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3 The use of taxon-specific PCR primers for ecological and diagnostic applications in forest mycology

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Abstract

The benefits and pitfalls of studying and diagnosing forest fungi using PCR-based methods are discussed in this article. It is argued that, because of the complexity of fungus-tree interactions, the benefits of tools such as taxon-specific primers are currently unparalleled. Recent technological advancements have largely increased the power of these approaches, with the inclusion of in-built verification of the DNA-molecule amplified and its quantification. The use of quantitative PCR allows to further the use of such tools in ecological studies. A good knowledge of the

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systematics of the targeted organism is a necessary prerequisite to design valuable PCR-based diagnostic assays. When such knowledge is present and an appropriate DNA region is available, fungal diagnostics needs to incorporate such new tools. Combined with more traditional techniques, DNA-based diagnostics will maximize our potential to diagnose tree fungi directly in *planta, even when culturing of the microorganism in question is not reliable.*

1. Introduction

Forest mycology, which I here define as the science focusing on beneficial or detrimental fungi in forests, poses unique challenges. Most of them are directly or indirectly determined by the fact that forests are extremely complex ecosystems at the temporal, structural, and spatial levels. Life spans of most trees exceed those of humans, and generational turnover often requires several decades: yet, every life stage of a forest is subject to associations with potentially different guilds of microorganisms. It is well known that some mycorrhizal fungal species are pioneers aiding forest establishment, eventually waning in favor of species associated with mature forests [1]. It is also known that inventories of saprobic and pathogenic fungi are markedly different when taken in mature or young forests [2]. Juveniles may be less or more susceptible to some diseases than their mature counterparts [3], and in many cases, successional events in a forest are accompanied and sometimes caused by successional turnover in the microbial components of such forests [4]. Forests have exceedingly diversified structures. Plant species diversity in forests, although variable depending on forest type and region, is always larger than in adjoining ecosystems. Both in the case of mycorrhizal associations and pathosystems, complex networks are being uncovered in forests in which different but important roles are played by different plant species. The cases of alternate hosts for the rust fungi, and of secondary hosts as inoculum reservoirs for many pathogens are obvious examples of such complexity. Finally, the spatial scale of forests can be dwarfing, with roots growing several meters underground, and canopies hovering tens of meters above the forest floor. Pathogens, symbionts, saprobes are extremely varied based on what cross section of the forest we are looking at. For instance wood decay organisms can often be differentiated based on their ecological preference for terminal portions of branches, basal portion of branches, or main stems [5]. Based on the high level of niche specialization suggested by the above example, it should be no surprise that above-ground and below-ground microbial communities often show little overlap. Transient habitats, such as the forest litter, bridge aboveground and belowground communities, compounding the complexity of the general picture.

For microbiologists, the revolutionary advent of molecular biology, and in particular of DNA technology can only be compared to the discovery of the

microscope. By allowing us to look at and quantify genetic differences, molecular biology has expanded the horizons of experimental science to levels unthinkable just 15 years ago. Besides allowing scientists to analyze the innermost biological and biochemical mechanisms within living organisms and to develop models of interaction between plants or animals and microbes, molecular biology is a way to see those differences that are invisible even to the most powerful microscope. Genetic variation among individuals, and within and between populations or species can be assessed and used to explain evolutionary histories, epidemiological routes, and reproductive strategies.

In the case of forest microbiology though, molecular biology has had far greater repercussions as it has allowed not only for the determination of the genetic structure of microbial populations and species, but it has allowed to see them where they would be practically invisible. The task of identifying fungi in a forest is not easy. Only portions of the fungi are visible or easily culturable, while the vast majority remains undetectable either as obligate symbionts, obligate pathogens, or as the cause of incipient or endophytic infection. The ability to detect and differentiate microorganisms using molecular biology techniques, and in particular DNA-based technology, has been a revolution leading to innumerable ecological, population, and community studies in forests. Although we could say that diagnosis is the essential contribution of these techniques, their contribution is way beyond simple diagnostics; instead, it represents the most powerful approach to disentangle the complex web of the microbial role and function in forests.

In this paper, I will focus on a simple but invaluable tool: taxon-specific (TS) PCR primers. PCR, an acronym for the Polymerase Chain Reaction, is the most broadly used molecular tool currently used in the world. Although I assume some knowledge of the basic technique, I will present more technical detail on Real Time (RT) PCR and Nested (N) PCR techniques, which may still be unfamiliar to many readers. An overview including both the theoretical (and empirical) bases of this technique, and a few studies involving the use of taxon-specific primers, will showcase the power of this tool, and at the same time outline further potential applications as well as drawbacks and pitfalls.

