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FOREST PATHOLOGY (A CARNEGIE, SECTION EDITOR)

Molecular Genetic Approaches Toward Understanding Forest-Associated Fungi and Their Interactive Roles Within Forest Ecosystems

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Abstract

Purpose of Review The continued, rapid development of novel molecular genetic tools is contributing to a better understanding of forest-associated fungi and their interactive roles within diverse forest ecosystems. This paper focuses on recent developments of DNA-based diagnostics/detection, phylogenetics, population genetics, genomics, and metagenomics tools that have been applied to forest-associated fungi to better understand their roles in forest ecosystems and provide key insights for managing forest health.

Recent Findings With the advent of new molecular technologies, we can better understand the biology of forest fungi by examining their genetic code. By utilizing genomics, fungal pathogens' biological functions can be deduced from its genomic content. Further, high-resolution marker systems allow the determination of a pathogen's population genetics and genomics, which provides important insights into its global movement and genetic shifts in local pathogen populations. Such genetic information has diverse applications for forest management to improve forest health. Lastly, new technologies in metagenomics will enhance the abilities to detect, describe, and utilize the complex interactions among fungal pathogens/symbionts, host trees, and associated microbial communities to develop novel management strategies for forest ecosystems.

Summary Continued development and applications of molecular genetic and genomic tools provide insights into the diverse roles of forest-associated fungi in forest ecosystems, but long-term, wide-scale research is needed to determine how ecological functions are influenced by complex ecological interactions among microbial communities, other forest ecosystem components, and the environment. Such approaches may foster a paradigm shift away from single microbial pathogens, decomposers, or symbionts interacting with a single host or substrate, and provide more holistic approaches toward understanding interactions among microbial communities that drive forest health processes.

Keywords Forest pathogens · Genomics · Transcriptomes · Metagenomics

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Introduction

Fungi play diverse roles in forest ecosystems, such as pathogens, decomposers, beneficial symbionts, and biocontrol agents. They interact with each other and other ecosystem components in complex and multidimensional ways within forest ecosystems.

Fungal pathogens cause the majority of devastating diseases of forest trees, such as Armillaria root disease (caused by *Armillaria* spp.), white pine blister rust (caused by *Cronartium ribicola*), chestnut blight (caused by *Cryphonectria parasitica*), and Dutch elm disease (caused by *Ophiostoma ulmi*, *O. novo-ulmi*), and countless other forest diseases, which result in growth losses, mortality, and/or threats to life, limb, and property. Fungi are the causal agents of diverse forest diseases, including diseases of the root/butt, vascular system, outer stem/branches, wood, and

foliage. Alternatively, fungi also play beneficial symbiotic roles in promoting forest health, such as ectomycorrhizae, arbuscular mycorrhizae, biocontrol agents, or endophytes/epiphytes that may confer resistance/tolerance against environmental stresses or pests [46•]. Furthermore, fungi are critical to decomposition and nutrient cycling processes within healthy forest ecosystems.

Because the literature on molecular genetics applications to forest-associated fungi is much too extensive for an all-inclusive review, our general goal is to provide a brief summary and selected examples of widely used approaches in (1) DNA-based identification and detection; (2) phylogenetics; (3) population genetics/genomics; (4) genomics/transcriptomics; and (5) metagenomics/metatransciptomics. Although molecular genetic tools are expected to rapidly evolve, this review is intended to provide a basic framework for understanding their applications to diverse fungi that are associated with forest diseases (e.g., foliar diseases, canker diseases, vascular diseases, root diseases, and/or wood rots) and other ecological roles in widely ranging forest ecosystems.

DNA-Based Identification and Detection

Accurate identification of fungi that are associated with ecological processes in forests, such as disease, symbioses, decomposition/nutrient cycling, and biological control is essential to understand the ecological roles of these diverse fungi. Fungal identification is sometimes difficult because diverse forest diseases can display similar symptoms, forest fungi with disparate ecological roles can display similar morphology, and obligate forest pathogens cannot be grown in culture. Determining appropriate disease management strategies are dependent on accurate identification of the pathogen, and other forest management strategies are dependent on knowing the ecological roles of key fungi that are not pathogens.

DNA-based identification of fungal pathogens relies on species-specific sequences of genomic regions, such as internal transcribed spacer (ITS) of rDNA, which is commonly used as a fungal barcode for species identification [79]. However, additional genomic regions, such as the small subunit (18S; SSU), large subunit (26S; LSU), and intergenic spacer (IGS) of rDNA, β-tubulin gene, translation elongation factor-1α (*tef1*) gene, ribosomal polymerase II (*RPB2*), Actin-1 (*actin-1*), glyceraldehyde 3-phosphate dehydrogenase (*gpd*), or a combination of different housekeeping DNA regions, are often used when the ITS region is inadequate to distinguish among closely related species. The most effective sequences for identification vary at the fungal species level, but are nearly identical at the population level within a species [79]. The utility of DNA regions for identification is frequently determined by phylogenetic analyses (see subsequent section). With well-studied fungal taxa, cursory identification can be attempted with sequence comparisons against a sequence database, such as GenBank (https://www.ncbi.nlm.nih.gov/genbank/) or others.

