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RECENT FINDINGS AND CURRENT PROSPECTS ON THE BIOLOGY AND MANAGEMENT OF *PHYTOPHTHORA RAMORUM*

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ABSTRACT

Exotic *Phytophthora* species represent a significant threat to native North American oaks. The diseases they cause may range from extremely virulent aggressive pathologies to secondary diseases in need of further weakening factors in order for plant mortality to occur. The severity of the disease may depend both on the pathogen and host species. Exotic *Phytophthoras* can further be divided in two groups; one of recently discovered introduction and one of species long known in the agricultural world, but only recently-discovered in wild environments in some North American regions. Population genetics information can be deployed to determine linkages between agriculture and wildlands, and at times surprising results about the role played by specific strains may be obtained, indicating that particular emphasis may be needed to prevent introductions of pathogen genotypes linked to some specific source.

Key words: oak disease, population genetics, sudden oak death

Phytophthora ramorum is the exotic pathogen responsible both for sudden oak death (SOD) in California and Oregon coastal forests (Rizzo and Garbelotto 2003), and for a *Phytophthora* blight disease of a wide array of ornamental plant species in commercial nurseries throughout North America and Europe. There are at least three review papers on this topic (Garbelotto and Rizzo 2005, Rizzo, Garbelotto and Hansen 2005), and I invite the reader to consult those for bibliographical references. Nonetheless, I will include those references that are not listed in any of the three review papers. Because of its high virulence, *P. ramorum* is highly regulated, and has been studied intensively since its role as the causal agent of SOD has been uncovered.

The origins of the organism are still unknown, but its genome sequence (Tyler et al. 2006) has revealed that the European and North American strains are characterized by heterozygosity levels typical of individuals originating from a sexually reproducing population. The whereabouts of these sexual native *P. ramorum* populations are still unknown. At least three studies employing a range of genetic markers, such as AFLPs and microsatellites, have clearly indicated though that sexual reproduction is not ongoing in North America and is not likely to be occurring in Europe. The high levels of heterozygosity evidenced by studies employing microsatellites suggest that the current known lineages of *P. ramorum* are the results of mating between genetically-divergent genotypes followed by prevalent clonal reproduction.

Two lineages of the pathogen were at first described: one present across European nurseries and one in California forests. These two lineages are genetically and phenotypically distinguishable, as indicated by their different allelic composition and mitochondrial DNA sequence, colony morphology, growth rate, and mating type (North American isolates belong to mating type A2 and with two exceptions all European isolates belong to mating type A1). While these differences indicate a lack of a direct connection between the California wildland infection and the European nursery infection, both lineages are reported to be present in some North

American nurseries. A further third lineage, genotypically and phenotypically distinct from the other two, is also present exclusively in some North American nurseries.

The microsatellite study by Ivors, Garbelotto et al. (Ivors et al. 2006) also identified individual genotypes within lineages: while the California infestation appeared to be caused almost exclusively by one genotype, multiple genotypes were identified in European and North American nurseries. This pattern may be explained by multiple introduction events of individuals belonging to distinct lineages into the commercial nursery trade in both continents, while the introduction into the wild can probably be associated with a single or multiple introduction of a few very closely-related genotypes. The fact that the California wild genotype is one of the several present in U.S. nurseries, and the fact that U.S. nurseries contain all three known lineages of *P. ramorum* suggest that nurseries may have operated as a stepping stone for the introduction of the pathogen into the wild.

We have furthered the genetic analysis of *P. ramorum* lineages and populations by using two distinct approaches. First, we have sequenced the flanking regions of five microsatellite loci and performed an analysis of the combined dataset for three genotypes, each belonging to one of the three lineages. Second, we have used three newly developed hypervariable tetrarepeat microsatellite loci to study hundreds of individuals from several populations scattered throughout the entire zone of infection in California, and of isolates found in nurseries across North America. In this analysis, we also included two sets of isolates that were obtained in 2002-2003 from several locations in two California counties (Santa Cruz and Sonoma), where SOD was reported since the late 1990s.

