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ORIGINAL PAPER

Species-Specific Detection of *Mycosphaerella polygoni-cuspidati* as a Biological Control Agent for *Fallopia japonica* by PCR Assay

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Abstract The ascomycete fungus *Mycosphaerella polygoni-cuspidati* has been undergoing evaluation as a potential classical biological control agent for the invasive weed *Fallopia japonica* (Japanese knotweed), which has become troublesome in Europe and North America. In advance of the potential release of a biocontrol agent into a new environment, it is crucial to develop an effective monitoring system to enable the evaluation of agent establishment and dispersal within the target host population, as well as any potential attacks on non-target species. Therefore, a primer pair was designed for direct, rapid, and specific detection of the Japanese knotweed pathogen *M. polygoni-cuspidati* based on the sequences of the internal transcribed spacer regions including the 5.8S rDNA. A PCR product of approximately 298 bp was obtained only when the DNA extracted from mycelial fragments of *M. polygoni-cuspidati* was used. The lower limit of detection of the PCR method was 100 fg of genomic DNA. Using the specific primer pair, *M. polygoni-cuspidati* could be detected from both naturally and artificially infected Japanese knotweed plants. No amplification was observed for other *Mycosphaerella* spp. or fungal endophytes isolated from *F. japonica*. The designed primer pair is thus effective for the specific detection of *M. polygoni-cuspidati* in planta.

Keywords Japanese knotweed · Leaf spot disease · Weed biocontrol · Molecular marker · rDNA-ITS region

Introduction

Japanese knotweed (*Fallopia japonica*) is a herbaceous perennial plant that originates from Japan and has become highly invasive and problematic in many parts of Europe and North America. This invasiveness often poses significant costs to biodiversity and to urban development [1]. Current control methods based on chemical applications and mechanical control are only effective in the short term as the plant has an extensive and resilient rhizome system [2]. In addition, the use of chemical application is restricted in riparian habitats predominantly invaded by Japanese knotweed, while mechanical control easily causes fragmentation of the rhizome, thereby further spreading through contaminated soil. In the UK, the cost of Japanese knotweed control has been estimated in excess of £1.5 billion ($2.1 billion) [3]. For those reasons, classical biological control, which is a more sustainable, economically viable and long-term strategy based on the use of highly specific and damaging natural enemies from the native range of the invasive species, is being considered part of an integrated management approach for this troublesome weed in its introduced range. Survey work has been previously conducted in Japan and selected arthropods and plant fungal pathogens associated with Japanese knotweed have been collected and assessed for their potential as classical biocontrol agents [4–6]. In 2011, the psyllid *Aphalara itadori* has been released in selected habitats as

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the first classical biological control agent of *F. japonica* following its extensive evaluation and the approval for release by the national authorities in 2010. The control efficacy of this agent against the weed in the field is currently under evaluation.

The hemibiotrophic leaf spot pathogen, *Mycosphaerella polygoni-cuspidati*, attacking Japanese knotweed in Japan, has been undergoing evaluation as an additional biological control agent for *F. japonica*, based on its apparent field host specificity and its severe impact on the weed in both the field as well as in greenhouse experiments [7].

From both a scientific and a regulatory point of view, it is crucial to monitor the fate and behaviour of any classical biological control agent once it has been released into the new environment. The same would apply to the leaf spot pathogen *M. polygoni-cuspidati* enabling an assessment of its establishment and impact on its invasive host Japanese knotweed as well as its dispersal within and between host populations. Furthermore, any potential attacks on non-target species near release sites would need to be rapidly detected to prompt required intervention. Tracking of the leaf spot pathogen can be undertaken using classical microbiological methods of fungal isolation from Japanese knotweed and non-target plant species; however, molecular techniques developed for a specific pathogen would considerably aid the detection. Thus, there are a need and an opportunity to develop a rapid, sensitive and accurate monitoring method that can detect *M. polygoni-cuspidati* and distinguish the fungus from the general microbial flora, such as endophytes present in the leaves of *F. japonica* [8]. In addition, such a method can track the population dynamic of the pathogen over time.

