Phytophthora ramorum and Sudden Oak Death in California: III. Preliminary Studies in Pathogen Genetics[1]

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Abstract

Sudden oak death (SOD) has been shown to be caused by a new species of *Phytophthora*, *P. ramorum*. A basic understanding of the genetics of *P. ramorum* is critical to any management strategy. We have initiated a number of studies to examine species concepts, population biology and mating behavior of the pathogen. Based on a number of morphological features (e.g., a combination of deciduous sporangia and chlamydospores), the *P. ramorum* does not match any of the currently described species of *Phytophthora*. Sequences of the internal transcribed spacer (ITS) of nuclear ribosomal DNA were identical for isolates from *Lithocarpus densiflorus*, *Quercus* spp., *Rhododendron* sp., *Vaccinium ovatum*, *Umbellularia californica*, and *Aesculus californica*. The sequences were also identical to a recently described species from Europe, *P. ramorum*. Based on ITS sequences, the closest species to *P. ramorum* is *P. lateralis*; ITS sequences between the two species differ by 12 nucleotides. We are now examining the population structure of *P. ramorum* using amplified fragment length polymorphisms (AFLPs) to determine variability within pathogen populations. This information will provide insights into whether *P. ramorum* is an exotic pathogen and whether sexual recombination is taking place in California populations. Finally, the use of genetic data allows for the development of species specific diagnostic probes. PCR primers based on the ITS region have been used to facilitate the rapid identification of the pathogen from plant tissue.

Introduction

The pathogen responsible for lethal stem cankers leading to the extensive dieback of California black oak (*Quercus kelloggii*), California coast live oak (*Q. agrifolia*), Shreve's oak (*Q. parvula var. shrevei*) and Tanoak (*Lithocarpus densiflora*) is easily identifiable as a species within the genus *Phytophthora* (Garbelotto and others 2000, Rizzo and others 2002a). A pathogen with similar morphology has also been shown to be responsible for extensive foliar blight and necrosis, often leading to twig and branch die-back, in several plant species including

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California bay laurel (*Umbellularia californica*), madrone (*Arbutus menziesii*), and *Rhododendron spp*. (Rizzo and others 2002b).

While the presence and morphology of sporangia (specialized reproductive structures containing motile zoospores) and chlamydospores (rounded, thick-walled resting propagules) provide sufficient information for the taxonomic placement of the pathogen within the genus *Phytophthora*, there is no obvious match with any other of the sixty known species belonging to this genus. The presence of sympodial semipapillate and caducous sporangia combined with the formation of terminal and intercalary chlamydospores is unreported within the genus (Garbelotto and others 2000, Rizzo and others 2002a). Only recently, similar traits have been described for a new *Phytophthora* species, *P. ramorum*, isolated from ornamental rhododendron plants in Germany and the Netherlands (Werres and others 2001).

While morphological descriptions can be diagnostic for many *Phytophthora* species, they are often equivocal. The problem is compounded by the variability in spore and propagule production as well as in somatic growth often observed among isolates within a species. This variability has been shown to be linked to intraspecific genetic variability, to different life histories (e.g., climate and hosts in which they have lived), and to growth medium and temperature conditions (Erwin and Ribeiro 1996, Brasier and others 2000).

In this paper, we describe: a) how species determination of the new SOD pathogen and its evolutionary placement within the genus *Phytophthora* was obtained through DNA-based phylogenetic analysis; b) how extremely species-specific DNA sequence data was used to design molecular probes that were then used with the aid of the polymerase chain reaction (PCR) to study the presence and distribution of the pathogen in the environment (e.g. plant hosts, soil, water); and finally, c) how multilocus genetic data is being generated using a technique called amplified fragment length polymorphisms (AFLP) (Vos and others 1995) to determine the genetic variability and the population structure of the pathogen. Based on an overall analysis of the above preliminary data, we suggest reasonably supported hypotheses regarding the biology of this pathogen.

Materials and Methods

Isolate Selection and Growth Conditions

Phytophthora ramorum isolates were chosen to represent a broad variety of both geographic locations and hosts. A few areas were selected to be sampled more intensively. *Phytophthora* isolates were kept on potato dextrose agar or 5 percent V8 agar media at 22° C. For DNA extractions, isolates were grown in 100 mL of potato dextrose broth or V8 broth (Erwin and Ribeiro 1996) in Erlenmeyer flasks. Flasks were kept on a shaker at 22° C for approximately 21 days. The material was harvested by vacuum-aided filtration on filter paper. Hyphal material was then frozen and lyophilized overnight.