The combined loci sequence analysis revealed that each one of the six haplotypes (two haplotypes for each diploid genotype) fell in one of two clades. One clade included one haplotype from the European lineage, one haplotype from the North American lineage, and both haplotypes of the third lineage. The other clade included one haplotype from the European lineage and one from the North American lineage. This analysis helps to better understand the history of the known lineages of *P. ramorum*, indicating that both the North American and European lineages are the result of mating events between different genotypes belonging to one of the two clades. On the other hand, the third lineage appears to be the result of mating between two haplotypes belonging to the same clade. The third lineage in fact is the only one to be characterized by some alleles displaying lower than expected heterozygosity levels.

By using the more sensitive tetra-repeat microsatellite markers, we have been able to increase our ability to differentiate genotypes. From the two originally identified, the number grew to over 40. It should be highlighted that these 40 genotypes are almost identical or quasi-clonal, and in the absence of sex they must have originated through mutation or somatic recombination events. The ability of an organism to generate new genotypes in the absence of sex will be directly correlated to a) the mutation or somatic recombination rate characteristic for that species, and to b) the size of its population. Because mutation rates are normally constant for a given species, the number of new genotypes is likely to be correlated to the actual size of the population. *P. ramorum* has been in an unchecked epidemic level for the last ten years, and its population in the wild has likely reached an enormous size (millions of trees have been estimated to have been infected yearly by the pathogen). Therefore, it is not surprising that the number of genotypes in the wild is currently larger than that in nurseries, where the disease is intensively managed and controlled.

A contingency analysis based on the frequency of genotypes detected in each of the study sites indicated that, with one exception (see below), the frequency of genotypes at each study site

was significantly different. This was also true for sites only 20 km apart, suggesting that the number of migrants between these sites is not sufficient to homogenize genotype frequency. Furthermore, over 50% of the genotypes were “private”, i.e., exclusively found in individual sites, indicating a great extent of population subdivision in the zone of infection in California. Although *P. ramorum* disperses aerially, the population structure here described indicates that long distance aerial movement is a rare event. This pattern is distinctively different from that described for other aerially-borne oomycetes. While the frequency of genotypes is distinctly different among sites, and many private genotypes were detected, some genotypes were considerably more common than others: those are likely to be the genotypes originally introduced.

The presence of identical genotypes in multiple locations suggests that some site to site movement is occurring, but that this movement may be rare or still unidirectional because the introduced pathogen has not yet reached its equilibrium in its new range. One striking exception was found when comparing the genotypic frequency among all locations: two sites, approximately 100 km apart, one North (Mount Tamalpais, Marin County) and one South (Scotts Valley, Santa Cruz County) of the Golden Gate were found to have an undistinguishable genotype frequency. There is corroborating evidence that infected ornamental plants were sold from the Santa Cruz to the Marin County area. Movement of infected plants between the two areas may explain the similarity in pattern between these two distant sites.

When we compared the frequency of genotypes found in nurseries, with those found in 2002-2003 in Santa Cruz and Sonoma Counties, we found no differences between genotype frequency of wild and nursery populations; this finding supports a close link between nursery populations and the early SOD epidemic. In light of the large number of *P. ramorum* individuals in the current pandemic in California, the contribution of possible current escapes from the nursery in the wild is probably insignificant. However, just recently a genotype associated with nursery plants was detected in a California stream outside the zone of infection, indicating that past introductions associated with nurseries may have not all been detected yet, or may be currently occurring. Some of the genotypes detected in the wild in 2002-2003 were not detected again after 2004; on the other hand novel genotypes are at times found. Because of potential extinction events and the creation of new genotypes through mutations, *P. ramorum* populations appear to be shifting as time goes by.

