A loop-mediated isothermal amplification (LAMP) assay for rapid and specific detection of airborne inoculum of *Uromyces betae* (sugar beet rust)

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Abstract

Sugar beet rust disease (causal agent *Uromyces betae*) represents a serious threat to worldwide sugar beet (*Beta vulgaris*) crops, causing yield losses of up to 10% in the UK. Currently, the disease is managed mainly by application of fungicides after rust disease symptoms appear. Development of a future forecasting system, incorporating data on environmental factors and *U. betae* inoculum levels, would enable better disease control by more targeted application of fungicides. In this study, we develop a first molecular diagnostic, targeted to cytochrome b DNA sequences and based on loop-mediated isothermal amplification (LAMP) technology, for rapid (<30 mins) and specific detection of *U. betae*. The new assay only detected *U. betae* strains (collected from across eastern England, the main sugar beet growing region in the UK); it did not detect other closely related pathogens (e.g. *Puccinia* sp., *U. fabae*) or others that are commonly found on sugar beet (*Cercospora beticola*, *Erysiphae betae*, *Ramularia beticola*). The assay could consistently detect down to small amounts of *U. betae* DNA (10 pg). Application of the new LAMP diagnostic to air spore tape samples collected between mid June – mid September from a single UK sugar beet field site revealed differences in temporal patterns of pathogen inoculum between the 2015 and 2016 seasons. The described LAMP assay could now be used as a component of a future automated inoculum-based forecasting system, enabling more targeted control of sugar beet rust disease.

Keywords: airborne inoculum, crop protection, diagnostics, disease forecasting, pathogen detection

Introduction

Sugar beet rust is a disease of sugar beet (*Beta vulgaris*) crops caused by the biotrophic basidiomycete fungus *Uromyces betae*. Typical foliar disease symptoms include circular orange-brown pustules that can occur on both upper and lower leaf surfaces and are typically around 1-2 mm diameter (Draycott 2008). Severe outbreaks of sugar beet rust can cause reduced green leaf area available for photosynthesis, and reported yield reductions can be ~1% root sugar content and ~15% of total root weight (EPPO 1994). The disease was first described in Canada in 1935 (Newton and Peturson 1943), and subsequently in Europe in 1988 (O'Sullivan 1997). In the UK, mild weather conditions throughout the year (i.e. cooler summers, warmer winters), are conducive for disease development (Draycott 2008). Indeed, in 2015, sugar beet rust was the most important disease of sugar beet crops in Britain (Mark Stevens, BBRO, unpublished data).

Effective control of sugar beet rust is achieved by integrated use of resistant cultivars, cultural practices (e.g. crop rotation) and mainly via use of fungicides including those with different modes of action (e.g. Escolta [cyproconazole + trifloxystrobin]). Previous studies have demonstrated that chemical control can increase sugar beet crop yields and improve quality (Soerensen and Marcussen 1996; O'Sullivan 1997). At present, decisions on fungicide use are based largely on visual rust disease symptoms, and are applied when the first symptoms are spotted (EPPO 1994; Draycott, 2008).

Molecular diagnostics already exist and enable rapid, sensitive and specific detection for a number of different phytopathogens, and when used alongside air spore trap samplers can be used to detect airborne pathogen inoculum (Williams et al., 2001). However, at present no molecular diagnostic is yet available that specifically targets *U. betae* (partly due to the biotrophic and hence unculturable *in vitro* nature of the fungus), with fungal species confirmation thus requiring relatively time consuming and expensive morphometric analyses and / or DNA sequencing. Development of a first molecular diagnostic for direct detection of *U. betae* could potentially be used for early detection of airborne inoculum (splash-dispersed urediospores that while airborne are further spread by wind) and could also allow detection even when disease is asymptomatic.

First described by Notomi et al. (2000) loop-mediated isothermal amplification (LAMP) assays have been developed for fast, sensitive and specific detection of a diverse range of plant pathogen species (Tomlinson et al., 2010, 2013; Bekele et al., 2011; Duan et al., 2014; Kogovšek et al., 2015). LAMP requires at least four specific primers; two outer primers (by convention FIP, BIP) and two inner primers (F3, B3) in combination with DNA polymerase enzyme with strand displacement activity. Furthermore, incorporation of additional loop primers can greatly increase the speed and sensitivity of the assay (Nagamine, 2002). LAMP technology also has several other potentially advantageous features including the requirement for only a single reaction temperature, quantification of pathogen DNA / inoculum (Aoi et al., 2006), and potential lyophilisation of reaction reagents.

