Eur J Plant Pathol (2016) 146:581–595
DOI 10.1007/s10658-016-0944-4

Development of a multiplex PCR diagnostic assay for the detection of *Stagonosporopsis* species associated with ray blight of *Asteraceae*

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Accepted: 18 April 2016 / Published online: 28 April 2016

Abstract Ray blight, a destructive disease of *Asteraceae* worldwide, is caused by three morphologically similar but phylogenetically distinct species; *Stagonosporopsis chrysanthemi*, *S. inoxydabilis* and *S. tanaceti*. *Stagonosporopsis chrysanthemi* has been reported as a specific pathogen of chrysanthemum while *S. inoxydabilis* has been found associated with various *Asteraceae*. *Stagonosporopsis tanaceti* has only been reported in Australia, causing substantial crop loss on pyrethrum. All three species were shown to infect and cause disease on in vitro grown pyrethrum plants, hence, *S. chrysanthemi* and *S. inoxydabilis* may pose a significant biosecurity threat to the Australian pyrethrum industry. All these *Stagonosporopsis* species are also Level 2 quarantine pathogens in Europe. Rapid and accurate detection and differentiation of these species is a priority for ray blight management in Australia and in Europe. Accordingly, three species-specific PCR-based assays, targeted to the intergenic spacer of the nuclear ribosomal DNA, were developed. The specificity of each assay was confirmed against 21 *Stagonosporopsis* spp. as well as 14 pathogenic and saprophytic fungal species commonly found in association with pyrethrum in Australia. The primers were highly sensitive and specific to the target species, detecting down to 4 fg of genomic DNA. These primers were further used in a multiplex PCR to differentiate the presence of the three *Stagonosporopsis* spp. based on variable sized amplicons in a single reaction.

Keywords *Chrysanthemi* · *Inoxydabilis* · *Tanaceti* · Intergenic spacer · Ribosomal DNA · Pyrethrum · Chrysanthemum

Electronic supplementary material The online version of this article (doi:10.1007/s10658-016-0944-4) contains supplementary material, which is available to authorized users.

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Introduction

Ray blight is a major disease of *Asteraceae* (Stevens 1907; Vaghefi et al. 2012), causing severe losses to the chrysanthemum (*Chrysanthemum* × *morifolium*) and pyrethrum (*Tanacetum cinerariifolium*) industries worldwide (Baker et al. 1949; Pethybridge et al. 2008b). The most conspicuous symptom of the disease in both hosts is discolouration and distortion of the flower buds and ray florets (Stevens 1907). Three morphologically similar but phylogenetically distinct species, *Stagonosporopsis chrysanthemi*, *S. inoxydabilis* and *S. tanaceti* have been found associated with ray

blight of *Asteraceae* (Vaghefi et al. 2012), collectively referred to as 'ray blight pathogens'.

Stagonosporopsis chrysanthemi (syn. *Phoma ligulicola* var. *ligulicola*) is considered a host-specific pathogen to chrysanthemum with a worldwide distribution (Boerema et al. 2004). *Stagonosporopsis inoxydabilis* (syn. *P. ligulicola* var. *inoxydabilis*) infects various *Asteraceae* genera including *Tanacetum*, *Zinnia* and *Matricaria* (Boerema et al. 2004; van der Aa et al. 1990). Both pathogens are important quarantine organisms in many European countries (EPPO 2015), however, due to historical confusion in their taxonomy (Vaghefi et al. 2012; Walker and Baker 1983), the reported host range and geographical distribution should be treated with caution (Rossi et al. 2014).

The taxonomy of the ray blight pathogens has undergone many changes, an extensive review of which is presented by Vaghefi et al. (2012). The pathogen causing ray blight of chrysanthemum was initially described as *Ascochyta chrysanthemi* (Stevens 1907), which was later reduced to a synonym of *Phoma chrysanthemi* (Garibaldi and Gullino 1971). This synonymy was later rejected by Walker and Baker (1983). In 1990, the pathogen was reclassified as *Phoma ligulicola*, and was divided into two varieties, namely var. *ligulicola* and var. *inoxydabilis* (Van der Aa et al. 1990). Studies on the host range of *S. chrysanthemi* and *S. inoxydabilis* (Chesters and Blakeman 1967; Peregrine and Watson 1964; van der Aa et al. 1990) were conducted prior to the separation of the two varieties of *P. ligulicola*, thus require further verification.

The two varieties of *P. ligulicola* were later elevated to species level and renamed as *S. chrysanthemi* and *S. inoxydabilis* (Vaghefi et al. 2012). A third species, *S. tanaceti*, was described as the ray blight of pyrethrum in Australia (Vaghefi et al. 2012). *Stagonosporopsis tanaceti*, also regarded as a quarantine pathogen in Europe (EPPO 2015), has only been reported in Australia associated with substantial crop losses in pyrethrum (Hay et al. 2015; Pethybridge and Wilson 1998; Pethybridge et al. 2007; Vaghefi et al. 2012). In glasshouse trials, *S. tanaceti* was also able to cause disease on *Tagetes patula* and *Chrysanthemum carinatum* (Pethybridge et al. 2008a).