DNA Extractions

DNA extractions were performed using the protocol described by Garbelotto and others (1998) amended in the following way. Instead of performing an ethanol

precipitation of nucleic acids, the Gene Clean II kit (Bio101, Carlstad, CA)[2] was employed, according to the manufacturer's directions. In each extraction, 10 uL of glassmilk were employed. DNA was finally resuspended in 35 uL of PCR water (Garbelotto and others 1993).

PCR Reactions, Sequencing, and AFLPs

The internal transcribed region (ITS) of the nuclear ribosomal operon was amplified in 50 uL volume reactions by 35 cycles of polymerase chain reaction (PCR) using primers ITS1 and ITS4 (White and others 1990) as described elsewhere (Garbelotto and others 1996). Four uL of each PCR product were loaded on a 1.5 percent agarose gel and electrophoresed for 1h and 30m at 4V/cm. Gels were stained with a 15 m Ethidium bromide wash followed by a 10 min rinse with water. Visualization of the gels was obtained with a digital camera and the software Kodak II. Positive amplifications were cleaned of all PCR reagents by using Qia-quick purification kit (Qiagen, Valencia, CA) and the pure DNA was sequenced with an automated ABI sequencer. Both DNA strands were sequenced for each isolate. Quality of each sequence was assessed by visual inspection of sequence chromatograms and congruity of sense and anti-sense DNA strands. Chromatograms were also inspected for the presence of double peaks, a feature that may indicate the presence of heterozygous ITS sequences. ITS heterozygosity is expected in first generation hybrids between two species characterized by divergent ITS sequences.

AFLPs were performed as recommended by the manufacturer of the AFLP microorganism kit (Invitrogen, Grand Island, NY). Visualization of AFLPs was obtained by polyacrylamide electrophoresis in an automated ABI 371 sequencer machine (Applied Biosystems, Foster City, CA). In each lane, both the molecular standard GeneScan (TM)-500 ROX(TM) Molecular Standard, and the final AFLP amplification product from each selected isolate. The final AFLP product was obtained using various primer combinations. The date here presented was obtained by using the primers EcoRI+GTA labeled with FAM and unlabeled MseI+A primer. Loading of the molecular standard and of the final product in the same lane allows for an unequivocal sizing of each AFLP fragment.

Sequence Alignment, Phylogenetic, and AFLP Analyses

Sequence alignments inclusive of the ITS1, ITS2 and 5.8S regions were obtained automatically with the program Sequencher and then manually corrected. The overall alignment deposited in GenBank by Cooke and others (2000) was used for phylogenetic placement of the new *Phytophthora* species. Phylogenetic analyses were performed using both the heuristic and the distance option in Phylogenetic Analysis Using Parsimony (PAUP) (Swofford 2000). Default settings were used for the heuristic search and the Neighbor Joining (NJ) algorithm was used for the distance search. Both methods yielded similar results and only the phylogenetic tree obtained by the heuristic search is here displayed and discussed *(fig. 1)*.

[2] Mention of trade names or products is for information only and does not imply endorsement by the U.S. Department of Agriculture.

