Chapter 3: Molecular Genetics of *Phellinus noxius*

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Molecular genetics studies provide critical information to establish the scientific foundation for many conclusions reached about *Phellinus noxius*. The following are some of the more important conclusions being drawn from these studies:

- DNA sequencing provides a reliable method to identify *P. noxius.*
- *P. noxius* possesses considerable genetic variation, and the species is composed of multiple genetic groups that may display different ecological behaviors.
- *P. noxius* can spread via airborne spores and by vegetative growth through root-to-root contact.
- *P. noxius* has numerous genes that encode diverse wood-degrading enzymes (e.g., enzymes that degrade cellulose, hemicellulose, and lignin), and other enzymes that help metabolize sugars and other compounds from the degraded wood for use in hyphal growth.

Approaches in molecular genetics, metagenomics, and metabarcoding offer promise of further insights into disease caused by *P. noxius.* These genetic approaches may assist in developing disease management methods by examining pathogen inoculum levels and changes in microbial communities in response to management treatments.

The growth and spread of *P. noxius* via root-to-root contact among adjacent trees, basidiospores, or relocation through human-related activities (e.g., the movement of infected breadfruit [*Artocarpus altilis*] root suckers or infected wood products) can be readily investigated. The impact of *P. noxius* on tree health can also be observed as wood decay progresses and tree structure is weakened. Molecular genetics can also provide additional insights on pathogen detection, movement, and disease development. Currently, several approaches are available that can be applied to the study of *P. noxius*, or brown root rot disease:

- DNA-based diagnostic tools to detect and identify *P. noxius.*

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- Phylogenetic studies to determine genetic groups and evolutionary relationships within *P. noxius.*
- Population genetic studies to determine gene flow and geographic movement of *P. noxius.*
- Genomic and transcriptomic studies into pathogenicity genes and other genes associated with growth and survival of *P. noxius.*
- Soil metagenomics and metatranscriptomic studies to examine soil and rhizosphere levels of *P. noxius*, biocontrol agents, and other associated soil microbes and their biological functions, and their interactions with environmental factors.

Recent applications of these molecular genetic approaches to study *P. noxius*, along with information generated from these applications, will be briefly covered in this chapter.

DNA-Based Diagnostic Methods to Detect and Identify *Phellinus noxius*

Fungal identifications for our survey of *P. noxius* on all the U.S.-Affiliated Pacific Islands included in this report (except Chuuk) were based on isolation and culture of butt-rot fungi from various forest trees, the isolation of DNA from these cultures, and the subsequent use of the appropriate DNA sequencing procedures to identify the species of fungus involved, especially *P. noxius*. Details on this process are provided below.

These fungi were isolated from recently infected woody tissue or basidiocarps (sporocarps, fruiting bodies, fructifications, conks, sporocarps, or basidiomata). Resupinate basidiocarps were often plentiful, relatively easy to find, and distinctive enough for observational identification (figs. 2.5 and 2.6). Conks, or shelf-like basidiocarps, were less common, but distinctive and more commonly found after periods of heavy rain (figs. 2.3 and 2.4). The chance of finding these conks can be improved by burrowing into the base of stumps killed by *P. noxius*-induced brown root rot disease. The heartwood at the base of the tree appears most susceptible to decay, so a hollow chamber is commonly formed in the center of the stump and below the ground line. The dark, humid conditions in these chambers are apparently conducive to conk production. Basidiocarps are optimal material for isolating *P. noxius*, according to plant pathologists Yuko Ota and Norio Sahashi, who have dedicated much of their professional careers to studying this fungal pathogen.

To isolate and establish *P. noxius* in culture for DNA extraction, Ota and Sahashi begin by surface sterilizing a small portion of the basidiocarp. Then they excise a small piece (ca. 5 × 2 × 1 mm) of this basidiocarp and use this to inoculate the culture medium. A preferred medium for isolating this fungus was developed by Tsai et al. (2017); it consists of potato dextrose agar (PDA) with 100 mg/L streptomycin and 10 mg/L benomyl. After a *P. noxius* isolate is established in the culture, it is subcultured onto the surface of a cellophane/nylon membrane that is then placed on top of the medium in a Petri plate. After about 1 week in culture at 28 °C, about 0.5 g of mycelium is collected by gently scraping the membrane surface and placing it in either liquid nitrogen or a DNA extraction buffer. After grinding the mycelium, DNA is then extracted using various methods, such as a CTAB (hexadecyltrimethylammonium bromide) method (Ibarra Caballero et al. 2019) or various commercially available DNA-extraction kits, such as the ZR Fungal/Bacterial DNA MiniPrep Kit™ (Zymo Research, Irvine, California) (Stewart et al. 2020, Tzean et al. 2016).

DNA-based detection and identification of *P. noxius* frequently relies on the use of polymerase chain reaction (PCR), which amplifies (multiplies) the desired target region of DNA. Standard target regions include the 28S region of rDNA (nuclear large subunit; LSU) and internal transcribed spacers 1 and 2 with 5.8S regions of rDNA (ITS) regions. These regions can be amplified using primer pairs LR0R (Rehner and Samuels 1994)/LR5 (Vilgalys and Hester 1990) and ITS1/ITS4 (White et al. 1990), respectively. For specific detection of *P. noxius*, the primer pair GIF (5'-GCCCTTTCCTCCGCTTATTG-3') and GIR (5'CTTGATGCTG-GTGGGTCTCT-3') have been used to amplify a specific 653-bp DNA fragment within the ITS region (Wu et al. 2009). PCR products (amplicons) are confirmed using agarose gel electrophoresis or an automated DNA fragment analyzer. Subsequently, PCR products are treated, by enzyme-mediated inactivation of excess dNTPs and primers (e.g., ExoSAP-IT® PCR Product Cleanup) in preparation for sequencing of the target region. DNA sequencing is typically performed by various commercial companies that offer this service (Stewart et al. 2020). The DNA sequences of the target regions (e.g., LSU, ITS) from each fungal isolate are compared to sequences from GenBank with the basic local-alignment search tool (BLASTN), to confirm the identification of *P. noxius* to species level. This DNA-based diagnostic approach, when used on the fungal isolates we collected as described in chapter 2, helped reveal that *P. noxius* was more prevalent on the U.S.-Affiliated Pacific Islands (i.e., American Samoa, Commonwealth of the Northern Mariana Islands [CNMI], Federated States of Micronesia [FSM], Guam, and Republic of Palau) than previously reported.

Phylogenetic Studies to Define Genetic Groups Within *Phellinus noxius*

Recently, phylogenetic studies were conducted on a large set of *P. noxius* isolates from geographically diverse locations, including the U.S.-Affiliated Pacific Islands, Australia, and eastern Asia (Stewart et al. 2020). This study was notable because it included 95 isolates from various U.S.-affiliated island states: American Samoa, CNMI (Saipan), Guam, FSM (Kosrae, Pohnpei, and Yap), and Palau. The other isolates in the study were from Japan, China (Taiwan and Hong Kong), Malaysia, and Australia. For each isolate, genetic sequencing was attempted at four loci: LSU; ITS; partial translation elongation factor -1 alpha (*tef1*); and partial RNA polymerase II, second largest subunit (*rpb2*). Details of this investigative process are provided in Stewart et al. (2020). Some important results of these analyses are shown in figures 3.1 and 3.2.