Stagonosporopsis chrysanthemi and *S. inoxydabilis* are not yet known to occur in Australia, and may be regarded as a biosecurity threat to the Australian pyrethrum industry if able to infect and cause disease on pyrethrum. Both pathogens are homothallic (Chilvers et al. 2014; Vaghefi et al. 2015a) and the environmental conditions conducive to their sexual reproduction are frequently observed in Tasmanian pyrethrum production areas (Pethybridge et al. 2008b). Therefore, if an incursion were to occur, rapid establishment and dissemination of *S. chrysanthemi* and *S. inoxydabilis* in Australian pyrethrum fields is highly probable (Pethybridge et al. 2008b). Understanding the ability of the aforementioned species to infect pyrethrum is, therefore, a high priority. Moreover, ray blight of chrysanthemum was previously recorded in Australia (Oxenham 1963; Simmonds 1996); therefore, an endemic source of *S. chrysanthemi* may exist.

Despite the biosecurity importance of the ray blight pathogens in Europe and Australia, no reliable assay is available for their rapid detection and identification. Current quarantine measures for identification of *S. chrysanthemi* rely on visual inspection of the plant material, cultural and morphological identification of the pathogen and NaOH spot test (production of red pigments following NaOH application, indicating the presence of metabolite 'E'; van der Aa et al. 1990) (EFSA PLH Panel 2013). However, visual inspection of plant material is not a reliable method of detection as *S. chrysanthemi* infection may be latent (Chesters and Blakeman 1966), and symptoms may be easily confused with other diseases and disorders (EFSA PLH Panel 2013). Differentiation of *S. inoxydabilis* and *S. tanaceti* based on morphology is also problematic and requires a high degree of expertise (Vaghefi et al. 2012). The need for a reliable molecular diagnostic assay for identification and differentiation of the three *Stagonosporopsis* species associated with ray blight of *Asteraceae* has been emphasised in several studies (EFSA PLH Panel 2013; Rossi et al. 2014; Vaghefi et al. 2012).

Of the small number of available sequences for *Stagonosporopsis* species (actin, β-tubulin, translation elongation factor 1-α, internal transcribed spacer and large subunit of ribosomal DNA), the actin sequence contained the highest variability and potential for species delineation (De Gruyter et al. 2012; Hyde et al. 2014; Vaghefi et al. 2012). Sequence information within the actin gene was used by De Gruyter et al. (2012) to develop TaqMan probes for the detection of *S. andigena* and *S. crystalliniformis*. However, our preliminary studies found that the actin sequence was not suitable for PCR-based differentiation of the three ray blight pathogens due to the limited number of consecutive

nucleotide differences (*unpublished data*). The intergenic spacer (IGS) of the nuclear ribosomal DNA (nrDNA) is highly variable and well suited for discriminating closely related species, and has been used for development of highly sensitive diagnostic assays for multiple fungal pathogens (Chilvers et al. 2007; Liew et al. 1998; Muller et al. 2013; Sampietro et al. 2010; Suarez et al. 2005). In some species, however, the IGS sequence is variable at an intra-specific level (Appel and Gordon 1996; Dissanayake et al. 2009; Kawabe et al. 2005; Srinivasan et al. 2011); therefore, suitability of the IGS for diagnostic purposes and differentiation of the three ray blight pathogens requires validation.

The objectives of this study were to: i) investigate the ability of *S. chrysanthemi* and *S. inoxydabilis* to cause disease on pyrethrum; ii) determine the suitability of the IGS region for diagnostic purposes for discriminating the three *Stagonosporopsis* spp. associated with ray blight; and iii) develop a multiplex PCR assay for detection and identification of the target *Stagonosporopsis* spp. in a single reaction.

Materials and methods

Pathogenicity assay

Pyrethrum plants

An in vitro system was developed to establish pathogen-free pyrethrum plants in a quarantine incubator at the Australian Quarantine and Inspection Services (AQIS) approved premises at the Faculty of Veterinary and Agricultural Sciences, the University of Melbourne, Australia. Seed of variety 'BR1' were surface-sterilised by shaking in 1 % (ai) sodium hypochlorite for two minutes, and rinsing in sterile distilled water three times. Air-dried seed were placed on Potato Dextrose Agar (PDA) (5 seeds/Petri dish) and incubated for two weeks at 22 °C under a 12 h photoperiod. Seed were checked daily and contaminated seed removed. After two weeks, single pathogen-free seedlings were transferred to double-autoclaved peat pellets (Jiffy-7® pot; 3 × 3 cm pots containing 75 % sphagnum peat and 25 % coir fibre), and individually placed in transparent screw cap plastic containers under sterile conditions. Plantlets were incubated at 20 ± 2 °C with a 12 h photoperiod. One millilitre liquid fertiliser (Miracle-Gro MaxFeed, Australia) was added to the containers every month. Three-month-old plants were used for pathogenicity trials.