Polymerase chain reaction (PCR) is a rapid and specific method, and its high sensitivity allows detection of target DNA in a complex mixture, offering an alternative to classical methods such as isolation or spore observation [9]. The development of molecular markers is required in order to detect and to exploit DNA polymorphism. The ribosomal internal transcribed spacer regions including the 5.8S gene of rDNA (rDNA-ITS) is suitable and extensively used for developing PCR primers specific to the target organism. The rDNA-ITS region has also a high copy number in the genome indicating the increase of the sensitivity in PCR amplifications. This region is present in conserved and variable regions of the genome [10, 11]. Comparison of the nucleotide sequences of the rDNA-ITS region has proved useful in delimiting and differentiating species [12–14]. Differences of the rDNA-ITS region between species have been used to develop species-specific primers, and this has become a common approach in molecular identification strategies [15]. With respect to plant pathogenic fungi, species-specific primers for *Mycosphaerella* species have already been developed for the

detection of *M. fijiensis* and *M. musicola* on banana [16–18], as well as *M. cryptica*, *M. lateralis*, *M. marksii*, *M. nubilosa* and *M. parva* on *Eucalyptus globulus* [19].

In order to enable future tracking of *M. polygoni-cuspidati* in its new environment and to differentiate the pathogen from other fungal species, a species-specific primer pair for the identification and detection of *M. polygoni-cuspidati* was developed, and the sensitivity and specificity of this primer pair were assessed using diseased *F. japonica* leaves.

Materials and Methods

Fungal Isolates

Eight different isolates of *M. polygoni-cuspidati*, which were previously isolated from lesions on diseased Japanese knotweed leaves sampled from various locations in Japan [6] and maintained on plugs of potato carrot agar (PCA; decoction of 20 g potato and 20 g carrot, and 20 g agar/l) under sterile distilled water, were selected (Table 1). For the specificity analysis of the primers designed in this research, *M. shimabarensis* isolated from *F. japonica* in Japan as an endophyte, and seven isolates of other *Mycosphaerella* species were investigated. These included *M. berkeleyi*, *M. chrysanthemi*, *M. delegatensis*, *M. graminicola*, *M. macrospora*, *M. mori* and *M. praecox*. These species isolated from various hosts worldwide were obtained from the Genetic Resources Collection, CABI (Egham, UK) (Table 1). Moreover, 23 fungal isolates belonging to 16 additional genera were included in the tests. These genera comprised *Alternaria*, *Annulohypoxylon*, *Aureobasidium*, *Bionectria*, *Biscogniauxia*, *Botryosphaeria*, *Cladosporium*, *Colletotrichum*, *Cynanchum*, *Irpex*, *Nemania*, *Nigrospora*, *Pestalotiopsis*, *Phoma*, *Phomopsis* and *Xylaria*, and were previously isolated as endophytes from *F. japonica* in Japan. All genera were identified based on morphological criteria and sequence analysis using the rDNA-ITS region (Table 1). The isolates of all respective fungal endophytes have been maintained on PCA plugs under the sterile distilled water at room temperature in the Laboratory of Plant Pathology, Kyushu University, Fukuoka, Japan.

Genomic DNA Extraction, PCR Amplification and Sequencing

Total genomic DNA was extracted from 14-day-old cultures of the respective fungal isolates grown on PCA with the DNeasy Plant Mini Kit (Qiagen, Valencia, USA) according to the manufacturer's instructions. The DNA