Numerous applications of DNA-based identification, which exemplify the utility with forest fungi, are much too extensive to cover comprehensively; however, a few examples demonstrate the diverse utility of DNA-based identification. After several decades devoted to controlling *Ribes* as a means to manage white pine blister rust, McDonald et al. [66] used ITS sequencing to determine that *C. ribicola* can also use non-*Ribes* species (*Pedicularis* sp. and *Castilleja* sp.) as alternate hosts to complete their lifecycle in northwestern USA. Stewart et al. [82, 83] used DNA-based characterization to determine that *Fusarium commune*, not *Fusarium oxysporum*, was the causal agent of damping-off of Douglas-fir (*Pseudotsuga menziesii*) seedlings in conifer nurseries; whereas, a subsequent report indicated that *F. oxysporum* has potential to protect Douglas-fir seedlings from root disease caused by *F. commune* [28]. Mmbaga et al. [68] used ITS sequences to distinguish between powdery mildew pathogens (e.g., *Erysiphe pulchra*, *Phyllactinia guttata*) on flowering dogwood (*Cornus florida*), and provided evidence that an emerging powdery mildew pathogen in the USA may have originated in Asia.

DNA-based identification can also be used to determine species diversity and global movement. These methods have played an essential role in identifying the myrtle rust pathogen, *Austropuccinia psidii* (formerly *Puccinia psidii*), and monitor pathogen spread and/or disease emergence in areas such as Hawaii, USA [89], Japan [47], Australia [14], South Africa [75], Indonesia [67], and Singapore [27]. Similarly, sequences of IGS, ITS, or *tef1* are routinely used to identify *Armillaria* species from mycelial and/or rhizomorph samples (e.g., [4, 11, 41, 48, 49, 51–53, 58, 59, 69, 78]). This identification is critical to understanding the natural distribution of *Armillaria* species because many look morphologically similar in culture. Furthermore, DNA-based identification has been used to document areas where exotic *Armillaria* species were introduced to an area (e.g., [19, 20]). Lastly, DNA sequences were used as evidence that *Heterobasidion* sp. (subsequently named *H. irregulare*; [96]) from North America were introduced to Italy [35–37].

In contrast, rapid and sensitive detection methods are also needed to determine if a known pathogen is present in samples. Early detection is especially critical for invasive pathogens to prevent their establishment in new geographic areas. DNA-based identification can serve as a valuable tool for early detection of forest pathogens, and many tools have been developed for the detection of fungal pathogens of forest trees. Tzean et al. [88] developed an eight-oligonucleotide, microarray platform to simultaneously detect 17 *Phellinus* spp., including the invasive *P. noxius*, in field samples, such as roots, wood, and soil. Lamarche et al. [57•] used several different species-specific DNA sequences and real-time PCR to

detect 10 different alien forest pathogens that represent an invasive threat to Canada. Also, real-time PCR methods also were developed for early detection of *A. psidii*, the invasive myrtle rust pathogen [7].

Phylogenetics

DNA-based phylogenetics of forest fungi examines evolutionary relationships within and among species. Such phylogenetic analyses typically rely on DNA regions similar to those used for identification, but more robust analyses can rely on more extensive genomic comparisons (phylogenomics). Phylogenetic approaches are also critical for recognizing cryptic species that are morphologically indistinguishable but genetically distinct, which is often reflected by an inability to mate in nature. Diverse mathematical inference methods are available to meet the demands of phylogenetic analyses, but these analyses typically result in a phylogenetic tree that allows evolutionary relationships to be visualized. Such analyses are a cornerstone of the phylogenetic species concept, on which fungal taxonomy is based.

Phylogenetic analyses are critical for determining evolutionary relationships among species and defining an organism's taxonomic placement. Phylogenetic analyses were critical to the finding of an undescribed *Armillaria* species in Mexico [31, 32, 54•] (Fig. 1; [54•]). Recently, two exannulate *Armillaria* species (*A. tabescens* and *A. ectypa*) were assigned to the genus *Desarmillaria*, which was newly described on the basis of phylogenetic analyses of six genetic loci [55••]. The myrtle rust pathogen (formerly known as *Puccinia psidii*) was reassigned to the cryptic genus *Austropuccinia*, which was newly described on the basis of LSU-SSU-based phylogenetic analyses [5]. Two Japanese *Heterobasidion* species, *H. annosum* sensu *lato* and an undetermined *Heterobasidion* sp., were revealed based on phylogenetic analyses of three gene loci, *tef1*, *gpd*, and heat shock protein [72]. Subsequently, this undetermined but phylogenetically distinct *Heterobasidion* sp. was newly described as *H. ecrustosum*, based on both cultures and dried specimens [87].

