Molecular Identification and Detection of *Phytophthora ramorum*[1]

Peter Bonants[2], Els Verstappen[2], Ineke de Vries[2], Katarzyna Wiejacha[3] , and Kelly Ivors[4]

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Introduction

The genus *Phytophthora* comprises over 70 described species, however many new species have been reported recently as a result of the discovery of previously undetected species or by the hybridization of known species. *Phytophthora ramorum*, one of the new *Phytophthora* species, is considered a high phytosanitary risk because it is currently responsible for large-scale oak mortality in coastal California forests. In Europe, the disease occurs mainly in nurseries on *Rhododendron, Viburnum* and *Camellia*. However, in landscapes, infected trees of *Quercus rubra, Q falcata, Q. ilex, Aesculus hippocastanum* and *Fagus sylvatica* were reported recently on sites previously identified with infected *Rhododendron* plants. In addition, numerous nurseries in the U. S. have recently been diagnosed with infected *Rhododendron* and *Camellia* spp.

P. ramorum is a heterothallic species and is known to exist as two distinct populations in California/Oregon (U.S. type) and Europe (E.U. type), respectively. In Europe, almost exclusively A1 mating type isolates have been found, while in the United States A2 type isolates are most often identified. A single A2 isolate was found in Europe and recently several A1 isolates were identified in nurseries in the United States.

Measures are in force to prevent spread of this pathogen, as well as to prevent co-existence of both types. Therefore, adequate detection and identification methods are urgently needed.

Several molecular techniques have been developed for detection and characterization of this new species of *Phytophthora*. Molecular differences between U.S.- and E.U.-isolates of *P. ramorum* exist and can be demonstrated with the techniques at hand. Data thus far obtained of isolates from throughout Europe and from the United States will be discussed.

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[2] Plant Research International, P.O. Box 16, NL-6700 AA Wageningen, The Netherlands. peter.bonants@wur.nl
[3] Research Institute of Pomology and Floriculture, Skierniewice, Poland
[4] Department of Plant Pathology, North Carolina State University, Mountain Hort. Crops Research & Extension Center, 455 Research Drive. Fletcher, NC 28732, USA

Results

For identification of *P. ramorum* several methods have been used:

AFLP: Amplified Fragment Length Polymorphism

This method consists of digesting genomic DNA with two restriction enzymes (a rare and a frequent cutter), ligation of adapters to the restriction fragments and two rounds of amplification: the first round using primers of the adapter sequence, which amplify all restriction fragments, and a second round using a specific amplification of a subset of restriction fragments using extra selective bases in the primers. One of the second round primers is fluorescently labeled; therefore, running the amplicons on a sequencing system will generate a DNA fingerprint pattern. Relatedness of isolates or species can be seen by scoring the number of identical and unique bands; these data can be presented in a dendogram. Large numbers of *P. ramorum* isolates have been analyzed with AFLP (Ivors and others 2004). Results show two clusters, with E.U. isolates and U.S. isolates grouping separately. A1 mating type isolates from the U.S. and the E.U. A2 mating type isolate cluster within the E.U. clade.

ISSR: Inter Simple Sequence Repeats

ISSR is a technique that uses primers to amplify regions between two microsatellites utilizing PCR. Microsatellites are Simple Sequence Repeats (SSR) occurring in the genome of many organisms (e.g. $(AG)_{12}$ or $(CTG)_7$). Several primers were tested on two E.U. and two U.S. isolates. Many E.U. and U.S. isolates of *P. ramorum* were then screened with polymorphic primers Although differences were identified between E.U. and U.S. populations, polymorphisms were not identified within each population with this technique.

Microsatellites (SSR: Simple Sequence Repeats)

Using the whole genome sequence of *P. ramorum*, SSR techniques were used for fingerprinting large numbers of *P. ramorum* isolates originating from different host species within Europe and the United States. Using a computer program developed at Plant Research International, 1334 potential microsatellite loci were identified. Primers were selected from over 110 flanking regions of SSRs and tested in PCR reactions to amplify repeats. Thirty-one polymorphic loci were identified and 14 primer sets were optimized for isolate genotyping. Many loci showed variation between the E.U. and U.S. populations. Minor variation was found within the E.U. populations and even less variation within the U.S. population. This information provided insight regarding the amounts of genetic variation within populations, identified new genotypes, and separated isolates into two distinct lineages correlated with continental provenance. Comprehensive data will be published soon (Ivors and others 2006).

Sequence analysis of several genes

In a recent study several genes of numerous *Phytophthora* species were sequenced (Kroon and others 2004). ß-tubulin and elongation factor 1_ as nuclear genes and NADH dehydrogenase I and Cox1 as mitochondrial genes were sequenced and phylogenetic trees were generated. Results show a close relationship between *P. ramorum* and *P. lateralis*.

For detection of *P. ramorum*, several other methods can be used:

ITS-PCR

ITS regions of the rDNA have been sequenced for many *Phytophthora* isolates. PCR-based detection methods using primers derived from those sequences have been developed for many *Phytophthora* species. PCR primers for *P. ramorum* have been developed and validated in this way. Detection of the pathogen in plant, water and soil samples has been successful.

TaqMan® PCR (also known as real-time PCR)

A TaqMan® probe has been developed based upon the ITS sequence (Ivors and Garbelotto 2002). A TaqMan probe is a probe with two fluorescent labels that can be used in PCR for real time analysis. In this way, quantification of the pathogen in infected material can be estimated.

PCR-RFLP

A single nucleotide difference in the Cox 1 gene has been observed between E.U. and U.S. isolates of *P. ramorum*. This single nucleotide polymorphism (SNP) exists at an *Apo*I restriction site. Based on this SNP, a PCR-RFLP method was developed to distinguish between E.U. and U.S. isolates of *P. ramorum*. This test can also be used on infected material (*in planta*) without a pure culture isolate.

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