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Use of hybridization melting kinetics for detecting *Phytophthora* species using three-dimensional microarrays: demonstration of a novel concept for the differentiation of detection targets

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ABSTRACT

Microarray-based detection is limited by variable and inconsistent hybridization intensities across the diversity of probes used in each array. In this paper, we introduce a novel concept for the differentiation of detection targets using duplex melting kinetics. A microarray assay was developed on a PamChip microarray enabling the differentiation of target *Phytophthora* species using the melting kinetics of probe-target duplexes. In the majority of cases the hybridization kinetics of target and non-target duplexes differed significantly. Analysis of the melting kinetics of duplexes formed by probes with target and non-target DNA was found to be an effective method for determining specific hybridization and was independent of fluctuations in hybridization signal intensity. This form of analysis was more robust than the traditional approach based on hybridization intensity, and enabled the detection of individual *Phytophthora* species and mixtures thereof.

Introduction

Plant diseases are a major limitation on plant production worldwide with the potential for disease epidemics to lead to the collapse of entire industries. The ability to detect the pathogenic micro-organisms responsible for plant diseases is a major weapon in disease management. Early and reliable detection is crucial for the containment of diseases and implementation of quarantine and chemical control measures when they are likely to be most effective (Eden *et al.* 2000).

Members of the *Phytophthora* genus are responsible for plant disease in a huge range of plant species worldwide. Many of these are caused by soil-borne species that infect the roots of the plant causing root and crown rots. Current baiting methods for the detection of soil-borne *Phytophthora* species are labour intensive, time-consuming, error prone, and require a high level of technical expertise. Consequently they are too inefficient and expensive for routine pathogen detection (Dobrowolski & O'Brien 1993). Baiting isolation techniques do not account for the possibility of *Phytophthora*

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species lying dormant in soils, resulting in the pathogen going undetected during testing (McCarren *et al.* 2005).

Distinguishing between different *Phytophthora* species can be difficult but necessary, particularly where more than one species of *Phytophthora* can be responsible for the same plant diseases. Jarrah Dieback in the south-west of Western Australia, although predominantly caused by *P. cinnamomi*, can also be caused by *P. citricola, P. cryptogea, P. megasperma var. sojae* and *P. nicotianae* (Shearer *et al.* 1987; Shearer *et al.* 1988). Also, oak decline in Europe has been attributed to several species of *Phytophthora,* including *P. quercina, P. cambivora, P. citricola, P. cactorum, P. gonapodyides* and *P. megasperma* (Jung *et al.* 2004). The causal organism must be identified as *Phytophthora* species vary in their potential impact on different vegetation types and areas, thus affecting the control methods implemented.

In recent years, there has been an increase in the application of PCR techniques to the detection of *Phytophthora* species in asymptomatic plant material (Coelho *et al.* 1997; Kong *et al.* 2003). These methods offer improved sensitivity, specificity, analysis time, and the potentially for high throughput applications (Coelho *et al.* 1997). However, in its conventional format PCR is limited to detection of one or a few pathogens in a single test, adding considerably to the cost of analysis.

The application of microarrays in the detection of bacterial and viral species in various environments has enabled parallel detection of multiple species in a high throughput format conducive to automation (Small *et al.* 2001; Loy *et al.* 2002). However, the application of microarray technology to pathogen detection has been impeded by inconsistencies between the hybridization intensities of some target and non-target probe duplexes (Schepinov *et al.* 1997). These inconsistencies have been put down to several factors including steric effects, secondary DNA structure, and the overall melting characteristics of both probe and target sequences (Schepinov *et al.* 1997). Furthermore, the two-dimensional microarray systems currently available are limited by the rate of diffusion of the target across the entire array and the inability to carry out real-time analysis of the hybridization. These two-dimensional systems require hybridization for between 12 and 24 h, as well as separate hybridizations for each experiment (Loy *et al.* 2002).

Many of these limitations have been overcome in this study using PamChip microarrays. These are a three-dimensional arraying platform consisting of a porous, flow-through substrate mounted within a hybridization chamber (Anthony *et al.* 2003). This system drastically reduces hybridization times, enables real-time monitoring of hybridization reactions, and enables the hybridization temperature or buffer to be varied throughout the experiment. Kinetic curves can be generated for each hybridization reaction to characterize the probe–target duplex with more stringency at a temperature that is discriminatory for each probe (Anthony *et al.* 2003).

This study demonstrates the concept of applying melting kinetics of the probe–target duplex as the determinant of species detection. Throughout this study PCR amplification products of several *Phytophthora* species were used as a model for the potential application of this method to the fields of plant pathology and environmental mycology.

Materials and methods

Isolates

Individual isolates of the eight *Phytophthora* species studied were sourced from the Dutch Plant Protection Services (Table 1). Isolates were cultured on pea agar (120 g frozen peas, 15 g agar, 2.5 g sucrose, 1 l tap water) at 24 °C for 3 d, sub-cultured into clarified pea broth (120 g frozen peas, 1 l tap water) and grown for a further 3 d at 24 °C. The mycelium was harvested, washed in sterile distilled water and lyophilized overnight. Genomic DNA was extracted using a Puregene DNA Purification Kit; DNA purification from 10–20 mg fungal tissue (Gentra Systems, Minneapolis, MN).