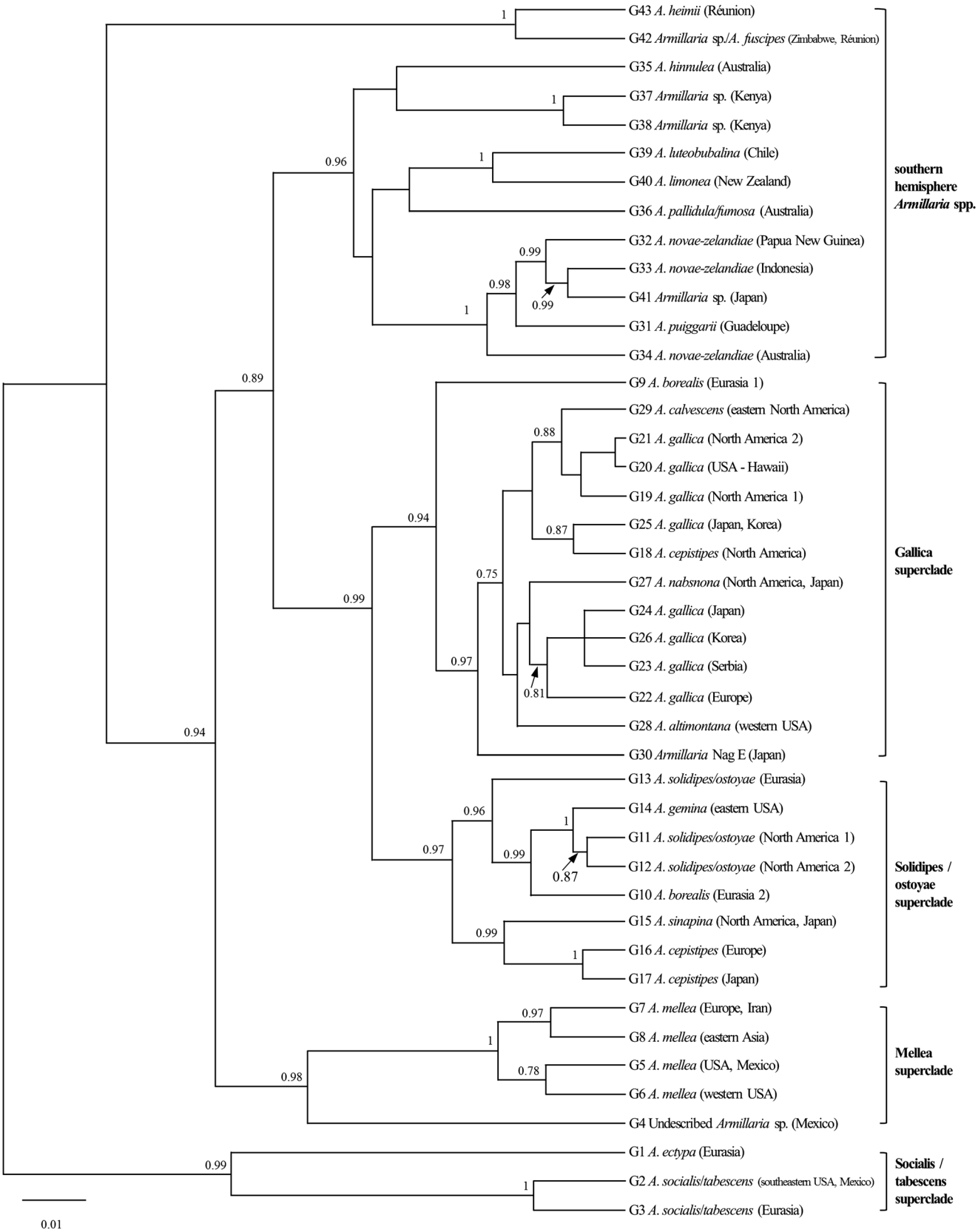
New species recognition is especially important when the ecology/biology (e.g., virulence, host range, climatic conditions) of the newly identified species varies from other species in the genus. Stewart et al. [82] revealed that damping-off in conifer nurseries is caused by *F. commune*, rather than *F. oxysporum*. *Fusarium commune* is difficult to morphologically distinguish from *F. oxysporum*. Although a few morphological characteristics (e.g., formation of polyphialides) can sometimes be used to separate *F. commune* and *F. oxysporum*, these characteristics are very subtle and occur only sporadically under specialized culture conditions. However, *F. commune* and *F. oxysporum* are distinct phylogenetically on the basis of three genetic loci (ITS, mtSSU, and *tef1*) [81, 82] and their ecological behavior (e.g., virulence) differs in forest nursery conditions [83]. Alamouti et al. [2] also used phylogenetics of 15 faster evolving genetic loci to discern a cryptic species, "Gs," closely related to *Grosmannia clavigera*, a tree pathogen vectored by bark beetles (*Dendroctonus* spp.). The authors found host preference where *G. clavigera* was associated with *Pinus ponderosa* and *Pinus jefferyi*; whereas, Gs was associated primarily with *Pinus contorta*.

The emergence of next-generation sequencing provides an enormous DNA sequencing capacity for phylogenetics. For example, restriction-sited associated DNA sequencing (RAD-Seq) or genotype-by-sequencing (GBS) tools can provide hundreds of thousands of short anonymous sequence data that potentially add more depth of phylogenetic trees. In spite of a few obstacles (e.g., alignment, standardization of sequencing data, linked loci, and unavailable reference genome sequence), RAD-Seq has proven useful for phylogenetic studies in some plant species including California white oaks (*Quercus* section *Quercus*) [30•, 33••, 43]. Fitz-Gibbon et al. [33••] demonstrated that both reference-aligned and de novo assembly pipelines produce a reliable phylogenetic inference of California white oaks using RAD-Seq data. Although phylogenetic analyses of RAD-Seq datasets have not yet been reported for forest-associated fungi, this approach offers great potential for high-resolution analyses to infer phylogenetic relationships among forest-associated fungi.

