FROM DIAGNOSTICS TO METAGENOMICS: APPLICATIONS OF DNA-BASED TOOLS IN FOREST PATHOLOGY

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INTRODUCTION

Advances in molecular technology provide an accessible set of tools to 1) help forest pathologists detect, identify, and monitor forest pathogens, 2) examine the evolutionary relationships and global distributions of forest pathogens and their hosts, 3) assess the diversity and structure of host and pathogen populations, and 4) evaluate the structure and function of genes, as well as their levels of expression, within species and within communities. This paper briefly outlines DNA-based tools, including molecular diagnostics, phylogenetics, population genetics, genomics, and metagenomics, and discusses how each has been or could be applied in the field of forest pathology.

MOLECULAR DIAGNOSTICS

DNA-based diagnostics use DNA-sequence data to identify or characterize biological organisms (e.g., Hoff et al. 2004, Glaeser and Lindner 2010). To help identify organisms, DNA-based diagnostics typically rely on comparisons with a worldwide database, such as GenBank® (http://www.ncbi.nlm.nih.gov/genbank/) that contains DNA sequences from known species. The utility of DNA-based diagnostics continues to increase, and DNA databases continue to expand. Morphology still plays a role in the identification of fungal species; however, members of different species can have similar morphology, and members of the same species can have very different morphology. Similarly, mating tests still play a role in the identification of fungi based on the biological species concept, but very different fungal species are sometimes inter-fertile. Thus, fungal identification continues to become more reliant on DNA-based methods. In forest pathology, DNA-based diagnostics are frequently used to detect, identify, and monitor forest pathogens and other microbes, largely because these methods are reliable, relatively quick, and economical.

Fusarium root disease in forest nurseries is one of the best examples of the utility of DNA-based diagnostics. *Fusarium oxysporum* is morphologically indistinguishable from *F. commune* (Stewart et al. 2006). *Fusarium commune* is an aggressive pathogen that causes damping off of Douglas-fir (Pseudotsuga menziesii) seedlings, whereas *F. oxysporum* is apparently non-pathogenic on Douglas-fir seedlings (Stewart et al. 2006, 2012). Furthermore, a recent paper by Dumroese et al. (2012) shows that some *F. oxysporum* isolates have biological control potential to protect seedlings from damping off disease caused by *F. commune*. DNA-based diagnostics is the only available method to distinguish these two fungal species.

Another example of the utility of DNA-based diagnostics lies with *Armillaria*. The pathogenic species *A. solidipes* [pending vote to conserve *A. ostoyae* (Redhead et al. 2011)] is often difficult to distinguish from the other, saprophytic species in the genus because it is typically observed in a vegetative state. Since DNA-based methods have become available to discriminate among North American *Armillaria* species (Kim et al. 2006, Ross-Davis et al. 2012a), these approaches have been used to detect the occurrence of *A. solidipes* throughout much of the west as part of a collaborative effort with USDA Forest Service, Forest Health Protection and Forest Inventory and Analysis (FIA) to predict where *A. solidipes* is likely to occur and cause disease under different forest management regimes and predicted conditions associated with a changing climate (e.g., Hanna et al. 2009, McDonald et al. 2011, Klopfenstein et al. 2012).

Similarly, DNA-based diagnostics were essential to determine that the white pine blister rust pathogen (*Cronartium ribicola*) could use non-*Ribes* alternate hosts, such as *Pedicularis* sp. and *Castilleja* spp., to

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complete its lifecycle (e.g., McDonald et al. 2006). This phenomenon may have been overlooked previously because infections on these non-*Ribes* hosts were perhaps attributed to *C. coleosporioides*, the cause of stalactiform rust of "hard" pines. It remains undetermined whether the use of non-*Ribes* alternate hosts by *C. ribicola* is a partial explanation for the shortcomings of the 50-year *Ribes* eradication effort to control white pine blister rust.