While long distance aerial movement is probably an infrequent, but not rare event, short-distance aerial movement is responsible for the frequent local spread of the pathogen. Although the extent of local movement has not been adequately studied, it appears to range between 10 m, in the absence of wind, to approximately 3,000 m. Several studies have indicated that sporulation of the pathogen mostly occurs during the rainy season, and that inoculum load is directly correlated with number of California bay laurel (*Umbellularia californica*) present in a site. The above information is based on collection of sporangia from rainwater under bay laurel and under other plant hosts, and on the observation of sporangia from symptomatic plant tissue collected in the wild. We have monitored inoculum levels for 2 years in 8 study plots using a different approach. A total of 16 buckets were placed in each site, and infection was monitored by baiting 5 bay leaves in each site. In the lab, we had previously determined that infection of up to 3 leaves corresponded to low and medium levels of inoculum, while infection of 4 or 5 leaves corresponded to high levels of inoculum. Leaves were left in the buckets for 3 weeks, and water was placed in the bucket, so that measurements would be independent of rainfall. Results indicated that low level of infection is possible in the absence of rain, but infection levels peaked

during the rainy season, but almost exclusively in the warm March to June months. Almost no infection was detected in the colder months of January and February in spite of the fact that rain events occurred in that period, indicating that while sporangial production may occur in colder weather, infection is favored by warmer temperatures. Infection levels were also much higher where bay density was higher. It should be highlighted that the sites with lower bay densities were characterized by the presence of tanoaks (*Lithocarpus densiflorus*). This indicates that tanoaks are not equivalents to bay laurels in sporulation ability.

Questions such as how long the pathogen sporulates on individual hosts and how long the pathogen may survive during the unfavorable dry summer and fall seasons are questions that have not fully been answered yet. The survival issue is one that may vary significantly year to year, and may be highly dependent on the type of substrate (soil vs. leaves vs. wood vs. water). One complexity of addressing the survival issue is that of differentiating samples in which the pathogen is dead from those in which the pathogen is dormant. Neither culturing nor DNA-based assays can differentiate between these two. We have developed a reverse transcriptase (RT-PCR) assay that targets mRNA of the COX mitochondrial region. COX mRNA is highly specific to *P. ramorum*, and is an indicator that the organism is breathing. RT-PCR results indicated that culturing significantly underestimates survival, while DNA-based assays overestimate it. By using this technique, we have determined that in 2005 an average of 40% of bay infections were still active in October, but this percentage varied greatly among sites. It should be noted that 2005 was a particularly favorable year for *P. ramorum*, and that survival percentages may be a lot lower in drier years.

One of the complexities of SOD is represented by its life cycle: while oaks (*Quercus* spp.) and tanoaks are lethally affected by the disease, they are not the most infectious hosts. California bay laurel, a species that only develops a foliar blight when infected, is the so-called SOD “superspreader.” The fact that the disease does not seem to significantly reduce the fitness of bay laurels is unfortunate, because when environmental conditions are favorable, epidemics can develop basically unchecked. Some ornamental host such as camellias (*Camellia* spp.) and rhododendrons (*Rhododendron* spp.) are also known to be able to spread the disease, and the few wildland infestations in Europe appear to be mostly associated with the presence of the latter genus. While the role of bay laurels in the epidemiology of SOD is supported by various lines of evidence, *P. ramorum* can infect an extremely broad range of hosts: the role played by most of these hosts in the disease epidemiology is still unknown. In order to determine the relative importance of each host in the wild as a substrate of colonization by *P. ramorum*, we have collected approximately 400 samples from 41 symptomatic plant species in 7 sites in California. Approximately 300 samples from 30 species were confirmed as infected by *P. ramorum* both by culturing and DNA-based assays.

The most important host for the pathogen was California bay laurel (57% of all confirmed infections), followed by tanoak (14%), redwood (*Sequoia sempervirens*) (5%), hazel (*Corylus* spp.) (4%), toyon (*Heteromeles arbutifolia*) (3.5%) and Pacific madrone (*Arbutus menziesii*) (3%). Although the sporulation potential by *P. ramorum* has not been determined for all of these hosts, it is likely that at least redwood, hazel and Pacific madrone may play a role in the spread of the disease. It is plausible that these hosts may be important in the *endemic* spread of the disease across the landscape, by allowing the pathogen to become established in new sites. These hosts in fact appear to be commonly infected and do not require the presence of bay laurels for infection to occur. Other hosts, such as Douglas-fir (*Pseudotsuga menziesii*), or many of the herbaceous plants and ferns normally appear to be infected only when infected bay laurels are in

the same area. These hosts may fundamentally “bait” the pathogen only when inoculum conditions are very high (epidemic levels driven by the presence of infected bay laurels), and may not play an important role in the spread of the disease in nature.