Population Genetics/Genomics

DNA sequences provide the foundation for examining genetic variation within and among populations of forest fungi. Population genetic studies can identify populations that may respond similarly, monitor gene flow among populations, determine if fungal pathogens were introduced, examine the global movement of forest pathogens, and identify genetic shifts in local fungal populations. Current examples of DNA-based markers used for population genetic studies include microsatellites (simple sequence repeats; SSRs), amplified fragment length polymorphisms (AFLPs), RAD-Seq or GBS, single nucleotide polymorphisms (SNPs), and other DNA sequence-based methods. Each type of DNA-based marker has strengths and weaknesses that should be considered when addressing specific issues and situations associated with a proposed study. Diverse and numerous studies in population genetics have significantly influenced our understanding of fungal pathogen interactions within forest ecosystems, but only few examples can be presented here.

Using AFLPs with *C. ribicola* derived from diverse host populations with white pine blister rust in the western USA, Richardson et al. [73] found considerable genetic diversity despite the rust pathogen having been introduced ca. 100 years



before. Furthermore, little population differentiation was apparent among six geographic locations (Idaho, Oregon, Colorado, and California, USA) with diverse aecial hosts [western white pine (*Pinus monticola*), whitebark pine

◀ **Fig. 1** Consensus phylogeny of coalescence-based Bayesian analyses estimated in Evolutionary Analysis by Sampling Trees (BEAST) under the strict clock with a GTR model of substitution on partial translation elongation factor 1-α consensus (50% strict) sequences of 43 phylogenic groups representing 242 *Armillaria* spp. isolates. Posterior support values > 0.5 are indicated at the nodes. Reprinted with permission from Klopfenstein et al. ([54•] Mycologia 109:75–91)

(*P. albicaulis*), limber pine (*P. flexilis*), foxtail pine (*P. balfouriana*), bristlecone pine (*P. aristata*), and sugar pine (*P. lambertiana*)], except in a sugar pine plantation that was screened for major gene resistance (*Cr1*). That study indicated that the type of host resistance placed the strongest selection on the rust pathogen population, despite the diversity in aecial hosts, available telial hosts, and environment. A subsequent SNP-based analysis confirmed a strong differentiation between eastern and western populations of *C. ribicola* in North America, which reflects separate introduction processes [9•]. It was also found that locally distinct population structures were likely influenced by host connectivity, landscape features, and anthropogenic movement of this invasive pathogen [9•].

Microsatellite analyses of the myrtle rust pathogen (*A. psidii*, reported as *P. psidii*) demonstrated the existence of at least two distinct biotypes in Brazil, one associated with guava (*Psidium* spp.) and another associated with eucalypts (*Eucalyptus* spp.) and rose apple (*Syzygium jambos*) [38]. Furthermore, coalescence analyses indicated that the eucalypt-infecting *A. psidii* biotype in Brazil did not originate via host jump from guava following the introduction of eucalypts to Brazil, as was long-believed. Thus, the source of the eucalypt-infecting *A. psidii* biotype in Brazil remains unknown. Continued analyses on *A. psidii* populations in Australia, New Caledonia, China—Hainan, and Indonesia showed little variation among the microsatellite-based genotypes [63, 67, 77], whereas, a unique *A. psidii* genotype was associated with myrtle rust emergence in South Africa [76]. Subsequent microsatellite analyses have determined that a "pandemic" *A. psidii* biotype occurs in Costa Rica, Jamaica, Mexico, Puerto Rico, USA—Hawaii, and USA—Florida [84•], which contains the genotype that is found in Australia, New Caledonia, and Indonesia [63, 67] (Fig. 2, [84•]). Furthermore, bioclimatic modeling indicated that *A. psidii* biotypes were associated with a different predicted suitable climate space, which likely reflects distinct invasive threats to some geographic regions [84•].

SNP-based analyses have been utilized to elucidate spread of invasive, introduced, and emerging pathogens. In studies of the ambrosia beetle (*Platypus koryoensis*)-vectored fungus (*Raffaelea quercus-mongolicae*) associated mortality of Mongolian oak (*Quercus mongolica*) in South Korea, Kim et al. [50•] used RAD-Seq to examine the population structure of the putative pathogen. Their analyses revealed low heterozygosity and no apparent population structure, which are consistent with the hypothesis that this putative pathogen was introduced to South Korea. Further, the population structure of the invasive ash dieback pathogen (*Hymenoscyphus fraxineus*) indicated that the pathogen can infect bark and survive saprophytically, and showed no differentiation between epidemic and post-epidemic populations and genetic diversity within the founding population in northeastern Europe was largely maintained in the front of the disease epidemic [12•].

Using a combination of microsatellite and SNP-based analyses, the dissemination, population structure, and evolution of the brown root rot pathogen, *Phellinus noxius*, has been elucidated. Chung et al. [17] examined mechanisms of *P. noxius* spread in Taiwan using microsatellites. Their study showed that tree-to-tree spread of *P. noxius* is largely clonal, but basidiospore-derived spread has resulted in little differentiation among populations in Taiwan. A similar approach has been applied for *P. noxius* populations in Japan, and it was found that *P. noxius* populations are genetically distinct on the Ryukyu and Ogaswawara islands of Japan [1]. These results suggest that *P. noxius* on the two island chains had different origins. Further sequencing at the whole genome level found that *P. noxius* in Asia Pacific is comprised of two lineages, both of which are extremely genetically diverse [18•].

Genomics

Sequencing of the complete genome (whole genome sequencing) determines the DNA sequences contained in the nuclear (chromosomal) and mitochondrial DNA of a fungal isolate. The availability of genome sequences for forest fungi has increased rapidly with the continued improvement in sequencing technologies [3]. As more fungal genomes are available, researchers can more easily determine the possible biology of pathogens by determining the function of genes found within each genome.