The utility of DNA-based diagnostics continues to grow in forest pathology. These techniques provide reliable identification of native and exotic pathogens, mycorrhizal fungi, symbionts, biocontrol agents, endophytes, decay fungi, and other microbes associated with forest ecosystems (e.g., Hoff et al. 2004, Glaeser and Lindner 2010). The accurate identification of forest microbes is essential to better understand their distribution and ecological relationships within forest ecosystems.

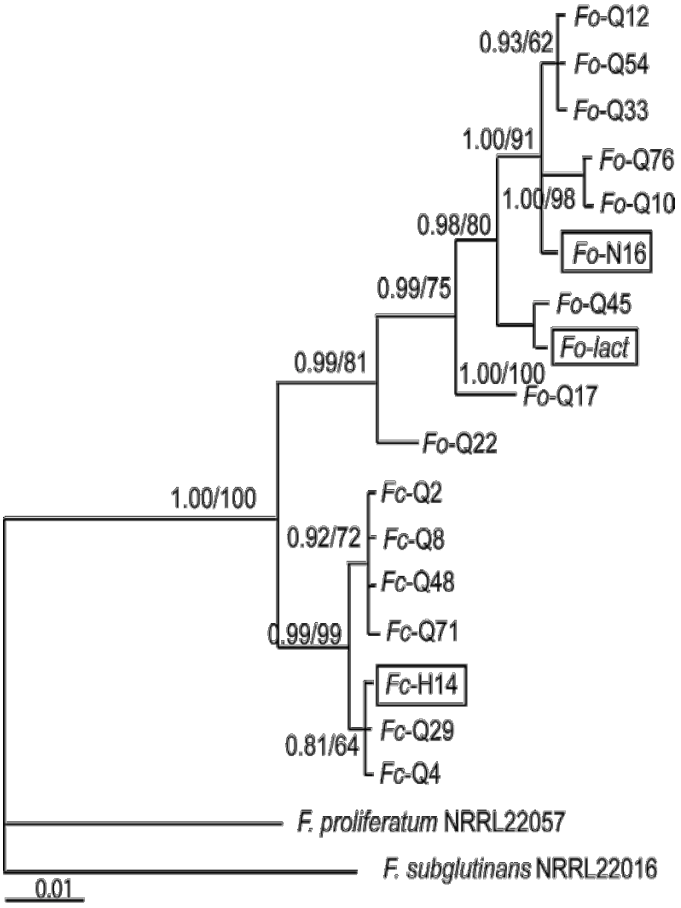

Figure 1. Phylogenetic relationship between the non-pathogenic *Fusaruim oxysporum* (*Fo*-) and the highly virulent *Fusarium commune* (*Fc*-) (from: Stewart et al. 2012).

PHYLOGENETICS

Phylogenetics is the study of evolutionary relationships among groups of organisms, and phylogenetic analysis is often based on DNA-sequence data. Our definitions of fungal species are becoming increasingly reliant on the phylogenetic species concept (Taylor et al. 2000), because it allows detection of species at a finer scale than most other speciation concepts and it seems to parallel the ecological species concept (Andersson 1990). As shown in Figure 1 (based on Stewart et al. 2012), phylogenetic analysis was able to determine a distinct genetic difference between the non-pathogenic *F. oxysporum* and the highly virulent *F. commune*, which fall into separate genetic groups when morphologically these two species are indistinguishable.

The genus *Armillaria* is another example where phylogenetics can contribute to our understanding of species delineations. *Armillaria* species have great variation in distribution and ecological behavior, with some species possessing the potential to be invasive pathogens. Each species may vary in their habitat, host range, and response to climate change. Morphology-based methods and compatibility-based methods have contributed greatly to the identification of *Armillaria* species, but phylogenetic methods are proving to be more precise. Currently, phylogenetic analysis based on the translation elongation factor-1α (*tef-1α*) gene sequences provides the best available method for distinguishing North American *Armillaria* species. Figure 2 depicts a phylogenetic tree of North American *Armillaria* species, based on DNA sequences from the *tef-1α* (Ross-Davis et al. 2012a), and efforts are now underway to examine the utility of this method for delimiting diverse *Armillaria* species from across the Northern Hemisphere (see Klopfenstein et al. this proceedings).