2. What are taxon-specific primers?

The Polymerase Chain Reaction (PCR) is a process used for the amplification of “target” DNA molecules. DNA amplification is possible thanks to an enzyme called *Taq* polymerase capable of replicating each DNA strand at temperatures high enough (e.g. 92 ^{0}C) to allow for the separation of DNA into single strands. The *Taq* polymerase will replicate a DNA strand by using individual nucleotides supplied in the reaction. The starting and finishing points of this process are usually within a few hundred base pairs (bp) from each other, and are determined by two blocks of artificially

synthesized DNA called primers. Primers are normally between 10 and 30 bp long, and must be an almost perfect match to the DNA sequence of the target molecule. Two primers, normally called "forward' and "reverse", are necessary for the reaction to occur. The reaction is completed through a succession of 20-45 cycles including three steps each: 92 ^{0}C to separate the strands, a variable annealing temperature to allow for the primers to anneal at the start and finish of the target molecule, and 72 ^{0}C for the *Taq* polymerase to add each nucleotide in the newly synthesized strand. The end product is highly concentrated, and DNA can be visualized through gel electrophoresis, digested with endonucleases, or sequenced.

Taxon-specific (TS) primers can be used in the PCR reaction, so that only the desired target sequence and organisms will be amplified. Primers must be in a DNA region where sequence divergence is sufficient to unequivocally match only the sequence of target organisms. The target may include individuals of the same species, or of the same genus, family, phylum, etc. This is usually achieved by identifying a DNA region unique to the target taxa through comparative DNA alignments. The specificity of such primers can be extremely varied and range from an entire kingdom (i.e. the fungi), to an individual allele (i.e. flanking regions of microsatellites). It should be emphasized that only one of the two primers needs to be taxon-specific to obtain specificity in the PCR reaction.

Although these primers can be used on laboratory cultures, their most powerful utilization involves the study of target organisms directly from environmental samples such as soil, water, and plant tissue. This allows for the identification of microorganisms without the need for culturing. This feature is obviously of enormous relevance when studying microbes that are hard or impossible to culture. These primers also allow to identify organisms long after their viability has disappeared, thus allowing for the study of short-lived organisms or of fossils and herbarium specimens.

3. The art and the science of primer design

Optimal primers can be designed by using one of the many software or web-based programs available. These programs will ensure that primers will not loop into hairpins, and that nucleotide complementarity will not result in primers binding to themselves or to the other primer rather than to the template DNA. They will also determine other important parameters such as optimal annealing temperature for each primer pair, and $MgCl_2$ concentration (see below). They will not, however, identify taxon-specific regions. These regions have to be identified by analyzing DNA alignments including the desired taxa and as many close relatives as possible. Unique polymorphisms should be placed at the 3'-end of the primer, where annealing is initiated. If possible, unique DNA deletions and insertions should be included in the primer region.

Although theoretically a single unique nucleotide polymorphism at the 3'-end should be enough to design a taxon-specific primer, it is preferable and sometimes necessary to have at least two unique polymorphisms at the 3'-end, and as many unique polymorphisms or deletion/insertions as possible in the remainder of the primer. Often, unique DNA regions may be hard to find. In this instance, the researcher may only have one or two choices for primer design. This may require accepting sub-optimal primers, with higher self-complementarity, hairpin secondary structure, or complementarity to the other primer. In most cases, moderate deviations from optimal parameters do not cause too many problems, but empirical tests are always needed before selecting a primer for a study or a diagnostic test.

When taxon-specific (TS) primers are used, amplification of other organisms will require a mismatch between primer and template. If reactions are performed in stringent conditions, this mismatch should result in lack of primer annealing to all organisms but the ones targeted by the assay. At any rate, amplification of non-target organisms will always be at a disadvantage, because energetically more costly that amplification of target organisms. In order to run the assay in stringent conditions, it is essential to optimize the parameters of TS-PCR reactions. The three basic parameters of PCR reactions are $MgCl_2$ concentrations, primer annealing temperature, and DNA template concentration.

$MgCl_2$ concentrations are normally empirically determined, and range between 15 and 25 μM of final concentration. Lower concentrations are normally used when higher specificity is desirable. When concentrations of the salt are low, however, even minute variations in the preparation of the reaction may result in absence of available salt for the enzyme. In this case, PCR conditions may be too stringent, resulting in a high number of false negatives.

Optimal annealing temperature for each set of primers differs based on primer length and its GC content, and can be calculated using different algorithms. In general, most algorithms will calculate the optimum annealing temperature; in TS assays, it may be desirable to use the maximum annealing temperature, which may be a couple of degrees higher. Theoretical calculations need to be backed up by empirical temperature optimization tests. Most PCR thermalcyclers are equipped with a temperature gradient option that can be activated in a single run. This feature is extremely helpful in the empirical determination of optimal annealing temperature. Alternatively, a touch-down approach can be used. In a standard touch-down protocol, starting annealing temperatures are high and they are progressively lowered on each cycle, until the theoretical optimal anneal temperature is reached. When temperatures are too high, the lack of primer annealing results in no amplification. As the temperature is lowered, the maximum annealing temperature is reached, and primers will anneal to template DNA in extremely stringent conditions, ensuring maximum specificity. Following cycles will use this highly specific

product as template for further amplifications, even as the temperature is further lowered, thus resulting in highly specific amplification product.