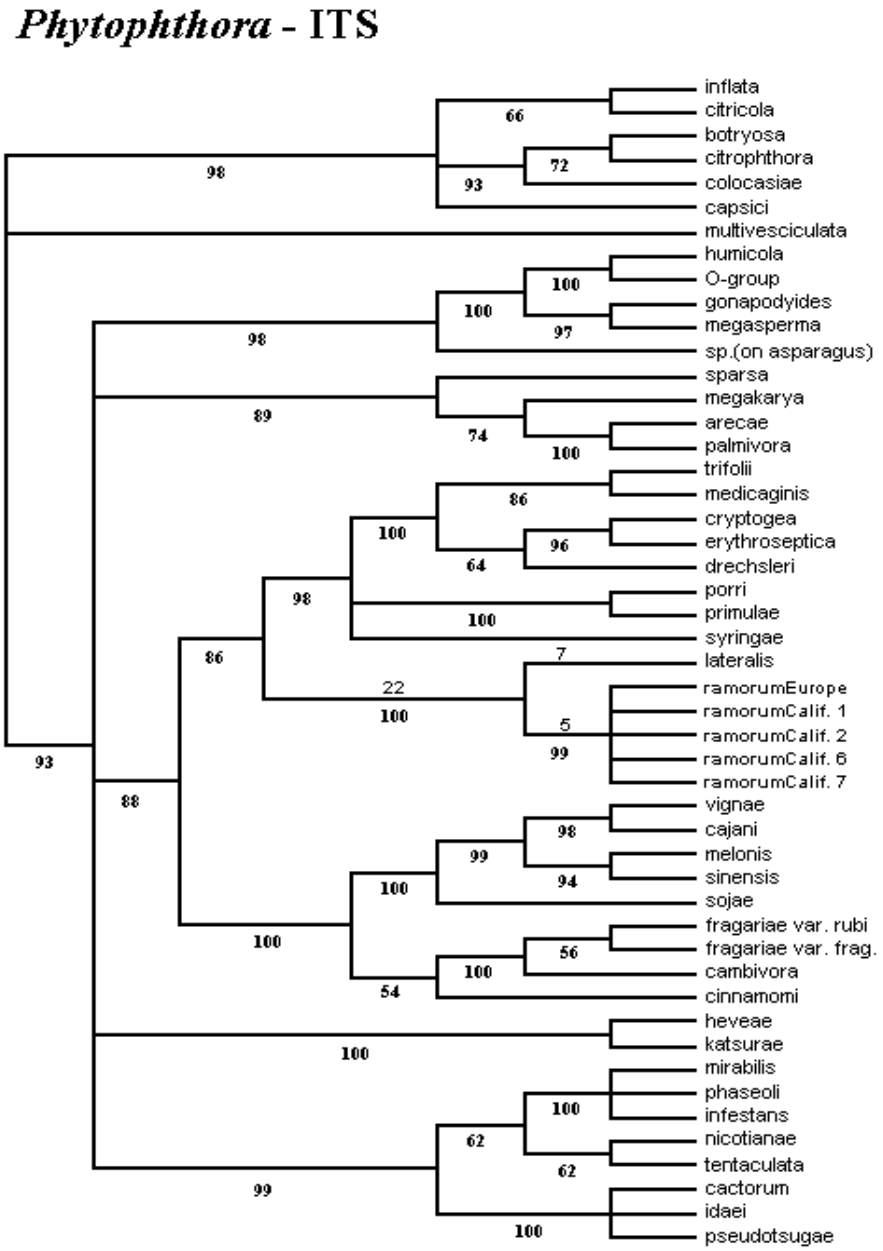

Figure 1—Cladogram showing phylogenetic positioning of the new *Phytophthora* species. The tree was obtained by parsimony analysis using the heuristic search option on PAUP (random addition sequence), and is one of 12 most parsimonious trees. Gaps were treated as missing, 860 characters were employed in the analysis. Tree parameters are as follows: consistency index=0.570, rescaled consistency index=0.834, homoplasy index=0.430, tree length=960. Boostrap values, shown in bold under branches, were obtained by 1000 replicates of the "fast" stepwise addition method. Branch lengths of terminal taxa are not shown, but there were no differences between *P. ramorum* and isolates from California.

AFLP bands were scored as present (1) or absent (0) for each isolate. The resulting binary matrix was transformed into a distance matrix using the Jaccard's coefficient of similarity. This transformation is necessary to reduce the error caused by the dominant nature of the AFLP markers as described in Garbelotto and others (1998). The resulting similarity matrix was then translated into a distance cladogram by using the neighbor-joining algorithm in PAUP. The tree was rooted by using the AFLP profile from an isolate of *Phytophthora lateralis*, the only close relative to the new species (see results below).

PCR Primer Design, Optimization of PCR Conditions, and Environmental Sampling

The alignments employed in the phylogenetic analyses were used to identify regions of sequence divergence (e.g., presence/absence of indels and base substitutions) between *Phytophthora ramorum* and all other *Phytophthora* species for which ITS sequences were available. Using the web-based Primer3 application (http://www.genome.wi.mit.edu/genome_software/other/Primer3.html) four specific PCR primers were designed. The first set of primers (Phyto1 and Phyto4) was designed to amplify a 687 BP fragment of the ribosomal operon of the new *Phytophthora* species. This fragment includes portions of the ITS1 and ITS2 and the entire 5.8S region. The second set of primers (Phyto2 and Phyto3) were designed to amplify a 291 BP portion of the ITS2 region, fully nested within the Phyto1-Phyto4 amplicon.

