# DNA Test Identifies DNA Wood Decay Fungi

By Matteo Garbelotto, Paolo Gonthier and Giovanni Nicolotti

Late last year, the University of Turin (Italy) had made a DNA-based assay available in the European market for the identification of wood decay organisms. This year, U.C. Berkeley (Berkeley, California) is providing the same service for the North American audience. The assay is described in three research papers recently published in the *Journal of Applied Microbiology* (103, 1490-1507), *FEMS Microbiology Letters* (282, 228-237), and *Arboriculture and Urban Forestry* (Nicolotti et al., in press). Although wood sampling approaches are already available, ongoing research lead by the University of Turin is currently studying how to optimize sampling of wood from standing trees: results will be available and publicized soon.

On the other hand, the basic assay that allows for the identification of major wood decay fungi from sampled wood is already developed and has been fully validated by testing its reliability and sensitivity on a range of samples of known infection status, with potential future changes dictated only by the inclusion of decay agents currently not targeted by the assay.

The principle behind this DNA-based test is simple: each living organism on this planet, including the fungi that are responsible for wood decay, has portions of its DNA that are unique and different from the DNA of all other organisms. The first step in the development of a diagnostic assay was to identify regions of DNA that would unambiguously differentiate one wood decay fungus from the other.

## Method of Analysis

In order to achieve this first goal, a large collection of wood decay agents and of close but harmless relatives was assembled in laboratories at both Berkeley and Turin. DNA sequences were obtained for each fungal species and compared with DNA sequences already present in public databases. Two different portions of the genome were then identified as being characterized by having DNA sequences that were conserved among different individuals of the same species, but variable when comparing different species. These DNA portions became the target of our assay.

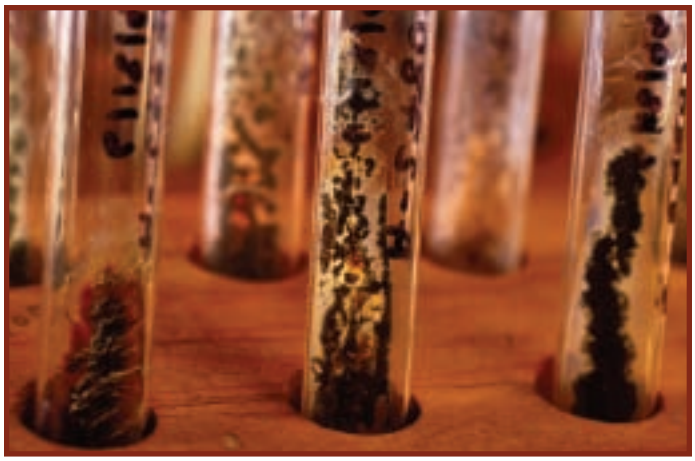

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**Figure 1. Isolated mycoparasites to be used in laboratory studies.**

The second step in assay development involved the design of probes that would unambiguously identify individual fungal species. DNA is a helix of two strands of nucleotides (i.e. the building blocks of DNA), each made of a long sequence of any of four possible ones. Each triplet of three nucleotides, codes for an amino acid, and during the process of protein synthesis, protein-assembling enzymes will decode each triplet thus determining the order in which amino acids will be assembled to form proteins.

We identified 20-30 nucleotide-long DNA stretches that were unique for each target fungal species, and synthesized artificial pieces of DNA to perfectly match such stretches. Each of these synthetic DNA blocks is called a primer, and for each targeted species, two primers were designed. Primers serve as probes to identify each decay agent when used in a process called the Polymerase Chain Reaction or PCR.

The PCR is a way to multiply one copy of a portion of DNA into millions of copies. Once a piece of DNA is "amplified" into millions of copies, it can be easily studied and in particular, its specific sequence and size can both be determined. The two primers determine the start and finish position of the amplification process. Only the DNA sequence included between the two primers will be amplified into millions of copies.

We designed PCR primers that not only had unique DNA sequence for each decay agent, but the distance between the two PCR primers was designed to be unique for each targeted species. If primers do not match the DNA sequence of the target organism, no DNA amplification occurs, and only when a target organism DNA is present does amplification occur. The size of the DNA amplified through the PCR process is unique and diagnostic for each targeted decay agent.

Next, the features of the assay were tested. This was done not only by testing the assay on fungal fruit bodies and fungal cultures, but also by testing wood from trees characterized either by failures or by the presence of obvious decay symptoms. The validation process showed that in 82% of the cases there was a match between the identification of the decay agent by the assay with that determined through identification of associated fruit bodies. Validation also indicated that the DNA-based assay could identify agents in the absence of fruit bodies.

In many cases, although fruit bodies of a single agent or a single type of decay pattern could be identified, the assay detected more than one decay agent in the same tree. The validity of the developed assay was confirmed when it was determined that its sensitivity in two tree species, namely California live oaks and London plane trees, was extremely high. In our trials, the assay could identify a decay agent in a wood chip the size of a thumb nail if it were ground into an Olympic-size swimming pool. Although tree species did affect the sensitivity of the assay, the threshold of detection was extremely high for both tested species.