Template DNA concentration is the final parameter that requires careful consideration. In general, problems may arise when DNA template concentration is either too high or too low. Because DNA extracts used for molecular ecology studies are rarely pure, the presence of PCR inhibitors, carried over during the processing of environmental samples such as plant roots, leaves or soil, may also affect the outcome of PCR amplifications. When inhibitors are present, increased dilutions of the template may have a beneficial outcome on PCR success, unless the amount of target DNA in the overall sample is minimal. In that case, further DNA purification steps may be required: these may include additional DNA precipitation, DNA chromatography or affinity steps, and Proteinase K treatment. When DNA concentrations are too high, specificity of primers can be lost. It is therefore important to empirically determine the range of template DNA concentration for which no undesired cross-reactivity occurs. If concentration-dependent cross-reactivity occurs, it may also be helpful to decrease primer concentration. Final primer concentrations in a PCR reaction generally range between 5 and 50 μM.

Specificity of any primer can be assessed only when a complete sequence database for the target organisms and their relative is available. GENBANK and in particular its POPSET database are invaluable sources of sequences and alignments for such purpose. It should be noted that, because taxonomic classification of fungi and oomycetes is in constant change, limiting the comparison to species within a genus may not be adequate. A perfect example regarding this issue is provided by the two wood inhabiting genera *Phellinus* and *Inonotus*, which are unresolved at the molecular level. In order to design a primer to identify a single species within either of these two genera, representatives from both would have to be included in the comparative alignment. This observation underlines the issue that often, an in depth knowledge of the taxonomic positioning of an organism is necessary to design good taxon-specific primers. The same knowledge is necessary when dealing with taxa or biological species within morphologically identical species-complexes. Unless the researcher is interested in a single biological species, a DNA region shared by all biological species should be targeted. Independently of theoretical primer design, it is always necessary to test the primers on a wide array of target and related organisms. Sometimes, unpredicted cross - reactivity may occur. Cross reactivity is not always fully understood, but it may be determined by a variety of causes, including changes in the stringency of the PCR reaction (a frequent occurrence when dealing with environmental samples), insufficient specificity of the primer sequence, contamination of the sample DNA, and presence of multiple and divergent copies of the same DNA region as it as been shown for some fungal groups [6].

The DNA region of choice for primer selection is often dictated by what is already available. Alternatively, if cultures or specimens representing the whole clade of interest are accessible, new sequence databases can be generated. In general, phylogenetic resolution of a DNA region is a good guide to determine resolution potential for taxon-specific primers. Many reviews and articles are available on the taxonomic level of resolution of several molecules [7]. The most developed databases include nuclear and mitochondrial ribosomal operons, as well as "housekeeping" genes such as elongation factor alpha-1, Beta-tubulin, etc. It should be noted that resolution power of sequences for taxonomic studies and taxon-specific primer design need not be the same. In general gene-coding regions, rich in third-base substitutions, may provide solid databases for phylogenetic studies of groups of related species, but may not provide small clusters of substitutions, or insertions/deletions that are needed for the design of TS primers. Other times, a conserved region characterized by the presence of insertions and deletions uniquely associated with some taxa, will be poor material for a phylogenetic analysis, but may help design powerful taxon-specific primers. A final consideration in the choice of a target region for primer design is the number of gene copies present in the genome. Multiple ribosomal gene operons are often selected because they considerably rise the threshold level of detection when compared to single-copy genes. This is a critical issue when dealing with environmental samples in which the target DNA may represent a very small portion of the total DNA of the sample.

4. Shortcomings and pitfalls of taxon-specific (TS) PCR

As stated above, cross reactivity is a distinct possibility even for the best designed TS-PCR protocol, especially in the course of molecular ecology studies based on real-world environmental samples. Furthermore, undetected malfunction of the thermalcycler may also result in unwanted cross-reactivity. It is therefore of paramount importance to implement a system of product confirmation, to verify no cross reactivity occurs. There are several options available to the researcher. If the target PCR product is unique in size, careful determination of amplicon size through gel electrophoresis may suffice. If amplicon length is not unique, nucleotide sequence needs to be. In order to ensure the amplicon belongs to the target organism, at least four approaches are currently available. If Real Time PCR technology is available (see below), it may be possible to select an amplicon characterized by a specific melt temperature [8]. If high resolution electrophoresis is available, it may be possible to use single strand conformation polymorphism (SSCP) to ensure product specificity [9]. The amplified fragment may be characterized by the

presence of unique endonuclease restriction sites and thus its identity could be verified by RFLP analysis [10]. Finally, sequencing the entire amplicon is now easy, fast, and inexpensive. If cross-reactivity occurred, the amplicon sequence will not match that of the target organism. There are intrinsic limitations to the size of amplicons that can be easily verified with each method. For the melt temperature approach, optimal length is about 200 bp (range 100-300), while for SSCP and sequencing it is fastest to use amplicons with sizes less than 500 bp. RFLP analysis has no significant size limitations.