$MgCl_2$ concentration and annealing temperature were optimized through sequential amplification of identical or comparable DNA extracts. Both the specificity and the sensitivity of the approach needed to be maximized. Cross reactivity with other *Phytophthora* species was checked by amplification of pure DNA extracts from the following species; *P. citricola, P. cinnamomi, P. cryptogea, P. cambivora, P. drechsleri, P. infestans, P. "ilicis*-like,*" P. pseudotsugae, P. megasperma, P. cactorum, P. capsici, P. megasperma f. sp. glycinea, P. bohmeriae, P. parasitica, P. erythroseptica, P. nicotianae,* and *P. lateralis.*

To maximize sensitivity, a nested PCR approach was devised. After 35 cycles of PCR using primers Phyto1 and Phyto4, the PCR product was diluted a hundred fold and used as a template for 35 cycles of nested PCR with primers Phyto2 and Phyto3. Agarose electrophoresis and Ethidium bromide staining were used as described above to visualize intermediate and final products.

Environmental sampling was conducted by extracting DNA as described above from symptomatic (e.g. cankered or necrotic) plant material. Leaves, wood, oozing sap from cankers, and bark of several plant species were tested. Water samples were processed by collecting 50 ml of water from either streams or rainwater, centrifuging them at 3,000 rpm for 15 min, and by collecting 1 ml from the bottom of the tube with a micropipette. The collected liquid was then placed in an Eppendorf tube and centrifuged for 3 min at 15,000 rpm. Approximately 200 uL were collected with a micropipette from the bottom of the tube and transferred to a new tube. DNA extractions were performed as described above. Soil was soaked in water overnight. The wash water was collected in 50 ml tubes and treated as described above. Live isolations on PARP medium were often performed from the same substrate to compare the sensitivity of the two methods.

Results and Discussion

Phylogenetic Analysis

All 12 sequenced isolates of *Phytophthora ramorum* from 6 host species (California coast live oak, tanoak, rhododendron, huckleberry, bay laurel and buckeye) and 3 California counties (Marin, Napa, Sonoma) had identical ITS sequences *(fig. 1)*. The ITS sequence of the California isolates was also a perfect match with the sequence of European *Phytophthora ramorum* isolates obtained from foliar blotches and branch and twig cankers on *Rhododendron* spp. This phylogenetic placement is therefore in agreement with our preliminary observations, which indicated a striking morphological similarity between isolates from the two continents.

While identical ITS sequences are suggestive of conspecificity between the European and American populations, it should be noted that evolution in the ITS region is highly variable and taxon-dependent. There are cases in which different species have identical ITS (O'Donnell, personal communication), and cases in which populations of the same species from different regions of the world have different ITS sequences (Garbelotto, personal observation).

The only close relative *(fig. 1)* of *P. ramorum* is *P. lateralis*, an aggressive pathogen of *Chamaecyparis lawsoniana* (Port-Orford cedar) thought to have been introduced in the American Pacific Northwest in the early 1900s (Hansen and others 2000). While *P. ramorum* and *P. lateralis* share similar climatic preferences (they both seem to thrive in cooler temperatures), and some morphological features, there are three striking differences: 1) *P. lateralis* is mostly found in the roots and root collar of infected trees, while *P. ramorum* appears not to infect trees below the soil line. 2*) P. ramorum* appears to have a significant aerial phase as suggested by infections on leaves and by cankers found up to 20 m from the ground. This appears as a consequence of the caducous sporangia of *P. ramorum*. Only rarely are the sporangia of *P. lateralis* caducous. 3) *P. lateralis* is only found on two tree species (Port Orford cedar and occasionally Pacific Yew), while *P. ramorum* is found on at least 14 species representing 8 plant families.

AFLP Analysis

The primer combination produced 112 polymorphic fragments. Fragments that were ambiguous or parsimony uninformative (e.g., one fragment present in one or two individuals and absent in all others) were excluded from the analysis. A total of 63 characters were thus employed. The sample size is currently too small to make definitive conclusions based on these preliminary data. The presence of several polymorphic alleles suggests that *P. ramorum* is not a clonal species. Nonetheless, most of the variability in our data was represented by the European isolates, while little genetic variation was detected in the California population. The reduced genetic variability is in agreement with the hypothesis of an introduced microorganism.