Table 1 Fungal isolates used to screen the specificity of the primer

Species	Isolate no.	Isolation source	Location of collection	PCR amplification	
				ITS1/ITS4	MP-F2/MP-R1
Mycosphaerella polygoni-cuspidati	IMI 401968	*Fallopia japonica*	Fukuoka, Japan	+	+
M. polygoni-cuspidati	IMI 393527	*F. japonica*	Kochi, Japan	+	+
M. polygoni-cuspidati	IMI 401910	*F. japonica*	Yamaguchi, Japan	+	+
M. polygoni-cuspidati	IMI 395027	*F. japonica*	Hiroshima, Japan	+	+
M. polygoni-cuspidati	IMI 395028	*F. japonica*	Nagasaki, Japan	+	+
M. shimabarensis	IMI 401914	*F. japonica*	Nagasaki, Japan	+	−
M. berkeleyi	IMI 169888	*Arachis hypogaea*	Trinidad and Tobago	+	−
M. chrysanthemi	IMI 147194	*Chrysanthemum* sp.	United Kingdom	+	−
M. delegatensis	IMI 362252	*Eucalyptus camaldulensis*	Ethiopia	+	−
M. graminicola	IMI 190859	Triticale	Ethiopia	+	−
M. macrospora	IMI 147030	*Iris* sp.	United Kingdom	+	−
M. mori	IMI 356555	*Morus alba*	India	+	−
M. praecox	IMI 292857	*Lactuca sativa*	Papua New Guinea	+	−
Alternaria alternata	FJJ24	*F. japonica*	Fukuoka, Japan	+	−
Al. azukiae	FJJ193	*F. japonica*	Oita, Japan	+	−
Annulohypoxylon squamulosum	FJJ206	*F. japonica*	Nagasaki, Japan	+	−
Aureobasidium pullulans	FJJ23	*F. japonica*	Fukuoka, Japan	+	−
Bionectria ochroleuca	FJJ180	*F. japonica*	Oita, Japan	+	−
Biscogniauxia capnodes	FJJ39	*F. japonica*	Fukuoka, Japan	+	−
Botryosphaeria berengeriana	FJJ29	*F. japonica*	Fukuoka, Japan	+	−
Bo. dothidea	FJJ64	*F. japonica*	Fukuoka, Japan	+	−
Cladosporium cladosporioides	FJJ69	*F. japonica*	Fukuoka, Japan	+	−
Colletotrichum acutatum	FJJ252	*F. japonica*	Oita, Japan	+	−
Co. crassipes	FJJ27	*F. japonica*	Fukuoka, Japan	+	−
Co. gloeosporioides	FJJ45	*F. japonica*	Fukuoka, Japan	+	−
Cynanchum auriculatum	FJJ242	*F. japonica*	Fukuoka, Japan	+	−
Irpex lacteus	FJJ143	*F. japonica*	Fukuoka, Japan	+	−
Nemania diffusa	FJJ219	*F. japonica*	Nagasaki, Japan	+	−
Nigrospora sphaerica	FJJ195	*F. japonica*	Oita, Japan	+	−
Pestalotiopsis sydowiana	FJJ267	*F. japonica*	Oita, Japan	+	−
Pe. vismiae	FJJ265	*F. japonica*	Oita, Japan	+	−
Phoma glomerata	FJJ21	*F. japonica*	Fukuoka, Japan	+	−
Ph. macrostoma	FJJ258	*F. japonica*	Oita, Japan	+	−
Phomopsis eucommicola	FJJ103	*F. japonica*	Fukuoka, Japan	+	−
Xylaria hypoxylon	FJJ208	*F. japonica*	Nagasaki, Japan	+	−
X. venosula	FJJ204	*F. japonica*	Nagasaki, Japan	+	−

IMI Genetic Resources Collection, CABI, Egham UK, *FJJ* Laboratory of Plant Pathology, Kyushu University, Fukuoka, Japan

samples were diluted to the concentration of 10 ng/μl and stored at −20 °C until use.

The rDNA-ITS region were amplified using the universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [20]. PCR reactions were performed in reaction volumes of 25 μl containing 10 ng of genomic DNA templates,

1× PCR Buffer (Toyobo, Osaka, Japan), 0.2 mM of dNTPs, 0.2 μM of each primer and 1.25 U Blend *Taq* (Toyobo). DNA amplification was performed in a MyCycler Thermal Cycler (Bio-Rad, Hercules, USA). The PCR profile consisted of denaturation at 96 °C for 5 min, followed by 36 cycles of 94 °C for 45 s, 50 °C for 30 s, and 72 °C for 90 s. The PCR-amplified DNA fragments were

fractionated in 2.0 % (w/v) agarose gels using 0.5 % (v/v) Tris-Acetate-EDTA buffer, and visualised by ethidium bromide staining and UV illumination. PCR products were purified and sequenced as described previously [6]. A newly generated sequence was deposited in DDBJ/EMBL/GenBank under the accession number LC146384.

