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Rapid nested PCR-based detection of *Ramularia collo-cygni* direct from barley

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Received 21 October 2005; revised 28 November 2005; accepted 16 December 2005. First published online 2 February 2006.

doi:10.1111/j.1574-6968.2006.00121.x

Editor: Geoffrey Gadd

Keywords
Ramularia collo-cygni; nested-PCR; ITS rRNA; detection; control; barley leaf spotting.

Abstract

Ramularia collo-cygni is a barley pathogen of increasing importance in Northern and Central Europe, New Zealand and South America. Accurate visual and microscopic identification of the pathogen from diseased tissue is difficult. A nested PCR-based diagnostic test has been developed as part of an initiative to map the distribution of the pathogen in Scotland. The entire nuclear ribosomal internal transcribed spacer and 5.8S rRNA gene regions from 14 isolates of diverse global origin exhibited complete homology following sequence characterization. Two pairs of species-specific primers, based on inter-specific sequence divergence with closely related species, were designed and empirically evaluated for diagnostic nested PCR. Nested primers Rcc3 and Rcc4 consistently amplified a single product of 256 bp from DNA of 24 *R. collo-cygni* isolates of diverse global provenance, but not from other *Ramularia* species, or other fungi commonly encountered in cereal pathosystems, as well as *Hordeum* or *Secale* DNA preparations. Using this approach, *R. collo-cygni* was successfully identified from naturally infected barley leaf, awn and grain samples of diverse geographical provenance, in particular from symptoms that lacked the presence of characteristic conidiophores. It is envisaged that this assay will become established as an important tool in continuing studies into the ecology, aetiology and epidemiology of this poorly understood yet economically damaging plant pathogen.

Introduction

In 1998, the spring barley (*Hordeum vulgare*) crop in Scotland suffered heavily from an unusual leaf spotting resulting in a detrimental effect on yield due to the premature loss of green leaf area (Oxley *et al.*, 2002). Examination of the spotting complex indicated a combination of biotic and abiotic factors were involved (Oxley *et al.*, 2002). The fungal pathogen *Ramularia collo-cygni* Sutton and Waller, was identified as a principle biotic component of the complex. This pathogen has previously been shown to cause yield losses of up to 20% in winter barley in Austria (Huss *et al.*, 1992) but, until recently, has been considered of only minor importance in the UK (Sutton & Waller, 1988). More recently *R. collo-cygni* has been identified in a number of countries and is known to be increasing in importance (Sachs *et al.*, 1998; Sheridan, 2000; Pinnschmidt & Hovmøller, 2003). A major impediment to the study of this pathogen, and its early recognition as a component in barley losses due to leaf spotting, has been its early, effective and accurate diagnosis. Conventional identification techniques rely on the microscopic identification of conidiophores on the leaf surface. However, conidiogenesis represents a relatively late stage in the infection process (Sutton & Waller, 1988). In addition, the slow growing nature of the fungus, even in the presence of a putative semi-selective media (Sachs, 2004), results in low frequencies of isolation (Frei & Gindrat, 2000), further frustrating research efforts.

In order to gain a better understanding and accuracy of the incidence and distribution of this pathogen from across the barley growing regions of Scotland, as well as its early detection, a reliable diagnostic assay is required. The internal transcribed spacer (ITS) regions of ribosomal RNA (rRNA) have been shown to be a suitable target for molecular detection assay development in fungi (Goodwin *et al.*, 1995; Langrell, 2002). The Nested PCR approach has been successfully utilised in the identification and detection of other plant pathogens, especially from recalcitrant vegetative tissues

(Hamelin *et al*., 2000; Langrell, 2005). Furthermore, the ready accessibility of these regions using universal primers (White *et al*., 1990) makes them particularly attractive for sequence characterisation and the eventual design of species-specific primers. Consequently, this study describes the development of a species-specific and sensitive nested PCR based method for the rapid detection of *R. collo-cygni* direct from barley as a tool to help elucidate its epidemiology.