Controlling SOD in the wild has not been an easy task. Even after relatively dry and unfavorable weather for the pathogen, rapid climbs in population levels have been noticed following just a couple of repeated rain events. On the coast of California, the percentage of infections surviving from one season into the next may be rather high. Two control approaches have been pursued: in Oregon, where infection was discovered in its early stage, a continuing effort to drastically reduce inoculum is attempted on a yearly basis. This effort includes cutting of all infected plant species, herbicide and fungicide treatments, and burning of infected material. Although the disease has not been eradicated, inoculum levels have remained consistently low.

In California, where the disease already occupied hundreds of miles when the causal agent was identified, mitigation efforts include protective chemical treatments of oaks and tanoak, reducing the number of bay laurels, and sanitation efforts using prescribed burns, chipping infected woody material, and composting. Phosphites have been mostly applied as bark sprays in conjunction with the organosilicate surfactant Pentrabark: Recent data have indicated that the effects of a single treatment last at least 20 months. Furthermore, when treating tanoaks, a reduction in foliar lesion development (those lesions are responsible for the sporulation of the pathogen), has been documented. Whether resistant oaks and tanoaks exist is still an open question, but the observation that oaks are infected only in the proximity of infected bay laurels and the reported presence of less susceptible individuals in each of nine populations studied, may indicate that oak species are not at risk of going extinct. On the other hand, although variation in susceptibility has also been recorded for tanoaks, it has been shown that some populations are on the whole more susceptible than others.

The high levels of mortality, locally approaching 100% of adult individuals, suggests that these susceptible populations are at risk of disappearing. A large common garden experiment has been started with the aim of identifying potential resistance traits (patterns of susceptibility indicate that infection of tanoaks and oaks by *P. ramorum* is a multi locus trait) that may be used to rear seedlings for restoration projects. In terms of sanitation, it has been shown that chipping and drying infected woody substrates is effective, and also that composting is effective, but more research needs to properly address the effects of fire. Finally, the impact of thinning California bay laurel is unclear, and studies are under way to address this issue.

Although a valiant effort to stop the disease is under way in the known areas of infection, the best strategy remains preventing its introduction in new areas. The reports in 2007 that *P. ramorum* was baited from two rivers in two states outside of California and Oregon is troubling. International and national agencies have placed a high priority in preventing further spread of the pathogen, and a series of surveys, including those testing entire watersheds by baiting *Phytophthoras* from rivers are routinely performed. To aid the surveying efforts, two general tools are available: a) risk assessment maps, which based on plant susceptibility and climate patterns indicate how favorable an area can be for *P. ramorum*, and b) a wide array of molecular diagnostic tools that will greatly enhance the ability to detect the pathogen. Certainly, no matter how good the diagnostic tools are, it is the sampling scheme that will determine the likelihood of success of detection of the pathogen. Aerial surveys, for instance, only identify dead and dying trees, but cannot identify the subtle symptoms of the most contagious host, California bay laurel.

This problem may be particularly complex for agencies from countries or regions that do not have the pathogen yet and are unaware of the potential susceptibility of their native flora. The

Ministry of Agriculture and Forestry of New Zealand has decided to fund research aimed at determining both the presence of hosts that could be lethally affected and that of hosts that could spread the disease (note that one species could play both roles).

Research at the University of California at Berkeley (UCB) has identified a few hosts of low susceptibility among the New Zealand flora, but also two hosts of high susceptibility. The red southern beech (*Nothofagus fusca*) was extremely susceptible and developed large stem lesions, although it did not support abundant sporulation. The Fuchsia tree (*Fuchsia excorticata*), on the other hand, developed large foliar lesions and was a better substrate for sporulation than Rhododendron. Evidence thus suggests that both types of hosts (“infectious” and “dead-end”) exist in New Zealand and that an epidemic could develop in that country. Furthermore, a study of the distribution of these two hosts indicated they coexist abundantly on the west coast of both the North and South islands. Similar approaches aimed at identifying areas containing both epidemiologically-important hosts and highly-susceptible hosts, may be important to identify areas at risk in other regions of the world, and to better focus our surveying schemes.

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