## Understanding the DNA Test

Naturally, the assay does not detect all decay agents, but it was developed only for those agents that were identified as relevant for landscape or urban situations in North America and in Europe. For instance, the widespread root decay agent *Heterobasidion annosum* was not included in the assay, because Annosus root disease is a problem in forest situations rather than in urban settings. Currently, the following genera or individual species are detected by the assay: *Armillaria spp., Ganoderma spp., Ganoderma resinaceum,* European *Ganoderma lucidum,* European *Ganoderma applanatum, Ganoderma adspersum, Hericium spp., Hypoxylon thouarsianum var. thouarsianum, Inonotus/Phellinus-group, Phellinus s.s. sp., Inonotus s.s. sp, Fuscoporia sp., Inonotus dryadeus, Fomitiporia sp., Inocutis sp., Laetiporus spp., Perenniporia fraxinea, Pleurotus spp., Schizophyllum spp., Stereum spp.* and *Trametes spp.*

The assay has an inbuilt control mechanism capable of detecting the presence of any fungus in the tested wood by using fungal universal DNA primers matching the DNA sequence of all fungi. This control ensures that the basic DNA amplification process has worked, and at times it may allow for the identification of decay agents that are not included in the standard assay. However, most non-target fungi detected by the control process are either endophytic or secondary colonizers not responsible for significant decay; nonetheless, their detection is a valid way to determine whether the negative results of the assay are true negatives due to the lack of any target organism or false negatives due to human error or technical malfunction.

## Laboratory Wood Sampling

It is essential to appropriately sample the wood. In the case of tree failures, the best way to collect wood is to cut out four quarter-sized pieces of wood right from the area where the breakage occurred and where obvious signs of decay such as stringy and discolored wood may be visible. The four samples should come from the four furthest points (imagine the four tips of a cross) within the area of the breakage and showing signs of decay. Once taken they can be pooled together and sealed in a paper bag or in a paper envelope. The use of paper containers minimizes the growth of molds; nonetheless, samples should always be kept in a cool place and away from sunlight, in order to minimize the breakdown of the DNA present in the sample.

**Figure 2. The double-fluted long drill bit used to collect sawdust from the base of standing trees.**

Alternatively, in the case of standing trees very small holes can be drilled at the root collar, either in the four cardinal directions or following three directions at 120 degrees, and the wood can be easily sampled by collecting the sawdust generated during the drilling. In the presence of obvious signs or symptoms of decay, one of the sampling directions should match the area of the stem displaying such signs or symptoms. Sawdust from the four or three holes can be pooled and collected in a paper container as described above for sampling of tree failures.

Use a 3/16" diameter, 16" long drill bit, with 12" long double flutes along the shaft to help clear the wood chips. The bit should be made of chrome vanadium steel for flexibility and strength, and have a cobalt/tungsten carbide tip for resistance to wear. The bits we currently use are manufactured by ARTU USA (Aberdeen, North Carolina). Any similar bit will work, as long as it is long enough to allow sampling most of the wood between the bark and the central core of the tree.

For laboratory assistance in this method of decay diagnosis, a tree sample submission form along with an accompanying tree sample can be sent to: Dept. of Environmental Science, Policy and Management; University of California; 137 Mulford Hall; Berkeley, California, 94720. A form can be downloaded from our website (Nature.Berkeley.edu/Garbelotto/English/Woodrot.php). Samples must be sent overnight using UPS or FEDEX, and the lab should be informed of the shipment as instructed in the form.

Once samples are received, they are immediately frozen for at least 24 hours, before being lyophilized. DNA is extracted from lyophilized wood and three rounds of PCR reactions are performed on the DNA extracts to determine the presence of the different fungal genera. If *Phellinus*, *Inonots* or *Ganoderma* are detected, then an additional PCR reaction is performed to identify which species is present in the sample. Genus or species identification is done by determining the size of any DNA fragment that was amplified during the assay (Figure 3).

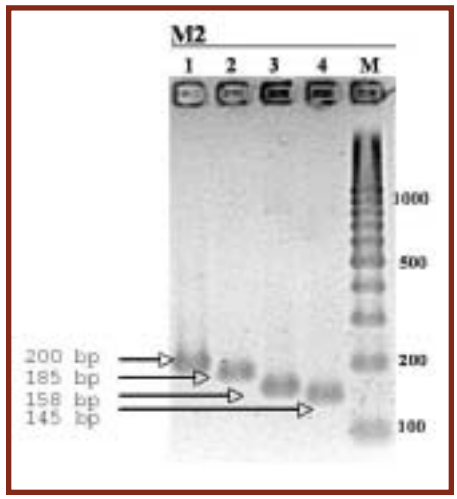


**Figure 3. The dark horizontal bands in the five lanes 1-M represent fragments of DNA of different length expressed in base-pairs or bp: each size corresponds to a different decay agent. Lane M is a standard used to determine the size of DNA bands, lane 1-*Hericium flagellum*, lane 2-*Armillaria mellea*, lane 3-*Pleurotus ostreatus*, lane 4-*Laetiporus sulphureus*.**