DNA sequences from each locus, such as ITS and 28S, are useful for phylogenetic analyses and can determine evolutionary and genetic relationships among the *P. noxius* isolates (Stewart et al. 2020). Additional DNA sequences for comparison are usually available in public databases, such as GenBank (https://www.ncbi.nlm.nih.gov/genbank/), or in association with published studies (Chung et al. 2017, Tsai et al. 2017). Various approaches and software are available for DNA sequence-based phylogenetic analyses. Leung et al. (2020) explain how to diagnose brown root rot disease and highlight phylogenetic variation among *P. noxius* isolates.

As discussed in the previous chapter, symptoms, and especially signs, are primary indicators of *P. noxius* as the cause of a disease, or mortality. However, morphological characteristics provide little or no evidence of genetic variation within *P. noxius* or the virulence of a given isolate. In their genetic analyses of *P. noxius* isolates, Stewart et al. (2020) used a *tef1*-based phylogeny to distinguish genetic groups of *P. noxius* (fig. 3.2). Some interesting patterns emerged from that analysis. For example, one haplotype (H3), present in Palau, Pohnpei, Kosrae, Saipan and Guam, was also present on the Ogasawara Islands of Japan. In addition, some *P. noxius* isolates from Guam and Saipan also appeared to be closely related, as would be expected because of their close geographic proximity. When haplotypes are identical or very similar on different islands, it suggests that *P. noxius* from one island may have been moved, or moved itself via spores, to another island. However, obtaining many *P. noxius* samples from both islands would help to determine on which island the fungus originated.

Figure 3.2 also shows that *P. noxius* isolates from western Pacific regions were contained within three widely defined clades: eastern Asia (Japan; Taiwan and Hong Kong, China; and Malaysia); western Oceania/Japan/Taiwan (including Australia

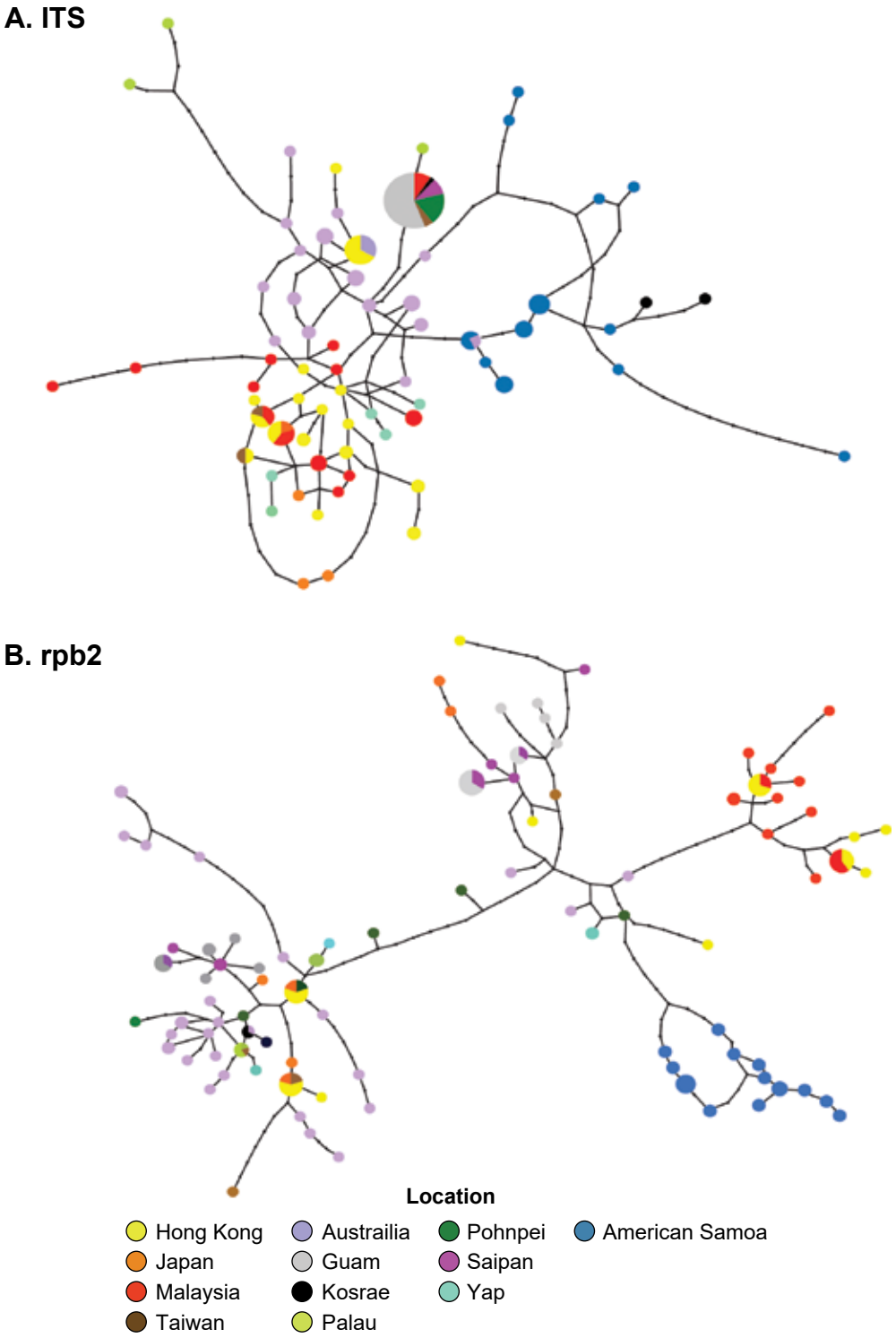

Figure 3.1—Haplotype networks of *Phellinus noxius* based on partial RNA polymerase II and second largest subunit (*rpb2*) concatenated sequences. Circle color represents origin of the isolates. Circle size is proportional to the number of isolates in each haplotype. Small, solid dots represent inferred intermediate haplotypes in the network. Pie charts indicate proportional representation of haplotypes. The number of mutations separating the haplotypes are reflected as segment lengths connecting the circles. (Figure adapted from Stewart et al. 2020)