Materials and methods

A collection of 24 authenticated *Ramularia collo-cygni* isolates of wide geographic and host origin was established, including a collection of other common fungal pathogens and late season pathogens of barley (Table 1). Fungal mycelia for DNA extraction was produced by cultivating colonies on cellophane membranes (BioRad Laboratories,

Table 1. Details of all *Ramularia collo-cygni* isolates and other fungal pathogens used in this study, including verification of primer pair and nested PCR specificity

Isolate	Species	Host	Year	Origin	Isolator	ITS1/ ITS4*	Rcc1/ Rcc5†	Rcc3/ Rcc4‡	Nested PCR§
R4‖	*R. collo-cygni*	*Hordeum vulgare*	1998	Scotland	E. Sachs	+	+	+	+
R9‖	*R. collo-cygni*	*H. vulgare*	1999	Scotland	E. Sachs	+	+	+	+
R16‖	*R. collo-cygni*	*H. vulgare*	1999	Scotland	E. Sachs	+	+	+	+
R19¶	*R. collo-cygni*	*H. vulgare*	2000	Ireland	E. O' Sullivan	+	+	+	+
R25¶	*R. collo-cygni*	*H. vulgare*	2001	Ireland	E. O'Sullivan	+	+	+	+
R1‖	*R. collo-cygni*	*Agropyron* sp.	1999	Germany	E. Sachs	+	+	+	+
R3‖	*R. collo-cygni*	*H. vulgare*	1998	Germany	E. Sachs	+	+	+	+
R5‖	*R. collo-cygni*	*H. vulgare*	1999	Germany	E. Sachs	+	+	+	+
R10‖	*R. collo-cygni*	*H. vulgare*	1999	Germany	E. Sachs	+	+	+	+
R8‖	*R. collo-cygni*	*Agropyron* sp	1999	Austria	E. Sachs	+	+	+	+
R12‖	*R. collo-cygni*	*H. vulgare*	1999	Austria	E. Sachs	+	+	+	+
R13‖	*R. collo-cygni*	*Secale cereale*	1999	Austria	E. Sachs	+	+	+	+
R24‖	*R. collo-cygni*	*H. vulgare*	2000	Norway	E. Sachs	+	+	+	+
R11‖	*R. collo-cygni*	*H. vulgare*	1999	Switzerland	E. Sachs	+	+	+	+
R14‖	*R. collo-cygni*	*H. vulgare*	2000	Czech Republic	E. Sachs	+	+	+	+
R23‖	*R. collo-cygni*	*H. vulgare*	2000	Argentina	E. Sachs	+	+	+	+
R22‖	*R. collo-cygni*	*H. vulgare*	2000	Uruguay	E. Sachs	+	+	+	+
R2‖	*R. collo-cygni*	*H. vulgare*	2000	New Zealand	E. Sachs	+	+	+	+
R7‖	*R. collo-cygni*	*H. vulgare*	2000	New Zealand	E. Sachs	+	+	+	+
IMI 240110 **	*R. indica*	*Rumex pulcher*	1979	New Zealand	G Laundon	+	–	–	–
Rsp2††	*R vallisumbrosae*	*Narcissuss sp.*	2004	United Kingdom	N. Havis	+	–	–	–
IMI 351012 **	*Didymella exitialis*	*H. vulgare*	1999	Ireland	P. H. Gregory	+	–	–	–
Pt1	*Pyrenophora teres*	*H. vulgare*	2000	Scotland	N. Havis	+	–	–	–
F1	*Fusarium culmorum*	*H. vulgare*	2000	Scotland	N. Havis	+	–	–	–
F2	*F. graminearum*	*H. vulgare*	2000	Scotland	N. Havis	+	–	–	–
D1	*Drechslera teres*	*H. vulgare*	2000	Scotland	N. Havis	+	–	–	–
RH1	*Rhynchosporium secalis*	*H. vulgare*	2001	Scotland	S. Piper	+	–	–	–
TH1	*Tapesia acuformis*	*H. vulgare*	2001	Scotland	S. Piper	+	–	–	–
S1	*Septoria tritici*	*T. aestivium*	1999	Scotland	N. Havis	+	–	–	–
RZ1	*Rhizoctonia cerealis*	*T. aestivium*	2000	Scotland	N. Havis	+	–	–	–
Hv1	*Hordeum vulgare*	–	2002	Scotland	N. Havis	+	–	–	–
Sc1	*Secale cereale*	–	2002	Scotland	N. Havis	+	–	–	–

*PCR amplification with universal primers internal transcribed spacer (ITS1) and ITS4 White *et al*. (1990).+, PCR amplicon generated.