Sequences and probes

The ITS1 region for each isolate of *Phytophthora* was sequenced using Big Dye Terminator Kit (Applied Biosystems) with ITS1 (5′-CTCGACCGTTAGCAGCATGA) and ITS2a (5′-TCATGCTGC TAACGGTCGAG) primers. These were aligned with sequences obtained from the Genbank database to confirm the identity of the isolates used.

The microarray probes were designed to target several diagnostic sequences within the ITS1 region of each species of *Phytophthora* studied (Table 1). A consensus sequence was generated by aligning multiple sequences of each species derived from Genbank along with those of the isolates being studied. The consensus sequences for each species were aligned with other *Phytophthora* species and regions of interspecies variability were identified. The probes were designed to target diagnostic sequences for each species such that each probe had a length of 18–24 nucleotides with a melting temperature of the probe–target duplex between 58 and 62 °C (Bodrossy 2003; Sambrook *et al.* 1989). Regions of variation within a species were noted and avoided as potential targets for candidate species probes. Where possible, probes were designed to have as many 5′ to centrally located

Table 1 – Isolates of *Phytophthora* species used. Isolates were obtained from the Plant Protection Services, Wageningen, the Netherlands

Species	Isolate number	Obtained from
Phytophthora cambivora	BBA 21/95-K-11	BBA Braunschweig, Germany
P. cactorum	PD 88/415	Plant Protection Services, Wageningen, the Netherlands
P. cinnamomi	BBA 62660	BBA Braunschweig, Germany
P. cryptogea	PD 20009183	Plant Protection Services, Wageningen, the Netherlands
P. erythroseptica	PD 92/133	Plant Protection Services, Wageningen, the Netherlands
P. infestans	PD 94	Plant Protection Services, Wageningen, the Netherlands
P. nicotianae	Coffey P 582	M. Coffey, USA
P. ramorum	PD 20019543	Plant Protection Services, Wageningen, the Netherlands

mismatches as possible (Loy et al. 2002). Using this rationale, several partially overlapping or non-overlapping probes were designed for each species of *Phytophthora* being studied (Table 2). Two genus-specific probes were designed to target all *Phytophthora* species. An internal standard probe was printed four times on each array for normalization.

Hybridization on microarray

The ITS1 region of each species of *Phytophthora* was amplified with the ITS1 and ITS2a primers in a 50 μl reaction containing 67 mM Tris–HCl pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 0.45% Triton X-100 (Invitrogen), 0.2 mM dNTPs, 0.25 μM primers, 1U Taq DNA polymerase, and 1 ng DNA template. The ITS2a primer was end-labelled with fluorescein. The PCR program involved an initial denaturation at 95 °C for 2 min, followed by 30 cycles of 95 °C for 1 min, 60 °C for 30 s, 72 °C for 1 min, and a final polymerization stage at 72 °C for 2 min.

Single-stranded amplification of the ITS1 region was performed using a fluorescently labelled ITS2a primer using the following amplification mixture: 0.3 mM dNTPs, 67mM Tris–HCl pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 0.45% Triton X-100 (Invitrogen), 0.36 μM ITS2aF, 0.5 mM $MgCl_2$, 1.5 U 0.5 μl Taq polymerase (Invitrogen), and 1 ng of the double-stranded PCR product. The PCR program included denaturation at 95 °C for 2 min, followed by 50 cycles of 95 °C for 1 min, 60 °C for 30 s, 72 °C for 1 min, and a final polymerization stage at 72 °C for 2 min.