The genomic sequence of *Phanerochaete chrysosporium*, a lignocellulose-degrading, wood-rot fungus, was an early example of genomic sequencing of a forest-associated fungus, which provided baseline data for subsequent sequencing of filamentous basidiomycetous fungi [65]. Since then, the genomic sequencing of wood-degrading fungi has rapidly increased [70], which has allowed large-scale genome comparisons with diverse applications, such as bioenergy production and bioremediation, and defines the genetic mechanisms behind different modes of wood decay (e.g., [34]). In addition, genomic sequencing has been completed for diverse other forest-associated fungi, including *Grosmannia clavigera* [25], *Melampsora larici-populina* [29, 40], *Heterobasidion* spp. [16, 71], *Armillaria* spp. (e.g., [21, 80]), *Dothistroma septosporum* (*Mycosphaerella pini*) [23•], *Diplodia pinea* [6], *Ophiostoma novo-ulmi* [22], *Mycosphaerella populorum*/

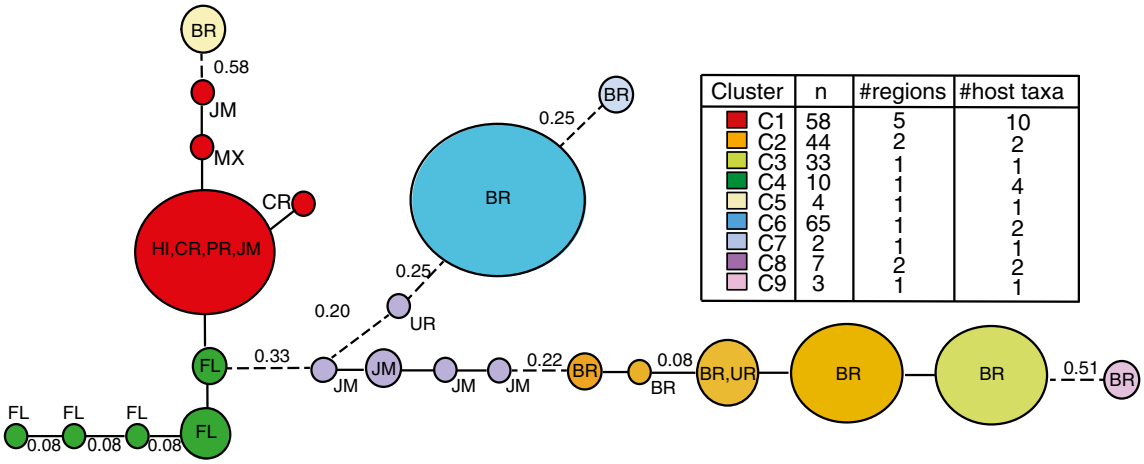


Fig. 2 Minimum-spanning network of *Austropuccinia psidii* microsatellite multilocus genotypes (MLGs) sampled from Brazil (BR), Costa Rica (CR), Jamaica (JM), Mexico (MX), Puerto Rico (PR), Uruguay (UR), and Florida (FL) USA, and Hawaii (HI) USA on 18 hosts. MLGs are represented by BAPS genetic clusters: C1 represents MLGs from Costa Rica on crimson bottlebrush (*Callistemon lanceolatus*), Jamaica, Mexico, Puerto Rico on rose apple (*Syzygium jambos*), and Hawaii, USA on ko olau eugenia (*Eugenia koolauensis*), broad-leaved paperbark (*Melaleuca quinquenervia*), pōhutukawa (*Metrosideros excelsa*), ōhi a lehua (*M. polymorpha*), common myrtle (*Myrtus communis*), rose myrtle (*Rhodomyrtus tomentosa*), Java plum (*S. cumini*), rose apple, and Malay rose apple (*S. malaccense*); C2 represents MLGs collected from Brazil on eucalypts (*Eucalyptus* spp.) and rose apple and from Uruguay on eucalypts (*Eucalyptus grandis* and *E. globulus*); C3 represents one MLG collected from Brazil on eucalypts; C4 represents MLGs collected from Florida, USA on broad-leaved paperbark, twin berry (*Myrcianthes fragrans*), rose myrtle, and rose apple; C5 represents one MLG collected in Brazil on Java plum; C6 represents one MLG collected in Brazil on guava (*Psidium guajava*) and Brazilian guava (*P. guineense*); C7 represents one MLG collected in Brazil on pitanga (*E. uniflora*); C8 represents MLGs collected from Jamaica on allspice (*Pimenta dioica*) and Uruguay on sweet flower (*Myrrhinium atropurpureum*); and C9 represents one MLG collected from Brazil on jabuticaba (*Myrciaria cauliflora*). Sizes of circles are proportional to MLG frequency. Connections are labeled with Bruvo genetic distances if different from 0.04, which corresponds to 1 mutational step at one locus. Broken lines connect MLGs that are separated by distances greater than 0.20. Reprinted with permission from Stewart et al. ([84•]. Forest Pathology, 48:e12378)

M. populicola [24••], and others. Aims of the genomic sequencing of forest fungi are ultimately related to forest management and risk assessment through understanding pathogenicity/symbiosis, adaptability, fungus-host/substrate interactions, etc. A few more detailed examples are presented below.