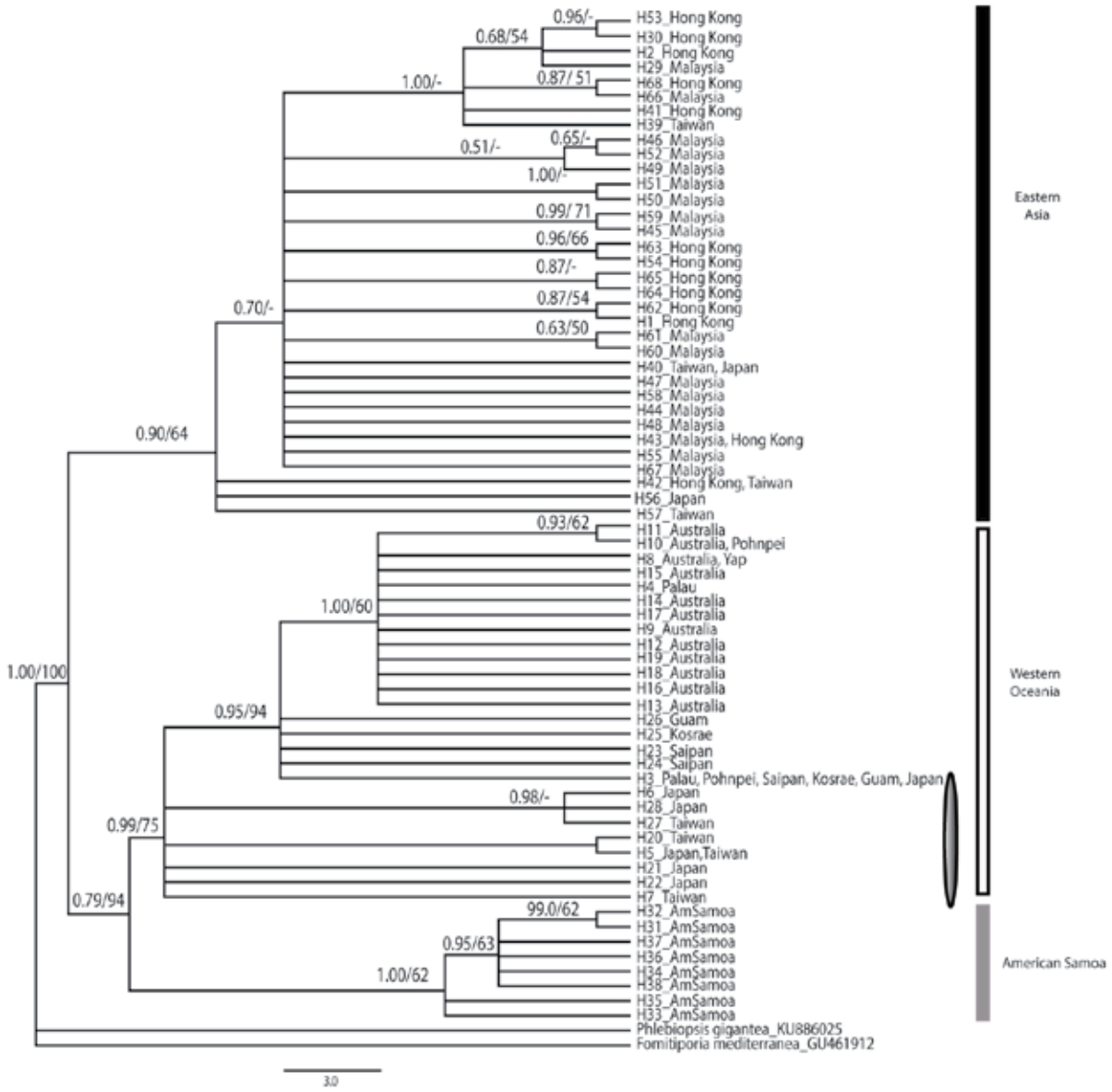

Figure 3.2—Phylogeny of *Phellinus noxius* isolates, based on Bayesian analysis of partial translation factor -1 alpha (*tef1*) sequences, with *Phlebiopsis gigantea* and *Formitoporia mediterranea* serving as outgroups. Node support is indicated as posterior probability/bootstrap. Distinct clades are presented as bars representing eastern Asia, Oceania/Japan/Taiwan, and American Samoa. Some isolates from Japan and Taiwan are grouped within the clade representing western Oceania isolates, which is illustrated by the vertical oval. (Figure from Stewart et al. 2020)

and the U.S.-Affiliated Pacific Islands of Palau, Guam, Saipan, Yap, Pohnpei, and Kosrae); and a distinct southern Oceania group from American Samoa. Isolates from one clade may behave more similarly ecologically, compared to isolates in either of the other two clades. For example, isolates from the genetically distinct American Samoa clade might be expected to appear and behave similarly, compared to isolates from the eastern Asia or western Oceania/Japan/Taiwan clades, but this has not yet

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Figure 3.3—The fascinating growth pattern of a thick, black, mucilaginous, mycelial crust of *Phellinus noxius* (known locally as "limu mea") growing up the outside of a ylang-ylang (*Cananga odorata*) tree (known locally as "moso'oi") near American Samoa Community College. The growth continually expands radially and eventually envelops the entire base of the tree. Each ridge of growth seems to correspond to a rainfall event and high humidity, which are common in American Samoa and probably conducive to the growth of this fungus.

been confirmed. The general appearance (e.g., mycelial growth pattern) of *P. noxius* on American Samoa is exemplified in figure 3.3, which appears quite different from the *P. noxius* in Pohnpei (fig. 1.1). It would be worthwhile to determine if responses to control measures are influenced by phylogenetic differences among *P. noxius* isolates. To date, no studies other than bioclimatic modeling have been conducted to determine if ecological behaviors are associated with distinct clades of *P. noxius*.

Population Genetic Studies Can Determine Gene Flow and Movement of *Phellinus noxius*

Population genetics is a subfield of genetics that considers genetic comparisons within and among populations. Population genetic studies of *P. noxius* are of interest because they can provide insight into the movement and spread history of this pathogen. For example, the genetic diversity of a fungus is expected to be high in the area where it originated. Conversely, the fungus typically has less genetic diversity in areas where it was recently introduced. This phenomenon is referred to as a genetic bottleneck, or "founder effect" (Dean and Ballard 2004) and occurs when all the fungal genotypes in a population are not introduced to a new region. Based on this principle, the area that has the larger number of fungal genotypes is considered as a potential source of the fungus. Furthermore, the pattern of fungal genotypes in each region can provide insight into direction of spread for the fungus. These principles are based on the assumptions that all, or most, of the genetic variation in the invaded locations are a subset of all the genetic variation from the source location.

Population genetics can also be used to determine other aspects associated with spread of the fungus. For example, vegetative or clonal (nonsexual) spread would result in a fungus at one location having the same genotype as a fungus at another

location. In the case of root-rot fungi, the same fungal genotypes on adjacent trees is an indication of growth of the mycelium from an infected tree growing onto the root of previously healthy trees. Distant trees with the same fungal genotype suggest spread by the physical movement of the vegetative fungus, including human-mediated transport, such as moving *P. noxius*-infected root suckers from one location to another or placing infected wood near a previously healthy tree.

Sexual reproduction and the spread of basidiospores, however, is suggested when the fungal genotype identified on one tree is distinct from the genotype found on another tree that is growing relatively close to the other infected tree.

Recent population genetic studies of *P. noxius* in Taiwan and Japan have produced interesting insights. From sequence-based analyses of DNA from *P. noxius* samples in Taiwan, fungal dispersal was attributed to both vegetative and basidiospore spread, and the highly diverse genotypes within the *P. noxius* populations attributed to a bipolar heterothallic reproductive system (Chung et al. 2015, 2017). Vegetative transmission has generally occurred through tree-to-tree spread via contact between separate root systems. Transmission by basidiospores was evident in new infection foci with different genotypes than those associated with other neighboring infection foci. Studies with invasive *Heterobasidion irregulare* basidiospores in Europe showed that although most airborne spores cause new infections generally close to the source (ca. 1.3 km year^{-1} or 0.8 mi year^{-1} (Gonthier et al. 2007), some spores, however, can apparently travel much farther (Gonthier et al. 2014), suggesting that *P. noxius* basidiospores might survive long-distance dispersal. In general, *P. noxius*-infected trees growing close to each other are most likely a result of vegetative spread through their roots. Conversely, separate areas of infection are most likely caused by sexually produced basidiospores (Akiba et al. 2015, Hattori et al. 1996). Understanding the mechanisms by which disease spreads is needed to develop and apply the most effective methods for monitoring and controlling *P. noxius* (see management section, chapter 4).

Focused population genetic studies of *P. noxius* specifically, have not yet been conducted for individual U.S.-Affiliated Pacific Islands, in part because the number of samples collected to date have been too small to support a robust study. When more samples are available, population genetic studies will help clarify the spread and ecology of *P. noxius*, both within an island and elsewhere. As tree-to-tree spread is often evident (fig. 2.11) and as basidiocarps are found on most/all the U.S.-Affiliated Pacific Islands, these transmission mechanisms (vegetative and airborne basidiospores) are both likely occurring in these regions. Until more definitive information is available, control measures for any island should consider that *P. noxius* is spreading both vegetatively by root-to-root contact and by airborne basidiospores.