Development of a first *U. betae* LAMP assay, capable of early detection of airborne pathogen inoculum, could potentially be incorporated as a component of a future inoculum-based forecasting system. Such a tool could be used to guide disease

management decisions by end user growers. Therefore, the specific aims of this study are: (1) develop and validate a rapid and specific LAMP assay for *U. betae*, and (2) demonstrate its applied use for inoculum detection in air environmental samples.

Materials and methods

Spore material and DNA extraction

Field strains of *U. betae* were collected from symptomatic leaves from five different locations throughout England representing the main sugar beet growing regions in the UK: Essex, North Lincolnshire, Nottingham, South Lincolnshire and Suffolk) (from commercial sugar beet field trials involved in the SporeID Technology Strategy Board project led by BBRO). *Uromyces betae* pustules were then scraped from the leaf surface with a sterile needle under a stereomicroscope (Olympus BH-2). Additionally, some leaves were air-dried and spores tapped out from the leaves and stored at -20°C until needed. Genomic DNA was subsequently extracted from frozen *U. betae* spores using a Qiagen DNeasy Plant Mini Kit (Qiagen, UK) and quantified using a nanodrop photospectrometer. Samples were diluted to the required concentration using PCR grade water.

Sequencing and LAMP assay primer design

Initial attempts to design a *U. betae*-specific LAMP assay based on internal transcribed spacer (ITS) region sequences were unsuccessful due to insufficient

variation between the target and other closely related species (data not shown). Thus the alternative cytochrome b gene region was instead selected, due to both its high gene copy number, and because variation in this gene had previously proved useful for development of molecular diagnostics for specific detection and phylogeny of pathogenic fungi (Wang et al., 1998; Biswas et al. 2001, Grasso et al., 2006).

Given that no *U. betae* cytochrome b sequences were yet available on GenBank, new primers were designed (PuccytB2F/R; Table 1) using PRIMER BLAST (Jian et al., 2012) to target an approximately 584 bp region of the gene in this species (these conserved primers were designed based on aligned sequences of closely related *Puccinia* and *Uromyces* species; DQ209276, AF511082, AF426199). For the development of LAMP assays, cytochrome b sequence data were obtained for five representative *U. betae* field strains obtained from the main sugar beet growing regions of the UK (Leicestershire, South Lincolnshire, Suffolk, Oxford, and Nottinghamshire) (Table 2). Twenty-five microliters PCR reactions contained 12.5 µl Mango Mastermix (1 x final concentrate; Bioline), 9.5 µl PCR grade water (HyClone), 1 µl of each primer PuccytB2F and R (with a final concentration of 0.4µM of each of them), and 1 µl crude DNA templates (between 20 and 30 ng) PCR reaction conditions were: 94°C for 2 min; followed by 30 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 2 min; and finally 72°C for 10 min. PCR products were visualised under UV light on an agarose gel incorporated with ethidium bromide. Amplicons were purified using a Qiagen MinElute PCR purification kit (Qiagen, UK), and sent for sequencing (using primer PuccytB2F) to MWG Eurofins Genomics. Newly obtained cytochrome b sequences were identical for all five of the *U. betae* strains (representative sequence deposited at GenBank under accession MF426925) and were subsequently aligned with closely related *Puccinia* and *Uromyces* species

(GenBank Acc. Nos. DQ209276, AF511082, AF426199) (van der Merwe, et al. 2007). Finally, LAMP primers targeted specifically to *U. betae* were designed using LAMP Designer Software (OptiGene Ltd, UK) (Table 1).

Validation of the *U. betae* LAMP assay

The analytical specificity of the newly designed LAMP assay to cytochrome b DNA sequence of *U. betae* was assessed by screening against a panel of other fungal species that were either closely related or often found on sugar beet plants (Table 2). LAMP reactions were done in 24 µl volumes, each containing: 12. 8 µl isothermal mastermix (ISO001, OptiGene Ltd, UK), 2.5 µl primer mix (at final reaction concentrations of FIP/BIP: 1.3 µM, LoopB/F: 0.42 µM, and B3/F3: 0.16 µM), 4.7 µl PCR grade water, and 4 µl DNA template (1 ng total). LAMP testing was done using a Stratagene Mx3000 Pro real-time PCR machine, with the optimal isothermal temperature being 61°C for 30 mins (FAM fluorescence measured every 30 secs); this was followed by melting curve analysis at 95°C for 1 min, 55°C for 30 secs and 95°C for 30 secs. Data were analysed on the log scale with the cycle threshold (Ct) adjusted manually to the exponential phase of the amplification curves. Each sample was run with three technical replicates, and considered positive only if all three gave a Ct value of <60 (i.e. within 30 mins).