†PCR amplification with *R. collo-cygni* specific primers Rcc1 and Rcc5.+, positive PCR signal of predicted 426 bp size; −, no amplification signal detected.

‡PCR amplification with *R. collo-cygni* specific primers Rcc3 and Rcc4.+, positive PCR signal of predicted 256 bp size; −, no amplification signal detected.

§PCR amplification with nested primer pairs, Rcc1 and Rcc5 followed by Rcc3 and Rcc4.+, positive PCR signal of predicted 256 bp size; −, no nested PCR amplification signal detected.

‖Isolates supplied by Dr E.Sachs, Institute for Plant Protection of Field, Crops and Grassland, Kleinmachnow.

¶Isolates supplied by E. O' Sullivan, Oak Park Research Station, TEAGASC, Carlow.

**Isolate sourced from CABI Bioscience, Egham, UK.

††Isolate obtained from infected leaf material supplied by T. O. Neill, ADAS Arthur Rickwood, Cambridgeshire.

Hercules, CA) placed directly over Vegetable agar (200 mL V8 Vegetable Juice (Campbell Soup Company, Cambridge, UK) + 20 g Technical Agar (Oxoid, Basingstoke, UK) L^{-1}) in 9 cm Petri dishes incubated at 20 °C. Total genomic DNA was extracted from harvested mycelial lawns using either the Nucleon® Phytopure Plant DNA extraction kit (Nucleon Biosciences, Deeside, UK) or the REDExtract-N-Amp™ Plant PCR kit (Sigma, Poole, UK) and quantified spectrophotometrically. The rRNA region between the small 18S and the large 28S sub-units, covering the entire ITS 1, 5.8S and ITS 2 regions, was amplified using universal primers ITS1 and ITS4 (White *et al.*, 1990). Total reaction volumes were 50 μL and comprised 5 ng template DNA, 10 μM each dATP, dCTP, dGTP and dTTP (Promega, Southampton, UK), 1 μmol each primer, 1.25 U Taq Polymerase and 10 × PCR reaction buffer (consisting of 15 mM $MgCl_2$, 500 mM KCl, 100 mM Tris-HCl and 1% Triton X-100) (all Promega, USA). Thermal cycling parameters, using a Multiblock system (MBU) thermal cycler (Hybaid, Basingstoke, UK), consisted of an initial denaturation step at 94 °C/4 min, followed by 30 cycles of: denaturation at 94 °C/1 min, annealing at 55 °C/1 min and extension at 72 °C/1 min, with a final extension step at 72 °C/10 min. PCR products were purified using the Wizard PCR Prep (Promega) prior to direct sequencing on both strands, using the same primers to initiate the reaction, with the ABI Prism™ Dye terminator Cycle Sequencing ready reaction Kit (Foster City, CA) and analysed at the Molecular Biology Support Unit, University of Glasgow. Sequence data was edited manually using both the Biology Workbench package (version 3.2, University of San Diego, http//workbench.sdsc.edu) and DNAStar modules (DNAStar, Madison, WI). A total of six putatively species-specific primers for *R. collo-cygni* were designed from the examination of inter-specific nucleotide divergent regions between closely related species through sequence alignment analysis using ClustalW (Thompson *et al.*, 1994) as revealed through BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST/). Primers were synthesized by Invitrogen™ (Life Technologies, Renfrew, UK). Details of all primers used in this study are given in Table 2. All forward and reverse primer combinations were empirically tested against three *R. collo-cygni* isolate DNA preparations (R1, R7 and R9) using identical conditions as outlined above, over a range of annealing temperatures (50.5–65.3 °C). Primer concentrations were reduced to 0.25 μmol for second round nested PCR using 5 μL of first round reaction as template. Evaluation of primer pair amplification efficiency was based on resulting amplicon intensity as revealed by standard gel electrophoresis, typically separated on 1% [weight in volume (w/v)] TBE – (89 mM Tris-borate, 2 mM EDTA at pH 8.0) agarose gels, stained with ethidium bromide and photographed over UV light (Sambrook *et al.*, 1989). Diagnostic nested PCR, with the final primer selection of Rcc1 and Rcc5 for first round amplification and Rcc3 and Rcc4 (using 5 μL of first round product as template) for second round amplification were both conducted at a final annealing temperature of 65 °C and visualized by gel electrophoresis as described above. The specificity of each selected primer combination, including nested PCR (as outlined above), was assessed by PCR with 5 ng of DNA of closely associated barley leaf and stem pathogens (e.g. *Rhynchosporium secalis*, *Fusarium culmorum*, *Fusarium graminareum*, *Pyrenophora teres*, *Didymella exitialis*), as well as closely related species (*R. vallisumbrosae* and *R. indica*), including host barley and cereal rye DNA (see Table 1). The sensitivity of selected primer combinations was assessed by testing 10 fold serial dilutions of *R. collo-cygni* genomic DNA (isolate R1) from 100 ng μL^{-1} down to 0.1 fg μL^{-1}. Any potential masking effect of barley DNA on primer function was evaluated by adding 50 ng (at 10 ng μL^{-1}) barley DNA to each *R. collo-cygni* dilution concentration and testing as described above. Appropriate positive and negative controls [sterile distilled water (SDW)] were included in all experiments.