Table 2 – Sequences of oligonucleotide probes used for analysis

Target species	Probe	Length	Probe sequence
Phytophthora cactorum	CAC2	22	GACGAAAGTCCTTGCTTTTAAC
	CAC2B	20	CGAAAGTCCTTGCTTTTAAC
	CAC5	24	GTAGCTTTTCTTTTAAACCCATTC
P. cinnamomi	CIN2	20	CCTATCACTGGCGAGCGTTT
	CIN3	22	CAATTAGTTGGGGGCCTGCTCT
	CIN3B	20	ATTAGTTGGGGGCCTGCTCT
	CIN4	19	ACGGCTGCTGCTGCGTGGC
	CIN4B	20	CGACGGCTGCTGCTGCGTGGC
	CIN5	23	CCTCTCTTTTAAACCCATTCTGT
	CIN5B	20	TCTCTTTTAAACCCATTCTG
P. cryptogea/P. erythroseptica	CRY1B	20	GGGCTAGTAGCGTATTTTTA
	CRY4	23	GGCTAGTAGCGTATTTTTAAACC
	CRY6	20	GACCGCTTGGGCCTCGGCCT
	ERY1	19	CGGTTTTCGGCTGGCTGGG
	ERY1B	20	CGGTTTTCGGCTGGCTGGGT
P. infestans	INF2	19	GGGGGTCTTACTTGGCGGC
	INF3	20	CCCTATCAAAAGGCGAGCGT
	INF4	21	TCTTACTTGGCGGCGGCTGCT
	INF4B	20	GTCTTACTTGGCGGCGGCTG
	INF5	20	GGGGGTCTTACTTGGCGGCG
	INF6	22	CCCTATCAAAAGGCGAGCGTTT
P. nicotianae	NIC1	22	CCTATCAAAAAAAAGGCGAACG
	NIC1B	20	CTATCAAAAAAAAGGCGAAC
	NIC3B	20	GCTTCGGCCTGATTTAGTAGT
	NIC4	22	GTCTTATTTGGCGGCGGCTGCT
	NIC4B	20	GTCTTATTTGGCGGCGGCTG
P. ramorum	RAM2	19	GAGCGCTTGAGCCTTCGGG
	RAM3	20	GCGCTTGAGCCTTCGGGTCT
All *Phytophthora* sp.	PHYT1	24	GCTTTTAACTAGATAGCAACTTCA
	PHYT1B	20	GCTTTTAACTAGATAGCAAC
Non-target probes			
P. capsici	CAP4	23	AAACCCATTTCACAAAACTGATT
P. citricola	CIC1	22	CTTGCTTTTTTGCGAGCCCTAT
P. fragariae	FRA4	22	GTAGCCCTTTTCTTTTAAACCC
P. gonopoloides	GON1	22	GGCGTGCGTGCTGGCCTGTAAT
P. megasperma	MES2	22	GTAATGGGTCGGCGTGCTGCTG
P. palmivora	PAL1	24	CGGTCTGAACTAGTAGCTTTTTTA
P. sojae	SOJ4	21	AGTCGGCGGCTGGCTGCTGTG
Internal standard	-	20	CTCGACCGTTAGCAGCATGA

ITS PCR products of target DNA was hybridized to the array in a thermostatically controlled four-array incubator (FD10, Olympus, Zoeterwoude, The Netherlands) in 30 μl of SSPE buffer (20x SSPE contains 3.0 m NaCl, 0.2 m NaH_2PO_4, and 0.02 m EDTA at pH 7.4) (Invitrogen, Den Bosch, The Netherlands). The PamGene system enabled the fluxing of the hybridization solution back and forth through the pores of the microarray substrate and real-time monitoring of the hybridization reaction with the incorporation of a CCD (charge-coupled device) camera (Beuningen *et al.* 2001). The array was initially washed three times with hybridization buffer with the SSPE pumped through the array five times during each wash. The hybridization mixture containing 30 μl SSPE buffer, 100 nM of the internal standard and the fluorescently labelled target DNA was hybridized to the array at 37 °C for 30 min, pumping two cycles of 50 μl min^{-1}; the additional 20 μl allowing the hybridization solution to pass completely through the array membrane with each pumping cycle. The hybridization mixture with the unbound target DNA and internal standard was removed and the array washed three times with fresh hybridization buffer. 30 μl SSPE buffer was added, the temperature of the hybridization chamber increased to 40 °C and held for 2 min. Pumping proceeded for a further 2 min at two cycles per minute. When the hybridization buffer was on the underside of the microarray membrane, it was photographed using the fluorescein filter for 1000 and 2000 ms. This cycle was repeated increasing the temperature by 5 °C intervals up to 75 °C.

Data analysis

Images were analysed using ArrayPro® Software (Media Cybernetics, Silver Springs, MD). Median intensities were calculated using local corners background removal for each probe signal, whereby the data were exported to Microsoft Excel® for preliminary analysis. All hybridization experiments were replicated three times on separate microarrays with individual probes printed twice in a systematically replicated pattern. All hybridization intensity data were transformed by taking Log2 of the hybridization intensities and then standardized as a percentage of the hybridization intensity of the internal standard at 40 °C. The melting curve of each probe was plotted and duplex stability analysed by determining the temperature (T_{60}) at which a 60% of the hybridization intensity at 40 °C was observed. This figure was extrapolated from the hybridization kinetics of each probe duplex (Fig 1). The probe efficiency was determined by taking the ratio of the T_{60} of the probe duplex to the all *Phytophthora* probe duplex on that array. The relative signal was then determined as the ratio of the probe efficiency of a given probe duplex to that probe on a standard hybridization in which 50 ng of the intended target species of a given probe was hybridized in 1 × SSPE (Fig 1). These data were analysed by analysis of variance (ANOVA) using Genstat 7 (Lawes Agricultural Trust, Oxford).

The application of duplex stability for microarray-based detection was addressed by comparing the T_{60} values, theoretical duplex stabilities and hybridization intensities of each of the eight *Phytophthora* species being analysed. T_m values for each of the target and mismatched duplexes were calculated using the m-fold algorithm (Zuker, 2003) and compared with both the T_{60} and hybridization intensities at 60 °C.