Fungal isolates and inoculum preparation

The ex-holotype strain of *S. inoxydabilis* (CBS 425.90) and reference strain of *S. chrysanthemi* (CBS 500.63; Boerema et al. 2004) were cultured on PDA and incubated at 22 °C under constant white light to induce sporulation. The ex-holotype strain of *S. tanaceti* (CBS 131484 = TAS1 = isolate PL1 in Jones (2009)), reported to be moderately aggressive on pyrethrum (Jones 2009), was cultured on V8 agar (200 mL V8 juice in 1 L sterile water, pH 6.25) and incubated at 22 °C under constant white light. Ten-day-old cultures were flooded with sterile distilled water and pycnidiospores were released by gently scraping with a sterile scalpel. The suspension was filtered through a sterile muslin cloth, and the spore concentration was adjusted to 10^4 spore mL^{-1} using a haemocytometer (Assistent®, Germany). Tween 20 was added to the inoculum at a concentration of 0.02 % (*v*/v).

Inoculation

To investigate the ability of *S. chrysanthemi* and *S. inoxydabilis* to infect and cause disease on pyrethrum, a replicated inoculation trial was performed on three-month-old pyrethrum plants in individual pots. The trial was conducted in a completely randomised design with four replicate plants inoculated with each of the *S. chrysanthemi* and *S. inoxydabilis* species. Four plants inoculated with *S. tanaceti* served as positive control, and four negative control plants were treated with sterile water containing 0.02 % (*v*/v) Tween 20. Depending on the size of the leaf, one to five drops (20 μL) of inoculum were placed on each leaf. After inoculation, all plants were incubated at 20 ± 2 °C under a 12 h photoperiod, and assessed for symptoms daily for three weeks. Days to symptom development was recorded for each plant, and isolations were carried out from the resultant necrotic lesions (three isolations per plant) to verify that symptoms were caused by the *Stagonosporopsis* spp. For this, infected tissue was excised and surface sterilised (0.4 % ai sodium hypochlorite), cultured on 2 % water agar, and incubated at room temperature in darkness for 3 to 4 days. Resultant fungal mycelia were transferred to V8 and PDA for species identification.

Species-specific PCR assay development

Fungal isolates and DNA extraction

Stagonosporopsis chrysanthemi, *S. inoxydabilis* and *S. tanaceti* isolates used for sequencing the IGS region are shown in Table 1. Additional fungal species used for testing the specificity of the developed assays are shown in Table 2. These included multiple pathogenic and saprophytic fungal species associated with pyrethrum in Australia (Pethybridge et al. 2003; Hay et al. 2015). Genomic DNA of each fungal species was extracted using a DNeasy Plant Mini Kit (Qiagen, Australia) and visualised on ethidium bromide-stained 1 % agarose gels. Genomic DNA of 18 *Stagonosporopsis* species, derived from ex-type or reference cultures according to Boerema et al. (2004), were obtained from Centraalbureau voor Schimmelcultures (CBS), The Netherlands. DNA of *S. citrulli* was kindly provided by Dr. M. T. Brewer, University of Georgia, Georgia, USA. Dilutions of 2 ng/μL were prepared for all DNA samples for use in PCR.

IGS amplification and sequencing

To assess the suitability of the IGS region of the nrDNA for marker development (lack of intra-specific variation), the entire region in seven *S. tanaceti* strains, isolated from various localities and years (Table 1), were sequenced by primer walking. The entire IGS region of *S. tanaceti*, *S. chrysanthemi* and *S. inoxydabilis* was amplified using the primers LR12R and invSR1R (Table 3) using the High-Fidelity Velocity PCR kit (Bioline, Australia). Fifty microliter PCR reactions contained Hi-Fi buffer including $MgCl_2$ at a final concentration of 2 mM (Bioline), 0.1 μM of each primer, 0.25 mM dNTPs (Bioline), 1 U Velocity DNA polymerase (Bioline) and 10 ng DNA template. The PCR cycle was 3 min at 98 °C, followed by 35 cycles of 30 s at 98 °C, 30 s at the 67 °C, 1 min at 72 °C, and a final extension of 10 min at 72 °C. This yielded products of ~3 kb in all species. PCR products were purified using a PCR purification kit (Qiagen, Australia) and sequenced at the Australian Genome Research Facility (AGRF, Melbourne, Australia). For sequencing the 5′ and 3′ ends of the IGS, primers LR12R and invSR1R were used, which provided ~700 bp from each end. Several nested primers (Table 3) were subsequently designed using Primer3 (Rozen and Skaletsky 1999) for sequencing the internal region (Fig. 1).

Qualitative PCR assay development

Sequenced fragments were visualised, trimmed and assembled using Geneious Pro v. 7.1.3 (http://www.geneious.com, Kearse et al. 2012). The entire IGS sequences of the three *Stagonosporopsis* species were aligned using MAFFT v. 7 (Katoh et al. 2013), and a variable region within the IGS, downstream of the 28S

Table 1 Isolates used for sequencing the entire IGS region of *Stagonosporopsis* spp. associated with ray blight of *Asteraceae*. Ex-type strains are shown in bold and with an asterisk. Voucher strains according to Boerema et al. (2004) are in bold

Species	Strain number[a]	Substrate	Year	Country	GenBank accession no.[b]
Stagonosporopsis chrysanthemi	ATCC 10748	*Chrysanthemum morifolium*	Unknown	USA	–
	CBS 500.63; MUCL 8090	*C. morifolium*	1963	Netherlands	KP161043
S. inoxydabilis	**CBS 425.90*; PD 81/520***	*C. parthenii*	1990	Netherlands	KP161042
S. tanaceti	**CBS 131484*; TAS 1***	*Tanacetum cinerariifolium*	2004	Australia	KP161044
	DAR 70020		1998		–
	TAS 55503		2004		–
	UM 011–0001		2011		–
	UM 011–0063		2011		–
	TAS 011–0021		2010		–
	TAS F63810B		2009		–