In a previous study, 43 *P. noxius* samples were collected from some U.S.-Affiliated Pacific Islands (Stewart et al. 2020), and phylogenetic relatedness among these samples was discussed in the previous section of this chapter. However, more samples of *P. noxius* from each location and higher resolution genetic markers (e.g., RAD-seq: Restriction site Associated DNA Sequencing) are needed to determine the history of how *P. noxius* has moved throughout the Pacific regions. Important questions are yet to be addressed. For example, *P. noxius* exists in the Philippines (CABI: 2021), and wood pallets came to Guam, Yap, and Saipan from the Philippines after World War II. Isolates of *P. noxius* from the Philippines, however, have not been collected and genetically analyzed so it remains undetermined if the Philippines could have been the source of *P. noxius* on Guam, Yap, and Saipan. Similarly, isolates of *P. noxius* from Saipan and the Ogasawara Islands of Japan appear to be genetically related (Stewart et al. 2020). A more focused study is needed to determine if the fungus was transferred from Saipan to the Ogasawara Islands of Japan or vice-versa. To answer critical questions about the evolution and movement of *P. noxius* among the Pacific Islands, larger numbers of *P. noxius* samples and high-resolution genetic markers are needed to identify the genetic bottlenecks, gene flow, and evolutionary history of this pathogen.

Population genetics can also be used in conjunction with bioclimatic modeling to predict geographic locations with suitable climatic conditions for a population to survive. To conduct bioclimatic modeling, the precise latitude and longitude for each fungal isolate is needed. A bioclimatic model of 179 locations of *P. noxius* isolates from eastern Asia, Australia, and the U.S.-Affiliated Pacific Islands used 19 climatic variables to predict the global regions that are at risk from different genetic groups of *P. noxius*. These regions include a large portion of the South American continent, Southeastern United States (e.g., Florida) and Hawaii, some parts of western Africa, eastern Asia, the eastern Australia coast, and many of the Pacific Islands (Stewart et al. 2020). Figure 3.4 shows that the differences in predicted distribution for the two genetic groups (eastern Asia for fig. 3.4c and western Oceania/Japan/Taiwan for fig. 3.4d) may reflect differences in ecological behavior between these two genetic groups. These data strongly suggest that different genetic groups of *P. noxius* pose distinct invasive threats. Geographic areas should consider different genetic groups of *P. noxius* and their suitable climate space when developing regulations to prevent the importation of invasive pathogens. For example, Florida and Hawaii are predicted to have a suitable climate for *P. noxius*, though the pathogen has not been reported in either region. This example shows how genetic characterization of forest pathogens coupled with bioclimatic modeling can help manage forest diseases. These types of information can be used to establish quarantine and other regulatory policies for management of *P. noxius*-caused brown root rot disease.

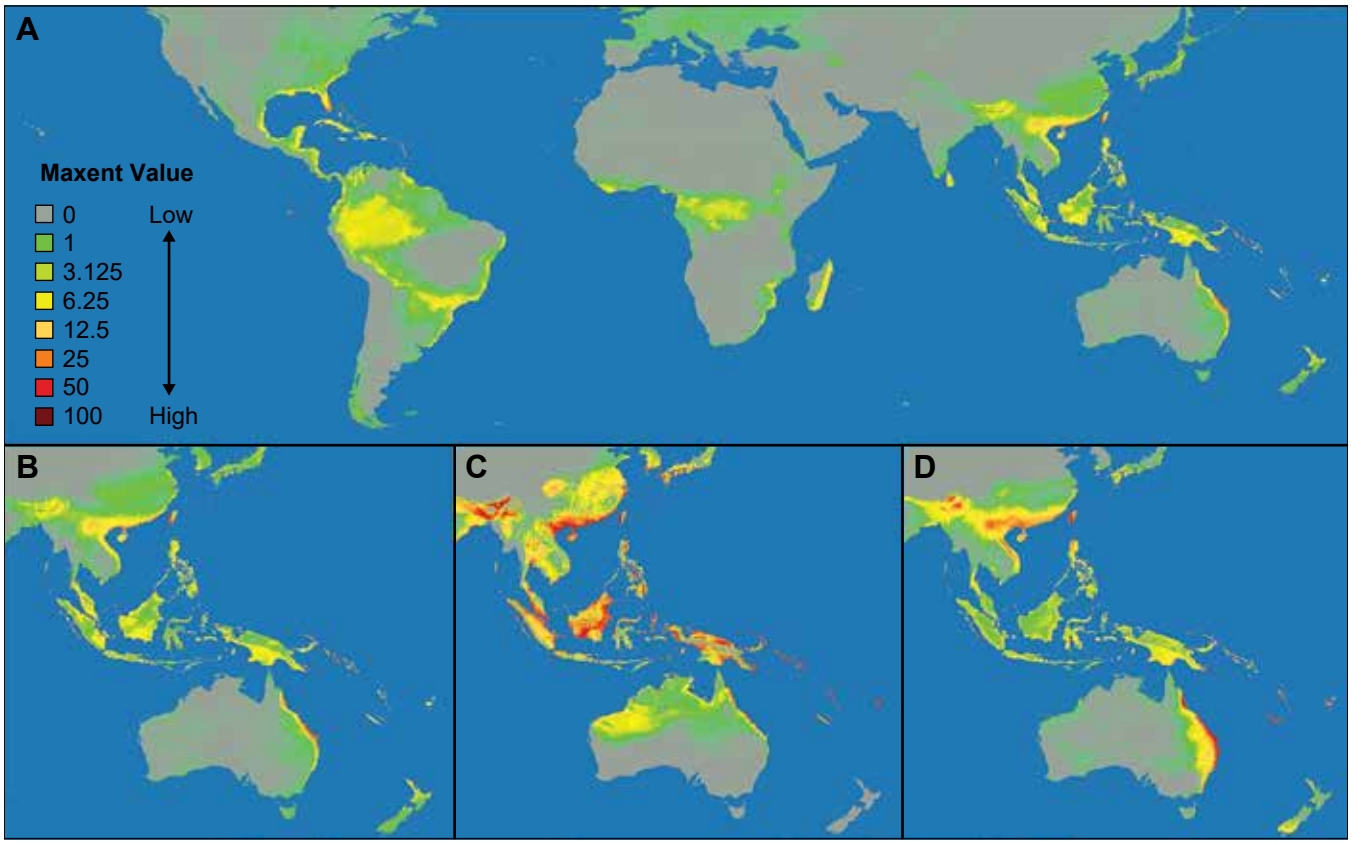

Figure 3.4—Predicted potential distribution (suitable climate space) of *Phellinus noxius* based on Maxent bioclimatic models. Maps 3.4a (global) and 3.4b (regional), 3.4c = eastern Asia (Group 1), and 3.4d = western Oceania/Japan/Taiwan (Group 2), are all based on confirmed occurrences of the *P. noxius*. Increasing Maxent values (probability) of suitable climate space (climate niches) for *P. noxius* are reflected as color shades, with increasing probability as colors range from green to red (figure from Stewart et al. 2020).

Genomic and Transcriptomic Studies Can Provide Insights Into Genes of Interest

Several institutions have conducted genomic sequencing of *P. noxius* (Ibarra Caballero et al. 2020). These efforts have improved our understanding of why *P. noxius* can attack such a wide range of tree species and cause rapid tree death and decomposition. Collaborative work among institutions and comparisons of *P. noxius* genomes have provided insights into its ecology, pathogenicity, and ability to cause bark and wood decomposition on living trees (Ibarra Caballero et al. 2020). Researchers discovered that some isolates were more aggressive than others in Taiwan, and then identified enzyme-encoding genes associated with increased tree mortality and wood decomposition (Chung et al. 2017, Ibarra Caballero et al. 2020). Chung et al. (2017) also determined that 74 percent of the predicted genes of *P. noxius* are the same as at least one other basidiomycete.