Table 2. Characteristics of designated primers used in this study

Primer	Sequence (5′-3′)	T_m (°C)*	Source
ITS1	TCC GTA GGT GAA CCT GCG G	55.4	White *et al*. (1990)
ITS4	TCC TCC GCT TAT TGA TAT GC	55.2	White *et al*. (1990)
Rcc1	ACT GAG TGA GGG AGC AAT CC	56.0	This study
Rcc2	TGC CGC GCA AGC GGC ATT CC	57.2	This study
Rcc3	TCA TTC AAA CAC TGC ATT CTT ACG	60.2	This study
Rcc4	CGT CGC GAG TCT CTC GCG CGC CT	62.5	This study
Rcc5	GCA GAG GAG TTC GAG TCG TCG C	60.2	This study
Rcc6	CAA AGG TTG ACC TCG GAT CA	55.6	This study

*As calculated by the manufacturers.

For assay application to suspect or infected winter and spring barley leaf tissue, total genomic DNA from naturally infected barley leaves (Table 3) was extracted using the Nucleon® Phytopure Plant DNA kit (Nucleon Biosciences, Deeside, UK) or the REDExtract-N-Amp™ Plant PCR kit (Sigma). Each barley sample exhibited varying degrees of leaf spotting (Table 3) as visually designated as mild, severe or absent (internal SAC field assessment approach for leaf spotting evaluation where; absent represented no symptoms observed, mild, where 0–50% leaf area exhibited symptoms, and severe, where > 50% leaf area exhibited typical symptoms). The presence or absence of characteristic conidiophores of *R. collo-cygni* was confirmed by microscopic examination at × 40 magnification using a Meiji (EMZ-5) stereo microscope (Axbridge, UK). A natural, asymptomatic sample of cv. Pewter and an artificially infected sample of cv. Chariot (glasshouse inoculation), made with a mycelial

Table 3. Evaluation of the nested-PCR detection assay on infected winter and spring barley

Country	Region	Variety	Year	Symptom severity*	ITS†	Presence of conidiophores‡	Nested PCR§
Scotland	Aberdeenshire	Optic	1999	N/A (grain)	+	−	+
Scotland	Aberdeenshire	Chariot	1999	N/A (grain)	+	−	+
Scotland	Aberdeenshire	Optic‖	2001	Severe	+	+	+
Scotland	Aberdeenshire	Troon¶	2004	N/A (grain)	+	−	+
Scotland	Berwickshire	Chariot	1999	Mild	+	+	+
Scotland	Berwickshire	Chariot	2000	Severe	+	+	+
Scotland	Berwickshire	Decanter	2000	Mild	+	−	+
Scotland	Berwickshire	Chariot	2001	Mild	+	+	+
Scotland	Berwickshire	Pewter‖,**	2003	Absent	+	−	+
Scotland	Berwickshire	Optic	2003	Mild	+	+	+
Scotland	Berwickshire	Pewter	2003	Absent	+	−	+
Scotland	Berwickshire	Doyen	2004	Absent	+	−	+
Scotland	Dumfries and Galloway	Cellar‖	2004	Absent	+	−	+
Scotland	Dumfries and Galloway	Riviera‖,¶	2004	N/A (grain)	+	−	+
Scotland	Fife	Troon¶	2004	N/A (grain)	+	−	+
Scotland	Midlothian	Cellar	2003	Absent	+	−	+
Scotland	Midlothian	Chariot	2003	Absent	+	−	+
Scotland	Midlothian	Cellar	2004	Mild	+	+	+
Scotland	Orkney	Tyne¶	2004	N/A (grain)	+	−	+
Scotland	Perthshire	Optic¶	2004	N/A (grain)	+	−	+
Scotland	N/A	Chariot††	1999	Absent	+	+	+
Germany	Bavaria	Autherre‖,‡‡	1999	Severe	+	+	+
Germany	Bavaria	Duet‖,‡‡	2003	Mild	+	−	+
Germany	Bavaria	Duetti‡‡	2004	Mild	+	+	+
Germany	Bavaria	Camera‡‡	2004	Mild	+	+	+
Germany	Gottingen	Tafeno‡‡	2002	Mild	+	+	+
Germany	Straubingen	Duet‡‡	2002	Mild	+	+	+
France	Loire	Unknown§§	2003	Mild	+	−	+
France	Raincheval	Unknown§§	2003	Mild	+	−	+
Ireland	Carlow	Century‖‖	1998	Severe	+	+	+
N Zealand	Mount Hutt	Chariot¶¶	2002	Severe	+	+	+