Design of *M. polygoni-cuspidati*-specific PCR Primers

Specific primers for *M. polygoni-cuspidati* were designed by comparison of the rDNA-ITS regions of 57 different Mycosphaerellaceae sequences, including 32 *Mycosphaerella* species, obtained from the GenBank database. The specific primer pair MP-F2 (5′-GCGTCGGAGTCT-TAATGAATTT-3′) and MP-R1 (5′-GCTCCGCAGC-GAAACATATA-3′) was designed, and the expected amplicon size for the PCR was 298 bp.

Primers MP-F2/MP-R1 Amplification

For PCR amplification with the primer pair MP-F2 and MP-R1, each 25 µl PCR reaction consisted of 10 ng of genomic DNA templates, 0.5 U KOD Hot Start DNA polymerase (Novagen/Toyobo, Darmstadt, Germany), 1× PCR Buffer, 0.2 mM of dNTPs, 1.0 mM $MgSO_4$ and 0.3 µM of each primer. To increase the specificity and sensitivity of the PCR assay, touchdown PCR was adopted using a Mastercycler (Eppendorpf AG, Hamburg, Germany) under the following programme: 98 °C for 2 min; 15 cycles of 94 °C for 30 s, 66 °C decreasing by 1 °C every 5 cycles for 30 s and 68 °C for 1 min; and 15 cycles of 94 °C for 30 s, 63 °C for 30 s and 68 °C for 1 min. PCR products were separated by electrophoresis in 1.5 % (w/v) agarose gels and stained with SafeView Nucleic Acid Stain (NBS Biologicals, Huntingdon, UK).

Specificity of Primers and Sensitivity of PCR

The specificity of the primer pair was evaluated with genomic DNA extracted from five isolates of *M. polygoni-cuspidati*, eight isolates of other *Mycosphaerella* species and an additional 23 different fungal species shown in Table 1. Before the specificity of the primer pair was tested, all genomic DNA were amplified with the universal primer pair ITS1 and ITS4 in order to avoid false negative results with *M. polygoni-cuspidati*-specific primers. The lower limit of specificity for PCR amplification using the primer pair MP-F2 and MP-R1 was determined by testing a dilution series of DNA concentration from 10 ng/ml to 1 fg/ml. PCR reactions were conducted as previously described with *M. polygoni-cuspidati*-specific primers.

Artificial Inoculation

A selected *M. polygoni-cuspidati* isolate (IMI 395028) was cultured in potato dextrose broth (PDB; 2.4 g PDB (Difco, Sparks, USA) and 100 ml of distilled water) by incubation on a shake in the dark at 18 °C for 14 days. The mycelial suspension for inoculation was obtained by blending the mycelial mass harvested from the flask at 15,000 rpm for 30 s (Model 8011, Warning, Torrington, USA) in 0.05 % Tween 80. The homogenised suspension was subsequently inoculated onto both surfaces of every leaves of three Japanese knotweed plants with the seven-leaf stage using a sterile paint brush. The inoculated plants were incubated in a customised dew chamber (Mercia Scientific, Long Itchington, UK) with 100 % relative humidity at 19.5 °C. After 48 h, the plants were transferred to a glasshouse chamber and maintained at a temperature regime of 21 °C day/19 °C night and regularly assessed for disease development.

### Detection of *M. polygoni-cuspidati* in Inoculated Diseased Leaves and Naturally Diseased Leaves

Inoculated leaves showing typical leaf spot symptoms (Fig. 1a) were harvested one month after inoculation. Approximately 20 mg of fresh leaf pieces cut from the lesions was surface sterilised by immersion in 70 % ethanol for 30 s, followed by immersion in 1.4 % sodium hypochlorite solution for 5 min. Leaf pieces were subsequently rinsed three times with sterile distilled water to avoid any DNA extraction from mycelial inoculum remaining on inoculated leaf surfaces. Leaf pieces from healthy Japanese knotweed plants grown in a glasshouse were used as negative control. Naturally diseased *F. japonica* leaves displaying leaf spot symptoms caused by *M. polygoni-cuspidati* were collected from a field site in Omura, Nagasaki Pref., Japan, in 2015 (Fig. 1b). After drying, approximately 10 mg of leaf pieces were segmented from asymptomatic and symptomatic areas of the diseased leaves. DNA was extracted from artificially and naturally infected leaves using DNeasy Plant Mini Kit according to the protocol. PCR amplification was performed with the designed primers following the protocol described above. DNA extracted from the selected *M. polygoni-cuspidati* isolate (IMI 395028) grown in vitro served as positive control.