[a] ATCC: American Type Culture Collection, Virginia, USA; CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; DAR: New South Wales Plant Pathology Herbarium, MUCL: Mycotheque de l'Universite catholique de Louvain; PD: Plant Protection Service, Wageningen, the Netherlands; TAS: Tasmanian Institute of Agriculture Fungal Collection; UM: University of Melbourne Fungal Collection

[b] No intra-specific variation was detected thus a single species-representative sequence was submitted to GenBank

Table 2 Isolates used for diagnostic assay specificity validation. Ex-type strains are shown in bold and with an asterisk. Voucher strains according to Boerema et al. (2004) are in bold

Species	Strain number[a]	Substrate	Country
Alternaria sp.	TAS 080–0030 TAS 080–0031	*Tanacetum cinerariifolium*	Australia
Boeremia exigua	TAS PE632 TAS PEMRA	*T. cinerariifolium*	Australia
Botrytis sp.	TAS 071–0090 TAS 071–0112	*T. cinerariifolium*	Australia
Chaetomium sp.	TAS 090–0002	*T. cinerariifolium*	Australia
Colletotrichum sp.	UM T5R3 UM T5R2 UM T8R3	*T. cinerariifolium*	Australia
Colletotrichum tanaceti	BRIP 57316 TAS 060–0382	*T. cinerariifolium*	Australia
Fusarium spp.	UM 0012 UM 0014 UM 0025	*T. cinerariifolium*	Australia
Microsphaeropsis tanaceti	UM T2-52 M UM T2-39 M	*T. cinerariifolium*	Australia
Paraphoma sp.	UM Para3 UM Para4	*T. cinerariifolium*	Australia
Pestalotiopsis sp.	UM PL UM PP	*T. cinerariifolium*	Australia
Sclerotinia minor	TAS 022–0075 TAS 022–0079	*T. cinerariifolium*	Australia
Sclerotinia sclerotiorum	TAS 021–0399	*T. cinerariifolium*	Australia
Stagonosporopsis actaeae	**CBS 106.96; PD 94/1318***	*Actaea spicata*	Netherlands
S. ajacis	**CBS 177.93; PD 90/115***	*Delphinium* sp.	Kenya
S. andigena	CBS 101.80; PD 75/909; IMI 386090	*Solanum* sp.	Peru
S. artemisiicola	CBS 102636; PD 73/1409	*Artemisia dracunculus*	France
S. astragali	CBS 178.25; MUCL 9915	*Astragalus* sp.	Unknown
S. caricae	CBS 248.90	*Carica papaya*	Chile
S. chrysanthemi	ATCC 10748	*Chrysanthemum morifolium*	USA
	CBS 500.63; MUCL 8090	*C. indicum*	Netherlands
	DSMZ 62547	*Chrysanthemum* sp.	Germany
	DSMZ 63133	*C. indicum*	Germany
	ICMP 10673	*Chrysanthemum* sp.	New Zealand
	ICMP 2287	*Chrysanthemum* sp.	New Zealand
S. citrulli	GA8005	*Citrullus* sp.	USA
S. crystalliniformis	**CBS 713.85; ATCC 76027; PD 83/826***	*Lycopersicon esculentum*	Colombia
S. cucurbitacearum	CBS 133.96; PD 79/127	*Cucurbita* sp.	New Zealand
S. dennisii	CBS 631.68; PD 68/147	*Solidago floribunda*	Netherlands
S. dorenboschii	CBS 426.90; IMI 386093; PD 86/551	*Physostegia virginiana*	Netherlands
S. heliopsidis	CBS 109182; PD 74/231	*Heliopsis patula*	Netherlands
S. hortensis	CBS 104.42	Unknown	Netherlands
S. loticola	**CBS 562.81; ICMP 6884 ***	*Lotus pedunculatus*	New Zealand
S. lupini	CBS 101494; PD 98/5247	*Lupinus albus*	UK
S. oculo-hominis	**CBS 634.92; IMI 193307***	Human	USA

Table 2 (continued)

Species	Strain number[a]	Substrate	Country
S. inoxydabilis	**CBS 425.90; PD 81/520***	*C. parthenii*	Netherlands
	PD 85/259	*Matricaria* sp.	Netherlands
	PD 90/2040	*Matricaria* sp.	
S. rudbeckiae	CBS 109180; PD 79/175	*Rudbeckia bicolor*	Netherlands
S. tanaceti	**CBS 131484; TAS1***	*T. cinerariifolium*	Australia
	DAR 70020		
	TAS 55503		
	UM 011–0001		
	UM 011–0063		
	TAS 011–0021		
	TAS F63810B		
S. trachelii	CBS 379.9; PD 77/675	*Campanula isophylla*	Netherlands
S. valerianellae	CBS 329.67; PD 66/302	*Valerianella locusta* var. *oleracea*	Netherlands
Stemphylium sp.	TAS 100–0010	*T. cinerariifolium*	Australia
	TAS 100–0018		
Ulocladium sp.	TAS 130–0003	*T. cinerariifolium*	Australia
	TAS 130–0004		