A poplar (*Populus* spp.) leaf rust pathogen (*Melampsora larici-populina*) provided the first available genomic sequence for a tree pathogen, and genomic sequencing was conducted by a whole genome shotgun method [29]. The genome analysis of this pathogen identified genes related to obligate biotrophy and host infection, and subsequent analyses identified genes encoding candidate effectors in the rust pathogen [39]. Insights into the ectomycorrhizal symbiosis of *Laccaria bicolor* were obtained by genomic sequencing, which identified gene sets involved in rhizosphere colonization, symbiosis, and nutrient cycling, but noting the absence of genes encoding enzymes to degrade polysaccharides contained in plant cell walls [64]. It is suggested that the genome comparison of symbiotic (*L. bicolor*) and pathogenic (*M. larici-populina*) basidiomycota fungi interacting with poplar can provide insights into pathogenicity/symbiosis mechanisms in evolutionary processes.

When the genome of a poplar canker pathogen (*Mycosphaerella populorum*) was compared to a closely related poplar leaf pathogen (*M. populicola*), relatively few genomic changes were found [24••]. Especially noteworthy is that changes in gene expression were associated with different disease etiology. It was further proposed that *M. populorum* gained the capacity to infect woody tissue via horizontal gene transfer and changes in gene dosage, which could have changed an innocuous, coevolved pathogen into a destructive pathogen of poplar plantations.

The genome of *Grosmannia clavigera*, an ascomycetous fungus associated with blue stain of conifers, was sequenced by combining data derived from different sequencing methods [25]. First insights into potential infection mechanisms of a pathogen associated with Armillaria root disease (*A. mellea*) was obtained through genomic and proteomic analysis [21]. This research found that *A. mellea* has a broad suite of carbohydrate-degrading enzymes, similar to both basidiomycete and ascomycete glycodegrative arsenals. The genome of *Dothistroma septosporum* (*Mycosphaerella pini*), the cause of red band needle blight of pines, was sequenced and compared to a closely related fungus to examine genes related to lifestyle adaptations and genes of common ancestry [23•, 42]. Genomic sequencing of the tip blight pathogen of pines (*Diplodia pinea*) provided the basis for a study of the *MAT* genes in pathogen populations [6]. Results suggested that *D. pinea*, which was previously considered exclusively asexual, may have a cryptic, heterothallic sexual cycle.

The myrtle rust pathogen (*A. psidii*) was found to contain sequences associated with transposable elements within ca.

27% of its genome, which may enhance its ability to generate genetic variability associated with adaptation to new hosts and environments [86]. The associated phylogenetic analyses of three DNA regions placed *A. psidii* within a separate branch with the Pucciniaceae lineage. Lastly, insights into the pathogenicity of the elm bark beetle-vectored Dutch elm disease pathogen were obtained by the annotation of the ascomycetous *Ophiostoma novo-ulmi* genome, which identified 1731 genes encoding proteins that were potentially involved in pathogenicity and diverse other genes related to carbohydrate utilization, electron transfer/detoxification, metabolism, growth/reproduction, signaling/plant defense relationships, etc. [22].

Transcriptome

The transcriptome of a forest fungus is the set of all expressed genes (transcribed as mRNA) associated with the fungal sample that reflects a specific time, tissue, developmental stage, abiotic/biotic environment, and other conditions under which the sample was collected. Brief examples of transcriptome sequencing of forest-associated fungi are presented below.

Root-Associated Fungi

Root disease pathogens are among the most actively investigated forest fungal pathogens using transcriptomic approach to examine diverse functions, such as pathogenicity and pathogen-host interactions. Transcribed genes in an active mycelial fan of *Armillaria solidipes* in association with grand fir (*Abies grandis*) were sequenced, which provided insights into putative signal peptides and genes with functions in pathogenesis, such as those encoding plant cell wall-degrading enzymes and responses to post-infection host environment, and other genes related to forest root/butt diseases (Fig. 3; [74]). Transcriptomes of invasive, vegetative, and reproductive developmental stages of *A. ostoyae* were compared, which provided detailed information on the regulation of pathogenicity-related genes, and evolutionary relationships of genes involved in wood-decay, morphogenesis, and complex multicellularity [80]. Gene expression studies of the laminated root-rot pathogen, *Phellinus sulphurascens*, revealed differential expression of genes encoding potential virulence factors, putatively secreted proteins, and many other enzymes [92]. Early studies on expressed genes of *Heterobasidium annosum* during infection of Scots pine (*Pinus sylvestris*) identified genes that were differentially expressed and genes with unknown function, which identified the need for genomic sequences of this and other forest fungi [45]. Genomic sequencing and transcript profiling of *H. irregulare*, a root/butt-rot pathogen of coniferous trees, was used to examine the differentially expressed genes associated with mechanisms for wood decay and parasitism [71]. Genes encoding putative ligninolytic enzymes in *H. irregulare* were shown to be differentially regulated in a substrate-specific manner [93]. Gene expression of Norway spruce (*Picea abies*) inoculated with *H. annosum* s.s. [62•] identified expression of several pathogen genes, including genes associated with virulence, in addition to a multitude of host genes.