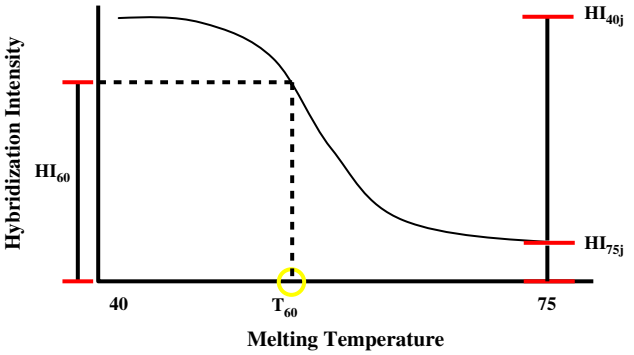

Fig 1 – Determination of T60 values extrapolated from real-time kinetic hybridizations on the PamGene microarray system. The T60 value was taken as the temperature at which 60 % of the total change in hybridization intensity from 40 °C to 75 °C was observed. Using

$$HI_{60} = 0.6(HI_{40j} - HI_{75j}) + HI_{75j}$$

Where: HI60 = hybridization Intensity at which 60 % of the initial probe–duplex hybridization intensity was present; HI40j = hybridization intensity of probe–target duplex j at 40 °C; HI75j = hybridization intensity of probe–target duplex j at 75 °C Calculation of probe efficiency and relative signal. Using

$$\text{Probe efficiency}_{x\,i} = T_{60x\,i}/T_{60PHYT1i}$$

$$\text{Relative Signal}_{x\,i} = \text{Probe efficiency}_{x\,i}/\text{Probe efficiency}_{x\,ii}$$

Where: x = the probe duplex being analysed; i = the array being analysed; ii = the standard array for the intended target of that probe.

Results

Characterization of hybridization properties and selection of probes with good specificity

Fifty nanograms of ITS PCR products from eight species of *Phytophthora* were individually hybridized to the microarray in 1 × SSPE hybridization buffer to assess the binding specificity of each probe. The species analysed were *P. cinnamomi*, *P. nicotianae*, *P. infestans*, *P. cryptogea*, *P. erythroseptica*, *P. cactorum*, *P. cambivora* and *P. ramorum* (Table 1). *P. cambivora* was included as a negative control as no probes targeting *P. cambivora* were present on the microarray. The results obtained by hybridization of target DNA from eight species of *Phytophthora* are listed in Table 3A. Of the 30 probes tested on the array, 26 showed specific hybridization to their target DNA. In each case the T_{60} value for hybridization to the target of the same species was significantly greater than the values for hybridization to DNA from different species. For example, the CAC probes hybridized to *P. cactorum* DNA with an average T_{60} of 61 ± 1.6 °C compared with an average of 50 °C for hybridization to non-target species. These correspond with the relative signal data displayed

Table 3 – Heat maps showing the T_{60} values of probe–Target duplexes hybridized with different target DNA (increments of 0.1 units are highlighted to aid in the comparison of duplex stabilities):

A) Relative signal values for each target and non-target duplex for PCR amplified DNA from eight species of *Phytophthora* independently hybridized to the microarray

Target DNA A.	PROBE CAC2	CA2B	CAC5	CIN2	CIN3	CIN3B	CIN4	CIN4B	CIN5	CIN5B	CRY1B	CRY4	CRY6	ERY1	ERY1B	INF2	INF3	INF4	INF4B	INF5	INF6	NIC1	NIC1B	NIC3B	NIC4	NIC4B	RAM2	RAM3	PHYT1	PHYT1B
CAC	1.0	1.0	1.0	0.7	0.4	0.5	0.6	0.6	0.5	0.7	0.0	0.4	0.3	0.4	0.4	0.4	0.5	0.8	0.6	0.5	0.6	0.0	0.0	0.4	0.8	0.6	0.0	0.0	1.0	1.0
CIN	0.5	0.5	0.5	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.0	0.3	0.0	0.1	0.1	0.4	0.4	0.8	0.6	0.0	0.5	0.0	0.0	0.0	0.7	0.6	0.0	0.0	1.0	1.0
CRY	0.3	0.5	0.2	0.2	0.0	0.0	0.0	0.0	0.2	0.4	1.0	1.0	1.0	1.0	1.0	0.4	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.5	0.1	0.0	0.0	1.0	1.0
ERY	0.4	0.5	0.5	0.0	0.0	0.0	0.0	0.0	0.1	0.2	1.0	1.0	1.0	1.0	1.0	0.0	0.0	0.0	0.1	0.1	0.0	0.0	0.0	0.0	0.6	0.2	0.0	0.0	1.0	1.0
INF	0.6	0.5	0.1	0.5	0.0	0.3	0.4	0.4	0.0	0.0	0.0	0.4	0.0	0.8	0.5	1.0	1.0	1.0	1.0	1.0	1.0	0.3	0.2	0.2	0.9	0.0	0.0	0.0	1.0	1.0
NIC	0.5	0.5	0.5	0.4	0.4	0.4	0.7	0.6	0.5	0.7	0.0	0.0	0.4	0.6	0.3	0.6	0.3	0.7	0.9	0.8	0.4	1.0	1.0	1.0	1.0	1.0	0.0	0.0	1.0	1.0
RAM	0.3	0.5	0.2	0.3	0.2	0.3	0.3	0.0	0.2	0.4	0.0	0.6	0.0	0.8	0.8	0.1	0.1	0.5	0.5	0.2	0.0	0.0	0.0	0.0	0.4	0.6	1.0	1.0	1.0	1.0
CAM	0.4	0.5	0.7	0.6	0.5	0.5	0.7	0.7	0.4	0.6	0.0	0.0	0.0	0.5	0.4	0.0	0.4	0.6	0.0	0.2	0.3	0.0	0.0	0.0	0.5	0.4	0.0	0.0	1.0	1.0