Genomic sequencing revealed that *P. noxius* has at least 488 genes that encode the production of carbohydrate- and lignin-metabolizing enzymes. The number of

carbohydrate-active enzymes (CAZymes) produced by *P. noxius* is significantly higher than the currently known number possessed by other related fungi. These genomic features of *P. noxius* are assumed to be responsible for its wide host range, including tree species with highly varied wood properties, and its capacity to quickly kill and decompose these hosts (Ibarra Caballero et al. 2020).

Enzymes associated with lignin degradation are among the enzymes encoded within the *P. noxius* genome. These enzymes contribute to the ability of *P. noxius* to cause a white rot (a rot caused by the degradation of all major structural components of wood: cellulose, hemicellulose, and lignin) and allow the fungus to attack and decompose a wide array of hardwood and coniferous trees.

The use of both genomics and transcriptomics can identify the genes in a fungus and determine which genes are expressed under specific developmental and environmental conditions, such as wood colonization and degradation. Before discussing these studies, it is important to understand why wood decomposition is a critical biological function.

Wood is an attractive substrate to micro-organisms such as fungi because it is so abundant (385 gigatons worldwide) (Bar-On et al. 2018). As wood forms (one of the results of photosynthesis), a tremendous amount of the sun's energy is captured and stored as carbon in the form of cellulose, hemicellulose, and lignin. This energy is available to any organism that can overcome a tree's defenses and successfully degrade these compounds.

Cellulose and hemicellulose are basic molecules. They are a linkage of many glucose-derived molecules into one large molecule. Although lignin is also composed of glucose-based molecules, it includes other components, and its complex structure is in turn bonded with cellulose and hemicellulose. Fungi that can decompose wood and derive energy for their own metabolism must therefore possess the capacity to decompose the bonds within cellulose, hemicellulose, or lignin. Fungi that are only able to decompose cellulose and hemicellulose create a brown rot because of the remaining lignin which is brown. In contrast, some fungi have additional wood-degrading enzymes and can degrade all three components of wood, leaving a whitish residue. *Phellinus noxius* is one of these fungi, which are known as white rot fungi.

Ibarra Caballero et al. (2020) have done extensive studies on genes encoding carbohydrate-decomposing enzymes in three *P. noxius* isolates from three geographic areas: Pohnpei, Hong Kong, and Japan. They found many enzymes contributing to the decomposition of cellulose, hemicellulose, and lignin (table 3.1). Collectively, these enzymes allow *P. noxius* to decompose the woody structures of diverse tree species.

We can only speculate why *P. noxius* has genes encoding so many different wood-degrading enzymes. One hypothesis is that tropical and subtropical forests are typically composed of dozens to hundreds of tree species with diverse wood properties and defense systems. While *P. noxius* likely requires one set of enzymes to attack/decompose one tree species under certain environmental conditions effectively, it likely requires a slightly different set of enzymes to attack/decompose another tree species under certain environmental conditions. The capacity to attack different tree species would be especially useful for *P. noxius*, which spreads by root-to-root contact within forests where the neighboring trees are frequently of a different species. Another speculation is that the extensive arsenal of *P. noxius* enzymes may allow it to overwhelm defense systems of some susceptible tree species (e.g., flame tree [*Delonia regia*]), which would also allow it to develop sufficient inoculum potential to easily infect other tree species.

Another interesting feature of *P. noxius* that could favor its rapid growth rate and spread is that it carries seven times more copies of the beta-glucan synthase gene than other basidiomycetes (Chung et al. 2017). This is consistent with the observation by Ann et al. (2002) that this fungus can grow about seven times faster than other basidiomycetes. Note: 1,3-Beta-glucan synthase catalyzes the formation of a beta-1,3-glucan polymer that is a major component of the fungal cell wall. The reaction catalyzed is as follows:

UDP-glucose + {(1,3)-beta-D-glucosyl}(N) = UDP + {(1,3)-beta-D-glucosyl}(N+1).

Therefore, not only is *P. noxius* well equipped with genes encoding enzymes that break down all components of wood, it is also able to use these products of wood degradation to rapidly synthesize its own hyphae and other tissues. Genomic and transcriptomic studies provide additional molecular evidence of potential invasive risks of *P. noxius* associated with the infection and degradation of diverse woody species.

Soil Metagenomic and Metatranscriptomic Studies

Soil metagenomic and metatranscriptomic methods can increase our understanding of soil microbial communities and their ecological function by determining the microbes present, their associated genes, and gene expression in relation to the environment (Stewart et al. 2018, 2021). These methods have not yet been used to understand the *P. noxius* pathosystem but could provide valuable information for management of brown root rot disease. These techniques were recently applied to *Armillaria solidipes*, the cause of Armillaria root disease in the United States. They

Table 3.1—Predicted secreted proteins, proteases, and plant cell wall (PCW) carbohydrate- and lignin-degrading enzymes annotated in *Phellinus noxius* genome assemblies

Enzyme	*P. noxius* isolates[a]		
	P919-02W.7	OVT-YTM/97	FFPR1411160
Total secreted proteins	562	497	518
Small secreted proteins	319	274	273
Proteases	61	54	64
PCW carbohydrate-degrading enzymes	112	102	106
Cellulose degrading	52	44	45
Hemicellulose degrading	31	35	32
Pectin degrading	38	34	38
Lignin- and lignin-derivative-degrading enzymes	30	26	28
AA1 Laccases	8	7	7
AA2 Peroxidases	13	9	12
AA3	5	5	4
AA4	0	0	0
AA5	3	2	2
Cytochrome P450	1	3	3

[a] *P. noxius* isolates from Pohnpei, P919-02W.7; Hong Kong, YTM/97; and Japan, FFPR141160.
Source: Ibarra Caballero et al. 2020.

provided information for the potential biological control of Armillaria root disease (Lalande 2019, Stewart et al. 2021). In general, metagenomics involves sequencing and analyses of DNA from all life forms, especially microbes, found in a soil or wood sample. This process can determine which microbial species are present, the relative level of each microbial species, and which genes are expressed under environmental conditions at a given place and time. This information is then used to assess microbial activities associated with healthy and diseased forest trees, which is explained further by Lalande et al. (2019) and Stewart et al. (2021).

Metabarcoding uses diagnostic DNA sequences to identify and establish the relative levels of each microbial species in a sample. It can also be used to determine the influence of various management techniques on the inoculum density of pathogens, such as *P. noxius*. Metabarcoding can examine the environmental or management influences on native microbes associated with natural biological control methods. This approach may be useful in developing tactics to manage brown root rot disease. An advantage of this approach is that not only can it determine if *P. noxius* is present in a soil or rhizosphere sample, it also can provide a quantitative estimate of how much *P. noxius* DNA is present. A limitation of this technique for *P. noxius* is that this fungus has never been isolated from soil (Wu et al. 2020). Therefore, metabar coding for *P. noxius* may require samples from roots and the

decayed remnants of root systems. Other applications of metagenomics are discussed in the management section in chapter 4 below, including its potential use for estimating the amount of *P. noxius* inoculum remaining in the soil after a treatment (e.g., fungicides, urea applications, pH changes).

Metatranscriptomics is based on the extraction and sequencing of mRNA produced by all life forms in a sample. It allows an examination of gene expression within microbial communities at specific time points. This approach can provide critical information on the ecological functions of microbes in relation to environmental conditions and the conditions that support healthy ecological functions within forests, which can help develop management approaches for brown root rot disease and maintaining healthy forests.