Because TS-PCR is designed to specifically amplify target organisms, while basically ignoring all non-target organisms, it can be a somewhat 'reductionist' and potentially misleading tool in the hands of the mycologist. This is particularly true when the TS assay is based on a single locus. There is mounting evidence of horizontal transfer of genes across taxa [11], hence associating the diagnosis of a species to the detection of a single gene may be deceiving, as the gene in question may be found in species different than the target one because of rare introgression events. This becomes a serious issue, when diagnosis is done directly on plant tissue, without the ability to support the diagnosis by observation of a live culture. It now understood that interspecific hybridization is a fairly common even amongst the fungi [12] and the oomycetes, and that first generation hybrids often bear phenotypic traits that are very different from those of either parents [13]. Unfortunately, when using a TS assay developed for species A, results will be identical to those obtained when processing individuals belonging to species A, and hybrid AB individuals. Using assays based on multiple and unlinked loci (e.g. a multiplex reaction based on two or more loci that are diagnostic for the target organism) may be helpful, but may ultimately not resolve the problem. A combination of genus-specific (as most hybrids do occur within a genus) and species-specific assay may be required to solve this issue.

In order to be sure that a negative result is really due to lack of DNA from the target organism, it is necessary to have a positive control primer set in the TS-PCR assay. This primer set should consist of primers that a)- will not interfere with the TS-primers, and b)- will universally amplify plant hosts and/or the entire group of microorganisms related to the target one. In this case, if no amplification at all will occur, it may be not necessarily due to the lack of target DNA, but simply to a bad DNA extraction.

Finally, although DNA of the target organism is detected in a sample, it is often unclear whether the pathogen may still be viable in the sample. This is an important detail when diagnosis is performed in the context of epidemiological studies or regulatory implementations. Quantification of template target DNA in the sample (see below) may provide a partial solution to this problem. It may be possible to determine for each pathogen/host combinations thresholds of DNA concentrations that correspond to viable, hence infectious, presence of the microbe.

5. Real time PCR

Real time (RT) PCR is a recent development, stemming from the traditional PCR approach. RT-PCR relies on the presence of a fluorophore whose activity is affected by the amplification of double stranded DNA (For a review, Klein [14]). By using different fluorophores, RT-PCR allows for the easy detection of multiple DNA targets in the same run (multiplex reactions). In RT-PCR, active fluorophores are detected during the PCR reaction by specialized laser-based sensors. During the PCR reaction, products can be visualized on a computer monitor linked to the thermalcycler. This feature eliminates the need for gel electrophoresis and saves considerable amounts of time.

By far, the main advantage of RT-PCR lies in its ability to quantify the amount of target DNA present in the template. DNA quantification, in turn, allows for important applications in more refined ecological studies, once limited by the plus/minus nature of the data provided by the traditional PCR approach. Applications of RT- PCR based quantification may include seasonal fluctuation of microbial flora in ecosystems, levels of endophytic and/or pathogenic fungal colonization; and quantification of airborne or insect-vectored inoculum. For diagnostic purposes, quantification may differentiate between viable and unviable inoculum, as well as between environmental contamination of samples (e.g. fungal spores present on the plant surface) and true plant colonization by the pathogen.

There are different basic fluorophore (or reporters) types used in RT-PCR. In some cases, e.g. when using SYBR green, the fluorophore is simply activated by the presence of ds-DNA. When using this type of non-specific approach, targeted DNA amplification can be obtained by using taxon-specific primers. Specificity of amplicons can be further verified in two ways; by traditional gel electrophoresis, and by determining the melt-temperature of the amplicon [8]. This approach relies on the principle that for each piece of ds DNA, the melting temperature necessary to separate the two DNA strands is highly dependent on both size and base composition of the DNA fragment. This approach has been shown to be more accurate than gel electrophoresis for amplicons between 100 and 300 bp in length.

Other RT-PCR approaches (e.g. Taqman, Molecular Beacons, etc.) generally rely on the interaction between fluorophores and quenchers designed inhibit expression of fluorescence when PCR is unsuccessful. Fluorophore/quencher modifications are usually applied to the termini of a probe, matching the DNA sequence of the target DNA sequence in between the primers. Primers and probe need to interact in these RT-PCR processes, hence they are generally designed to be adjacent to one another. Because the combination of primers and probes generally requires an annealing area of almost perfect nucleotide match between 80 and 110 bp, these approaches can be extremely more taxon-

specific than traditional PCR where specificity is provided simply by the overall combined lengthy of the primers.

6. Shortcomings and pitfalls of RT-PCR

The same problems listed above for traditional PCR apply to RT-PCR. In addition, it should be noted that the modifications imposed on primers and probes (e.g. adding fluorophores and quenchers) may result in a lowered level of specificity for the target DNA. It has been our experience that single or even double base pair differences in either the primers or the probe may not suffice to confer specificity to the assay. When using SYBR green, increased amounts of $MgCl_2$ need to be used. It is essential to empirically optimize $MgCl_2$ concentration in the buffer for each primer combination, in order to avoid unspecific DNA amplification.

Taqman represents one of the RT-PCR approaches that are easiest to design. Although this approach has been widely used with great success, at times there appears to be an inhibition of the Taqman chemistry (or a significant reduction in detection threshold), when DNA templates are obtained not through DNA extraction of pure cultures but by DNA extractions of environmental samples or from infected plant material. Currently, the bases of these problems have not been fully evaluated and understood.

