as "highly susceptible" (McPherson, personal communication). These issues make attempts to understand and control the pathogen a top priority. In order to curb the spread of *P. ramorum*, an understanding of its life history is of the utmost importance. Determining whether *Monarthrum* and *Pseudopityophthorus* are vectors for *P. ramorum* is a vital piece of information. These usually rare beetles and their hypothesized association with the exotic pathogen, *P. ramorum*, are of ecological interest to the scientific community (McPherson, personal communication). Although there is a strong association between beetles and oak mortality, many researchers contend that they are only secondary in the death of the tree (Garbelotto et al. 2001). Despite these debates, the possible role that beetles play in the demise of oaks from SOD remains an important and unknown piece of the puzzle.

Methods

Study Site Four mature coast live oaks (*Quercus agrifolia*) were felled in China Camp State Park (latitude 38.0144N and longitude 122.460W) located in Marin County, California, USA, on the shores of the San Francisco Bay. The 1,640-acre state park has a coastal-temperate climate with temperatures ranging from 21-27^{o}C in the summer with winter temperatures lingering around 10^{o}C. The state park receives on average 67 days of rain a year with an average annual precipitation of 20.52 centimeters (Climate of San Francisco, 2002). Felled oaks were located within 30 meters of the state park's campground and access road. The oaks were located on slightly sloped, moderately forested sites in association with Bay trees (Lauraceae). Felled oaks were sawed into logs and transported to a University of California Berkeley laboratory facility.

Sampling Procedure Oak selection was based on several factors. Oaks had to (1) be approved by the China Camp State officials for removal, (2) be infected with *Phytophthora ramorum* as evidenced by seeping, (3) show signs of recent bark or ambrosia beetle activity, and (4) have live green crowns. Logs were sawed into 1½' - 2' sections, 4 sections from each oak (16 total sections), and placed in greenhouse isolation chambers located at the Oxford Research Unit in Berkeley, CA. The log sections were incubated in greenhouse isolation chambers for 4 weeks at 27^{o}C and emerging beetles were collected. Breeding chambers were similar to those used in the Rich (2001) project. Breeding chambers were constructed from plywood. A Lucite strip ran along the back panel of these chambers allowing light to enter the otherwise dark enclosure. The emerging beetles were drawn to the light and with the assistance of a circulating fan, were swept into a connecting pipe leading to a refrigerated storage device at 4^{o}C.

Beetles that emerged were collected daily from the refrigerated storage units and then plated on selective media. Two techniques were employed in plating beetles. Beetles were both crushed with sterilized forceps and plated whole or homogenized. The selective media used to isolate the pathogen was the *Phytophthora* selected media known as PARP. Petri dishes (10 cm diameter), each containing 17 grams of Difco corn meal and 950 mL of distilled water, were amended with the antibiotics Pimaricin (0.4 mL), Ampicillin(0.25 g), and Rifampin (0.01g) dissolved in DMSO (1.0mL). Groups of 15-35 beetles were homogenized in 1.0 mL sterile distilled water and spread across the pimaricin-ampicillin-rifampicin-PCNB agar (PARP)

prepared plates. Plates designated for whole body plating consisted of 60-70 beetles per plate and special care was taken to entirely submerge the beetle bodies within the media as suggested in the Rich study (2001). Plates were stored at room temperature to allow for fungal growth. At assessment, usually 7-10 days following plate preparation, suspected *Phytophthora* fungal growths were identified by a pathologist and were isolated onto newly prepared PARP plates. Suspected *Phytophthora* fungal growths were those that morphologically resembled samples of pure, isolated cultures of the pathogen. The true identities of the suspected *Phytophthora ramorum* growths were determined using polymerase chain reaction (PCR) techniques (Campbell et. al., 1999). Following the denaturation of the suspected *Phythophthora*'s DNA using heat, known primers (Garbelotto, unpublished) of *Phytophthora ramorum* were used to amplify any existing complimentary strands of DNA. If amplification occurred, the presence of *Phytophthora ramorum* was assumed. This was an acceptable assumption since SOD researchers have used this technique without reporting difficulties (Garbelotto, unpublished and Rizzo et. al., 2002). Furthermore, the technique provides more certainty in the findings since it does not depend solely on identification by taxonomic features.