The aforementioned studies and many others clearly demonstrate that phylogenetic studies are essential to better understand genetic and evolutionary relationships among forest microbes and forest hosts. The application of more powerful phylogenetic tools will contribute to improved definitions of host and microbe species. Furthermore, phylogenetic tools are useful for determining sources of invasive pathogens or predicting sources of potentially invasive pathogens before they are introduced.

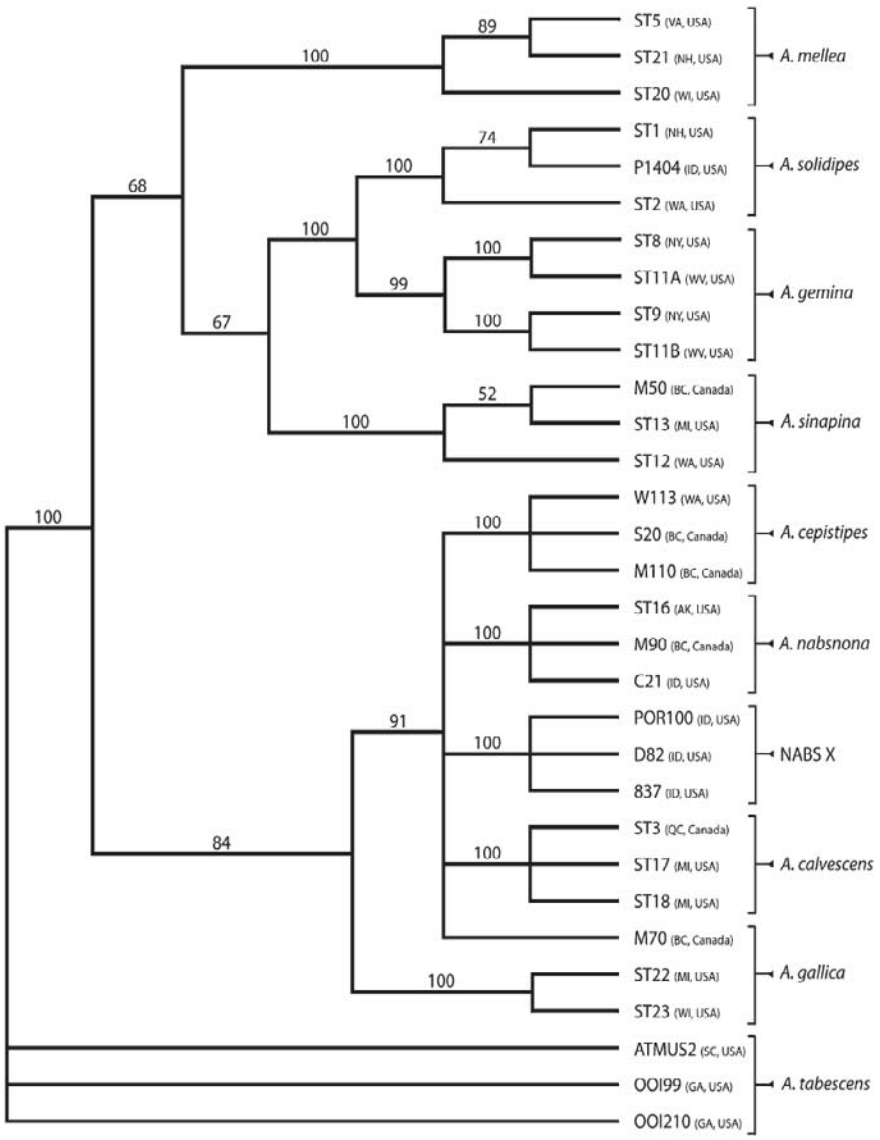

Figure 2. Phylogenetic relationship among North American *Armillaria* species (from: Ross-Davis et al. 2012a).