(B) Relative sensitivity of micorarray detecting decreasing amounts of *Phytophthora cinnamomi* DNA

50 ng	0.5	0.5	0.5	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.0	0.3	0.0	0.1	0.1	0.4	0.4	0.8	0.6	0.0	0.4	0.0	0.0	0.0	0.7	0.6	0.0	0.0	1.0	1.0
25 ng	0.4	0.5	0.4	0.9	0.9	0.9	0.8	0.8	1.0	0.9	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.7	0.5	0.0	0.5	0.4	0.2	0.0	0.5	0.2	0.0	0.0	1.0	1.0
12.5 ng	0.3	0.4	0.4	0.8	0.8	0.8	1.0	0.9	1.0	0.9	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.7	0.5	0.0	0.0	0.0	0.0	0.3	0.6	0.5	0.0	0.0	1.0	1.0
6.25 ng	0.4	0.4	0.4	0.7	0.4	0.7	0.6	0.8	0.9	0.9	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.3	0.4	0.0	0.1	0.0	0.0	0.1	0.2	0.3	0.0	0.0	1.0	1.0

(C) Mixtures of 50 ng of *Phytophthora nicotianae* DNA hybridized in the presence of decreasing amounts of *P. cinnamomi* DNA

CIN	0.5	0.5	0.5	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.0	0.3	0.0	0.1	0.1	0.4	0.4	0.8	0.6	0.0	0.4	0.0	0.0	0.0	0.7	0.6	0.0	0.0	1.0	1.0
NIC	0.5	0.5	0.5	0.4	0.4	0.4	0.7	0.6	0.5	0.7	0.0	0.0	0.4	0.6	0.3	0.6	0.3	0.7	0.9	0.8	0.4	1.0	1.0	1.0	1.0	1.0	0.0	0.0	1.0	1.0
1NIC: 1CIN	0.4	0.5	0.3	0.9	0.8	0.8	0.8	0.8	0.9	1.0	0.0	0.3	0.0	0.5	0.2	0.4	0.3	0.8	0.7	0.6	0.4	0.9	0.9	0.9	0.7	0.6	0.0	0.0	1.0	1.0
1NIC: ½ CIN	0.4	0.5	0.4	1.0	0.8	0.8	0.8	0.7	1.0	1.0	0.0	0.4	0.0	0.5	0.3	0.5	0.2	0.9	0.7	0.7	0.5	0.9	1.1	0.9	0.8	0.8	0.0	0.0	1.0	1.0
1NIC: ¼ CIN	0.4	0.5	0.1	0.9	0.5	0.7	0.8	0.6	0.9	0.6	0.2	0.4	0.0	0.4	0.2	0.5	0.3	0.9	0.7	0.6	0.4	1.0	0.9	1.0	0.9	0.8	0.0	0.0	1.0	1.0
1NIC: 1/8 CIN	0.3	0.5	0.3	0.8	0.3	0.8	0.6	0.6	0.8	0.8	0.0	0.0	0.0	0.5	0.3	0.5	0.2	0.9	0.7	0.6	0.3	0.9	0.9	0.8	0.7	0.7	0.0	0.0	1.0	1.0

(**D**) Equal amounts of *Phytophthora cinnamomi, P. nicotianae* and *P. infestans* DNA hybridized concurrently in different concentrations of hybridization buffer (SSPE)

5x SSPE	0.4	0.5	0.5	0.9	0.8	0.8	0.7	0.7	0.9	1.0	0.0	0.3	0.0	0.6	0.3	0.9	0.8	0.8	0.8	1.0	0.9	0.9	0.9	0.8	0.8	0.7	0.0	0.0	1.0	1.0
2.5x SSPE	0.3	0.5	0.4	0.5	0.6	0.5	0.5	0.5	0.9	0.8	0.0	0.3	0.0	0.6	0.5	0.8	0.7	0.8	0.8	0.8	0.8	0.6	0.6	0.7	0.6	0.6	0.0	0.0	1.0	1.0
1x SSPE	0.6	0.5	0.0	0.5	0.6	0.6	0.4	0.4	0.8	1.1	0.0	0.5	0.0	0.5	0.4	0.4	0.5	0.5	0.5	0.4	0.6	0.4	0.6	0.5	0.3	0.5	0.0	0.0	0.9	1.1
0.5x SSPE	0.5	0.3	0.0	0.4	0.4	0.4	0.3	0.3	0.7	0.9	0.0	0.3	0.0	0.4	0.3	0.6	0.5	0.5	0.4	0.5	0.5	0.2	0.2	0.3	0.4	0.4	0.0	0.0	1.0	1.0

Intended Target of Probe	*P. cactorum*	*P. cinnamomi*	*P. cryptogea*	*P. infestans*	*P. nicotianae*	*P. ramorum*	*All*
SED = 0.03							

in the first column of Table 3A in which the hybridization of CAC2 clearly differentiates *P. cactorum* from the remaining species of *Phytophthora*.