[a] ATCC: American Type Culture Collection; BRIP: Queensland Department of Agriculture and Fisheries Plant Pathology Herbarium; CBS: Centraalbureau voor Schimmelcultures; New South Wales Plant Pathology Herbarium, NSW, Australia; DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen; ICMP: International Collection of Micro-organisms from Plants; IMI: International Mycological Institute; MUCL: Mycotheque de l'Universite catholique de Louvain; PD: Plant Protection Service; TAS: Tasmanian Institute of Agriculture Fungal Collection; UM: University of Melbourne Fungal Collection

rDNA, was targeted for assay development. Primer3 (Rozen and Skaletsky 1999) was used to design one forward primer common to the three species (StagFd1) and three species-specific reverse primers (ScSR2 for *S. chrysanthemi*, SiSR3 for *S. inoxydabilis* and StSR03 for *S. tanaceti*) that amplified specific-sized amplicons

Table 3 Primers used for sequencing the entire IGS region in *Stagonosporopsis chrysanthemi*, *S. inoxydabilis* and *S. tanaceti*

Primer	Sequence (5′ to 3′)	Species	No.[a]	Target	Reference
LR12R	GAA CGC CTC TAA GTC AGA ATC C	Universal primer	1	IGS amplification	Vilgalys lab[b]
invSR1R	ACT GGC AGA ATC AAC CAG GTA	Universal primer	7	IGS amplification	Vilgalys lab[b]
StagIGSF02	CAC CTT CCT AAA TAG GCA AG	*S. chrysanthemi*, *S. inoxydabilis* and *S. tanaceti*	2	IGS Sequencing	This study
inoIGSF1	GAA GCG CCC TAA CAT AGC AG	*S. inoxydabilis*	9	IGS Sequencing	This study
inoIGSF3	GCT AGC AAG CGT TTA GTG GAT T	*S. inoxydabilis*	10	IGS Sequencing	This study
StagIGSR1	GCC TGC CTG CTC TTC TCA TA	*S. chrysanthemi*, *S. inoxydabilis* and *S. tanaceti*	6	IGS Sequencing	This study
StagIGSR2P3	TTT GGC AAA CCT TCT ACA ATT T	*S. tanaceti*	5	IGS Sequencing	This study
StagFd1	TGC ARA GTA CAM GGC AGA GG	*S. chrysanthemi*, *S. inoxydabilis* and *S. tanaceti*	3	Diagnostic assay	This study
ScSR2	CCATTGATTAACGATACCTCGAC	Specific to *S. chrysanthemi*	8	Diagnostic assay	This study
SiSR3	GGC ACG CAC AAT AAA GAG TG	Specific to *S. inoxydabilis*	11	Diagnostic assay	This study
StSR03	TAC CCT CAC CTT TAG GGG AAA T	Specific to *S. tanaceti*	4	Diagnostic assay	This study