POPULATION GENETICS

In addition to determining species and their relationships, defining the relationships of pathogen population structure in association with host, environment, and geography allows a more precise examination of the biological processes of forest disease. Population genetics allows us to examine genetic relationships among populations by studying allele frequency distribution and change under the influence of four main evolutionary forces (natural selection, genetic drift, mutation, and gene flow and transfer). Several previous studies have examined the population structure of pathogens. For example, Hamelin et al. (2000) found distinct *Cronartium ribicola* populations in eastern and western North America, and Richardson et al. (2008) found that major gene resistance appears to have imposed strong selection pressure on the blister rust pathogen. Winton et al. (2006) identified two lineages of Swiss needle cast pathogen (*Phaeocryptopus gaeumannii*) in Oregon and Washington, where only one lineage was associated with disease epidemics. Zhang and Blackwell (2002) reported that the dogwood anthracnose pathogen (*Discula destructiva*) exists as distinct eastern and western populations. In addition, Baumgartner et al. (2010) demonstrated the existence of two geographically isolated, divergent genetic pools of *A. mellea* in the USA.

In addition to pathogen species, we can also examine the population structure of host species, which is important for delimiting seed transfer zones and focusing conservation efforts. Kim et al. (2011) showed that southern western white pine (*Pinus monticola*) populations (below 45°N latitude) are more genetically diverse than northern populations, but most of this genetic variation is attributable to differences among individuals within populations rather than differences among populations. This is not surprising given that this is a wind-pollinated species so gene flow is unobstructed. The definition of western white pine populations is essential for species management and conservation programs that are addressing the threats of white pine blister rust, climate change, and other environmental threats.

Aspen (*Populus tremuloides*) is the most widely distributed tree species in North America and one that is currently threatened by a sudden aspen decline (Worrall et al. 2010). Mock et al. (2008) used genetic markers to determine genetic diversity and clonal structure of aspen stands. In a subsequent continental-scale study of aspen, Mock et al. (2012) found that triploidy was common in the central Rocky Mountains and rare in almost all other parts of aspen's range. The proportion of triploidy in local populations was highest in areas characterized by relatively high summer temperature and low precipitation (Figure 3). Although sudden aspen decline has been associated with climate stress (Rehfeldt et al. 2009, Worrall et al. 2010), the resistance/susceptibility of triploid aspen remains unknown.

Population genetics allows us to define units of hosts and pathogens that may respond similarly to environmental threats, identify valuable and unique populations for species management and restoration, make predictions of potential effects of environmental threats, and identify mechanisms behind invasions by exotic forest pathogens. Furthermore, understanding population structure of forest trees and pathogens is essential for efforts to restore, conserve, and sustain forest tree species during an era of climate change.

THE "-OMICS"

Genomics is the study of the structure and function of genes. A genome is the entirety of an organism's hereditary information (both coding and non-coding). Much progress has been made since the mid-1970s when the first complete nucleotide sequence of a viral RNA-genome was established (Fiers et al. 1976) and then the first DNA-based genome was sequenced (Sanger et al. 1977). In the mid-1990s the first bacterial genome was sequenced (Fleischmann et al. 1995) followed by the first eukaryotic genome of the budding yeast (http://www.yeastgenome.org/). In 2003 the 13-year effort to map and sequence the human genome was completed (International Human Genome Sequencing Consortium 2004). When accessed in October 2012, the genome online database (http://www.genomesonline .org/cgi-bin/GOLD/index.cgi) listed 18,378 genome projects, of which 3,762 had been completed. At the same time, the Department of Energy (DOE) Joint Genome Institute (JGI) Fungal Genome Program (http://genome.jgi.doe.gov/programs/fungi/ index.jsf) listed 744 fungal genome projects, many of which had been completed, including several species of white rot fungi, brown rot fungi, and other pathogenic species. The 1000 Fungal Genomes Project (http://1000.fungalgenomes.org/home/) is a 5-year collaborative project to sequence 1000 fungal genomes across families allowing for the comparative genomics of fungal pathogens to look for common pathogenicity mechanism strategies and new ways to combat pathogens.