Results

Design of *M. polygoni-cuspidati*-specific Primers

The universal primers ITS1 and ITS4 amplified an approximately 500-bp fragment of genomic DNA on *M.*

Fig. 1 Disease symptoms caused by *Mycosphaerella polygoni-cuspidati* on *Fallopia japonica*. **a** Artificially infected leaves of *F. japonica* in a greenhouse. **b** Naturally infected leaves of *F. japonica* at Omura, Nagasaki, Japan
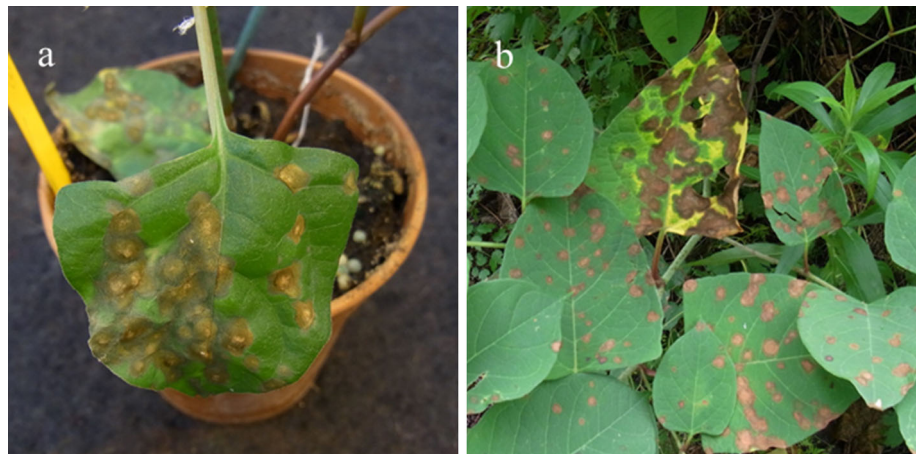
polygoni-cuspidati isolates. The sequence identity was 100 % for all the assessed isolates. By contrast, these *M. polygoni-cuspidati* isolates showed less similarity with other fungal species. Out of these other species, the sequence of *M. sumatrensis* was the one most similar to that of the *M. polygoni-cuspidati* isolates with 97 % identity. On the basis of alignment of the sequences of the rDNA-ITS region with other fungal isolates of 57 Mycosphaerellaceae obtained from GenBank, the primer pair MP-F2/MP-R1 was designed for specific amplification. These primer sequences shared 100 % identity only with sequences of *M. polygoni-cuspidati*. Amplification of DNA from *M. polygoni-cuspidati* with the primer pair MP-F2/MP-R1 produced a PCR product of 298 bp. The PCR product was sequenced and had the same sequence as the original sequence.

Specificity and Sensitivity of the Designed Primers

All isolates tested had a positive PCR amplification using the universal primers ITS1 and ITS4 (Table 1). PCR products of 298 bp amplified with the specific primers MP-F2 and MP-R1 were obtained only when DNA was extracted from isolates of *M. polygoni-cuspidati* obtained from various field sites in Japan (Table 1, Fig. 2). No amplification was observed for other fungal species tested, including other *Mycosphaerella* spp. and fungal endophytes isolated from *F. japonica*. The lowest limit of detection of the PCR method determined for *M. polygoni-cuspidati* (IMI 395028) was established as 100 fg of total DNA (Fig. 3).

Detection of *M. polygoni-cuspidati* in Infected Japanese knotweed Leaves

The primer pair successfully detected *M. polygoni-cuspidati* from lesions of both artificially and naturally diseased leaves (Fig. 4). A weak band was also observed at 298 bp on some of the DNA samples extracted from asymptomatic areas of diseased leaves. The specific PCR product was sequenced to confirm that the designed primers have amplified the expected consensus region of the target organism. No PCR products were amplified with DNA from healthy leaves.