The analytical sensitivity of the developed assay was initially evaluated by screening against differing amounts of total *U. betae* genomic DNA: 10 ng, 1 ng, 100 pg, 10 pg and 1 pg. Subsequently, sensitivity was investigated by using the known number of spores measured by a haemocytometer suspended in sterile distilled water.. The

spore suspension was applied as 10 µl droplets (containing known total amounts of spores as follows 10000,1000, 100, 10, 1 spores) and air dried in a sterile laminar flow hood for 1 hour onto Melinex tape segments DNA was extracted using a MASTERPURE Yeast DNA Purification kit (Epicentre, USA) according to the manufacturer's instructions.

Each LAMP reaction contained the DNA dilution equivalent to: 100, 10, 1, 0.1 or 0.01 spores. Three DNA extract replicates from each of each of the different amounts of *U. betae* spores were tested, with three technical replicates for each sample tested with LAMP. Reaction components and conditions were as above.

Application of the *U. betae* LAMP assay to air spore tape samples

Burkard spore traps were positioned within a sugar beet field located in Suffolk (52°19'17.1"N, 0°48'36.7"E) in the 2015 and 2016 field seasons. The crop was drilled in mid March in both seasons. Spore traps operated with a 2 mm air inlet, powered from 12V batteries recharged by a solar panel and collected spores between mid June and mid September. The air flow was set to 10 L/min (14 m³ per day). Adhesive used to collect air particles on transparent Melinex tape was as follows: 23% petroleum jelly (Vaseline), 14% paraffin wax and 3% phenol, which was applied as a suspension in hexane (adapted from Lacey and West 2006). Tapes were replaced weekly, and exposed drums sent to the RRes laboratory for testing. Tapes were cut into two equal replicate sections covering each 24 hour sampling period and stored at -20°C until processed. DNA was extracted from one of these replicate tape samples using a Master Pure Yeast DNA extraction kit (into a final volume of 100 µl). LAMP assay testing was done as previously described. Each run included standards

of known amounts of *U. betae* DNA (10 ng, 1 ng, 100 pg, 10 pg and 1 pg), and the equation from the resulting standard curve was used to estimate amounts of pathogen DNA in each sample. Additionally, to test the possibility of inhibition in environmental samples, selected negative samples were spiked with 1 ng of pure *U. betae* DNA.

Results

LAMP assay validation

The new LAMP assay targeted to *U. betae* cytochrome b DNA sequence amplified specifically this target species, and could detect pathogen strains from main sugar beet growing regions from across England and two sites in Denmark. These positive samples all yielded a single dissociation curve of 84-85°C (Fig. 1). By contrast, the assay did not detect several closely related fungi, nor a broad range of other commonly occurring sugar beet pathogens (Table 2). Sensitivity screening indicated, the *U. betae* LAMP assay could consistently detect down to 10 pg of pure pathogen DNA (Fig. 1a, b); although a signal was also sometimes given with as little as 1 pg DNA this result was obtained only approximately 50% of the time and was hence not consistently repeatable (data not shown).

When Ct values obtained from testing varying amounts of pure *U. betae* genomic DNA were plotted against those from known numbers of spores (from which DNA had been extracted), a positive linear correlation was observed.

LAMP assay applied to air spore tape samples

The LAMP assay was successfully applied to air spore tape samples collected over two alternate growing seasons (2015/16) by the spore trap placed near the untreated plots at the fungicide trial in Suffolk. The differences in amounts of airborne pathogen inoculum were observed between both years. As indicated in 2015 the first arrival of *U. betae* spores was detected later in the season (early July) than in 2016 (mid June) (Fig. 3). Overall 2015 was a year with higher presence of inoculum in the air with the peak on a day in mid August. In contrast, during sampling in 2016, the amount of pathogen's DNA detected from early August until the end of sampling in mid September remained sporadic and low.

Discussion

This study describes the first molecular diagnostic, based on cytochrome b DNA sequences, for rapid, specific and direct detection of *U. betae,* the cause of sugar beet rust and a major threat to sugar beet crops in the UK. Prior to this, identification of *U. betae* was based on microscopic and morphological analyses or required further sequencing (Mahlein et al. 2010; 2012). The developed LAMP assay detected only *U. betae* strains, and not those from other closely related *Uromyces* or *Puccinia* species, nor other commonly occurring sugar beet pathogens (e.g. *C. beticola, E. betae* and *R. beticola*). Although LAMP assays have previously been described for several economically important phytopathogens e.g. *Botrytis cinerea* (grey mold) and *Guignardia citricarpa* (citrus black spot) (Tomlinson et al. 2013;

Duan et al. 2014), this study describes the first such assay for a sugar beet pathogen.