Controls A large variety of insects were collected from the refrigerated storage units in addition to the desired beetle species. These insects were either homogenized or plated whole, and then analyzed in the same manner proposed for the beetle species. The objective was to determine if there existed a background level of pathogen spores in the environment, which were being transmitted by organisms in contact with infected material or if any observed vectoring was restricted to *M. scutellare*, *M. dentiger*, and *P. pubipennis*.

Larvae In addition to determining whether beetles vectored the *Phytophthora* pathogen, the project also attempted to determine if the pathogen was present on beetle gallery walls and on larval bodies. To explore the possibility of beetle larvae as pathogen carriers, one 1 ½'- 2' log section was taken from two of the four felled oaks and carefully debarked with a hammer and ¾ inch chisel to expose the surface of the vascular cambium. Exposed larvae of the bark beetle, *P. pubipennis*, were removed and place in refrigerated storage units at 4^{o}C. The debarked logs were

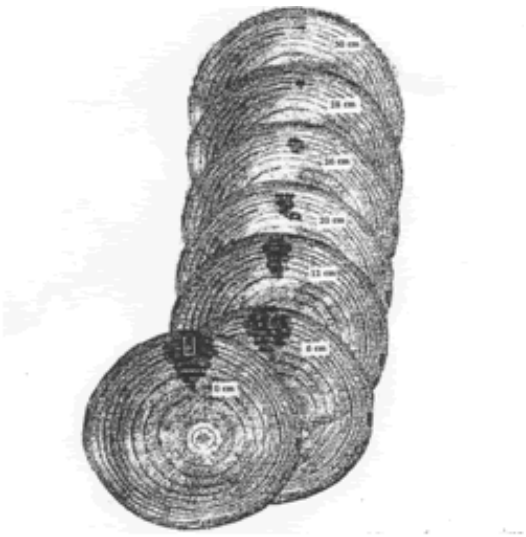

Figure 2. Illustration of the 1-2 inch cross sections
(Uzunovic, A. et. al., 1998)

then cut transversely into 1"-2" sections (Figure 2). All exposed ambrosia beetle (*M. scutellare* and *M. dentiger*) larvae were removed from the exposed galleries and placed in refrigerated storage units at 4^{o}C. Within 2-3 days after collection, the larvae of each species were separately homogenized, plated 5 larvae per plate, and analyzed like those of the beetles described earlier.

Egg Galleries and Tunnel Walls From the two logs selected for debarking, shavings were taken from all identified beetle entrance and exit holes found on the bark surface. After logs were debarked with a hammer and ¾ inch chisel, shavings were taken from each bark beetle gallery or tunnel found on the exposed vascular cambium surface. Likewise, following the transverse sectioning of a log and the removal of ambrosia beetle larvae, shavings were taken from galleries and tunnels exposed on the transverse surfaces. Shavings taken from the surface of bark exit and entrance holes, bark beetle tunnels/galleries, and ambrosia beetle tunnels/galleries were approximately 1-2mm thick and 2mm long. Shavings were taken at the midpoints of all exposed tunnels and galleries. If tunnel or gallery branching occurred, shavings were also taken at the midpoints of the branches. If an exposed tunnel or gallery exceeded 5 cm in length, shavings were taken at one-third and at two-thirds the length of the tunnel or gallery.

All shavings were immediately plated after extraction. Entire shavings were plated in groups of 5-7 on PARP media according to their source and underwent the same scrutiny to detect *Phytophthora ramorum*, as described above for the beetles and larvae.

Statistical Techniques There exists only a single nominal variable, the product of which is either "pathogen present" or "pathogen not present". Since there is no comparison or correlation of multiple variables a statistical test is not applicable. In order to determine an adequate sample size, the following formula was used as presented by Martin et. al. (1987):

$$n = ((1-\beta)^{(1/D)})(N-(D-1)/2).$$

The variables are defined as follows:

n = Required Sample Size N = Population Size D = Number of beetles with pathogen

(1-β) = Probability of observing at least one beetle vectoring the pathogen in a sample when the disease affects at least D/N in the population.