References

Ahmad Nazarudin, M.R.; Mohd Farid, A. 2019. *In vitro* evaluation of Paclobutrazol against selected pathogenic soil fungi. Journal of Tropical Plant Physiology. 11(2): 13–21.

Akiba, M.; Ota, Y.; Tsai, I.J.; Hattori, T.; Sahashi, N.; Kikuchi, T. 2015. Genetic differentiation and spatial structure of *Phellinus noxius*, the causal agent of brown root rot of woody plants in Japan. PLoS One. 10(10): e0141792. https://doi.org/10.1371/journal.pone.0141792.

Ann, P.-J.; Chang, T.-T.; Ko, W.-H. 2002. *Phellinus noxius* brown root rot of fruit and ornamental trees in Taiwan. Plant Disease. 86(8): 820–826. https://doi.org/10.1094/PDIS.2002.86.8.820.

Bar-On, Y.M.; Phillips, R.; Milo, R. 2018. The biomass distribution on Earth. Proceedings of the National Academy of Sciences, USA. 115(25): 6506–6511. https://doi.org/10.1073/pnas.1711842115.

Bondartseva, M.A.; Herrera, S. 1992. *Phellinidium noxium* (Corner). Mikologiya I Fitopatologiya. 21(1): 13. In Russian.

Brooks, F.E. 2002a. Brown root rot. The Plant Health Instructor. https://doi.org/10.1094/PHI-I-2002-0923-01.

Brooks, F.E. 2002b. Brown root rot disease in American Samoa's tropical rain forests. Pacific Science. 56(4): 377–387. https://doi.org/10.1353/psc.2002.0031.

Cannon, P.G. 2013. Forest pathology in Micronesia. 84 p. Unpublished report. On file with: U.S. Department of Agriculture, Forest Service, Pacific Northwest Research Station, Forestry Sciences Laboratory, 3200 SW Jefferson Way, Corvallis, OR 97331.

Cannon, P.G. 2014. Forest pathology visit to American Samoa. 14 p. Unpublished report. On file with: U.S. Department of Agriculture, Forest Service, Pacific Northwest Research Station, Forestry Sciences Laboratory, 3200 SW Jefferson Way, Corvallis, OR 97331.

Cannon, P.G. 2015. Forest pathology in the Pacific (FSM, CNMI, Guam, and Hawaii). 74 p. Unpublished report. On file with: U.S. Department of Agriculture, Forest Service, Pacific Northwest Research Station, Forestry Sciences Laboratory, 3200 SW Jefferson Way, Corvallis, OR 97331.

Cannon, P.G. 2017. *Phellinus noxius* and other activities in Hong Kong, Micronesia and CNMI during March/April of 2017. 54 p. Unpublished report. On file with: U.S. Department of Agriculture, Forest Service, Pacific Northwest Research Station, Forestry Sciences Laboratory, 3200 SW Jefferson Way, Corvallis, OR 97331.

Cannon, P.G. 2019. Highlights of the *Phellinus noxius* trip to Taiwan, May 18–June 25, 2019. 14 p. Unpublished report. On file with: U.S. Department of Agriculture, Forest Service, Pacific Northwest Research Station, Forestry Sciences Laboratory, 3200 SW Jefferson Way, Corvallis, OR 97331.

Centre for Agriculture and Bioscience International [CABI]. 2021. *Phellinus noxius* (brown tea root disease). In: Invasive Species Compendium. https://cabi.org/isc/datasheet/40154. (11 December 2020).

Chang, T.-T. 1995. A selective medium for *Phellinus noxius*. European Journal of Forest Pathology. 25(4): 185–190. https://doi.org/10.1111/j.1439-0329.1995.tb01002.x.

Chang, T.-T. 1996. Survival of *Phellinus noxius* in soil and in the roots of dead host plants. Phytopathology. 86(3): 272–276. https://doi.org/10.1094/Phyto-86-272.

Chang, T.-T.; Chang, R.J. 1999. Generation of volatile ammonia from urea fungicidal to *Phellinus noxius* in infested wood in soil under controlled conditions. Plant Pathology. 48(3): 337–344. https://doi.org/10.1046/j.1365-3059.1999.00349.x.

Chou, H.; Xiao, Y.-T.; Tsai, J.-N.; Li, T.-T.; Wu, H.-Y.; Liu, L.-Y.D.; Chung, C.-L. 2019. In vitro and in planta evaluation of *Trichoderma asperellum* TA as a biocontrol agent against *Phellinus noxius*, the cause of brown root rot disease of trees. Plant Disease. 103(11): 2733–2741. https://doi.org/10.1094/PDIS-01-19-0179-RE.

Chung, C.-L.; Huang, S.-Y.; Huang, Y.-C.; Tzean, S.-S.; Ann, P.-J.; Tsai, J.-N.; Yang, C.-C.; Lee, H.-H.; Huang, T.-W.; Huang, H.-Y.; Chang, T.-T.; Lee, H.-L.; Liou, R.-F. 2015. The genetic structure of *Phellinus noxius* and dissemination pattern of brown root rot disease in Taiwan. PLOS One. 10(10): e0139445. https://doi:10.1371/journal.pone.0139445.

Chung, C.-L.; Lee, T.J.; Akiba, M.; Lee, H.-H.; Kuo, T.-H.; Liu, D.; Ke, H.-M.; Yokoi, T.; Roa, M.B.; Lu, M.-Y.J.; Chang, Y.-Y.; Ann, P.-J.; Tsai, J.-N.; Chen, C.-Y.; Tzean, S.-S.; Ota, Y.; Hattori, T.; Sahashi, N.; Liou, R.-F.; Kikuchi, T.; Tsai, I.J. 2017. Comparative and population genomic landscape of *Phellinus noxius*: a hypervariable fungus causing root rot in trees. Molecular Ecology. 26(22): 6301–6316. https://doi.org/10.1111/mec.14359.

Corner, E.J.H. 1932. The identification of the brown root fungus. The Gardens' Bulletin; Straits Settlements. 5(12): 317–350.

Daly, C.; Halbleib, M. 2001. Pacific Islands (Pohnpei) average monthly and annual temperature and mean dewpoint temperature, 1971 - 2000 (oregonstate.edu) (islandatlas.org).

Daly, C.; Nielson, R.P.; Phillips, D.L. 1994. A statistical topographic model for mapping climatological precipitation over mountainous terrain. Journal of Applied Meteorology. 33: 140–158. https://doi.org/10.1175/1520-0450(1994)033<0140:ASTMFM>2.0.CO;2.

Dean, M.D.; Ballard, J.W.O. 2004. Linking phylogenetics with population genetics to reconstruct the geographic origin of a species. Molecular Phylogenetics and Evolution. 32(3): 998–1009. https://doi.org/10.1016/j.ympev.2004.03.013.

Fu, C.-H.; Hu, B.-Y.; Chang, T.-T.; Hsueh, K.-L.; Hsu, W.-T. 2012. Evaluation of dazomet as a fumigant for the control of brown root rot disease. Pest Management Science. 68(7): 959–962. https://doi.org/10.1002/ps.3303.

Gonthier, P.; Anselmi, N.; Capretti, P.; Bussotti, F.; Feducci, M.; Giordano, L.; Honorati, T.; Lione, G.; Luchi, N.; Michelozzi, M.; Paparatti, B.; Sillo, F.; Vettraino, A.M.; Garbelotto, M. 2014. An integrated approach to control the introduced forest pathogen *Heterobasidion irregulare* in Europe. Forestry. 87(4): 471–481. https://doi.org/10.1093/forestry/cpu015.