7. Some examples of taxon-specific primers

The first and most consequential taxon-specific primers were those designed to amplify the fungal internal transcribed spacer region (ITS) of the nuclear ribosomal operon [15]. The ITS region was selected because of its high phylogenetic informative value. In general, ITS sequences for fungi are conserved within species and variable between them. These primers were to be used not only to optimize PCR amplification of fungi in culture, but for a variety of applications including DNA amplification from ancient herbarium specimens and from plant tissue. The last application constituted a milestone for the study of plant pathogens. Probably, the most influential ecological study to make use of such or slightly modified primers was the analysis of the composition of mycorrhizal communities directly from the usually unculturable root tips [16]. In a follow-up paper Gardes & Bruns [17]. were able to provide the first unequivocal picture of the composition and structure of the underground component of the mycorrhizal community. The authors were also able to determine that the above-ground mycorrhizal community (e.g. based on analysis of mycorrhizal fruitbodies) did not necessarily match the below-ground one(e.g. based on the analysis of mycorrhizal root tips).

This ecological application started an endless number of studies aimed at specific groups of organisms, mostly using the developing ITS sequence

database. Although ITS sequences are generally variable between species, in some cases, different species may have the same sequence. In other cases, sequence divergence between species may be minimal. Because primers can misanneal (e.g. they can anneal even when their sequence does not perfectly match the target DNA sequence), regions with significant sequence divergence should be used for the design of TS primers. This is particularly true when working with herbarium specimens, or environmental samples, in which the quality of the DNA is variable and often subject to degradation, potentially leading to less than perfectly stringent PCR conditions. Taxon-specific competitive-priming PCR is an approach designed to circumvent the problem of limited sequence divergence [18]. The approach was devised to molecularly identify two sympatric taxa, of the pathogen complex *Heterobasidion annosum.* The two taxa, characterized by a marked difference in host range, only differ at a few bp in the ITS region. Two primers were designed, each perfectly matching only the sequence of one of the two taxa, by placing the few polymorphic nucleotides at the 3'-end of each primer. Primers were in different positions, so as to amplify different size bands. The size of the amplicon was thus diagnostic for the species. Both primers were designed as forward, and the universal ITS 4 was used in reverse. When each specific primer was used by itself in conjunction with ITS4, it was not possible to selectively amplify only the target species, as inevitably, the other species would amplify as well. When both primers were used together, only the one matching the target taxon would produce significant amplification. Despite the presence of both primers, only the one perfectly matching the DNA sequence of the tested sample would anneal. Taxon diagnosis is based on the size of the amplicon. The method also included a positive control for DNA quality. By adding the universal primer ITS1 to the PCR reaction, it was possible to determine through a multiplex reaction, whether the sample tested was that of another fungus (the universal ITS1-ITS4 band would have amplified), or the quality of the DNA was too poor to produce any amplicon (ITS1-ITS4 would not amplify).

By using this method, it was possible to rapidly type hundred of isolates. Because there are no morphological differences between cultures of the two species, this approach was immensely useful, bypassing the need for the otherwise inevitable mating studies [19] or isozyme analyses [20]. The authors were also capable of typing dead fruitbodies of this pathogen, estimated to be 10-20 years old. By doing so, it became possible to reconstruct the history of the disease in many California sites. The first homobasidiomycete fungal hybrid was found using this method [18]. TSCP-PCR on several isolates resulted in the amplification of both taxon-specific bands at the same time. It was verified by ITS RFLPs and by isozyme analysis that these isolates were all hybrids between the two species. TSCP-was also developed to differentiate

two *Heterobasidion* species from Europe [21]. It has recently been modified, [22] to include the identification of a third European species. It is now possible to type any isolate as belonging to one of the three European species with a single PCR reaction.

While TSCP can obviously be used from DNA of plant extracts, amplification is quite effective simply by suspending a few hyphal cells or conidia in sterile water, vortexing, and spinning down the crude cell debris. This method, allows for extremely fast diagnosis of cultured samples.

Despite the availability of taxon-specific primers, it is often difficult to successfully amplify DNA from old plant material, old infections, and from tough substrates such as wood. A nested-PCR approach was devised to amplify target DNA from wood. Old wood infections on European Larch and Swiss Stone Pine in the Western Alps had all the characteristics of *Heterobasidion* decay. No cultures were obtainable and TSCP PCR on the woody substrate was unsuccessful. However, PCR-assisted diagnosis was possible in the following way. First, 20 cycles of PCR were run on DNA extracted from the decayed wood using the universal fungal primers ITS1F-ITS4. Second, the PCR product was diluted 1:500 and a second round of 35 cycles of PCR was performed using a *Heterobasidion* specific primer (ITS S1, [18] fully nested within the ITS1f-ITS4 region. ITS4 was used as a reverse primer. Third, amplified products were fully sequenced and sequences were blasted in the GENBANK database and in our own sequence database. Both larch and Swiss stone pine were added to the list of natural hosts of *H. parviporum* by using this method [23, 24]. The following three key points should be noted: 1- Extractions from wood need to be optimized, by using silica beads chromatography and potentially proteinase K treatments; 2- Extreme caution needs to be exercised when using PCR products as templates for PCR reactions. DNA concentration of PCR products can be so high, that it may be easy to contaminate entire work spaces, including other DNA extracts and reagents; 3- When doing nested PCR it is best to use two primers fully nested within the first two in the second round. Alternatively, at least one primer needs to be fully nested within the two priming sites of the first round, without even partial overlap. Without full nesting, it is a lot more difficult to optimize amplification conditions, and the result may be a smear of amplified DNA, rather than a discrete band.