Several probes that had hybridization signal intensities considerably lower than other target probes, such as RAM2 and RAM3, could be clearly differentiated by T_{60} evaluation. In some cases non-target probes formed duplexes with thermal stabilities that were marginally less than that of the target species. Of the eight species tested, *P. nicotianae* showed high levels of cross-hybridization with several probes targeting *P. infestans* and *vice versa*. Comparison of these duplexes showed single base pair sequence variation and high melting stabilities for the mismatched duplex. However, NIC1, NIC1B, NIC3 and NIC4B all clearly differentiate *P. nicotianae* from *P. infestans*. Due to high levels of cross-hybridization NIC4, INF4B and INF5 would all be excluded from future analysis. The ubiquitous probes targeting all of the *Phytophthora* species displayed consistently strong thermal stability across all of the species analysed.

Although some individual probes showed cross-hybridization to non-target species (ERY1, INF4, NIC4), when we take the average of the T_{60} values for the probes for each species the specificity becomes apparent (Table 3A). For each of the eight species tested the average T_{60} values are significantly higher than those to non-target species.

The relative sensitivity of detection was assessed by hybridizing 50 ng, 25 ng, 12.5 ng and 6.25 ng of single-stranded *P. cinnamomi* ITS amplicons to the microarray in 1 × SSPE hybridization buffer. This analysis was carried out to determine the effect of differing target concentration on detection using hybridization kinetics. Serial dilution of target DNA resulted in uniform reduction in T_{60} values with target species differentiation maintained down to 6.25 ng of target DNA (Table 3B). This was not the case when analysing the hybridization intensities of each probe at each temperature with variable concentrations of target DNA (data not shown).

Detection of artificially mixed populations of Phytophthora *species*

Amplification products of *P. cinnamomi* and *P. nicotianae* were hybridized concurrently on a single array in 1 × SSPE to analyse the detection of multiple *Phytophthora* species using the PamGene system. The consistency and relative sensitivity of two species detection was assessed by hybridizing 50 ng *P. nicotianae* with 50 ng, 25 ng, 12.5 ng and 6.25 ng *P. cinnamomi*. Two species mixtures of *P. nicotianae* and *P. cinnamomi* displayed consistent thermal stabilities for the probes targeting *P. nicotianae* and non-target *Phytophthora* species, which closely reflect the single species hybridization of *P. nicotianae* (Table 3A). Incrementally lower thermal stabilities were observed for the probes targeting *P. cinnamomi* (Table 3C). The reductions in T_{60} values of the CIN probes and those primarily hybridized by *P. cinnamomi* were closely correlated with the T_{60} values of *P. cinnamomi* dilutions (Table 3B).

The potential of using the system for multiplex detection was extended to three species using *P. cinnamomi*, *P. nicotianae* and *P. infestans* hybridized in various concentrations of hybridization buffer. Each of the three species of *Phytophthora* was analysed on a single array by hybridizing 50ng of each in 5 × SSPE, 2.5 × SSPE, 1 × SSPE and 0.5 × SSPE. In addition, this experiment contrasted the consistency of detecting multiple species using different hybridization buffers as increased non-target DNA may potentially influence the stringency of DNA hybridization. Mixtures of *P. cinnamomi*, *P. nicotianae* and *P. infestans* produced T_{60} values that were highest for each of the three target species probes (Table 3D). In each case, these probes produced lower T_{60} values in the three species mixture when hybridized in 1 × SSPE than in the individual species study (Table 3A). SSPE concentration had a significant influence on the T_{60} equilibrium with increased values for the CIN, INF and NIC probe duplexes in 5 × SSPE. This was not accompanied by an increase in non-target duplex stability as there was minimal increase in the T_{60} values of the CAC, CRY and RAM probes with increased SSPE concentration. This indicates that hybridization stringency was increased in the presence of larger amounts of non-target DNA.

A strong linear relationship ($p < 0.001$) was observed between the matched and mismatched T_{60} values of all eight species of *Phytophthora* and the duplex T_m predicted by the m-fold method (Fig. 2A). Mismatched duplexes displayed higher levels of variation than perfectly matched duplexes. In contrast, analysis of hybridization intensity of target duplexes at individual temperatures did not demonstrate any clear relationship with the predicted duplex stability (Fig. 2B). Hybridization intensity data accounted for an optimum of 50% of the variation across all target duplexes at any given temperature.

Discussion

This study has demonstrated that kinetic-based microarray analysis using the PamGene microarray system has good potential in the detection and differentiation of microbial species. Previous investigations into the design and hybridization characteristics of probes for detection arrays have sought to design probes that have uniform hybridization behaviour (Bodrossy *et al.* 2003). However, several studies have shown that probes often display hybridization characteristics contrary to their intended design (Schepinov *et al.* 1997). This has been attributed to indirect factors such as the density of probes, physical interference of the array surface, secondary structures of DNA, and the inability to accurately predict the hybridization kinetics of DNA bound to solid surfaces (Bodrossy 2003; Dai *et al.* 2002; Peterson *et al.* 2001; Schepinov *et al.* 1997). Detection analysis is then determined based on differences in the signal intensity associated with DNA hybridization and the use of mismatch controls to account for variations in hybridization characteristics. These problems have been overcome in the present study using the kinetic profile of target hybridization. Due to minimal sequence variation between some of the species of *Phytophthora* studied, it was not possible to design an ideal set of probes for each species. Analysis of the T_{60} melting kinetics enabled target and non-target hybridization to be differentiated, allowing the use of several probes that display consistently low hybridization intensities. Calculation of T_{60} data in mixed populations were observed to be compounded by cross-hybridization of non-target species at lower temperatures; artificially decreasing the T_{60} values in mixed targets. Mixed target hybridizations were also accompanied by a reduction in all hybridization intensities indicating