Use of the LAMP assay enabled fast detection of the pathogen within <30 mins, and more often <20 mins. Sensitivity testing revealed a robust signal down to 10 pg *U. betae* pathogen DNA, with spurious detection down to 1 pg which was therefore excluded from subsequent analyses. A clear linear relationship (R^2=0.964) (Fig. 2a) was observed in screening of known amounts of pathogen DNA (10 ng, 1 ng, 100 pg, 10 pg) and Ct (crossing threshold) value. A similar result was found in testing against DNA extracted from known numbers of *U. betae* spores that had been deposited onto pieces of waxed Melinex tape, with a positive linear correlation (R^2 = 0.94) (Fig. 2b). Previous studies with DNA extracted directly from Melinex tape samples has been used previously to quantify amounts of DNA of fungal pathogens in air samples and showed to be efficient enough despite possible presence and consequently qPCR/LAMP inhibition, of dust or other microorganisms. (Rittenour et al. 2012, Wieczorek et al. 2014).

However, it should be noted that similar Ct values were obtained from DNA extracted from $\leq$1 spore, indicating that below this threshold very small amounts of pathogen DNA cannot be reliably quantified using the assay. Furthermore, it is possible that gene copy number may vary between different *U. betae* strains and thus the results might therefore be best considered semi-quantitative (i.e. high versus low) at the present time. Nevertheless, similar Ct values were obtained using the new LAMP assay from DNA extracted from known amounts of spores from three different *U. betae* field strains, and thus this does not appear to be the case (data not shown).

Testing of the new LAMP assay against DNA extracted from known number of *U. betae* spores revealed a positive linear correlation (R^2 = 0.94), indicating that the

assay could be used to estimate amounts of pathogen inoculum in environmental samples by using the established model. Subsequently, the assay was successfully applied to air spore tape samples collected from a single sugar beet site in Suffolk over two consecutive growing seasons. Pathogen inoculum levels, as determined by LAMP, increased markedly with numerous spike periods between mid August to mid September in the 2015 but not 2016 season. Interestingly, field disease severity data (percentage of leaf cover with visible sugar beet rust symptoms) from both years in August / September were low (≤3%), but were considerably higher in October 2015 (25%) than October 2016 (2%) (Mark Stevens, BBRO, unpublished data). These observations suggest that early detection of airborne *U. betae* inoculum, as opposed to simply visual inspection of sugar beet crops alone when the pathogen may be growing asymptomatically might, subject to more extensive testing and validation, allow potential disease severity later in the growing season to be predicted.

LAMP offers several potential advantages over conventional PCR that could allow field-based use. These include the requirement of only a single reaction temperature that can be combined with lyophilized reagents. Moreover, given the speed of LAMP (<30 mins) this opens up the prospect of real-time monitoring of pathogen inoculum. The LAMP assay described here for *U. betae* could be used as a component of a sugar beet rust disease forecasting system that incorporates data on pathogen inoculum present in air samples. Such information could be combined in models with additional agronomic (e.g. cultivar resistance ratings, fungicide usage) and weather (e.g. temperature, humidity) data that are likely to influence progression of the disease (Draycott, 2008; http://bbro.co.uk). This could form the basis of a future disease forecasting system, guiding end users to ensure more precise and integrated strategies for better control of sugar beet rust.

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Figure and table legends

Figure 1 Representative results obtained using the new *Uromyces betae* LAMP assay developed in this study. (A) Amplification (on log scale) of a dilution series of *U. betae* DNA (10 ng – 0.01 ng) and a no template control (X axis denotes time in minutes). (B) The corresponding dissociation plots for the four positive samples (peaks at 84-85°C), and the single no template control (NTC; no clear peak evident). Fluorescence values (*R*) are in arbitrary units.

Figure 2 Screening of the new *Uromyces betae* LAMP assay against different amounts of serially diluted amounts of pathogen DNA or (B) known dilutions of spores. Results are shown on the log scale. Ct = crossing threshold. Representative results shown are the mean of three technical replicates.

Figure 3 Use of the new *Uromyces betae* LAMP assay to estimate the amount of DNA per day in air samples collected form a single sugar beet field site in alternate 2015 and 2016 seasons.

Table 1 List of the primers targeted to cytochrome b DNA sequences used in this study.

Table 2 Fungal strains screened against the developed *Uromyces betae* LAMP assay.