[a] The number assigned to the primer in Fig 1

[b] http://biology.duke.edu/fungi/mycolab/primers.htm

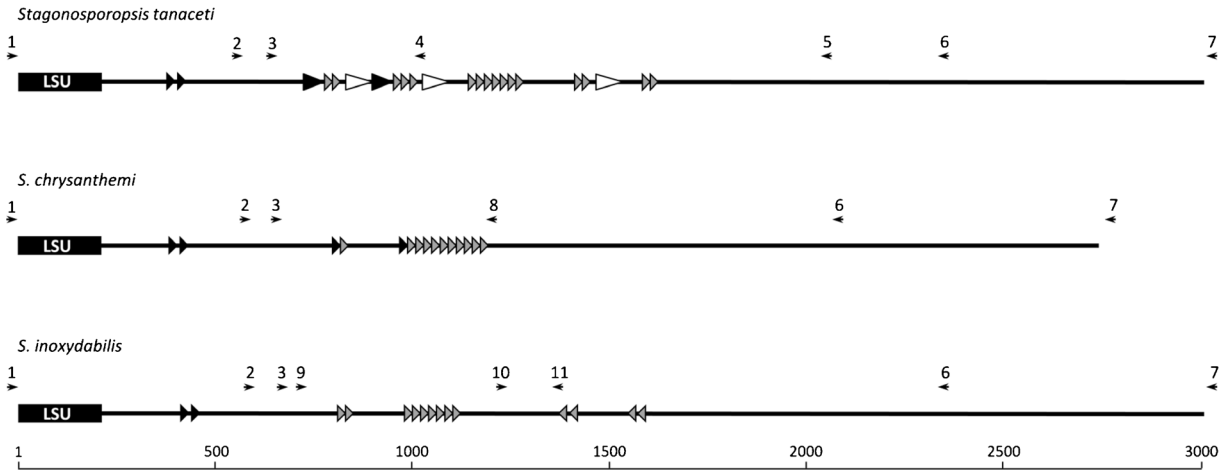

Fig. 1 Schematic presentation of the partial ribosomal DNA large subunit (LSU) and the entire Intergenic Spacer (IGS) sequence in *Stagonosporopsis tanaceti*, *S. chrysanthemi* and *S. inoxydabilis*. Position and orientation of primers used for amplifying and sequencing the IGS as well as the species-specific primers are indicated by arrows bearing the primer numbers specified in Table 3. The position and orientation of the repeat units are indicated by *black*, white and *grey* triangles in each species. Distances and lengths of repeat units are to scale. Approximate nucleotide positions are indicated by scale at the top of the figure. The repeat unit sequences are 5′ – GCR TTA GTA GGT TGB GRM R – 3′ (*small black*), 5′ – GCR TTA GTA GGT TGY GRC R – 3′(*grey*), 5′ – GTR GAG CCC CTA GCT TTG GCG RGT ACC GCC CAA AGG KTT TGR GRG GTC ASG G – 3′ (*large black*) and 5′ – GGG GGG TAG RCG CCY KRG CWT AGG GGC TCG ACY GCC TGY ACT AAG CGA GCA KAC CGC CTA GAG TTR GGG G – 3′ (white) in *S. tanaceti*; 5′ – GCG TTA GTA GGT TGS GGS R-3′ (*black*) and 5′ – GCR TTM GTR GGY TGY GRC R – 3′ (*grey*) in *S. chrysanthemi*; 5′ – GCG TTA GTA GGT TGG GGM R – 3′ (*black*) and 5′ – GCR TYA GYA RGT TGH RRY A – 5′ (*grey*) in *S. inoxydabilis*

when paired with the forward primer (Fig. 1). The designed primers were checked for specificity to NCBI GenBank database to minimise the chance of non-specific priming, and were synthesised by Sigma-Aldrich, New South Wales, Australia.

Each of the species-specific primer pairs were tested in separate conventional PCR reactions for amplification in a range of temperatures with variable primer and $MgCl_2$ concentrations, and specificity against the DNA of the other two *Stagonosporopsis* species. PCR mixtures contained 10 ng genomic DNA, MangoTaq reaction buffer (Bioline), 1 or 2 mM $MgCl_2$, 0.1 or 0.2 μM of each primer, 0.1 mM dNTPs (Bioline) and 0.7 unit MangoTaq DNA polymerase (Bioline). The PCR cycle included 3 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at a range of annealing temperatures (58 to 63 °C), zero or 30 s at 72 °C, and a final extension of 3 min at 72 °C. Each series of amplification reactions included sterile MilliQ water as a negative control as well as genomic DNA from the reference strains of *S. chrysanthemi* (CBS 500.63), *S. inoxydabilis* (CBS 425.90) and *S. tanaceti* (CBS 131484) as positive and negative controls. PCR reactions were conducted in a MyCycler thermal cycler (Bio-Rad, Australia) and the products were visualised on ethidium bromide-stained 1 % agarose gels. The optimised PCR assay for the three species-specific primer pairs compromised 15 μL reactions containing 10 ng genomic DNA, MangoTaq reaction buffer (Bioline), 1 mM $MgCl_2$, 0.1 μM of each primer, 0.1 mM dNTPs (Bioline) and 0.7 unit MangoTaq DNA polymerase (Bioline). The PCR cycle included 3 min at 95 °C, followed by 35 cycles of a two-step amplification (30 s at 95 °C and 30 s at 60 °C), and a final extension of 3 min at 72 °C.

After ensuring the specificity of each primer pair for the target species at a range of temperatures, multiplex PCR reactions were carried out using a multiplex PCR kit (Qiagen, Australia). PCR mixtures included 0.8 or 1 × multiplex buffer (Qiagen), 0.3 or 0.6 μM of the forward primer (StagFd1), 0.1 or 0.2 μM of each reverse primer (StSR03, SiSR3 and ScSR2) and 10 ng genomic DNA. The PCR cycle included 5 min at 95 °C, followed by 30 cycles of 30 s at 95 °C, 30 s at a range of annealing temperatures (58 to 63 °C), 30 or 90 s at 72 °C, and a final extension of 5 min at 68 °C. PCR reactions were conducted in a MyCycler thermal cycler (Bio-Rad) and the products were visualised on ethidium bromide-stained 1 % agarose gels. The optimised multiplex PCR assay compromised 15 μL reactions of 0.8 × multiplex buffer (Qiagen), 0.3 μM of the forward primer

(StagFd1), 0.1 μM of each reverse primer (ScSR2, SiSR3 and StSR03) and 10 ng genomic DNA. The PCR cycle was 5 min at 95 °C, followed by 30 cycles of 30 s at 95 °C, 30 s 60 °C, 30 s at 72 °C, and a final extension of 5 min at 68 °C.

The optimised multiplex PCR assay was further employed to verify the identity of 407 *S. tanaceti* isolates collected from commercial pyrethrum fields along the northern coast of Tasmania, Australia (Vaghefi et al. 2015b). Also, five additional *S. chrysanthemi* isolates and two additional *S. inoxydabilis* strains imported from international culture collections (Table 2) were screened using the multiplex PCR assay to ascertain the absence of intra-specific length variation in the PCR products.