Using *P. lateralis* as the outgroup *(fig. 2)*, it appears that European and California *P. ramorum* isolates are representatives of two different populations. This is supported by a 100 bootstrap value of the branch separating isolates from the two continents. The topology of the NJ tree could be explained in at least three ways: 1) the two populations are genetically isolated, but belong to the same species; 2) the two populations represent two species recently evolved from a common ancestor, and

3) the sampled individuals are representatives of the same population, but the oversampling of a single genotype, due to its successful spread throughout California, results in an apparent separation of the two populations. This final hypothesis would require the presence of individuals that may have been moved from one continent to the other. One *P. ramorum* isolate from Marin County (Pr-3) was strongly associated with the European isolates. Further studies need to verify whether such an isolate may represent a potential link between continents.

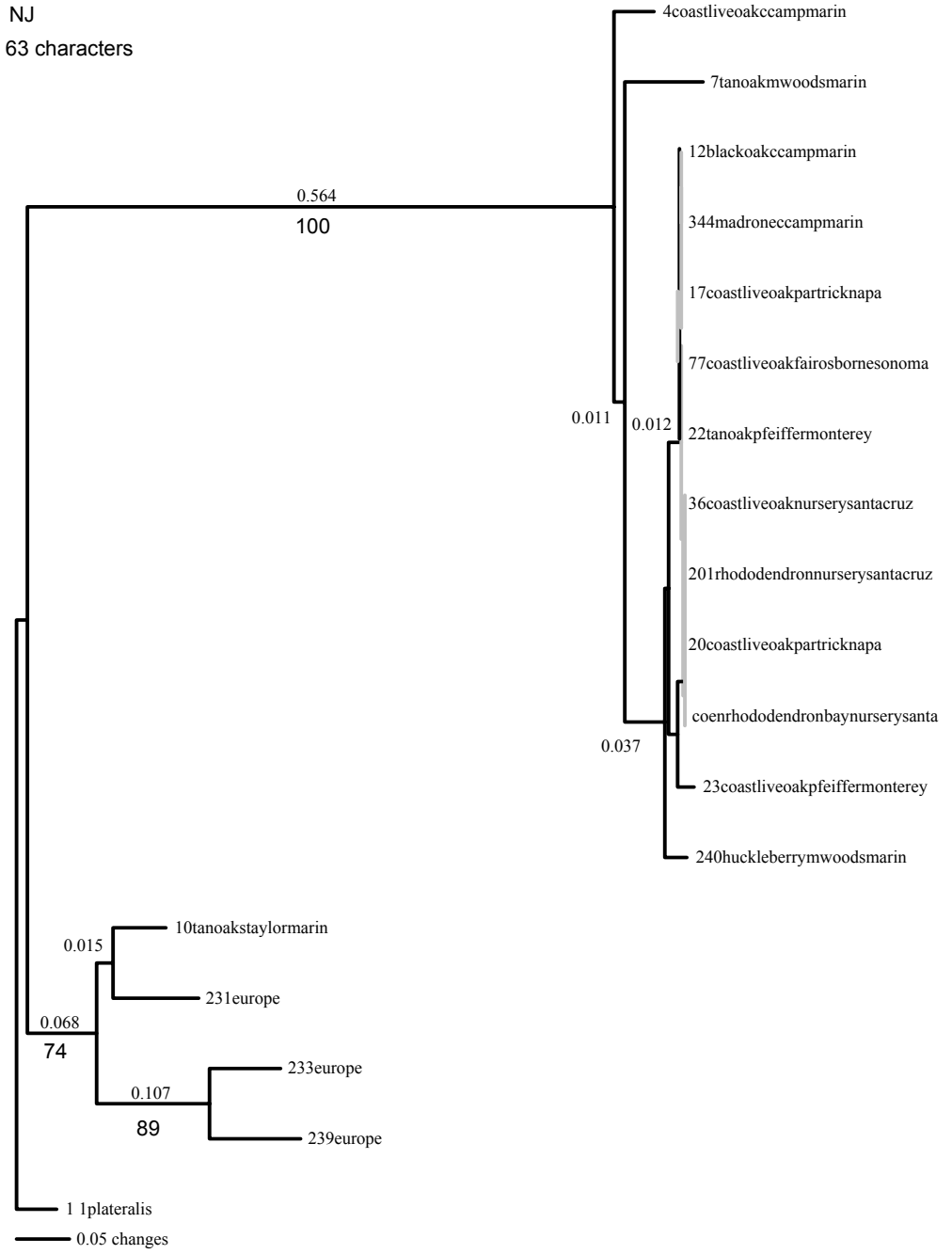

Figure 2—Neighbor Joining (NJ) phylogram obtained by analysis of the AFLP data. NJ distances greater than 0.01 are shown above branches. Bootstrap support values obtained by 1,000 replicates are shown under branches, when larger than 50.

There was no significant structuring of the California population based on host or location. This is in agreement with the hypothesis that *P. ramorum* was introduced recently on the West Coast of the U.S.A, and has had no time to diversify yet. Lack of structuring in our data could also be explained if *P. ramorum* were strictly clonal, The presence of some polymorphisms seems to suggest though that *P. ramorum* is not strictly clonal. One possible scenario explaining our data is that of multiple introductions (hence the lack of pure clonality) followed by the successful spread of only one or two individuals. It is interesting to observe that most of the variability in California is found in Marin County, the area where the disease was first reported.

More primer combinations and larger sample size are necessary to draw final conclusion on the genetic relatedness of European and California *P. ramorum* populations as well on the genetic structure of populations within California. Molecular analysis will also need to be supported by pathogenicity and mating tests.

These preliminary data though strongly suggest that in epidemiological terms, *P. ramorum* moves from one host to the other. The NJ tree, in fact, shows no clear separation between isolates on oaks and on non-oaks. On the contrary, isolates from all species are intermingled. Sporangia and chlamydospores of *P. ramorum* have been commonly found on bay leaves, but they are yet to be found on infected oak trees. For this reason, we suggest that infestation of a site may start with infection of the foliar hosts. This infection may lead to a build-up of the inoculum in the area and to successive infection of oaks. If this hypothesis were correct, most infected oaks would not be in pure oak stands, but in stands where foliar hosts such as bay or madrone are present. Our preliminary observations seem to corroborate such hypothesis.