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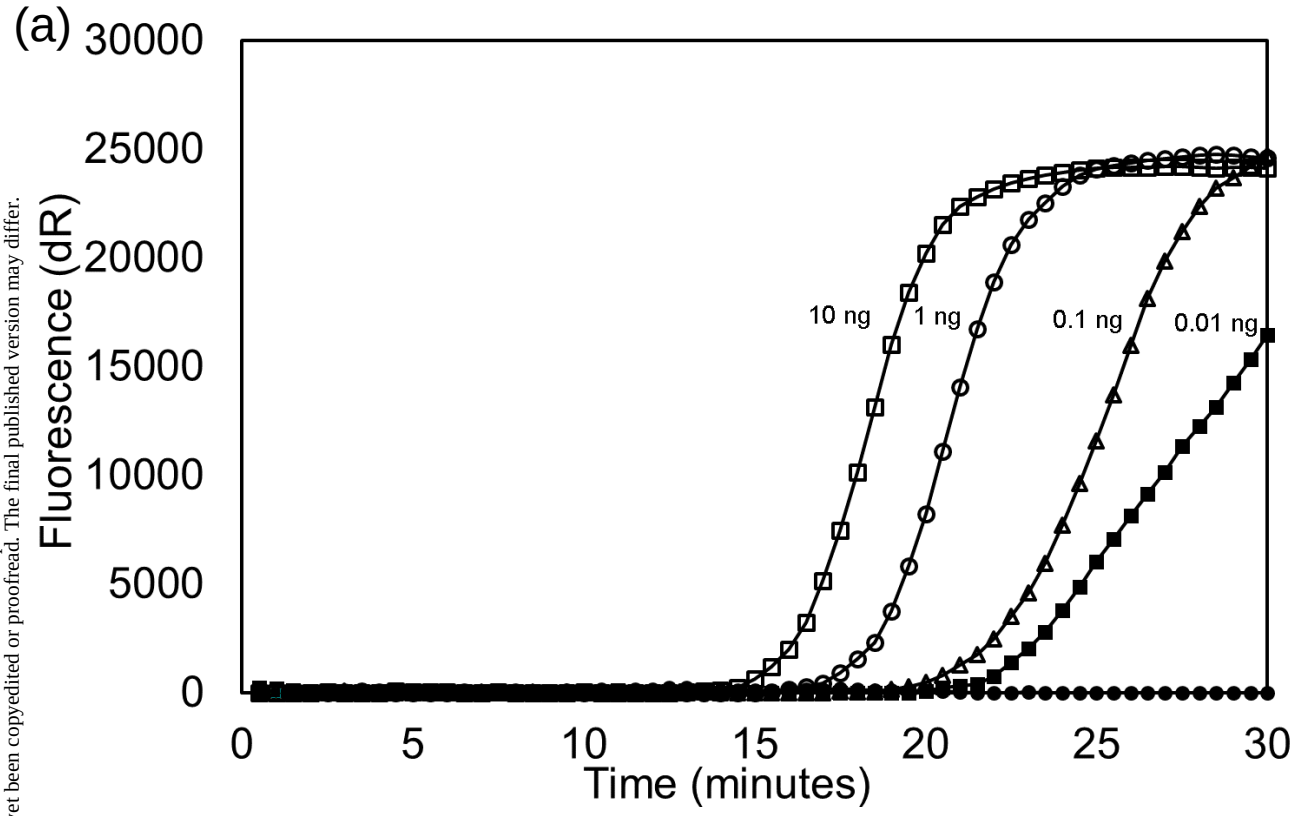