*Symptom severity designated as mild, severe or absent (internal SAC field assessment approach for leaf spotting evaluation where absent represented no symptoms observed, mild, where 0–50% leaf area exhibited symptoms, and severe, where > 50% leaf area exhibited typical symptoms).
†PCR amplification with universal primers ITS1 and ITS4 White *et al*. (1990). +, PCR amplicon generated.
‡Presence of conidiophores as assessed by microscopic examination at X 40 magnification using a Meiji (EMZ-5) stereo microscope, where +, characteristic *R. collo-cygni* conidophores observed, and, −, where no conidiophores observed.
§Nested PCR amplification with primers Rcc1 and Rcc5 (first round) and Rcc3 and Rcc4 (second round). +, positive PCR signal of predicted 256 bp size; −, no amplification signal detected.
‖ See Fig. 2, lanes 12–16.
¶Barley grain samples supplied by V. Cockerell, Scottish Agricultural Science Agency, Edinburgh.
**Natural asymptomatic sample.
††Artificially inoculated asymptomatic sample.
‡‡Barley leaf material supplied by E. Sachs, Institute for Plant Protection of Field, Crops and Grassland, Kleinmachnow.
§§Barley leaf material supplied by S. Pepin, Syngeta Agro SAS, St Sauveur, France.
‖‖ Barley leaf material supplied by E. O'Sullivan, Oak Park Research Station, TEAGASC, Carlow.
¶¶Barley leaf material supplied via P. Bury, Syngenta Seeds, Cambridge.
N/A, not applicable.

suspension of Scottish isolate R9 (exhibiting atypical symptoms), were also included (see Table 3). All total DNA extracts were subjected to ITS amplification with universal primers ITS 1 and ITS4 (White *et al*., 1990) as a precheck against the presence of PCR inhibitors prior to diagnostic nested PCR as detailed above.

Results and discussion

DNA extraction of pure fungal cultures and plant material was achieved using the Nucleon kit, which proved the most reliable and reproducible method, consistently yielding good quality DNA of high molecular weight, although the

REDExtract-N-Amp™ Plant PCR kit (Sigma) gave similar yields over shorter times (20 min instead of 4 h) without the requirement for liquid nitrogen. Amplification of the rRNA region between the small 18S and large 28S sub-units of all 24 isolates of *R. collo-cygni* using primers ITS1 and ITS4 (White *et al*., 1990) produced a product of approximately 600 bp. No size variation was observed. Full ITS sequence from 14 isolates, representing maximum host and geographic diversity from the core collection of *R. collo-cygni* isolates, were sequence characterized and compared with each other using Clustal W alignment analysis (Thompson *et al*., 1994). The size of each entire ITS 1, 5.8S and ITS 2 generated fragment was 535 bp. Complete intraspecific homogeneity was observed between each of the 14 sequences generated [R4; AJ536178, R9; AJ536179, R19; AJ536180, R1; AJ536181, R3; AJ536182, R5; AJ536183, R10; AJ536184, R8; AJ536185, R12; AJ536186, R13; AJ536187, R11; AJ536188, R14; AJ536189, R2; AJ536190, R7; AJ536191] and with a previously published ITS sequence of *R. collo-cygni* (AF222848) isolated from winter barley grown in Bavaria, Germany (Crous *et al*., 2001). Interspecific nucleotide divergence with closely related species obtained from BLAST database searches, (in particular *Septoria passerini*, *Leptosphaeria herpotrichoides*, *Microsphearia baumleri* and *Phaeosphaeria avenaria* f. sp. *tritici*) and other common pathogens of barley (e.g. *Fusarium oxysporum*, *Claviceps purperea*, *Blumeria graminis, Rhynchosporium secalis*, *Pyrenophora* spp., *Drechslera teres*, *Puccinia graminis* and *Microdochium nivale*) were exploited in the design of primer pairs specific for *R. collo-cygni*. A total of three putatively species-specific primers were designed in the forward direction of rRNA transcription (Rcc1, Rcc2 and Rcc3) and three in the reverse direction (Rcc4, Rcc5 and Rcc6, see Table 2).