Gonthier, P.; Nicolotti, G.; Linzer, R.; Guglielmo, F.; Garbelotto, M. 2007. Invasion of European pine stands by a North American forest pathogen and its hybridization with a native interfertile taxon. Molecular Ecology. 16: 1389–1400. https://doi.org/10.1111/j.1365-294X.2007.03250.x.

Hattori, T.; Abe, Y.; Usugi, T. 1996. Distribution of clones of *Phellinus noxius* in a windbreak on Ishigaki Island. European Journal of Plant Pathology. 26(2): 69–80. https://doi.org/10.1111/j.1439-0329.1996.tb00711.x.

Hodges, C.S.; Tenorio, J.A. 1984. Root disease of *Delonix regia* and associated tree species in the Mariana Islands caused by *Phellinus noxius*. Plant Disease. 68(4): 334–336. https://doi.org/10.1094/PD-69-334.

Ibarra Caballero, J.R.; Ata, J.P.; Leddy, K.A.; Glenn, T.C.; Kieran, T.J.; Klopfenstein, N.B.; Kim, M.-S.; Stewart, J.E. 2020. Genome comparison and transcriptome analysis of the invasive brown root rot pathogen, *Phellinus noxius*, from different geographic regions reveals potential enzymes associated with degradation of different wood substrates. Fungal Biology. 124(2): 144–154. https://doi.org/10.1016/j.funbio.2019.12.007.

Ibarra Caballero, J.R.; Jeon, J.; Lee, Y.-H.; Fraedrich, S.; Klopfenstein, N.B.; Kim, M.-S.; Stewart, J.E. 2019. Genomic comparisons of the laurel wilt pathogen, *Raffaelea lauricola*, and related tree pathogens highlight an arsenal of pathogenicity related genes. Fungal Genetics and Biology. 125: 84–92. https://doi.org/10.1016/j.fgb.2019.01.012.

Jayasuriya, K.E.; Thennakoon, B.I. 2007. Biological control of *Rigidoporus microporus*, the cause of white root disease in rubber. Ceylon Journal of Science (Biological Sciences). 36(1): 9–16.

Johnson, B.; Niederholzer, F.; Milliron, L.; Rizzo. D.M. 2021. Improved understanding of the threat posed by *Phellinus* heart-rot in California prune orchards. ISHS Acta Horticulturae. XII international symposium on plum and prune genetics, breeding and pomology. 1322: 295–302. https://doi.org/10.17660/ActaHortic.2021.1322.41.

Kadowaki, K.; Leschen, R.A.B.; Beggs, J.R. 2010. Periodicity of spore release from individual *Ganoderma* fruiting bodies in a natural forest. Australasian Mycologist. 29: 17–23.

Kliejunas, J.K. 1989. Borax stump treatment for control of Annosus root disease in east-side pine type forests of northeastern California. In: Otrosina, W.J.; Scharpf, R.F., tech. coords. Proceedings of the symposium on research and management of annosus root disease (*Heterobasidion annosum*) in western North America. Gen. Tech. Rep. PSW-GTR-116. Berkeley, CA: U.S. Department of Agriculture, Forest Service, Pacific Southwest Forest and Range Experiment Station: 159–166. https://www.fs.usda.gov/psw/publications/documents/psw_gtr116/.

Kozhar, O; Kim, M.S.; Ibarra Caballero J.R.; Klopfenstein, N.B.; Cannon, P.G.; Stewart, J.E. 2022. Long evolutionary history of an emerging fungal pathogen of diverse tree species in eastern Asia, Australia, and the Pacific Islands. Molecular Ecology. 31: 2013–2031. https://doi.org/10.1111/mec.16384.

Knutsen, T.R.; Sirutis, J.J.; Zhao, M.; Tuleya, R.E.; Bender, M.; Vecchi, G.A.; Villarini, G.; Chavas, D. 2015. Global projection of intense tropical cyclone activity for the late twenty-first century from dynamical downscaling of CMIP5/RCP4.5 scenarios. Journal of Climate. 28: 7203–7224. https://doi.org/10.1175/JCLI-D-15-0129.1.

Lalande, B. 2019. Abiotic and biotic factors influencing Western United States coniferous forests. Fort Collins, CO: Colorado State University. 93 p. M.S. thesis.

Lalande, B.; Abdo, Z.; Hanna, J.W.; Page-Dumroese, D.S.; Warwell, M.V.; Tirocke, J.M.; Kim, M.-S.; Klopfenstein, N.B.; Stewart, J.E. 2019. Metagenomic approaches to determine soil microbial communities associated with Armillaria root disease. In: Cleaver, C.; Palacios, P., comps. Proceedings of the 65th annual western international forest disease work conference. [Place of publication unknown]: Western International Forest Disease Work Conference: 123–126.

Landers, M.A.; Khosrowpanah, S. 2004. Rainfall climatology for Pohnpei Islands, Federated States of Micronesia. Technical Report Number 100. Mangilao, Guam: Water and Environmental Research Institute of the Western Pacific, University of Guam. 51 p. http://www.weriguam.org/docs/reports/100.pdf. (2 February 2022).

Liao, T.-Z.; Chen, Y.-H.; Tsai, J.-N.; Chao, C.; Huang, T.-P.; Hong, C.-F.; Wu, Z.-C.; Tsai, I.J.; Lee, H.-H.; Klopfenstein, N.B.; Kim, M.-S.; Stewart, J.E.; Atibalentja, N.; Brooks, F.E.; Cannon, P.G.; Farid, A.M.; Hattori, T.; Kwan, H.-S.; Lam, R.Y.C.; Ota, Y.; Sahashi, N.; Schlub, R.L.; Shuey, L.S.; Tang, A.M.C.; Chung, C.-L. [In press]. Translocation of fungicides and their efficacy in controlling brown root rot disease. Plant Disease.

Liebhold, A.M.; Brockerhoff, E.G.; Garrett, L.J.; Parke, J.L.; Britton, K.O. 2012. Live plant imports: the major pathway for forest insect and pathogen invasions of the U.S. Frontiers in Ecology and the Environment. 10(3): 135–143. https://doi.org/10.1890/110198.

Leung, K.-T.; Chen, C.-Y.; You, B.-J.; Lee, M.-H.; Huang, J.-W. 2020. Brown root rot disease of *Phyllanthus myrtifolius*: the causal agent and two potential biological control agents. Plant Disease. 104(11): 3043–3053. https://doi.org/10.1094/PDIS-02-20-0412-RE.

Lopez-Real, J.M.; Swift, M.J. 1977. Formation of pseudosclerotia ('zone lines') in wood decayed by *Armillaria mellea* and *Stereum hirsutum*: III. Formation in relation to composition of gaseous atmosphere in wood. Transactions of the British Mycological Society. 68(3): 321–325. https://doi.org/10.1016/S0007-1536(77)80183-6.

Mohd Farid, A.; Lee, S.S.; Maziah, Z.; Patahayah, M. 2009. Pathogenicity of *Rigidoporus microporus* and *Phellinus noxius* against four major plantation tree species in Peninsular Malaysia. Journal of Tropical Forest Science. 21(4): 289–298.

Nandris, D.; Nicole, M.; Geiger, J.P. 1987. Variation in virulence among *Rigidoporus lignosus* and *Phellinus noxius* isolates from West Africa. European Journal of Forest Pathology. 17(4/5): 271–281. https://doi.org/10.1111/j.1439-0329.1987.tb01026.x.