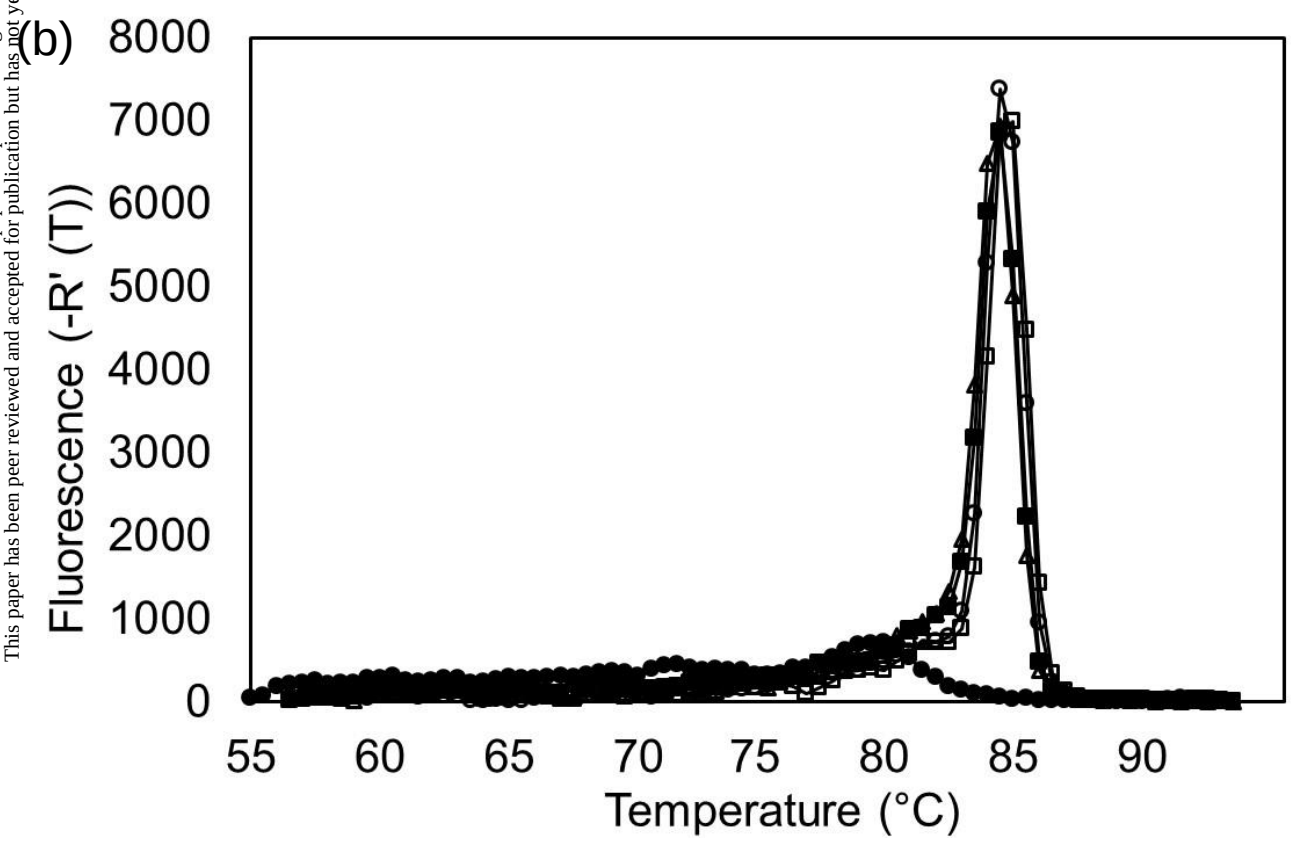
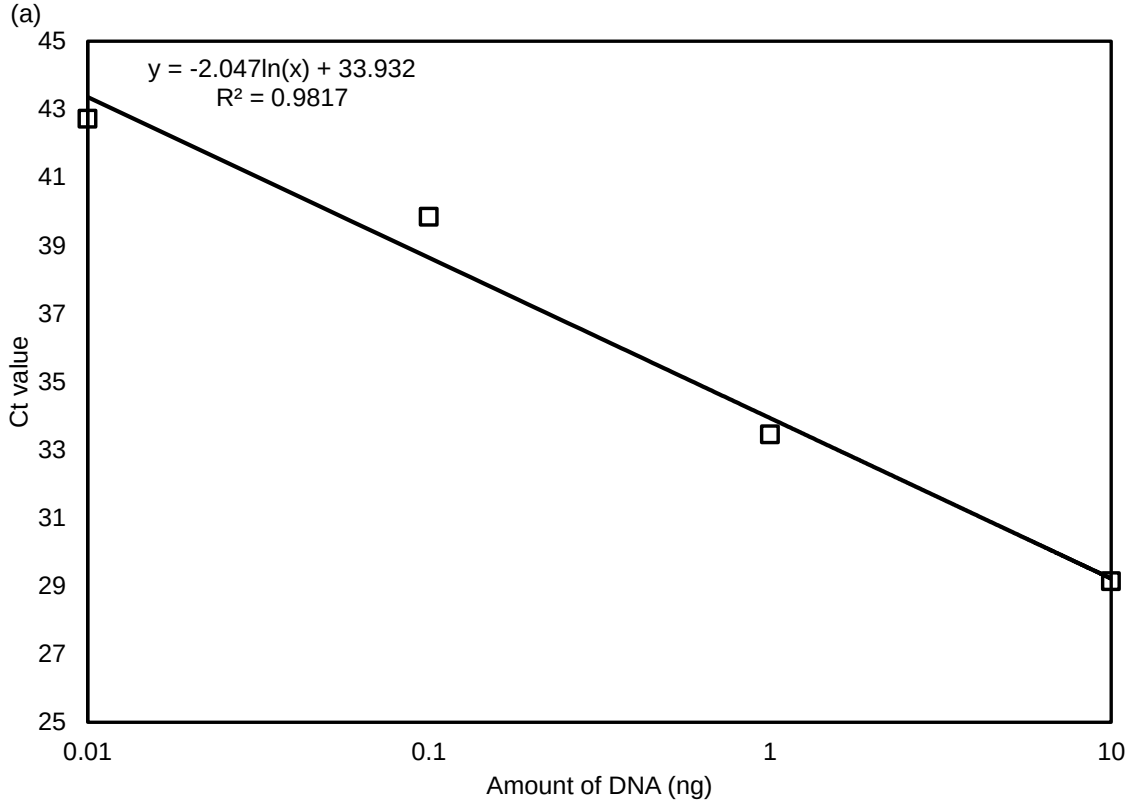




