DEVELOPMENT OF A DIAGNOSTIC DNA ARRAY FOR PATHOGENIC *PHYTOPHTHORA* SPECIES

Daniel O'Gorman, Arthur W.A.M de Cock*, C. André Lévesque

Agriculture & Agri-Food Canada,
Pacific Agri-Food Research Centre,
Summerland, B.C., Canada, V0H 1Z0
tel: (250)-494-6396, fax: (250)-494-0755

* Centraalbureau voor Schimmelcultures,
Yeast Division, Julianalaan 67, 2628 BC
Delft, Netherlands

Introduction

Among *Phytophthora* species, there is considerable overlap of the morphological characters used for species identification. These shared characteristics are potential sourcesof errors in morphology-based identification. Molecular techniques can be useful in identifying these species more reliably. A ribosomal DNA sequence database for *Phytophthora*, based on (neo-)type strains, has been compiled in our laboratory. This sequence database has allowed the development of a comprehensive DNA array consisting of taxonomic-specific oligonucleotides, for the detection of *Phytophthora* species. The oligonucleotides are bound to a solid support, in an array, and are used in a reverse dot blot hybridization (RDBH) reaction. Amplified, labeled sample DNA is used as a probe in the hybridization reaction with the array. Species identification is thereby determined as positive hybridization signals which appear as dark dots on exposed x-ray film. RDBH was first used in the medical field to identify mutations associated with cystic fibrosis (1) and since then it has been applied to bacterial and fungal identification in both ecological and agricultural studies (2, 3). The technological advantages of a diagnostic system such as this one for *Phytophthora* species, would included the elimination of the need for fungal isolation from field samples, avoidance of difficulties associated with morphology-based identification, and the ability to detect multiple pathogens in a single test.

The objective of this study was to develop a DNA array capable of identifying a wide range of pathogenic *Phytophthora* species. Our results to date are presented here.

Materials and Methods

All *Phytophthora* and *Halophytophthora* (neo-)type strains that are presently available as pure cultures were used in this study. Sequencing reactions, of ribosomal internal transcribed spacers (ITS) region of these cultures, were performed using the Big Dye Terminator Cycle Sequencing Kit and the ABI 310 automated sequencer (Applied Biosystems, Foster City, CA). ITS sequences were aligned using GCG 9.1 (Genetics Computer Group Inc., Madison, WI). Parsimony analyses were performed using PAUP 4.0b2 (Sinauer Associates, Sunderland, MA).

Using sequence alignments of this data, taxanomic-specific oligonucleotides were designed which correspond to various *Phytophthora* and *Halophytophthora* species. The oligonucleotides were synthesized with amino modifiers for attachment. In total, 110 amino-terminated oligonucleotides were arranged in duplicate using a 6 row by 20 column array in a 384 well plate. The oligonucleotides were diluted to 40 µM in 0.5M sodium bicarbonate buffer pH 8.4 and spotted directly onto pre-activated nylon membranes (Immunodyne ABC; Pall, GlenCove, NY) using a 384 pin replicator (V&P Scientific, Inc., San Diego, CA). After blotting, membranes were stored in 2X SSC at 4°C until required for use in reverse dot blot.

Total DNA obtained from pure cultures was simultaneously amplified and labeled with alkaline labile digoxygenin (DIG; Roche Diagnostics, Laval, Québec). The resulting DIG-labeled PCR products were quantified by comparison to a known standard using gel electrophoresis, and subsequently used as probes in hybridization reactions with the array. Hybridization results were scanned and automatically analyzed using the public domain software, NIH Image. Average gray values for each dot were recorded as numbers ranging from 0 (completely white) to 255 (completely black). These intensity values were converted into five distinct categories, and reported in Table 1.

Results and Discussion

The hybridization data generated confirmed the specificity of most oligonucleotides. In Table 1, both the species (rows) and oligonucleotides (columns) are arranged according to phylogenetic relationship. As a result, successful hybridizations are depicted in Table 1 by a diagonal trend of positive reactions . In some instances, where group-specific oligonucleotides were designed to identify one or more groups of closely related species, vertical lines or blocks are evident within the diagonal, which correspond to specific groups of fungi. An example of this is the group 5/6 oligonucleotide which identifies *P. cambivora* and *P. fragariae* (group 5) as well as *P. cinnamomi* isolates(group 6).