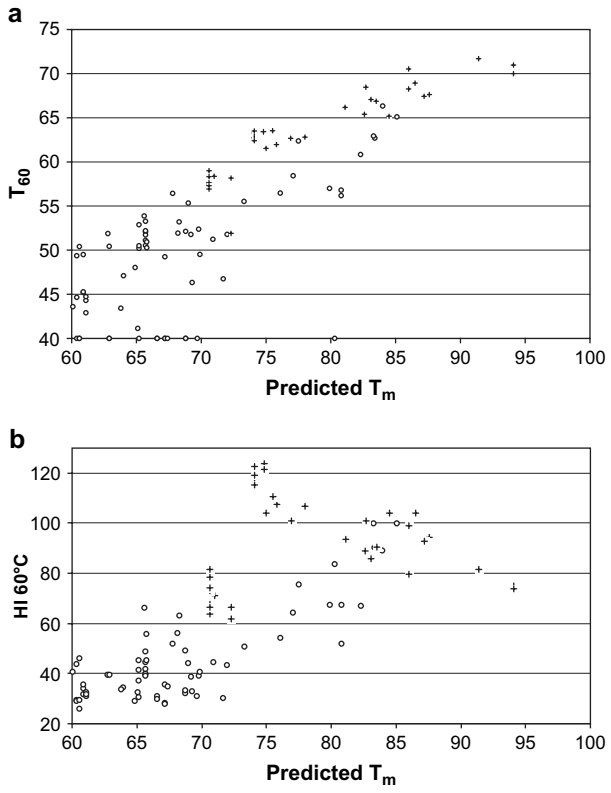

Fig 2 – (A) The relationship between the theoretical thermal stability of both perfectly matched and mismatched duplexes (R^2 = 0.58). This correlation was strongest for the perfectly matched duplexes (R^2 = 0.74), while mismatched duplexes were found to be considerably more variable (R^2 = 0.33). Target probe duplexes are indicated with (+) symbols, while non-target duplex interactions are indicated with a (○) symbol. (B) Correlation of the predicted thermal stability of target and non-target duplexes with the Hybridization intensity at 60 °C. Although there is a weak correlation between the predicted and observed value (R^2 = 0.47), there is no apparent relationship between the hybridization intensity of perfectly matched duplexes and their theoretical thermal stability. Target probe duplexes are indicated with (+) symbols, while non-target duplex interactions are indicated with a (○) symbol.

some level of competitive hybridization between target DNA and non-target probes. As a consequence it is likely that the uniformity of the analysis could be further improved by introducing non-labelled heterologous DNA during analysis to block non-reacted probes and improve hybridization stringency (Sambrook *et al.* 1989). Such intricacies were not addressed here as this study was established to demonstrate the feasibility of using hybridization kinetics to differentiate between pathogenic species. However, improved modelling of the melting kinetics of mixed populations is likely to further improve the interpretation of multiplex samples.

The ability to use all of the available probes is critical when designing detection arrays for closely related species as probe design is governed by the sequence variation available (Bodrossy 2003). The real-time kinetics of the PamGene microarray meant the majority of probes could be used to effectively identify their intended target species. On a conventional microarray system, parallel hybridizations would have needed to be carried out on separate arrays at nine different temperatures to gain the equivalent kinetic data that were generated during this analysis. Automated fluxing, reduced hybridization times, and temperature control of the PamGene system all attribute to the highly reproducible kinetic analysis performed on this system.

Comparison of the kinetic data generated on the PamGene microarray showed close correlation with established kinetic models predicting duplex kinetics (Zuker 2003). In contrast, the hybridization intensity of the duplexes did not always correlate closely to predictions of hybridization behaviour. The relationship between the predicted and observed T_{60} values may be improved with further modelling of the melting kinetics of probe–target duplexes on the PamGene system. In addition, fragmenting the target DNA and using hybridization facilitators may minimize the variation in secondary structure and steric inhibition of the target (Small *et al.* 2001). Establishment of species identification based on the kinetic nature of probe–target duplexes could be used to predict the behaviour of each probe with various target and non-target sequences before running microarray experiments. Furthermore, the focus of probe design changes to selection of those probes with target duplex stabilities readily distinguishable from non-target duplexes rather than those that produce high hybridization intensities.

In developing a microarray system for multiplex detection of *Phytophthora* species, it is critical that variable proportions of different targets can be identified within a single assay. Hybridization intensities were observed to vary greatly with changes in the amount of target DNA, which implies that analysis of hybridization intensities requires uniform concentrations of each DNA target be present. This may not be practical for multiplex detection from environmental samples due to variations in the amount initial target material for each target species. The comparative consistency of T_{60} analysis with variable amounts of target DNA means that hybridization kinetics display greater potential for multiplex species detection than conventional microarray analysis.