(a)



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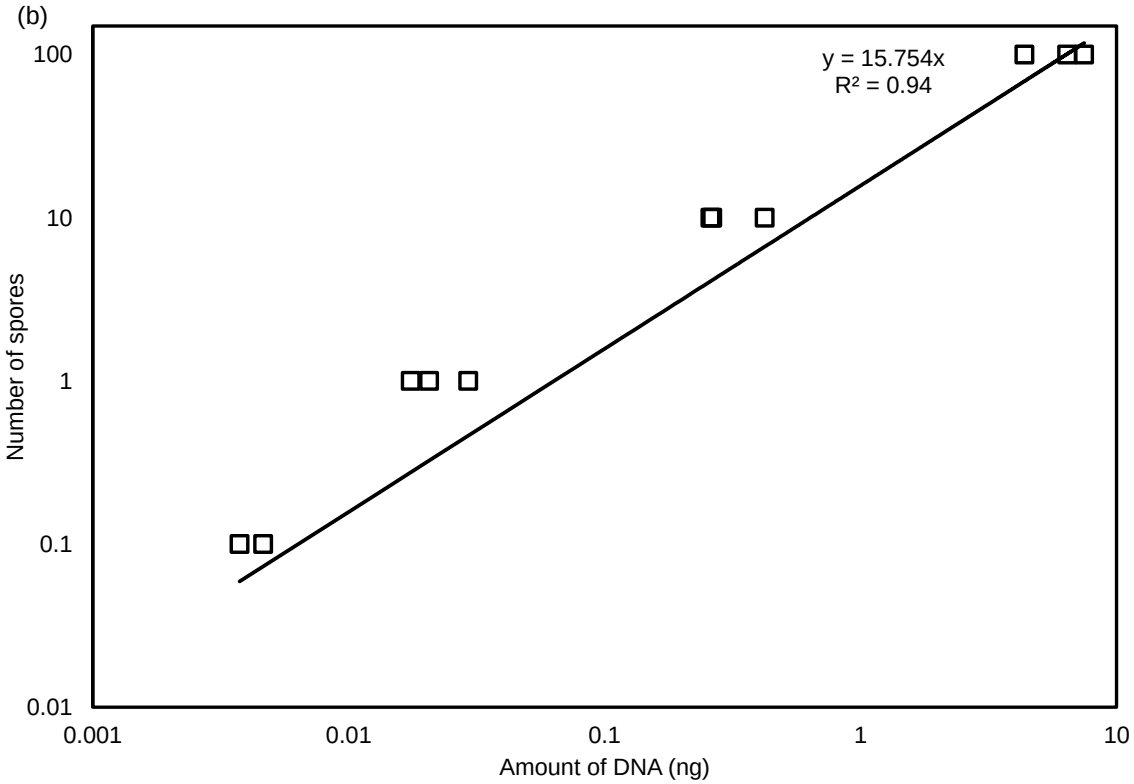