Based on the Rich study (2001), it was believed that if beetles vectored the pathogen, only a small percentage of the beetle population did so. As the number of beetles with the pathogen (D) in a large population (N = 1,000,000) increases, the sample size required for detection (n) decreases. In this estimation, β was set at .05, which gives a 95% certainty of seeing at least one beetle vectoring the pathogen among those sampled. For this research project, it was assumed 1 out of every 1000 beetles carried the pathogen and therefore the required sample size to detect at least one beetle with the pathogen with 95% certainty was approximately 3000 beetles (Figure 3).

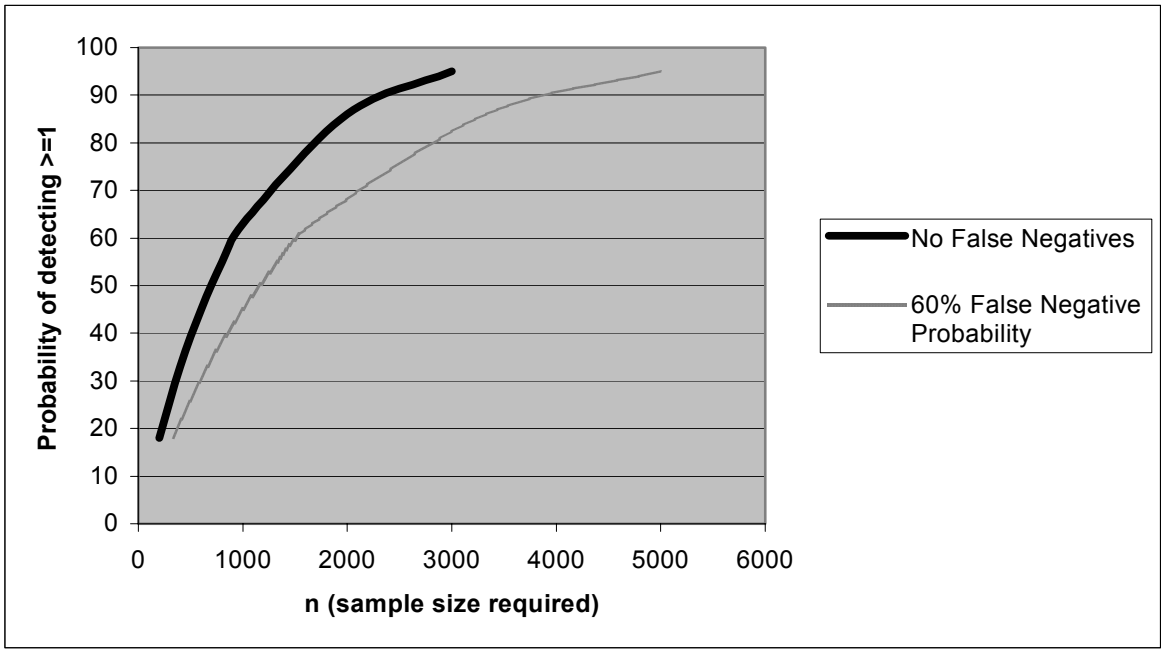

FIGURE 3. The probability of detecting at least one beetle with the pathogen based on sample size (n) with and without consideration of "false negatives" (See Discussion). Assumptions include a large population size (N) of 1,000,000 beetles and a vectoring rate of 0.1%.

Results

Association of beetles with *Phytophthora ramorum* Throughout the four-week collection period, 1,344 beetles and 157 miscellaneous insects were gathered after emerging from logs stored in greenhouse isolation chambers. The beetles collected consisted almost entirely of the species *Monarthrum scutellare* (98%) with the remainder identified as the species *Monarthrum dentiger* (2%). Logs in isolation did not generate the bark beetle, *Pseudopityophthorus pubipennis*. The ooymcyete *P. ramorum* was not detected on either species of ambrosia beetle. However, beetles were observed to carry other fungi from the Phylum Ascomycota and Basidiomycota. Phylum classifications were based on morphological characteristics of the isolated fungi (including sporangia, conidia, crosswalls, etc). Of the 157 miscellaneous insects that emerged from the logs, none were shown to be associated with the *P. ramorum* pathogen.