Wood-Associated Fungi

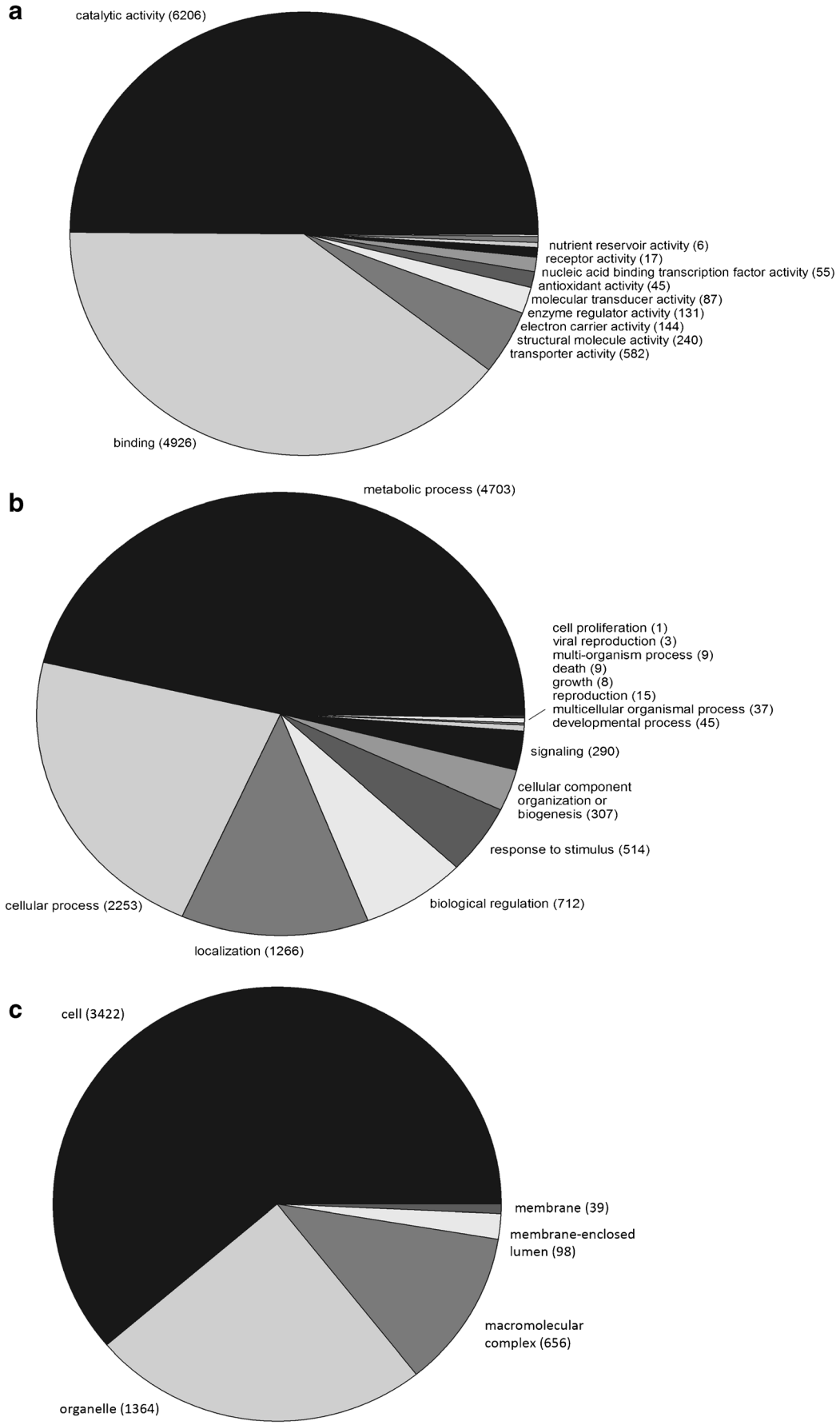
Analysis of the genome and transcriptome of a wood-rot fungus, *Phlebiopsis gigantea*, that is capable of colonizing freshly cut conifer stumps, revealed potential gene-based mechanisms that allow tolerance for resinous compounds while degrading complex polymers of wood [44]. Genome and transcriptome sequencing was performed on *Grosmannia clavigera*, the fungal symbiont of mountain pine beetle (*Dendroctonus ponderosae*) and causal agent of blue stain disease of lodgepole pine (*Pinus contorta*), which provided insights into how this pathogen tolerates conifer-defense chemicals during tree colonization [26].

Foliage/Branch/Stem-Associated Fungi

Transcriptome analyses of the white pine blister rust pathogen predicted the secretome, candidate effectors, other pathogenicity determinants, and genes that were differentially regulated during different life-cycle stages [61]. Differential gene expression of *Dothistroma septosporum*, a needle endophyte and pathogen of radiata pine (*Pinus radiata*), was examined at different stages of infection [8••]. Genes encoding wall-modifying enzymes and signaling proteins appeared upregulated in the initial biotrophic stage and genes encoding enzymes associated with secondary metabolism were upregulated in later necrotrophic stages. Recent genomics and transcriptomics studies of *Marssonina brunnea* infection of poplar leaves examined changes in gene expression of the pathogen and host, which demonstrated differential regulation of pathogen virulence genes in association with differential regulation of host resistance genes, which exemplified the complex host-pathogen interactions that occur during the infection process [15, 94].