Discussion

In the present study, we developed species-specific PCR primers for the identification of *M. polygoni-cuspidati*, a potential biological control agent for *F. japonica*, based on sequence information of the fungus from the rDNA-ITS region. The primer pair MP-F2 and MP-R1 amplified a single fragment of approximately 298 bp in size only with DNA extracted from mycelia of *M. polygoni-cuspidati* cultured on PCA. This primer pair was also used to detect the presence of *M. polygoni-cuspidati* in infected Japanese knotweed leaves. These results confirm that this primer pair is suitable for the specific detection of *M. polygoni-cuspidati* and thus for monitoring the presence of this fungus in *F. japonica* field populations after a potential future release into a new environment.

To establish a species-specific PCR detection method with high accuracy, designing primers based on ubiquitously conserved known genes with sequence variation are of great importance. In this study, sequences of the rDNA-ITS region were selected as a target region because this region of the genome evolves fast, may vary among species within a genus or even among species populations [21–25] and is present in many copies in fungal genomes [26, 27]. In addition, a large amount of the rDNA-ITS sequence data provided by public databases can be used for comparison of sequences of *M. polygoni-cuspidati* with other *Mycosphaerella* species, which facilitates the design of
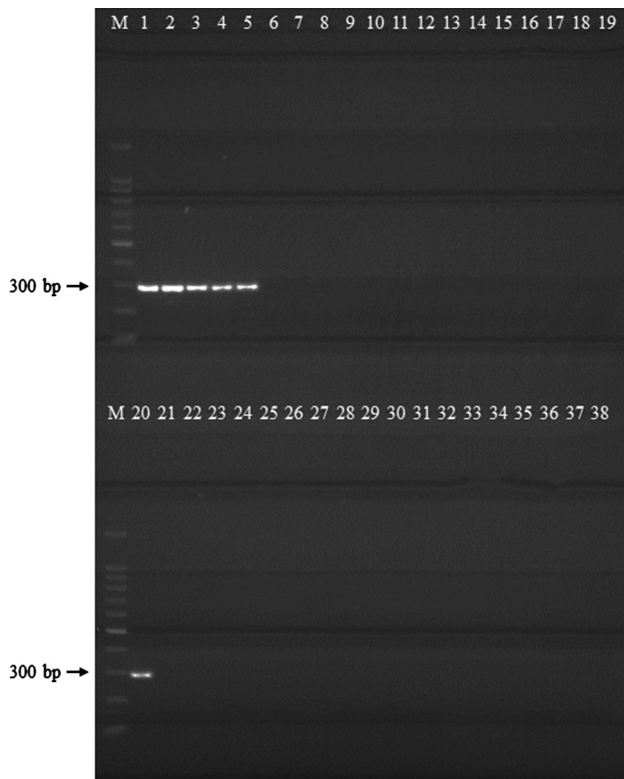
Fig. 3 PCR sensitivity assay using the primer pair MP-F2/MP-R1 for detection of *Mycosphaerella polygoni-cuspidati*. *Lane M*—Molecular marker (100-bp DNA ladder), *Lane 1*—10 ng/μl, *Lane 2*—1 ng/μl, *Lane 3*—100 pg/μl, *Lane 4*—10 pg/μl, *Lane 5*—1 pg/μl, *Lane 6*—100 fg/μl, *Lane 7*—10 fg/μl, *Lane 8*—1 fg/μl, *Lane 9*—negative control (without DNA)