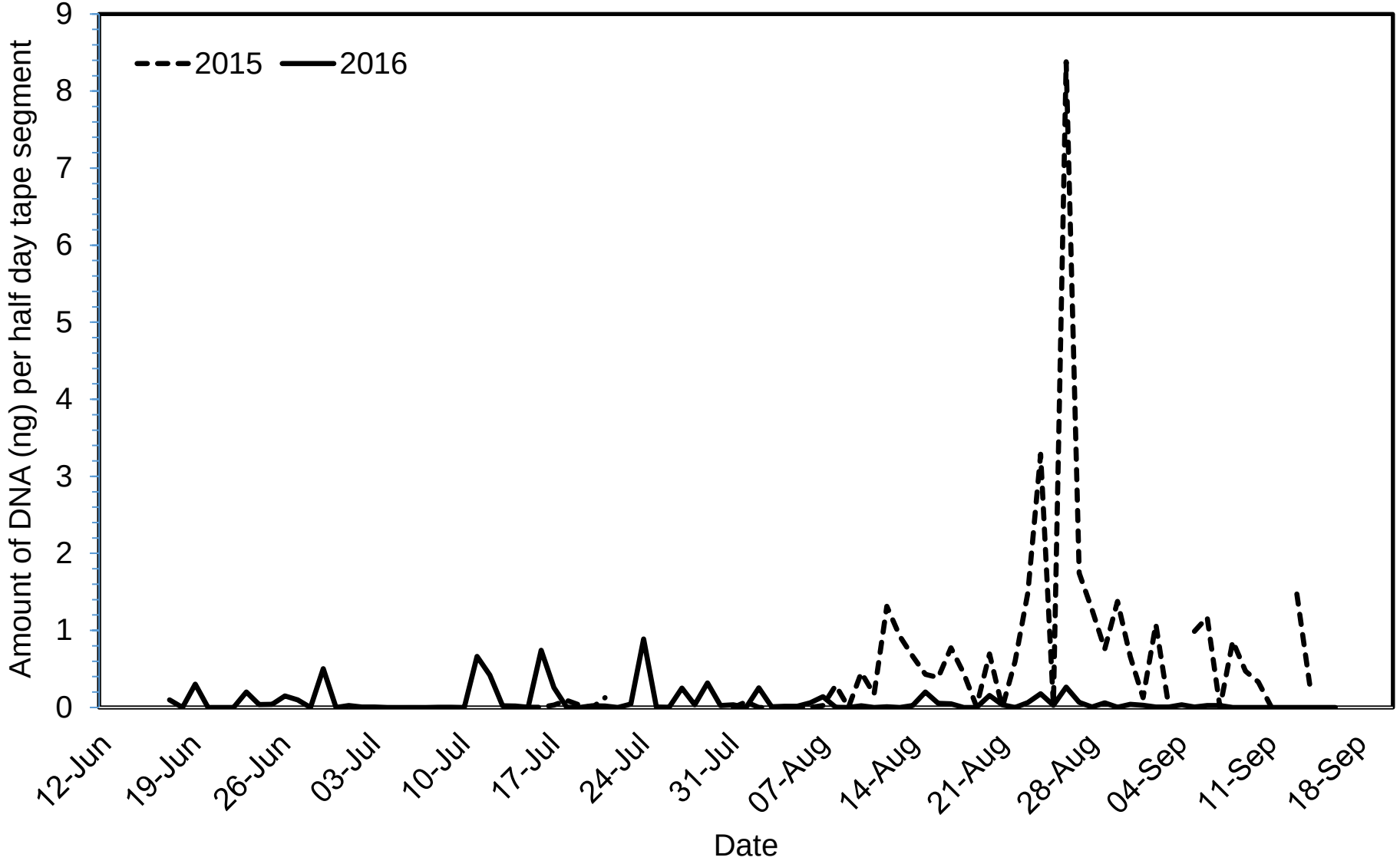

Table 1 List of the primers targeted to cytochrome b DNA sequences used in this study.

Technique / primer name	**Sequence**
Conventional PCR:	
PuccytBp2F	TCTGAGTAATAGGTGACGGT
PuccytBp2R	AAAGGAATGTGAGTTAGCGT
Loop-mediated isothermal amplification (LAMP):	
RCytBF3	GTACACGTCTAACTCAAGTTCT
RCytBB3	TCATCAGTGTCGCCCTTA
RCytBFIP (F1+F2)	GCAAGATACCATATGCGGTCGTTGTGAAGTC AAGTGTACAATCA
RCytBBIP (B1+B2)	GGGAGAGTAGCACAGTTAAATGCCGCTTCTA TACATTAGGCTAT
RCytBLoopF	GTAGATCGAACATCGATTCAGC
RCytBLoopB	AGTCCTTATAGTCCGAACAGAT

Table 2 Fungal strains screened against the developed *Uromyces betae* LAMP assay

Fungal species (1ng DNA per reaction)	**Origin (all UK unless specified)**	**LAMP result**
Puccinia triticina (wheat leaf rust – both yellow and brown)	University of Nottingham (UoN) Rothamsted Research Institute (RRes)	-
U. betae (sugar beet rust)	UoN, RRes Leicestershire (Sutton Bonington), South Lincolnshire (Barkston), Suffolk (Garboldisham), Oxfordshire (Oxford) Nottingham (Bracebridge) Aarhus University and NBR (Denmark) Flakkebjerg Glasshouse isolates	 + + + + + + +
Erysiphe betae (sugar beet powdery mildew)	UoN	-
Botrytis cinerea (Botrytis storage rot)	RRes	-
Erysiphe sp (Tomato mildew)	RRes	-
Uromyces viciae-fabae (Bean rust)	RRes	-
Alternaria alternate (leaf spot)	RRes	-
Cercospora beticola (Cercospora leaf spot of sugar beet)	UoN	-
Ramularia beticola (Ramularia of sugar beet)	UoN	-