Association of beetle larvae with *Phytophthora ramorum* The debarking and subsequent cross sectioning of log sections did not yield larvae of *M. scutellare*, *M. dentiger*, or *P. pubipennis* in either the tunnels or egg galleries of the vascular cambium. However, three larvae of three different, unidentified species were found within phloem tunnels. *P. ramorum* was not detected on these larval bodies.

Tunnel walls, Egg Galleries and *Phytophthora ramorum* Between the two dissected logs, a total of 48 shavings were taken from egg galleries, tunnel walls, and entrance/exit holes. *P. ramorum* could not be isolated from the shavings.

Discussion

Attempts to isolate *P. ramorum* on *Phytophthora* selective PARP media from 928 whole but crushed beetles were unsuccessful. Similarly the 414 homogenized beetles did not reveal an association with the pathogen. Data from both plating techniques strongly suggest that *M. scutellare*, which accounted for 98 % of the sampled beetles, is not capable of vectoring the pathogen, *P. ramorum*. Therefore, it can be concluded that this species of ambrosia beetle is unlikely to play an important role in the dispersal of the pathogen.

Despite the overwhelming evidence that suggests that the pathogen is not associated with *M. scutellare*, there are a few factors that may have prevented the detection of the beetle-pathogen relationship. One explanation for the lack of detection may be the artificial lab conditions required for the study. Logs in the greenhouse isolation chambers at 27^{o}C were observed to be,

in some cases, under considerable stress due to desiccation as evidenced by the splitting of the wood along xylem rays. Perhaps a beetle-pathogen association exists yet went undetected because the spores of *P. ramorum* became unviable due to artificially generated environmental stress. Yet, in preliminary laboratory tests, the pathogen was shown to survive when petri dishes were exposed to 40^{o}C for 4 hours and 45^{o}C for 1 hour (M. Garbelotto, unpublished data). These findings suggest that the pathogen is particularly heat tolerant and therefore may be more likely to withstand the drought-like conditions of the greenhouse isolation chambers. On the other hand, *P. ramorum* spore viability may have been threatened by cold exposure. After emergence and prior to collection, beetles were exposed to the 4^{o}C conditions of the refrigerated storage devices. However, in an effort to maintain spore viability, the study involved semi-immediate plating (i.e. within 24 hours of emergence) which reduced cold exposure and eliminated the need for further extensive cold storage. Whether the very limited exposure to cold impacted the viability of any spores on emerged beetles is unknown and warrants further investigation.

Yet another possible factor preventing the detection of the beetle-pathogen association may be attributed to the lag time between beetle emergence and plating as strongly suggested by undergraduate researcher, Tamara Rich (2001) who in an earlier, similar study likewise explored the possibility of ambrosia beetles as vectors of the *P. ramorum* pathogen. Research suggests that on average a beetle will alight on a new potential host in a matter of minutes after emerging (Jactel, 1991). It is very difficult to mimic natural lag times between emergence and plating (equivalent to alighting), however the maximum experimental lag time of 24 hours is unlikely to have lead to spore inviability. Studies of other *Phytophthora* species have shown spore viability ranges from 7-180 days (Malajczuk, 1983). Unmistakably, further research concerning *P. ramorum* biology is required to merit this conclusion and to ensure that future experimental designs do not inadvertently undermine spore viability.

Conclusions concerning *M. scutellare* beetles cannot be extended to the beetle species *M. dentiger* and *P. pubipennis*. Only 28 of the 1,344 beetles collected were identified as *M. dentiger* and there was no emergence of *P. pubipennis*. Plating results of *M. dentiger* exhibited no evidence of *P. ramorum*. However, the sample size is inadequate to determine whether there exists a beetle-pathogen association between *M. dentiger* and *P. ramorum*. Likewise, due to a lack of data no conclusions could be made concerning *P. pubipennis*.