An exciting opportunity for the use of taxon-specific primers was provided by the discovery of *Phytophthora ramorum*, a previously undescribed *Phytophthora* causing the forest epidemic known in California as Sudden Oak Death (SOD) [25, 26]. SOD may represent the first case in which discovery of most new hosts and range of distribution for a new organism was aided by PCR-based detection. Using *P. ramorum*-specific primers, it was discovered that hosts other than oaks were commonly infected by *P. ramorum*, and in

some cases even more abundantly than oaks [27]. These discoveries lead to the under-standing that in California, the epidemiology of SOD is driven not by oaks, but by bay laurels, where the pathogen sporulates abundantly and for long periods.

While symptoms on oaks are dramatic and almost inevitably leading to tree death, symptoms on other hosts are hard to identify, being often limited to spots or blotches on leaves. Pathogen isolation from the plant tissue of most hosts is unreliable because of the extreme variability in isolation success among plant species, plant parts, and time of year. The PCR-based method allowed for a much more sensitive and reliable diagnostic approach, and significantly reduced the numbers of false negatives [28]. Because of the ephemeral nature of the somatic structure of *Phytophthora* spp., the amount of pathogen DNA in the colonized tissue can be scarce. A nested PCR approach was then designed to maximize our detection threshold. Again, the ITS was the region of choice because a)- the ITS sequences of most *Phytophthora* spp. were already available, and b)- the multicopy nature of the ITS should increase the detection threshold of the assay. However, we also realized that a traditional nested approach consisting of one set of generic primers and of a second set of specific primers was problematic. When the first round of PCR produced high amounts of DNA, cross-reactivity with a *Phytophthora* species other than *P. ramorum* was observed. The problem was circumvented by designing a nested approach in which the outer set was species-specific, and the nested primer set was semi-specific. In this way, only *P. ramorum* DNA would be amplified on the first PCR round, eliminating the risks of cross-reactivity.

Because nested PCR is extremely sensitive, the question arises of whether the technique may be too sensitive, picking up what could be considered an environmental contaminant, e.g. sporangia on the leaf surface, rather than true infection. Two approaches make the diagnosis stronger: the observation and description of appropriate symptoms on the plant part tested [27], and the ability to quantify the amount of *P. ramorum* DNA in the sample using an approach called real time (RT) PCR. Although comparisons across different host species may be difficult, RT-PCR allows for an exclusion of those positive samples in which the amount of target DNA in the template are exceedingly low, suggesting the possibility of contamination. By using a similar approach, we have been also able to differentiate plant samples where the pathogen was consistently viable (low PCR thresholds corresponding to high DNA concentration) from those samples where pathogen viability was uncertain or variable (high thresholds corresponding to low DNA concentrations). By allowing quantifying DNA in templates, RT-PCR opens the doors to innumerable studies on the biology and ecology of microorganisms.

For instance, a RT-PCR assay has been developed to collect and quantify spores of *Fusarium circinatum*, the causal agent of Pitch canker disease of pines [31].

Rather than on selective media, which only allows collections for a limited amount of time (e.g. 1-3 days), spores are collected on inert surfaces for longer periods (e.g. 1-6 weeks). Cumulative spore deposition rates can then be determined by RT-PCR, using *F. circinatum*-specific primers. These were designed in the non transcribed spacer region (IGS) of the nuclear ribosomal operon, and were tested in the laboratory against all those species that were the closest relatives to *F. circinatum* according to an IGS-based phylogeny. By using this simple approach, we have started a year-long continuous sampling of *F. circinatum* spores in two areas in California. These data will be unique, and invaluable in building epidemiological models for this pathogen.

Finally, an obvious application of taxon specific primers is for all those microorganisms that are extremely hard to culture. Fastidious bacteria, mycoplasmas, viruses, and even some fungi need to be detected by immunological assays, plant infection trials, and DNA-based diagnostics. PCR is used routinely for most of these organisms, but is still relatively underused for most fungi. A case study exemplifying the power of PCR-based techniques for the identification of fungi is that of blackstain root disease, caused by the pathogen *Leptographium wagenerii*. Three closely related pathogens cause an almost identical disease on Douglas-fir, Ponderosa pine, and pinion pine. The epidemiology of the three diseases appears to be quite different. Insect involvement has been hypothesized for the disease in ponderosa pine, but numerous attempts to isolate the pathogen from beetles have repeatedly failed. We have designed primers specific for *L. wagenerii*, and were able to detect its DNA on most beetles of several species of the genus *Hylastes* trapped in areas where the disease is abundant (Garbelotto, unpublished). This finding suggests beetles may be carrying the pathogen, although they may not necessarily be infectious. In this case, more work is needed to define disease vectoring by beetles, but the approach allowed to set the direction of future research.