Validation of the species-specific PCR assays

The conventional uniplex and multiplex PCR assays were tested for specificity, sensitivity, repeatability and reproducibility; the key performance criteria outlined by the OEPP/EPPO guideline (EPPO 2010) for validation of diagnostic assays. Specificity of each assay was tested against 10 ng genomic DNA from 21 *Stagonosporopsis* spp. as well as 14 fungal species associated with pyrethrum in Australia (Table 2), using PCR conditions described above, including 1 mM $MgCl_2$, 0.1 μM of each primer, and an annealing temperature of 60 °C. To ensure that the DNA samples may be amplified by PCR, all samples were tested in an ITS PCR containing 10 ng genomic DNA, MangoTaq reaction buffer (Bioline), 2 mM $MgCl_2$, 0.2 μM of each primer (V9G: 5′ – TTA CGT CCC TGC CCT TTG TA – 3′ and ITS4: 5′ – TCC TCC GCT TAT TGA TAT GC – 3′) (De Hoog and Gerrits van den Ende 1998; White et al. 1990), 0.1 mM of each dNTP (Bioline) and 0.5 U MangoTaq DNA polymerase (Bioline). Conditions for amplification included an initial denaturation step of 5 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 52 °C and 30 s at 72 °C, and a final denaturation step of 7 min at 72 °C. PCR reactions were visualised on ethidium bromide-stained 1 % agarose gels.

Sensitivity of the PCR assays was determined using two-fold serial dilutions of genomic DNA of *S. chrysanthemi* (CBS 500.63 and ICMP 2287), *S. inoxydabilis* (CBS 425.90) and *S. tanaceti* (CBS 131484 and DAR 70020) in sterile Type I water. The initial concentration of the DNA samples was determined using a Qubit fluorometer and a dsDNA BR Assay Kit (Life Technologies, Grand Island, NY, USA). Each of the PCR assays was conducted on a total of 8 ng to 4 fg genomic DNA to quantify the detection limit of each assay. A similar dilution series was prepared using a mixture of the DNA samples for testing the detection limit of the multiplex PCR assay.

Repeatability of the assays was tested by conducting the PCRs again under the same conditions and using the same reagents and PCR equipment as above. Reproducibility of the assays was investigated by performing the PCRs with minor variations, i.e., using different PCR reagents and equipment in a different laboratory. PCR reactions contained 10 ng genomic DNA, Standard PCR buffer (New England Biolabs Inc., Ipswich, MA, USA), 1 mM $MgCl_2$ (New England Biolabs Inc.), 0.1 μM of each primer (synthesized by Integrated DNA Technologies Inc., CA, USA), 0.1 mM dNTPs (New England Biolabs Inc.), and 1 unit Taq polymerase (New England Biolabs Inc.). The PCR cycle was 3 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 60 °C, 30 s at 68 °C, and a final extension of 3 min at 68 °C. Each series of amplification reactions included sterile Type I water as a negative control. PCR reactions were conducted in a T100 thermal cycler (Bio-Rad, Hercules, CA, USA) and the products were visualised on a 1 % agarose gel amended with 0.5× (*v*/v) nucleic acid stain GelRed (Biotium Inc., Hayward, CA, USA). The multiplex assay was also performed as above, using 0.3 μM of the forward primer and 0.1 μM of each of the reverse primers.

Results

Pathogenicity assay

Stagonosporopsis chrysanthemi and *S. inoxydabilis* were pathogenic to pyrethrum and caused symptoms similar to *S. tanaceti*. Pyrethrum plants inoculated with *S. inoxydabilis* (CBS 425.90) and *S. tanaceti* (CBS 131484) developed symptoms after three to four days. The initial symptoms included small necrotic spots on the leaves. Plants inoculated with *S. chrysanthemi* (CBS 500.63) developed necrotic spots seven days after inoculation (Fig. 2). Subsequently, leaf spots expanded to larger lesions, which eventually killed the leaves and spread into the petioles. Two weeks after inoculation, plants started to develop necrosis at the base (crown) and white aerial mycelia were visible on most