PCR evaluation of all possible primer combinations indicated all were capable of amplifying *R. collo-cygni* DNA. However, optimal pairing, in terms of amplification intensity and clarity, appeared to be Rcc1 and Rcc5, generating a fragment size of 426 bp as predicted from the determined sequence data. This combination resulted in consistent levels of visualized PCR band intensity over a wide range of annealing temperatures, including at high stringency (65 °C).

PCR amplification of the dilution series of *R. collo-cygni* DNA resulted in visible product on ethidium bromide-stained gels down to *c.* 5 pg target DNA. This compares favourably with other one step PCR diagnostic steps for other fungal pathogens (Fraaje *et al*., 1999; Lee & Tewari, 2001; Langrell, 2002). Refinement of the assay through introduction of a second-step, nested PCR amplification approach, incorporating primer pair Rcc3 and Rcc4 in the second round amplification, resulted in an expected diagnostic sized fragment of 256 bp, increased robustness and increased overall sensitivity to 0.5 fg (Fig. 1). However, in order to ensure no potential masking effect from the presence of host plant DNA in the total extracts, so to further assess the robustness of the system, additional nested PCR reactions were performed in the presence of relatively high concentrations of barley DNA. Examination of the results indicates sensitivity of the assay is not impaired by the presence of host DNA and a clear, unambiguous diagnostic band of expected size was still visible as low as 0.5 fg in the presence of 50 ng barley DNA (data not shown). Of the infected leaf samples evaluated with the assay, each produced a clear, unambiguous nested-PCR product of predicted 256 bp size upon gel electrophoresis, regardless of symptom expression (see Table 3). Of significance here is the fact that the assay is capable of detecting the presence of the pathogen not only in the absence of characteristic conidiophores, but also in the complete absence of typical symptoms, including with harvested grain (template prepared from total ground seed/grain powder using either DNA extraction kit as described), as evidenced with much of the Scottish material reported from Table 3.

Despite increased attention to the study of *R. collo-cygni*, its life cycle on barley remains poorly understood (E. Sachs, Federal Biological Research Centre for Agriculture and Forestry, Kleinmachnow, pers. comm.). Although a number of potential additional hosts have been identified (Sutton &

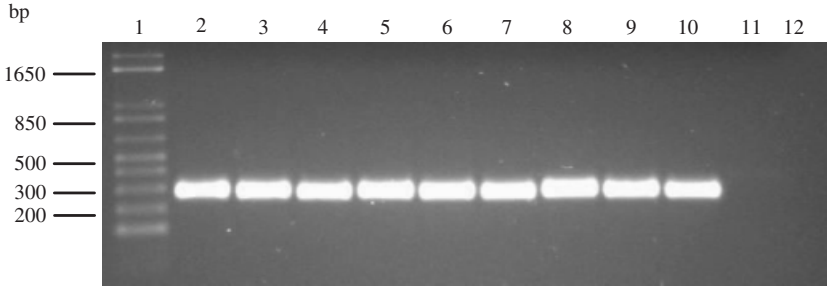

Fig. 1. Nested PCR against 10-fold serial dilutions of *Ramularia collo-cygni* DNA (isolate R1). Lane 1, 1 kb Plus DNA Ladder marker (Invitrogen); 2, 50 ng; 3, 5 ng; 4, 500 pg; 5, 50 pg; 6, 5 pg; 7, 500 fg; 8, 50 fg; 9, 5 fg; 10, 0.5 fg; 11, negative control (SDW), 12, nested negative control (using SDW as first round start template).