Transcriptomics is also known as expression genomics and is the study of that portion of the genome that is transcribed into mRNA molecules at any given time and in a given environment. It allows us to study gene expression in different tissues, at different time points, and under different conditions. By comparing how gene expression differs we can begin to identify genes associated with certain functions. For example, more than 1000 genes in hybrid poplar are up-regulated in response to insect attack as reported by Ralph et al. (2006). In the *Populus-Melampsora* pathosystem, differential regulation of genes involved in pathogeneses have been revealed via transcript profiling in susceptible versus incompatible interactions (Rinaldi et al. 2007), susceptible interactions over time (Miranda

et al. 2007) and susceptible interactions as infected with two different *Melampsora* species (Azaiez et al. 2009).

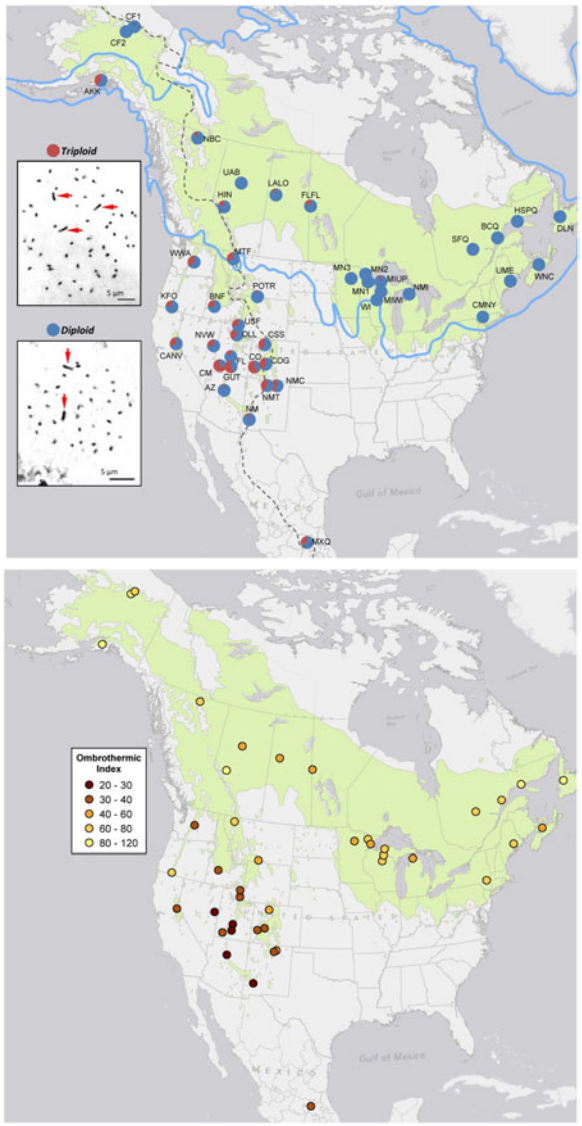

Figure 3. Top. Map of diploid:triploid proportions for 42 local populations of aspen in North America. **Bottom.** Ombrothermic index for aspen population centroids (low = warm-dry summer, high = cool-wet summer). (from: Mock et al. 2012).