Both tanoak stems and leaves appear to be infected by the pathogen. We believe the presence of both types of available infection sites, combined with a high genetic susceptibility are two of the key factors that may explain the high impact of *P. ramorum* on tanoak (Garbelotto and others 2000, Rizzo and others 2002a).

DNA-based Diagnostics

Our specific primers were extremely successful in identifying the pathogen, even when it was not detected by culturing. Hosts such as bay, madrone, buckeye, and the susceptibility of tanoak leaves were actually discovered thanks to positive PCR-based diagnosis (Rizzo and others 2002b). The nested technique greatly enhanced the sensitivity of our assays. For instance on a total of 89 leaf samples of unknown status 18 positive samples were added by the nested PCR round to the 8 samples that were positively diagnosed as *P. ramorum* in the first PCR round. While a precise quantification was not attempted, the PCR diagnostic was extremely more reliable than standard culturing techniques when analyzing leaf material. In the case of wood the technique was extremely successful, with a 53 percent increase in success (based on 79 samples), compared to standard culturing techniques.

Diagnosis from 51 oozing sap samples from cankers was highly successful (57 percent), while standard culturing of the same sap yielded only one positive isolation. This result suggests that while DNA diagnosis is extremely sensitive, it may not always indicate the presence of infectious propagules. Seasonal variability in the success of PCR diagnosis from oozing sap is suggested by our preliminary data (not shown). It appears that both culturing and PCR identification are more successful

during the cooler and wetter months than in the summer time. Positive identification of *P. ramorum* from ooze may represent an excellent way to diagnose the disease. PCR amplifications were also positively performed from water and soil samples.

To our knowledge, this is the first time the study of a new fungal disease has been aided by DNA-based technology from its discovery. Molecular techniques were pivotal in providing rapid evidence of a connection between the American and European isolates of *P. ramorum*, in identifying plant material from species other than oaks as potential hosts of the disease (e.g., rhododendrons and other ornamentals), in broadening our understanding of the host range and of the biology and epidemiology of the disease, and finally in providing us with an invaluable tool that complements and improves the standard diagnosis technique based on live culturing of the pathogen.

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