The number of microsatellite repeats (short repeats of tandem DNA sequences) in loci where they are found, is often extremely variable amongst individuals of a population. At any rate, variation in microsatellite repeats has been equaled to allelic variation [29]. Microsatellites can thus be used as a powerful tool for the identification of individual genotypes and for population genetics. Microsatellite variation is usually identified by designing primers to match the sequence of the regions flanking the microsatellite repeats [30]. These flanking sequences are often extremely species specific, although there are cases of primers capable of amplifying closely related species. In this regard, microsatellite primers can be viewed as extremely powerful taxon-specific primers that can be used for population genetic analysis of individuals

within a species. These primers can also be used directly on infected plant material, thus bypassing the need for pure fungal cultures.

8. Towards an integrated diagnosis of plant pathogens

TS-PCR is an invaluable tool for the diagnosis of pathogens, fungi in general, and for molecular ecology studies. Because of its recent broad application, TS-PCR still awaits the definition of clear standards and procedural protocols. It is extremely important that researchers exhaust all possible explanations of TS-PCR results, as well as perform all necessary positive and negative controls, before translating such results into diagnostic conclusions. Nevertheless, PCR-assisted diagnostic is the most powerful tool to identify fungi available to us, and needs to become the golden standard of all research or regulatory action in which detection of fungi is involved. The value of diagnosis based on culturing is not in discussion here. Molecular diagnostics are needed especially where culturing is unsatisfactory or impractical. To avoid the risk of creating two systems without cross-referencing, one based on isolations and one on DNA-assays, I suggest DNA-based assay should be developed and standardized for all organisms of interest. This approach may create a body of compatible and highly comparable diagnostic systems. Other approaches will be needed at times, depending on the type of questions asked, and may be easily superimposed on the molecular approach.

With the understanding of the potential limitations and pitfalls of molecular diagnostic, it would seem inevitable that regulatory agencies would rely heavily on these highly discerning diagnostic approaches. In actuality, molecular approaches in the US are standard only for groups of pathogens such as mycoplasmas and viruses, which cannot be cultured. Diagnosis of fungi and oomycetes relies almost exclusively on morphological description of structures on infected plants or of cultures obtained from infected plants. This translates into a serious limitation when diagnosing organisms that although culturable, are often difficult to obtain because of great spatial heterogeneity of their somatic body or because of marked seasonality in its life cycle. Organisms like many *Phytophthora* species that can be cultured but with variable success rates because of the effects of seasonal patterns and substrate are not routinely diagnosed through DNA-based assays. Diagnosis of obligate parasitic or endophytic fungi that do not fruit readily would also be greatly aided by molecular diagnostics.

9. Discrepancy between accepted technology in science circles and regulatory agencies

Regulatory agencies are traditionally slow paced, and, in many respects, this is a good thing. Because of the economic and environmental

implications of decision made by such agencies, it is appropriate they demand that new diagnostic approaches be thoroughly tested for specificity, simplicity of execution, and reliability in face of slight protocol changes. These attributes are essential for a widely used diagnostic assay, and are not necessarily a priority for scientists who often develop these assays in laboratories with state-of-the-art equipment. Simpler assays may have broader application as they will be employed by more laboratories. For instance, in the case of *Phytophthora ramorum*, protocols used for the nationwide survey are based on a traditional nested approach, rather than on Real Time (SYBR-green or Taqman) technology, in spite of the fact that these protocols are already availabl1e. In the case of *P. ramorum*, the more advanced technology is used by research groups studying the biology and the epidemiology of this newly-discovered pathogen, while State and Federal surveys are heavily dependant on simpler, but also more widely tested, techniques. The adoption of DNA-based diagnostics is nevertheless a huge step forward for agencies that largely ignored DNA-based evidence for about two years, in spite of the fact that the vast majority of new host and geographic range discoveries were made possible thanks to this technique [27].

This incongruence between basic research and practical surveying approaches, on the surface appears justifiable because of the need for a verification process. A more in depth look though, suggests a fundamentals schism between research organizations and regulatory agencies. Regulators, in fact, are extremely concerned about getting “false positive” diagnosis, while researchers are more concerned about “false negative” results. Although I recognize the previous statement is invalid because of its crude and absolute categorization, its validity can be partially verified by an historical look at policies embraced by regulatory agencies in the US and in most other industrial countries. In spite of the fact that these agencies are proposed to safeguard both the economic interests of groups directly involved with agricultural and natural resources and the natural resources themselves, historically there has been a natural tendency of these governmental agencies to act conservatively when direct economic interests of some groups are at stake (false positive would be viewed extremely negatively because they would unjustly economically affect one or more interest groups), while wildly ignoring potential threats to natural ecosystems (false negatives are accepted although that may result in the introduction of a potentially devastating microbe). This is often achieved by imposing burden of proof on those claiming the potential destructive effects of introduced microbes and pests, rather than reversing the picture and demanding assurance these introductions will not be devastating to native ecosystems. The issue of the importation of green lumber in the U.S., is one that well exemplifies this conflict between government-backed private interest (advocating the need of such import) and a

group of advocates, including scientists and environ-mentalists, extremely concerned about the dangers of importing exotic organisms with shipments of untreated lumber from far away regions of the world. In light of well accepted co-evolutionary theories, suggesting that the most devastating animal and plant epidemics have been caused by introduced diseases, the validity of the general attitude of regulatory agencies deserves at least to be discussed at a broader public level.