CASE STUDY

We embarked on a study to characterize a transcriptome of a mycelial fan of a widespread, virulent clone of *A. solidipes* (isolate RNA1), and focus particularly on the identification of genes involved in degrading plant cell wall components and responding to the host environment (Ross-Davis et al. 2012b). RNA was stabilized, isolated, and then sequenced to generate over 24 million short reads which were then assembled *de novo* (since a reference genome for *Armillaria* does not yet exist) to generate almost 40,000 contigs – or consensus regions of DNA. We then compared these contigs to genes in the NCBI database and found almost 20,000 significant alignments, over 12,000 of which have known functions. One concern with *in planta* transcriptomics is isolating the pathogen RNA from the host tissue. Of the significant alignments, most contigs aligned best to fungal sequences, mostly within the phylum Basidiomycota, and the order Agaricales into which *A. solidipes* is classified. Only 54 of the alignments were to sequences from *Armillaria* species due to the limited availability of DNA sequences from this taxon in the database. The ability to use the host as a carbon source plays an essential role in pathogenesis. White rot fungi like *A. solidipes* are capable of efficiently degrading all components of plant cell walls including cellulose, hemicellulose, pectin, and lignin. In characterizing this transcriptome, we found evidence for the use of an array of enzymes involved in plant cell wall degradation (Table 1).

Metagenomics is also known as environmental genomics, ecogenomics, community genomics, and megagenomics and is the study of genetic material recovered directly from environmental samples. Microbial genome sequencing traditionally relied on clonal cultures but early environmental gene sequencing, which cloned specific genes to produce a profile of diversity in a natural sample, revealed that most of the biodiversity had been missed by these culture-based methods. This is not surprising given the estimate that a single gram of soil contains billions of microbial cells, only a fraction of which can be grown in culture. The term metagenomics first appeared in publication in 1998 (Handelsman et al. 1998) and has been used to examine the microbiome of marine environments, beginning with Craig Venter's work almost a decade ago (Venter et al. 2004) and now with 179 marine metagenomics published papers catalogued in the Thomson Reuters Web of Knowledge^{SM}. Much work is also being done on human systems. For example, the Human Microbiome Project (http://commonfund.nih.gov/Hmp/) seeks to describe the diversity of microbial species associated with health and disease. Since metagenomics allows us to tap into the metabolic potential of uncultivated microbes we can now discover novel genes and novel metabolic pathways with clear applications for biotechnology,

biofuel production, and bioremediation. Most applicable to the field of forest pathology is the application of metagenomic approaches to understanding soil microbial communities and how these relate to disease suppression (van Elsas et al. 2008a and b, Hernandez-Leon et al. 2010, Mendes et al. 2011, Klein et al. 2013). This is a very active area of research with over 200 papers currently published in the area of soil metagenomics as catalogued in the Thomson Reuters Web of Knowledge[SM].

Essentially, the three general steps involved in a metagenomics project are (1) sequencing, (2) processing - which includes (a) assembling the multitude of sequencing reads, (b) annotating these assembled contigs to assign function, and (c) assigning contigs to specific taxa, and then finally (3) analysis - which refers to examining the functional and metabolic diversity of microbial communities. One challenge associated with metagenomics is the generation and analysis of massive amounts of sequence data. While the efficiency associated with high-throughput sequencing has changed metagenomics so that sequencing DNA is no longer the limiting factor, a bottleneck now lies in the area of computational analysis and interpretation. Nonetheless, international collaborations for sharing information and coordinating sequencing and bioinformatics activities, such as terragenome (http://www.terragenome.org/), exist so that these efforts can be met with success.

Disease-suppressive soils are exceptional ecosystems in which crop plants suffer less from specific soil-borne pathogens than expected because of the activities of other soil microorganisms. Metagenomics of the rhizosphere microbiome coupled with culture-dependent functional analyses identified key bacterial taxa and genes involved in suppression of a fungal root pathogen (e.g., Mendes et al. 2011, Klein et al. 2013). Such studies show that specific constituents of the microbial community help protect plants against pathogen infections. Similar approaches in forest ecosystems can begin to uncover the role of the soil microbial community, particularly certain taxa and genes, in suppressing various forest diseases. And, by linking metadata (or specific environmental factors like fire, thinning, climate) to the presence of beneficial or harmful taxa and to the expression of specific genes, we can begin to identify management practices that can effectively address root disease.

Table 1. Enzymes involved in plant cell wall degradation expressed in *Armillaria* solidipes isolate, RNA1.