Cross-hybridization reactions were detected at various degrees of signal strength for certain oligonucleotides. These cross-reaction are most likely due to the fact that the ITS region is less variable within the genus *Phytophthora* than in other genera such as *Pythium* (4). The lack of variability in the ITS region created challenges in the design of some oligonucleotides. There are approximately five oligonucleotides (#353 *cryptogea*, #361 *syringae*, #390 Group 17/18, #393 *infestans*, and #412 *lateralis*) which show cross-hybridizations with the majority of *Phytophthora* species tested. The removal or modification of these oligonucleotides would greatly decrease the overall number of cross-reactions. Additionally, there are species that did not react or reacted poorly with their corresponding oligonucleotides. The modification or re-design of all poorly reacting or cross-hybridizing oligonucleotides is being investigated to produce more definitive results.

As part of a continuing effort, this system is presently being tested for its ability to detect and identify pathogenic *Phytophthora* species from infected plant tissue(5). This diagnostic system for *Phytophthora* species will ultimately have practical, and valuable applications in the detection and management of Phytophthora root diseases, for a wide variety of crops.

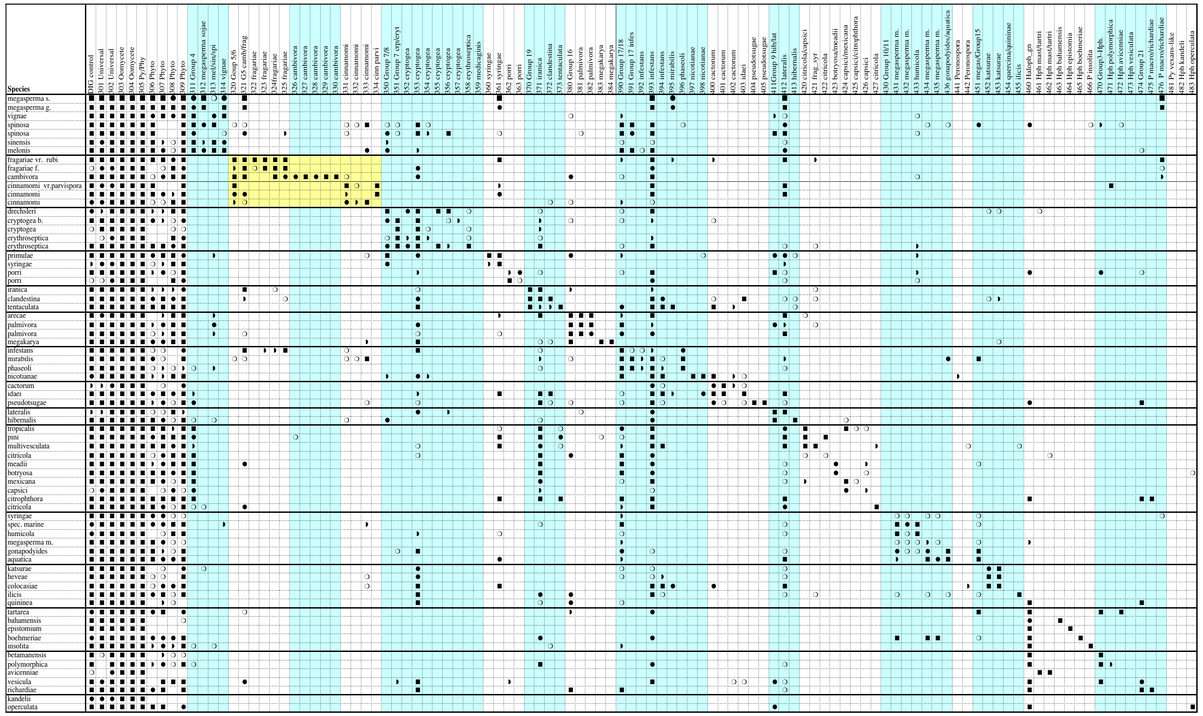


Table 1. Hybridization results of the DNA array with pure cultures of *Phytophthora* and *Halophytophthora*. Species (rows) and taxonomic-specific oligonucleotides (columns) are arranged according to phylogenetic relationship. Hybridization signals are reported as scanned gray values and classified into five classes of reactions (refer to the following legend). Phylogenetic clusters are between the solid lines for the isolates, and within shaded columns for the oligonucleotides. Positive hybridization results are depicted as a diagonal trend across the table. The yellow shaded area shows scanned gray values for species in group 5 and 6. The actual blots for the hybridizations for these species can be seen in Fig. 2.