One of the inevitable consequences of such approach is the lack of a truly preventive policy. Dangerous trades are still allowed; basic phytosanitary rules are not enforced, education on the subject is lacking, and introductions are rarely prevented. Although it is believed by most, in both science and government, that prevention is a much less costly approach, there are few models that can be adopted by large countries with strong and dynamic economies. These observations also underline the significant differences in dealing with threats to human, animal, and environmental/plant health, with a significant decline in urgency from the first to the last. Hopefully, models used to deal with human and animal epidemics may be adjusted for plant protection as well.

A less obvious problem caused by this schism between government and science, is the presence of different, if not implicitly discordant, information. While this is generally not an obvious problem for obscure or technical issues, allowing for plenty of time for a reconciliation of the two, it may be a problem for topics that are “hot” for the public. This has been an issue for the first two years of Sudden Oak Death, when the rate of discoveries, mostly by University of California researchers, was extremely fast paced. It is interesting to observe that this occurred in spite of an extremely open information channel between U.C. researchers and California Department of Food and Agriculture (CDFA) officials. The problem was compounded by the fact that, because of the urgency of the matter, information was released after an internal review process rather than a normal peer review process. When CDFA started applying its own standards to selectively accept or reject data originated by U.C. researchers, and exerted pressure to re-direct University research and its approaches, it became apparent that an uncoupling of research and regulation was absolutely necessary. Allowing regulatory agencies to control basic research equates to combine the legislative and the executive powers of the government.

In many respects, DNA-based diagnostic has started at a slow pace because of the need to build up expertise within the agencies, especially at the state level. As people with molecular expertise join traditional plant pathologists, these new approaches are bound to become increasingly popular and accepted. In some instances, DNA-based diagnosis has fallen victim of internal inconsistencies; for instance, regulatory agencies will regulate all life stages of

an insect pest, but will not care about spores or infectious propagules of a pathogen. Only infected plant material is regulated leaving a huge unregulated area. Because DNA-based diagnostic can potentially detect the presence of spores, this can be seen as a negative feature rather than as a useful tool, from a regulatory perspective. Another issue of DNA-based diagnosis relates to pathogen viability. To address these issues, we have developed some guidelines correlating concentration of target DNA in the template with viability. These lines of research can be further developed, especially now that substantial information on the whole genome of organisms is being produced. It should also be noted that while it is true that non viable pathogen will be detected by PCR, it is also true that culturing will often not detect pathogens that are still viable, but dormant at the moment the plant was sampled. I believe that a diagnosis based on presence of disease symptoms and DNA identification should suffice.

Finally, the path to full embracement of molecular diagnostics by regulatory agencies has been made more arduous by scattered cases of mistaken identity. PCR primers designed to be specific for *Tilletia indica*, the causal agent of karnal bunt, unknowingly also amplified (i.e. detected) a native and undescribed species of *Tilletia*, later on named *T. walkerii*. Molecular databases used or created to design highly specific DNA diagnostic assays, can only include known species of microorganisms. Even extremely variable DNA molecules may discriminate among most closely related species, but not necessarily all of them. Thus, if the locus chosen for the molecular detection of a species ends up being identical to that of a new species, the assay will diagnose both species as the target one. The false positive diagnosis of *T. indica* was particularly worrisome, because the two species could be distinguished morphologically [32]. The karnal bunt "experience' reinforced the belief of the superiority of the classical morphological diagnostic approach over the molecular one. This was unfortunate, because the resulting conservative outlook on diagnosis of plant pathogens, is in sheer contrast with the enormous amount of evidence gathered solely through molecular (mostly DNA) information on the presence of innumerable different microbial species undistinguishable at the morphological level. In many cases, these cryptic species identified through molecular data, are characterized by significant differences in phenotypic traits of great importance from a regulatory perspective such as pathogenicity, host range, temperature optima, etc. The lack of precise new guidelines for species definition in the absence of morphological difference, leaves regulatory agencies in a diagnostic limbo. More importantly, it allows for lack of appropriate regulations when apparently conspecific organisms from different regions of the world are known to belong to genetically isolated taxa. In biological terms these organisms should be regarded as exotic and need to be regulated.

10. Conclusions

TS-PCR is an invaluable tool for the diagnosis of pathogens, fungi in general, and for molecular ecology studies. Because of its recent broad application, TS-PCR still awaits the definition of clear standards and procedural protocols. It is extremely important that researchers exhaust all possible explanations of TS-PCR results, as well as perform all necessary positive and negative controls, before translating such results into diagnostic conclusions. Nevertheless, PCR-assisted diagnostic is the most powerful tool to identify fungi available to us, and needs to become the golden standard of all research or regulatory action in which detection of fungi is involved. The value of diagnosis based on culturing is not in discussion here. Molecular diagnostics are needed especially where culturing is unsatisfactory or impractical. To avoid the risk of creating two systems without cross-referencing, one based on isolations and one on DNA-assays, I suggest DNA-based assay should be developed and standardized for all organisms of interest. This approach may create a body of compatible and highly comparable diagnostic systems. Other approaches will be needed at times, depending on the type of questions asked, and may be easily superimposed on the molecular approach.

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