Fig. 2 Specificity of PCR with the primer pair MP-F2/MP-R1 for detection of *Mycosphaerella polygoni-cuspidati*. *Lane M*—Molecular marker (100-bp DNA ladder), *Lane 1*—*M. polygoni-cuspidati* IMI 401968, *Lane 2*—*M. polygoni-cuspidati* IMI 393527, *Lane 3*—*M. polygoni-cuspidati* IMI 401910, *Lane 4*—*M. polygoni-cuspidati* IMI 395027, *Lane 5*—*M. polygoni-cuspidati* IMI 395028, *Lane 6*—*M. shimabarensis*, *Lane 7*—*M. berkeleyi*, *Lane 8*—*M. chrysanthemi*, *Lane 9*—*M. delegatensis*, *Lane 10*—*M. graminicola*, *Lane 11*—*M. macrospora*, *Lane 12*—*M. mori*, *Lane 13*—*M. praecox*, *Lane 14*—*Alternaria alternata*, *Lane 15*—*Al. azukiae*, *Lane 16*—*Annulohypoxylon squamulosum*, *Lane 17*—*Aureobasidium pullulans*, *Lane 18*—*Bionectria ochroleuca*, *Lane 19*—*Biscogniauxia capnodes*, *Lane 20*— *M. polygoni-cuspidati* IMI 395028, *Lane 21*—*Botryosphaeria berengeriana*, *Lane 22*—*Bo. dothidea*, *Lane 23*—*Cladosporium cladosporioides*, *Lane 24*—*Colletotrichum acutatum*, *Lane 25*—*Co. crassipes*, *Lane 26*—*Co. gloeosporioides*, *Lane 27*—*Cynanchum auriculatum*, *Lane 28*—*Irpex lacteus*, *Lane 29*—*Nemania diffusa*, *Lane 30*—*Nigrospora sphaerica*, *Lane 31*—*Pestalotiopsis sydowiana*, *Lane 32*—*Pe. vismiae*, *Lane 33*—*Phoma glomerata*, *Lane 34*— *Ph. macrostoma*, *Lane 35*—*Phomopsis eucommicola*, *Lane 36*—*Xylaria hypoxylon*, *Lane 37*—*X. venosula*, *Lane 38*—negative control (without DNA)

Fig. 4 Detection of *Mycosphaerella polygoni-cuspidati* from artificially and naturally infected *Fallopia japonica* samples. *Lane M*—Molecular marker (100-bp DNA ladder), *Lanes 1*—*M. polygoni-cuspidati* IMI395028, *Lanes 2–4*—artificially infected leaf, *Lanes 5–7*—naturally infected leaf (asymptomatic part), *Lanes 8–10*—naturally infected leaf (symptomatic part), *Lanes 11*—negative control (healthy leaf)

species-specific PCR primers. Thus, a specific primer pair for *M. polygoni-cuspidati* was designed on the basis of these features.

Specificity test confirmed that the designed primers MP-F2 and MP-R1 could amplify only DNA extracted from *M. polygoni-cuspidati*, but not from other fungal species including *M. shimabarensis* isolated from *F. japonica* and other *Mycosphaerella* species. In addition, all assessed isolates of *M. polygoni-cuspidati* collected from a wide
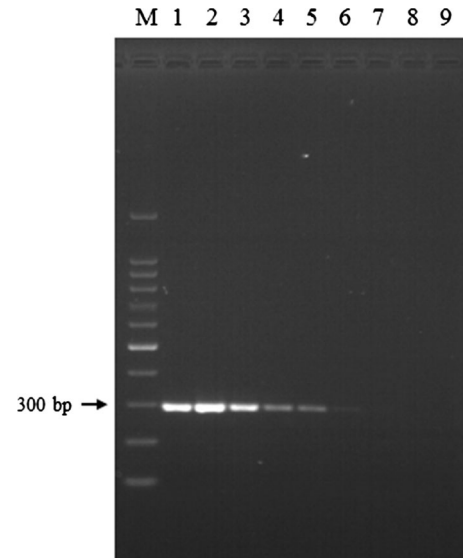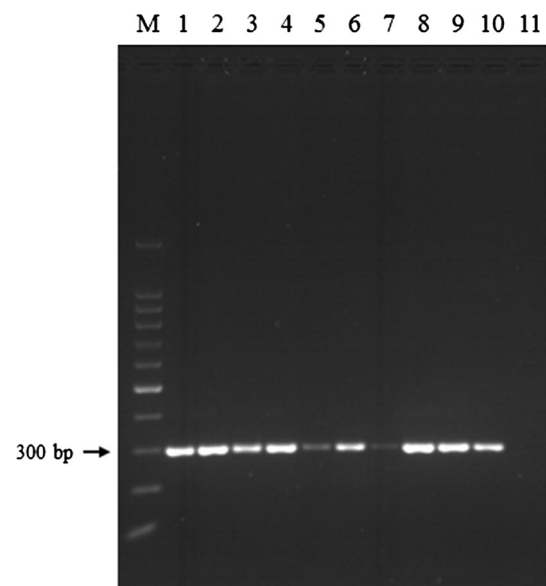
range of field sites in Japan were detected with the specific primer pair, suggesting that it can amplify *M. polygoni-cuspidati* DNA across different populations. This primer