blank	=intensity nil or at background level (average gray value 0 to <6)
❍	=low signal (average gray value 6 to <50)
◗	=moderate signal (average gray value 50 to <90)
●	=high signal (average gray value 90 to <175)
■	=very high signal (average gray value 175 to 255)

Acknowledgments: We would like to thank Anita Quail for her technical assistance in the preparation of the DNA arrays. The funding for this research project was provided by Ocean Spray Cranberries Inc., the BC Cranberry Growers Association and Agriculture and Agri-Food Canada.

References:

1. Kawasaki, E. S., Saiki, R., and Erlich, H. 1993. Genetic analysis using polymerase chain reaction-amplified DNA and immobilized oligonucleotide probes: reverse dot-blot typing. Methods Enzymol. 218:369-81.
2. Voordouw, G., Shen, Y., Harrington, C. S., Telang, A. J., Jack, T. R., and Westlake, D. W. S. 1993. Quantitative reverse sample genome probing of microbial communities and its application to oil field production waters. Appl. Environ. Microbiol. 59:4101-13.
3. Lévesque, C. A., Harlton, C. E., and de Cock, A. W. A. M. 1998. Identification of some oomycetes by reverse dot blot hybridization. Phytopathology 88:213-22.
4. Quail, A., de Cock, A.W.A.M., Lévesque, C.A. 2000. *Pythium* identification by hybridization to a DNA arrays. Poster. CPS and APS (Pacific division) joint meeting.
5. Lévesque, C. A., Lazaroff, W., Quail, A., O'Gorman, D., Macdonald, L., Elmhirst, J., Hudgins, E., Mazzola, M. 2000. The use of DNA arrays for direct detection of oomycetes from roots and soils. Poster. CPS and APS (Pacific division) joint meeting.

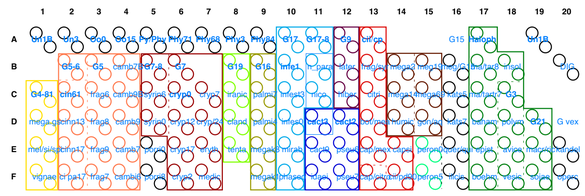
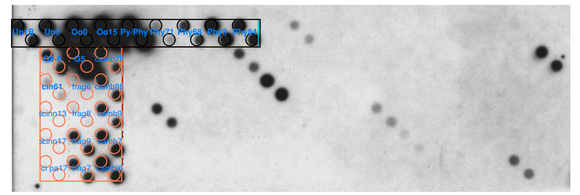
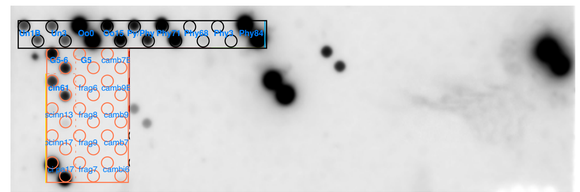


Fig. 1. Template pattern for the DNA array of oligonucleotides. Each block represents a phylogenetic cluster to which the oligonucleotide(s) in bold react. Hybridizatrion controls are universal and oomycete specific oligonucleotides (row A1- A9) and a DIG control for the chemiluminescence detection procedure (20B).

A- *P. cambivora*



B- *P. cinnamomi*



C- *P. fragariae*

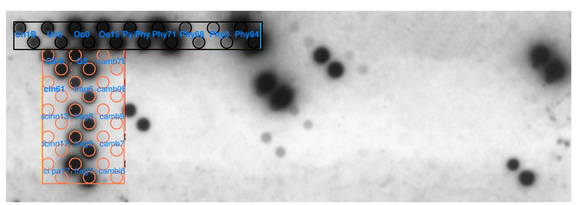


Fig. 2 Hybridizations results of DNA arrays with DIG-labeled PCR products amplified from pure culture of *P. cambivora* **(A)**, *P. cinnamomi* **(B)**, and *P. fragariae* **(C)**. These isolates are representatives of group 5 and group 6 (also highlighted in Table 1). Some positive dots are known cross reactions and can be observed as dots which are outside of the blocked template pattern for group 5/6 and the block of control oligos.