Enzymes	Orthologs
Ligninolytic and related enzymes	
Laccase (EC 1.10.3.2)	10
Manganese peroxidase (EC 1.11.1.13)	5
Versatile peroxidase (EC 1.11.1.16)	4
Aryl alcohol oxidase (EC 1.1.3.7)	4
Alcohol oxidase (EC 1.1.3.13)	1
(S)-2-hydroxy-acid oxidase (EC 1.1.3.15)	1
D-arabinono-1,4-lactone oxidase (EC 1.1.3.37)	4
Cytochrome P450s	37
Cellulolytic, hemicellulolytic, and related enzymes	
Cellulase (EC 3.2.1.4)	9
β-glucosidase (EC 3.2.1.21)	3
Cellulose 1,4-β-cellobiosidase (non-reducing end) (EC 3.2.1.91)	8
Feruloyl esterase (EC 3.1.1.73)	1
β-galactosidase (EC 3.2.1.23)	18
α-mannosidase (EC 3.2.1.24)	7
Mannan endo-1,4-β-mannosidase (EC 3.2.1.78)	4
Endo-1,4-β-xylanase (EC 3.2.1.8)	4
Xylan 1,4-β-xylosidase (EC 3.2.1.37)	1
α-N-arabinofuranosidase (EC 3.2.1.55)	3
Xylan α-1,2-glucuronosidase (EC 3.2.1.131)	2
xyloglucan-specific exo-β-1,4-glucanase (EC 3.2.1.155)	1
Cellobiose dehydrogenase (EC 1.1.99.18)	1
Pectinolytic enzymes	
Pectinesterase (EC 3.1.1.11)	2
Feruloyl esterase (EC 3.1.1.73)	1
Polygalacturonase (EC 3.2.1.15)	13
Pectate lyase (EC 4.2.2.2)	9

DNA-BASED ANALYSES AND STRATEGIC FOREST INVENTORIES

The availability of environmental data is essential to provide ecological interpretations for genetic analysis of forest pathosystems at diverse spatial scales. Even as methods for DNA-based analyses continue to progress for application in forest ecosystems, meaningful ecological insights are dependent on obtaining geographically distributed, representative samples with accompanying environmental data. Even in well-studied areas like western North America, the distributions of some pathogens remain only partially mapped.

The USDA Forest Service FIA program samples a wide variety of forest characteristics using a sampling grid with approximately 5-km spacing in all U.S. States and Territories (approximately 135,000 forested plots nationwide). Individual plots are remeasured on a 5- to 10-year cycle, depending on the state. By linking DNA-based analyses to such an inventory system, the presence, absence, and effects of pathogens may be linked to environmental variables and associated stand/site characteristics. Furthermore, responses of forest pathogens to any changes in the environment, whether though successional processes, common disturbance events (e.g., insect infestations or fire), or long-term climatic change may be monitored during successive inventory cycles. The combination of high-resolution genetic data with extensive environmental metadata, offers great potential to understand forest ecosystem processes, including forest diseases, at an unprecedented level. However, methods must be developed to allow the efficient collection and analyses of genetic data from well-characterized sites. Once field and laboratory methods can be scaled up for application in a production inventory environment, the potential for understanding genetic and environmental interactions, as they relate to forest disease, will increase substantially. Thus, the availability of a permanent plot network with associated environmental data, such as those of FIA, provide an invaluable opportunity to derive increased ecological information from the application of DNA-based tools to better understand interactions among forest pathogens, hosts, other components of the abiotic and biotic environment, and stand history.

CONCLUSION

In summary, we can (1) detect, identify, and monitor forest pathogens and other organisms through molecular diagnostics, (2) determine the evolutionary relationships and global distributions of forest pathogens and their hosts through phylogenetics, (3) assess the diversity and structure of host and pathogen populations through population genetics, and (4) evaluate the structure and function of genes within species and communities with genomics and metagenomics. Although, the power and utility of these genetic tools continues to grow at a very fast pace, their utility is also dependent on the availability of environmental metadata at diverse spatial scales.

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