The minimal emergence and absence of *M. dentiger* and *P. pubipennis*, respectively, can be accounted for with a variety of explanations. One possibility may be that eggs and/or larvae of the two species perished due to their exposure to the highly desiccated environment of the greenhouse isolation chambers. Additionally, *M. dentiger* and *P. pubipennis* species may have had a low population size as a result of cyclical population dynamics commonly seen in insect populations. Density independent factors such as drought and temperature can also influence population size by reducing environmental favorability (Berryman, 1986). Examination of *M. dentiger* and *P. pubipennis* population dynamics and past environmental conditions may help to explain their absence in this study. Furthermore, it is possible that unlike *M. scutellare*, *M. dentiger* and *P. pubipennis* are not attracted to the trees exhibiting the symptoms of SOD. Further research is required concerning the chemical signals utilized by *M. scutellare*, *M. dentiger*, and *P. pubipennis* to locate and colonize host trees and whether these species are capable of locating SOD stricken oaks via some unknown, unique chemical signal.

Of the 157 miscellaneous insects that were plated in PARP media, none displayed *P. ramorum* growth. These results suggest that the "noise" level of *P. ramorum* in the environment is insignificant. The haphazard and inadvertent transport of *P. ramorum* by a cornucopia of insects does not appear likely. To verify these conclusions, a larger sample size is required.

Larvae and *Phytophthora ramorum* Debarking and cross sectioning of sample logs did not produce larvae of *M. scutellare*, *M. dentiger*, or *P. pubipennis* as desired. No conclusions could be made concerning the possibility of larvae transporting the pathogen further within the vascular cambium of the host.

The lack of larvae of all three targeted species may be attributed to the highly desiccated environment of the greenhouse isolation chambers as suggested earlier or as a result of inappropriate timing. Logs were dissected during the last week of March, a period in which daily emergence began to decline. It is possible that most larvae had already undergone complex metamorphosis and emerged. In future studies, dissection occurring earlier in the season, late January or early February, may yield more successful results. A third explanation for the lack of larvae may be attributed to the exceptionally destructive sampling techniques employed in the study. Chainsaws, hammers, and chisels were used to violently debark and section the extremely dense oak samples. These methods may have destroyed tiny and fragile larvae and

eggs located within tunnels and egg galleries. Alternative techniques to expose larvae are needed to ensure that they are not obliterated in the violence of dissection

Tunnel Walls, Egg Galleries and *Phytophthora ramorum* Attempts to isolate *P. ramorum* from tunnel walls and egg galleries were unsuccessful. These results suggest that the pathogen does not or is unable to utilize tunnels and galleries to spread throughout the vascular cambium of the host. Further research is required to better understand the biological requirements of the pathogen. Competition between microorganisms and soil-borne *Phytophthora* species for limited resources, such as nutrients, space, and oxygen, has been observed to impact both population growth and infection (Malajczuk, 1983). While having a devastating impact on oak woodland communities, *P. ramorum* may be a poor competitor against species of basidiomycetes and ascomycetes, which are commonly found within beetle galleries. Whether *P. ramorum* is excluded from tunnel colonization due to competition is unknown. Furthermore, recent research findings have suggested that oaks may be a "dead-end" host, implying that the pathogen is incapable of reproduction while inhabiting these host species (Garbelotto, unpublished).

The inability to detect *P. ramorum* within galleries and tunnels further supports the conclusion that *M. scutellare* is not a vector of the pathogen and further suggests that transmission via larvae is unlikely. These findings suggest that beetles, in order to be designated as vectors, must be exposed to and acquire pathogen spores from a location other than within the tunnels and galleries of the vascular cambium. Since larvae do not leave their tunnels until after metamorphosis, exposure to inoculum from an alternative source is doubtful.

Although the evidence suggests that the pathogen was not present within the tunnels, detection may have been impeded due to procedural flaws. As previously discussed, destructive sampling methods may have destroyed viable spores located within tunnels as a result of heat and friction generated by motorized tools as well as hand tools. To reach more confident conclusions, a less destructive dissection technique should be employed for future studies.

Preliminary statistical analysis determined an ideal sample size to be 3,000 beetles, however the study's sample size only consisted of 1,344 beetles. A consequence of this less than ideal sample size was a decrease in certainty that the test was sufficiently powerful to detect the presence of the hypothesized beetle-pathogen association. For a 95% detection certainty, 3,000 beetles were required. With a reduction in sample size to 1,344 beetles, the certainty is reduced

to 74% (Figure 3). Moreover, our certainty is further reduced if the problem of "false negatives" is factored into the analysis.