Figure 4 is a flowchart that explains the entire diagnostic process. While the standard assay is limited to the fungal agents mentioned above, it may be possible to look for particular agents. This process may be facilitated if a fruit body of the target agent is provided with the shipment. This is a non-profit service, in order to cover expenses we ask for a voluntary contribution of US$90 per tree for diagnosis of fungi already included in the assay (plus

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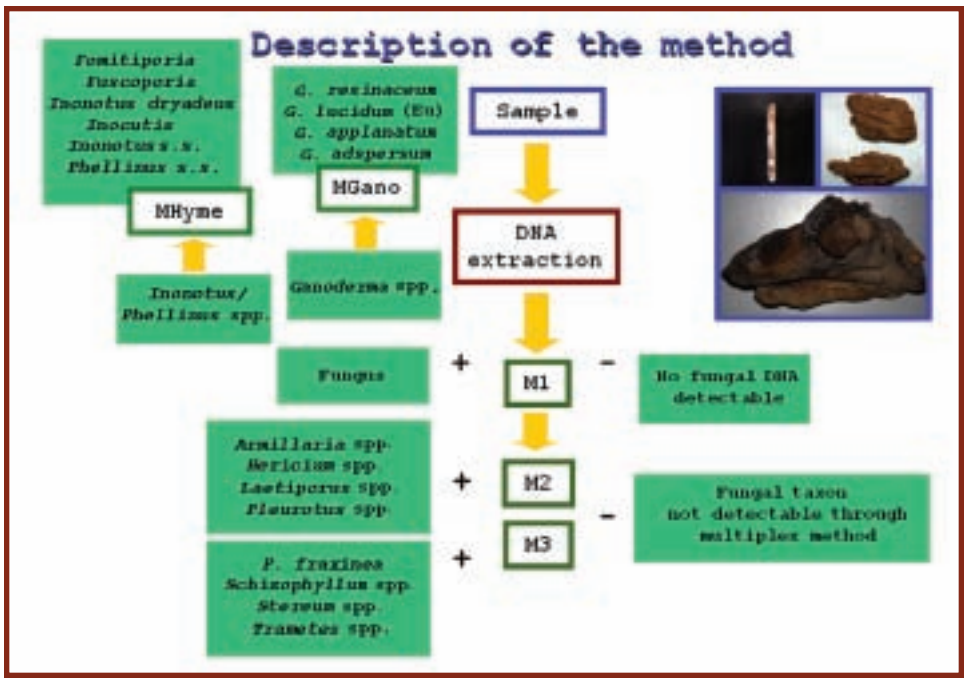


**Figure 4. A flowchart of the DNA-based assay developed to identify some wood decay agents. M1, M2, and M3 represent three DNA amplifications always performed, while MGano and MHyme are DNA amplifications performed only if M1 resulted positive for *Phellinus/Inonotus* spp., or *Ganoderma* spp., respectively.**

US$90 to attempt identification of each new fungal species) that will be used to further research and professional education in forest pathology.

The diagnostic service here described does not require the presence of any symptoms or signs of decay fungi for their successful detection, and as such, it is superior to other methods currently available. It is also a method that allows for the identification of multiple decay agents in the same tree. The ability to identify standing trees harboring decay agents allows tree managers to red-flag these trees as individuals that need to be carefully monitored through time. Because the method allows the differentiation between more and less aggressive agents, tree can also be prioritized in terms of monitoring needs. This method is invaluable to managers dealing with large number of trees as it allows for a rational definition of a subset of trees requiring closer attention. Wood decay agents can also be identified for some time after failures or windthrows thanks to the fact that the method does not require living fungal tissue: this can be extremely important in the case of tree failures resulting in substantial damages to lives or property. Nonetheless, it should be highlighted that the success of the diagnosis decreases as time passes. Attempting to identify decay agents in trees fallen for a long time may be inconclusive.

Despite the power of this new DNA-based assay, the method does not provide any information on the extent of the decay, nor does it exclude failure of trees diagnosed as negative for decay agents. Wind, shape of the tree, soil factors, decay agents not included in the assay, or incorrect or limited wood sampling, may all contribute to failures in trees for which the assay could not detect any target decay agent. Tree care specialists need to continue their assessment of tree stability using traditional approaches, such as Visual Tree Assessment and available instrumentations capable of determining extent of decay. Managers and tree care specialists are supported through this assay in their decision-making by knowing which trees positively harbor the most common and important wood decay fungi.

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