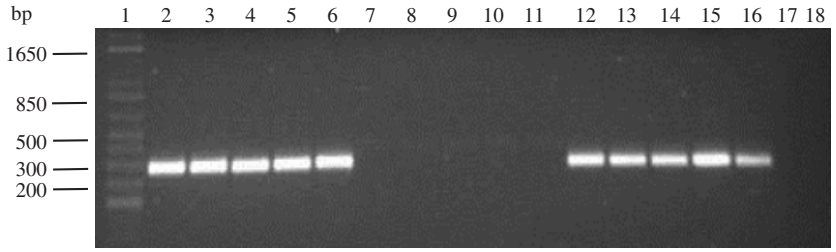

Fig. 2. Nested PCR approach showing specificity for *Ramularia collo-cygni* from axenic cultures and species-specific detection from preparations of infected and suspect barley tissue of various provenances, respectively. Lane 1, 1 Kb Plus DNA Ladder marker (Invitrogen); lanes 2 – 6, *R. collo-cygni* R4 (Scottish), R19 (Irish), R10 (German), R2 (New Zealand), R23 (Argentinian), respectively; 7, *R. vallisumbrosae* (Rsp2); 8, *R. indica* (IMI 240110); 9, *Didymella exitialis* (IMI 351012); 10, *Rhynchosporium secalis* (RH1); 11, *Pyrenophora teres* (Pt1); 12–16, *H. vulgare* tissues of varying disease severity, Scotland (severe), Germany (mild), Scotland (absent), Scotland (absent), Scotland (grain), respectively (see Table 3); 17, negative control (SDW); 18, nested negative control (using SDW as first round template).

Waller, 1988; Heuser & Zimmer, 2002) the movement of the pathogen from these into cereal crops remains inadequately researched, due, mainly, to its low frequency of isolation and slow growth in culture impeding investigations. Application of this assay should help circumvent these limitations and help resolve the host status of such species and further help define their respective role(s) in the epidemiology of this disease.

Further to this, the two additional *Ramularia* species, *R. vallisumbrosae and R. indica*, both of which are present in Britain, were primarily included in this study to evaluate possible cross reactivity of the diagnostic assay. Neither species produced a diagnostic product of 256 bp upon nested PCR (see Table 1 and Fig. 2, lanes 7 and 8, respectively). Further, in repeated inoculation studies, *Ramularia indica* and *Ramularia vallisumbrosae* failed to induce disease symptoms on a range of barley varieties, including cvs. Chariot, Optic and Pewter, under conditions conducive to disease development.

Positive diagnostic nested PCR results were also derived from barley grain samples harvested in 1999 and 2004 in Scotland (see Table 3). Although only limited anecdotal evidence exists for a seed-borne stage of this pathogen (H. Huss, Versuchsstation Lambach-Stadl-Paura, Bundesamt für Agarbiologie, Austria, pers. comm.) detection of the presence of *R. collo-cygni* at this stage in the crop cycle suggests seed from infected crops could act as a further source of infection and disease spread. Here, application of this assay may further assist in research efforts into this hitherto over-looked, and potentially significant, aspect of its enigmatic epidemiology.

It is envisaged that this assay will continue to find direct application as a routine research tool in studying the ecology, aetiology and epidemiology of this recalcitrant species not only in Scotland, but in all barley growing regions of the world where *R. collo-cygni* is problematic. Increased knowledge, generated through application of this assay, should aid in the design of effective integrated control strategies as well as offering the potential for the development of advanced forecasting or monitoring schemes.

Acknowledgements

This work is financially supported by the Scottish Executive Environment and Rural Affairs Department Project No 627718. We would like to thank Dr E. Sachs, Institute for Plant Protection of Field, Crops and Grassland, Kleinmachnow, Germany and Dr E. O'Sullivan, Oak Park Research Station, TEAGASC, Ireland, for supplying isolated cultures used in this project (imported and maintained under SEERAD Plant Health Licence No: PH/15/2004). The authors would like to acknowledge the work of Mr. K. Szymoniak, Mr M. Pezaki and Ms S. Reignoux at SAC Edinburgh for technical assistance.

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