A significant problem associated with this type of research is "false negatives". A "false negative" occurs when a pathogen is present but cannot be cultured. In fact, 60% of all samples taken from SOD infected trees test negative for the pathogen (Storer et. al., 2001). If the 60% probability of "false negatives" is taken into account, to obtain 95% certainty a sample size of approximately 5,000 beetles is required. The sample size of 1,344 beetles reduces confidence to 55% when "false negatives" are considered (Figure 3).

A high probability of "false negatives" is not unique to *P. ramorum* research. A study of *Phytophthora cinnamomi* by Huberli et. al. (2000) exhibited a 11% probability of "false negatives". The high "false negative" rate suggests that in order to detect the pathogen in a population, an exceptionally large sample size is required, especially when the pathogen is in low abundance. Further research to reduce "false negative" levels is necessary. Although consideration of "false negatives" reduces confidence in the results from 74% to 55%, the findings of this project are not entirely invalidated. The underlying assumption of the project was that one out of a 1000 beetles vectored the pathogen; therefore results confirm that if beetles vector *P. ramorum*, only a very small percentage of the population participates. No conclusive statement concerning the vectoring status of the beetles can be made. In order to conclusively determine whether the beetles are vectors, an additional project with a much larger sample size would be required. There also exists the inescapable however, unlikely possibility of a 100% "false negative" rate. The possibility exists given that the "false negative" rate was not established in this project but based on findings of Storer et. al. (2001). Future studies should establish "false negative" rates independently.

Future Research Unfortunately, the dispersal mechanism of *P. ramorum* remains unknown. However, this study suggests that *M. scutellare* either does not vector the pathogen or does so at an extremely low frequency. Whether *M. dentiger* and *P. pubipennis*, two beetle species commonly found in association with weakened oaks, act as vectors was not determined. Whether beetle research concerning SOD should continue is arguable. The conclusions expressed here and in a recently published paper by Rizzo et. al. (2002) allege that beetles play at most a secondary role in the death of a host infected with SOD. Results suggest that oaks are infected by *P. ramorum* and severely weakened as result of the pathogen's girdling effects and

only then are they secondarily colonized by beetles, which may hasten host death but are not responsible for pathogen transmission. In short, ambrosia beetles and bark beetles are being pigeonholed as opportunistic organisms like the ascomycete, *Hypoxylon thouarsianum*. Although questions remain concerning the role of beetles in the SOD disease complex, efforts and resources may be better directed toward understanding alternate means of pathogen dispersal.

Phylogenetic studies further suggest that research efforts be redirected towards the exploration of other possible mechanisms of *Phytophthora* dispersal. Phylogenetic research places *P. ramorum* within the clade that includes *P. lateralis*, *P. drechsleri*, *P. cryptogea*, and *P. syringae* based on synapomorphic, morphological characters and DNA sequencing (Cooke et. al., 2000). This clade consists of soil-borne species, which are primarily dispersed via soil and water splash. *P. ramorum* is distinct from other *Phytophthora* species due to exclusive aerial biology. However, in addition to customary root cankers, aerial cankers of *P. citricola*, *P. cactorum* and *P. cinnamomi* have been observed (Mircetich, 1977). Aerial dispersal of *P. ramorum* via rain splash and wind driven rain is not an unlikely means of dispersal. In fact, J.M. Davidson (unpublished) claims to have successfully recovered *P. ramorum* from rainwater collected around diseased trees. Clearly, this mechanism of dispersal warrants further investigation.

While conclusions of this study suggest redirecting SOD research efforts towards alternate mechanisms of *P. ramorum* dispersal, it would be premature to entirely discount the potentially important role beetles may play in this epidemic. Although this study is unable to conclusively state whether beetles act as *P. ramorum* vectors, it does suggest that the beetle's role in pathogen dispersal is at most minimal. Furthermore, given the limited funding available and the continuing spread of the pathogen, research priority should be given to other possible mechanisms of dispersal. Time is running out. In fact, SOD expert, Matteo Garbelotto has likened the discovery of *P. ramorum* to "the sudden finding of a very poisonous snake that can fly" (Ritter, 2001). Future research must 'clip the wings' of this deadly and devastating pathogen and do so swiftly in order to preserve biodiversity. If this is not adequate incentive, a selfish need to protect economic interests should be inspiration enough.

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