This study was established as a ''proof of concept'' investigation into the use of probe-target melting kinetics as a determinant of species identification. As such, we have demonstrated that the reduced hybridization times and real-time kinetics of the PamGene microarray system is of great benefit in differentiating between microbial species. The kinetic output is critical in differentiating between closely related species and distinguishing target and non-target probe duplexes. As each probe–duplex interaction displays different thermal stability, melting kinetics were found to be more systematic in assessing hybridization specificity than hybridization intensity.

The practical application of this technology will require further modelling of probe–duplex interactions across an expanded range of closely related and non-related species representative of populations likely to be encountered in real-life detection samples. This series of experiments has verified the potential of hybridization kinetic analysis for use in the detection of multiple *Phytophthora* species and could be

applied to plant pathology, bio-security and population diversity studies.

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REFERENCES

Anthony R, Schuitema A, Chan A, Boender P, Klaster P, Oskam L, 2003. Effect of secondary structure on single nucleotide polymorphism detection with a porous microarray matrix; implications for probe selection. *Biotechniques* **34**: 1082–1089.

Beuningen RV, Damme HV, Boender P, Bastiaensen N, Chan A, Kievits T, 2001. Fast and specific hybridization using flow-through microarrays on porous metal oxide. *Clinical Chemistry* **47**: 1931–1933.

Bodrossy L, 2003. Diagnostic oligonucleotide microarrays for microbiology. In: Balock E (ed), *A Beginners Guide to Microarrays*. Kluwer Academic Publishers, New York, pp. 43–92.

Bodrossy L, Stralis-Pavese N, Murrell J, Radajewski S, Wellharter A, Sessitsch A, 2003. Development and validation of a diagnostic microbial microarray for methanotrophs. *Environmental Microbiology* **5**: 566–582.

Coelho A, Cravador A, Bollen A, Ferraz J, Moreira A, Fauconnier A, 1997. Highly specific and sensitive non-radioactive molecular identification of *Phytophthora cinnamomi*. *Mycological Research* **101**: 1499–1507.

Dai H, Myer M, Stepaniants S, Ziman M, Stoughton R, 2002. Use of hybridization kinetics for differentiating specific from non-specific binding to oligonucleotide microarrays. *Nucleic Acid Research* **30**: e86.

Dobrowolski M, O'Brien P, 1993. Use of RAPD-PCR to isolate a species speciic DNA probe for *Phytophthora cinamomi*. *FEMS Microbiology Letters* **113**: 43–48.

Eden M, Hill R, Galpoththage M, 2000. An efficient baiting assay for quantification of *Phytophthora cinnamomi* in soil. *Plant Pathology* **49**: 515–522.

Jung, T, Blaschke, H. & Oswald, W., (2004) Involvement of *Phytophthora* species in the complex decline of oaks in central Europe. Abstract in IUFRO meeting. In *Phytophthora* in Forests in Natural Ecosystems. Freising, Germany, www.phytophthora-freising-2004.de

Kong P, Hong C, Richardson P, 2003. Rapid Detection of *Phytophthora cinnamomi using* PCR with primers from the Lpv putative storage protein genes. *Plant Pathology* **52**: 681–693.

Loy A, Lehner A, Lee N, Adamczyk J, Meier H, Ernst J, Schleifer K, Wagner M, 2002. Oligonucleotide arrays for 16 S rRNA gene-based detection of all recognized lineages of sulfate-reducing Prokaryotes in the environment. *Applied and Environmental Microbiology* **68**: 5064–5081.

McCarren K, McComb J, Hardy G, Shearer B, 2005. The role of chlamydospores in the survival of *P. cinnamomi*. *Australasian Plant Pathology* **34**: 333–338.

Peterson A, Heaton R, Georgiadis R, 2001. The effect of surface probe density on DNA hybridization. *Nucleic Acid Research* **29**: 5163–5168.

Sambrook S, Fritsch E, Maniatis T, 1989. *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Springs Harbour Laboratory Press, New York.

Schepinov M, Case-Green S, Southern E, 1997. Steric factors influencing hybridization of nucleic acids to oligonucleotide arrays. *Nucleic Acid Research* **25**: 1155–1161.

Shearer B, Michaelsen B, Somerford P, 1988. Effects of isolate and time of inoculation on invasion of the secondary phloem of *Eucalyptus* spp. and *Banksia grandis* by *Phytophthora* spp. *Plant Disease* **72**: 121–126.

Shearer B, Michaelsen B, Warren H, 1987. Comparative behaviour of *Phytophthora* species in the secondary phloem of stems and excised roots of *Banksia grandis* and *Eucalyptus marginata*. *Australian Journal of Botany* **35**: 103–110.

Small J, Call D, Brockmn F, Straub T, Chandler D, 2001. Direct detection of 16 S rRNA in soil extracts by using oligonucleotide microarrays. *Applied and Environmental Microbiology* **67**: 4708–4716.

Zuker M, 2003. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acid Research* **31**: 3406–3415.