pair also successfully detected the pathogen through the direct amplification of DNA from *F. japonica* leaves, both naturally and artificially infected with *M. polygoni-cuspidati*. Although naturally diseased Japanese knotweed leaves are also colonised by a range of endophytes, the designed primer pair MP-F2/MP-R1 amplified no PCR product from DNA extracted from any of these endophytic fungal species, which further demonstrates its specificity.

The detection sensitivity with the primer set MP-F2/MP-R1 was 100 fg of genomic DNA. This high sensitive detection is probably due to the high copy number of rDNA genes in any genome. Furthermore, touchdown PCR used in the present study resulted in higher sensitivity with the primer compared to normal PCR with consistent annealing temperature (data not shown). This is more sensitive than the previous reports including that the detection limit of *M. graminicola* or *M. parva* is as low as 10 pg [19, 28]. In addition, the primers could detect *M. polygoni-cuspidati* from 1 pg of genomic DNA extracted from the lesions (data not shown), which showed sufficient and higher sensitivity than that shown in other report [19]. Amplicons were also successfully detected from asymptomatic areas of the diseased leaves. A difference in the detection sensitivity with the primer pair was observed between DNA extracted from symptomatic and asymptomatic areas of the diseased leaves (Fig. 4). The most likely reason for this is the higher proportion of target DNA in relation to the total DNA extracted from developed leaf lesions. Moreover, one of the samples extracted from asymptomatic areas of the diseased leaves showed higher sensitivity compared to the other two samples (Fig. 4). It suggests that the samples showing higher sensitivity may be cut more close to the symptomatic area compared to the other samples. Because the PCR assay is successful in detecting *M. polygoni-cuspidati* in infected leaves, it may also be suitable to research pathogen movement with the host plant. In order to track the disease development of the pathogen in the latent infection period, more information about the PCR detection from asymptomatic *F. japonica* leaves at the earlier stages after artificial infection would be required.

After a potential future release of *M. polygoni-cuspidati* into a new environment, it would be important to track the fungus in order to study its dispersal within and between populations of *F. japonica*. Using the primer pair MP-F2/MP-R1 with fungal genomic DNA extracted from symptomatic and asymptomatic areas of leaves collected in the field as the template, *M. polygoni-cuspidati* was specifically detected, indicating that this primer pair can be useful for monitoring the presence of the fungus in the field. To our knowledge, there are no reports about developments of species-specific detection for foliar plant pathogens used as weed biological control agents. For the majority of such fungal pathogens, this detection method may not be applicable due to their easy identification in the field and unproblematic re-isolation from diseased plant material. In the case of *M. polygoni-cuspidati*, however, an early and quick confirmation of infection of the target weed or potentially any non-target species will be required to evaluate establishment and spread of the pathogen as well as to assess potential risks to non-target species. Re-isolation of the fungus from infected leaves and subsequent identification will be time consuming due to the slow growth rate of *M. polygoni-cuspidati* in vitro. Moreover, the lack of ascospore formation on artificial media and on artificially inoculated diseased leaves does not easily support the identification of the pathogen based on morphological characteristics. The rapid and accurate method of *M. polygoni-cuspidati*-specific PCR which we established will help overcome these limitations. In order to ensure this method is reliable in tracking the pathogen in a new environment, it will also be necessary to prove the specificity by using DNA extracted from leaves of *F. japonica* plant growing in the UK because the endophytic mycobiota differs from the one present in Japanese knotweed in Japan.

In conclusion, our research has successfully designed a rapid, sensitive and accurate method for the specific detection of *M. polygoni-cuspidati* from DNA extracted from pure cultures as well as diseased Japanese knotweed leaves by PCR assay. Should at any point in the future *M. polygoni-cuspidati* be released into a new environment as a classical biological control agent for *F. japonica*, this reliable detection assay will be a useful tool to monitor the establishment and spread of this pathogen in the introduced range.

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