Metagenomics/Metatransciptomics

Metagenomic and metatranscriptomic studies elucidate the bacterial and fungal (and other) organisms within microbial communities and the function of those organisms within environmental samples through DNA and RNA sequencing. These approaches can determine the phytobiome (microbes associated with plants) within or associated with forest foliage, branches/stems, wood, roots, or rhizosphere using


a
catalytic activity (6206)
nutrient reservoir activity (6)
receptor activity (17)
nucleic acid binding transcription factor activity (55)
antioxidant activity (45)
molecular transducer activity (87)
enzyme regulator activity (131)
electron carrier activity (144)
structural molecule activity (240)
transporter activity (582)
binding (4926)
b
metabolic process (4703)
cell proliferation (1)
viral reproduction (3)
multi-organism process (9)
death (9)
growth (8)
reproduction (15)
multicellular organismal process (37)
developmental process (45)
signaling (290)
cellular component organization or biogenesis (307)
response to stimulus (514)
biological regulation (712)
cellular process (2253)
localization (1266)
c
cell (3422)
membrane (39)
membrane-enclosed lumen (98)
macromolecular complex (656)
organelle (1364)

◀ **Fig. 3** Classification of sequences from de novo transcriptome assembly of *Armillaria solidipes* RNA1 based on predicted gene ontology (GO) terms. **a** Molecular function. **b** Biological process. **c** Cellular component. GO terms were determined using Blast2GO PRO V.2.6.4 with an *e*-value cutoff of $1e^{-3}$, filtered by fungal homology, and are sorted based on level 2 classifications. Reprinted with permission from Ross-Davis et al. ([74] Forest Pathology 43:468–477)

DNA barcoding, e.g., tagged amplicon sequencing of the ITS region, or shotgun (whole community) sequencing. The utility of metagenomic studies to help understand ecological interactions within forest ecosystems is greatly increased by the collection of environmental metadata (e.g., temperature, moisture, biotic environment, soil physical and chemical properties, and site history) and a well-designed study with suitable sampling [60••]. Such studies can determine the identity and role of microbes in forest ecosystems processes, such as disease, decomposition, and symbiosis. An early metagenomic study on forest soils found a high diversity of fungal species in six different forest soils, with the majority of species belonging to Ascomycota and Basidiomycota [10], and DNA barcoding was used to identify diverse fungal taxa associated with wood decay in tropical forests [91•]. Forest harvesting was associated with a reduction in genes associated with biomass decomposition [13]. Uroz et al. [90••] examined relationships among bacteria and fungi in temperate and boreal forests in association with nutrient recycling and other ecological functions. Štursová et al. [85••] showed that death of Norway spruce (*Picea abies*) forests caused by bark beetle (*Ips typographus*) resulted in profound changes in the fungal communities in the soil with decreased biomass and reduced fungal symbionts of tree roots, and relative increases of saprophytic taxa. Recently, Žifčáková et al. [95] used metagenomic and metatranscriptomics approaches to examine soil and litter microbes, including fungi, and ecological functions in coniferous forest soil, which were substantially different during summer and winter, especially in the soil. That study also showed reduced activities of ectomycorrhizal fungi in winter, an indication that photosynthetic output is a driver of changes in microbial function of soil microbes in coniferous forests. Metagenomic approaches have also been used to examine wood decomposition. For example, studies by Kubartová et al. [56] revealed different patterns in fungal communities within decaying logs. Such approaches are essential to understand intricate microbial interactions and successional processes in complex ecological activities in forests, such as decomposition and nutrient cycling.

The clear trend from research on microbial communities within forest ecosystems and in other systems as well is that ecological processes are much more complex and networked than was previously recognized. Metagenomics studies are radically transforming traditional forest pathology paradigms by demonstrating that ecological functions, such as root/canker diseases, wood rots, and symbioses, are the result of complex interactions among microbial communities, and not the direct result of a single microbial pathogen, decomposer, or symbiont interacting with a single host or substrate. Though in its infancy, the field of metagenomics shows great promise for helping researchers understand complex interactions among forest fungi (e.g., pathogens, symbionts, decomposers, and biocontrol agents), hosts, and associated microbial communities that will aid in the development of novel tools for forest management in the future.

Conclusions

This review is a brief summary with selected examples of widely used approaches in (1) DNA-based identification and detection; (2) phylogenetics; (3) population genetics/genomics; (4) genomics/transcriptomics; and (5) metagenomics/metatransciptomics. The selection of suitable genetic/genomic approaches for application to forest-associated fungi is situation specific and depends on various parameters (i.e., research objective, quality/quantity of available DNA, supporting genetic information for the fungus, cost considerations, available labor skills and equipment, etc.). Genetic and genomic technologies continue to become more powerful and affordable, which allows new and expanded applications to forest-associated fungi, and these applications will likely continue to grow in a manner that reflects advances in next-generation sequencing technologies, computational power, and bioinformatics. Such genetic and genomic approaches will expand our understanding of ecological processes in forest ecosystems by determining the diverse roles of microbial communities in processes such as pathogenesis, biological control, symbiosis, decomposition, or other critical processes in relation to forest-associated fungi and the environment. This highlights the significance of genetic and genomic approaches which could foster a paradigm shift away from single microbial pathogens, decomposers, or symbionts interacting with a single host or substrate and provide more holistic approaches toward understanding interactions among microbial communities that drive forest health processes. Diverse molecular genetic approaches offer the potential to better understand the ecological interactions of forest fungi with biotic/abiotic components within forest ecosystems, and this information can be used to foster forest health, productivity (carbon sequestration), sustainability, and resilience.

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Compliance with Ethical Standards

Conflict of Interest Dr. Stewart, Dr. Kim, and Dr. Klopfenstein have no conflicts of interests to declare.

Human and Animal Rights and Informed Consent This article contains no studies with human and animal subjects performed by the authors.

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