O'Malley, M. 2010. The regulatory evaluation of the skin effects of pesticides. In: Krieger, R., ed. Hayes handbook of pesticide toxicology. 3rd ed. New York: Academic Press: 701–787. Chapter 28. https://doi.org/10.1016/B978-0-12-374367-1.00028-8.

Pang, Y.-Y. 2020. Control of brown root rot disease and abiotic stresses of trees with the endophytic fungus *Serendipita indica*. Taipei, Taiwan: National Taiwan University. M.S. thesis. In Chinese.

Rehner, S.A.; Samuels, G.J. 1994. Taxonomy and phylogeny of *Gliocladium* analyzed from nuclear large subunit ribosomal DNA sequences. Mycological Research. 98(6): 625–634. https://doi.org/10.1016/S0953-7562(09)80409-7.

Ribera, J.; Tang, A.M.C.; Schubert, M.; Lam, R.Y.C.; Chu, L.M.; Leung, M.W.K.; Kwan, H.S.; Bas, M.C.; Schwarze, F.W.M.R. 2016. In vitro evaluation of antagonistic *Trichoderma* strains for eradicating *Phellinus noxius* in colonized wood. Journal of Tropical Forest Science. 28(4): 457–468.

Rizzo, D.M.; Harrington, T.C. 1988. Root movement and root damage of red spruce and balsam fir on subalpine sites in the White Mountains, New Hampshire. Canadian Journal of Forest Research. 18: 991–1001. https://doi.org/10.1139/x88-152.

Sahashi, N.; Akiba, M.; Ishihara, M.; Miyazaki, K.; Kanzaki, N. 2010. Cross inoculation tests with *Phellinus noxius* isolates from nine different host plants in the Ryukyu Islands, southwestern Japan. Plant Disease. 94(3): 358–360. https://doi.org/10.1094/PDIS-94-3-0358.

Schwarze, F.W.M.R.; Jauss, F.; Spencer, C.; Hallam, C.; Schubert, M. 2012. Evaluation of an antagonistic *Trichoderma* strain for reducing the rate of wood decomposition by the white rot fungus *Phellinus noxius*. Biological Control. 61(2): 160–168. https://doi.org/10.1016/j.biocontrol.2012.01.016.

Stewart, J.E.; Kim, M.-S.; Klopfenstein, N.B. 2018. Molecular genetic approaches toward understanding forest-associated fungi and their interactive roles within forest ecosystems. Current Forestry Reports. 4: 72. https://doi.org/10.1007/s40725-018-0076-5.

Stewart, J.E.; Kim, M.-S.; Lalande, B.; Klopfenstein, N.B. 2021. Pathobiome and microbial communities associated with forest tree root diseases. In: Asiegbu, F.O.; Kovalchuk, A., eds. Forest microbiology—tree microbiome: Phyllosphere, Endosphere, and Rhizosphere. Vol 1: London, United Kingdom: Academic Press, Elsevier, Inc.: 277–292. Chapter 15. https://doi.org/10.1016/B978-0-12-822542-4.00004-8.

Stewart, J.E.; Kim, M.-S.; Ota, Y.; Sahashi, N.; Hanna, J.W.; Akiba, M.; Ata, J.P.; Atibalentja, N.; Brooks, F.; Chung, C.-L.; Dann, E.K.; Mohd Farid, A.; Hattori, T.; Lee, S.S.; Otto, K.; Pegg, G.S.; Schlub, R.L.; Shuey, L.S.; Tang, A.M.C.; Tsai, J.-N.; Cannon, P.G.; Klopfenstein, N.B. 2020. Phylogenetic and population genetic analyses reveal three different lineages of the invasive brown root-rot pathogen, *Phellinus noxius*, and bioclimatic modeling predicts differences in associate climatic niches. European Journal of Plant Pathology. 156: 751–766. https://doi.org/10.1007/s10658-019-01926-5.

Thies, W.G.; Nelson, E.E.; Zabowski, D. 1994. Removal of stumps from a *Phellinus weirii* infested site and fertilization affect mortality and growth of planted Douglas-fir. Canadian Journal of Forest Research. 24(2): 234–239. https://doi.org/10.1139/x94-034.

Tsai, J.-N.; Ann, P.-J.; Liou, R.-F.; Hsieh, W.-H.; Ko, W.-H. 2017. *Phellinus noxius*: molecular diversity among isolates from Taiwan and its phylogenetic relationship with other species of *Phellinus* based on sequences of the ITS region. Botanical Studies. 58: 9. https://doi.org/10.1186/s40529-017-0162-1.

Tzean, Y.; Shu, P.-Y.; Liou, R.-F.; Tzean, S.-S. 2016. Development of oligonucleotide microarrays for simultaneous multi-species identification of *Phellinus* tree-pathogenic fungi. Microbial Biotechnology. 9(2): 235–244. https://doi.org/10.1111/1751-7915.12341.

U.S. Department of Commerce, National Oceanic and Atmospheric Administration [USDC NOAA]. 2021. Hurricanes FAQ. NOAA's Atlantic Oceanographic and Meteorological Laboratory. https://www.aoml.noaa.gov/hrd-faq/.

Vilgalys, R.; Hester, M. 1990. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. Journal of Bacteriology. 172(8): 4238–4246. https://doi.org/10.1128/jb.172.8.4238-4246.1990.

White, T.J.; Bruns, T.; Lee, S.; Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A.; Gelfand, D.H.; Sninsky, J.J.; White, T.J., eds. PCR protocols: a guide to methods and applications. New York: Academic Press: 315–322. Chapter 38. https://doi.org/10.1016/B978-0-12-372180-8.50042-1.

Wikipedia. 2018. 2018 Pacific typhoon season. https://en.wikipedia.org/wiki/2018_Pacific_typhoon_season.

Worrall, J. 2004. Armillaria root disease, shoestring root rot. The Plant Health Instructor. https://doi.org/10.1094/PHI-I-2004-0706-01.

Wu, J.; Peng, S.L.; Zhao, H.B.; Tang, M.H.; Li, F.R.; Chen, B.M. 2011. Selection of species resistant to the wood rot fungus *Phellinus noxius*. European Journal of Plant Pathology. 130: 463–467. https://doi.org/10.1007/s10658-011-9774-6.

Wu, M.-L.; Chang, T.-T.; Jaung, L.-M.; Hung, T.-H.; Lin, L.-D. 2009. Establishment of PCR rapid detection technique for tree brown root rot disease. Quarterly Journal of Chinese Forestry. 42: 239–247. In Chinese with abstract in English.

Wu, Z.-C.; Chang, Y.-Y.; Lai, Q.-J.; Lin, H.-A.; Tzean, S.-S.; Liou, R.-F.; Tsai, I.J.; Chung, C.-L. 2020. Soil is not a reservoir for *Phellinus noxius*. Phytopathology. 110(2): 362–369. https://doi.org/10.1094/PHYTO-08-19-0314-R.

Zhang, L.; Karnauskas, K.B.; Donnelly, J.P.; Emanuel, K. 2017. Response of the north Pacific tropical cyclone climatology to global warming: application of dynamic downscaling to CMIP5 Models. Journal of Climate. 30(4): 1233–1243. https://doi.org/10.1175/JCLI-D-16-0496.1

Zhang, W.; Vecchi, G.A.; Murakami, H.; Delworth, T.L; Paffendorf, K.; Jai, L.; Villarini, G.; Gudgel R.; Zeng, F.; Yang, X. 2016. Influences of natural variability and anthropogenic forcing on the extreme 2015 accumulated cyclone energy in the western North Pacific. In: Explaining extreme events of 2015 from a climate perspective. Bulletin of the Meteorological Society. 97(12): S131-135. https://doi.org/10.1175/BAMS-D-16-0146.1.