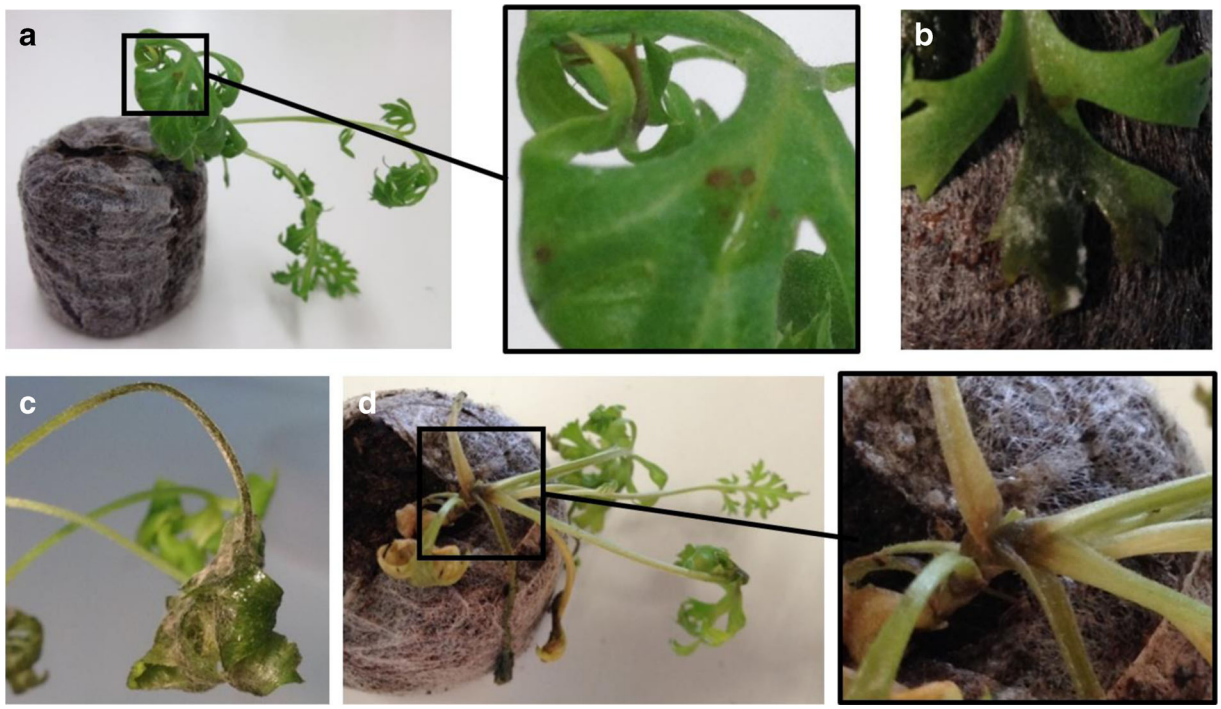

Fig. 2 Pathogenicity of *Stagonosporopsis chrysanthemi* to pyrethrum in an in vitro inoculation trial; **a** necrotic spots on leaves one week after inoculation (wai), **b** necrotic lesions and dieback, **c** death of leaves and spread of necrosis into the petiole **d** necrosis in the crown area at 2 wai

symptomatic leaves. All plants collapsed completely after three weeks. The three *Stagonosporopsis* species developed reproductive structures on diseased pyrethrum tissue (Fig. 3). Both pycnidia and pseudothecia were formed on plants inoculated with *S. chrysanthemi* and *S. inoxydabilis* while only pycnidia developed on the *S. tanaceti*-inoculated plants (Fig. 3). Isolation frequency of the *Stagonosporopsis* spp. from infected tissue was 100 %, confirming that the lesions were caused by the intended fungal species.

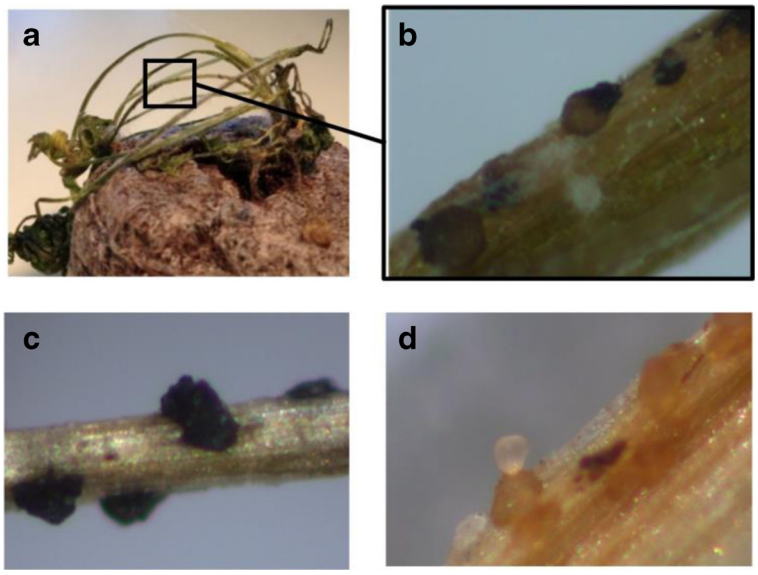

Fig. 3 Pycnidia and pseudothecia development on pyrethrum plants inoculated with *Stagonosporopsis* spp.: **a** death of pyrethrum plants three weeks after inoculation with *Stagonosporopsis chrysanthemi*; **b** pseudothecia of *S. chrysanthemi*; **c** pseudothecia of *S. inoxydabilis*; and **d** pycnidia of *S. tanaceti* on pyrethrum petiole

Species-specific PCR assay development

IGS amplification and sequencing

Sequencing of the entire IGS region resulted in a fragment of ~2.7 kb in *Stagonosporopsis chrysanthemi* and ~3.0 kb in *S. tanaceti* and *S. inoxydabilis*. The IGS sequences were identical among the seven isolates of *S. tanaceti*, and between the two *S. chrysanthemi* isolates. Alignment of the *S. tanaceti* IGS with *S. chrysanthemi* and *S. inoxydabilis* detected 78 % and 75 % sequence similarity, respectively. Sequences were deposited in the NCBI database (GenBank accession numbers KP161042 to KP161044).

Qualitative PCR assay development

The uniplex PCR assays amplified species-specific sized amplicons at a range of temperatures (Supplementary Fig. 1). Primers StagFd1 and StSR03 resulted in a ~ 400 bp band in *S. tanaceti* and no amplicon in either *S. inoxydabilis* or *S. chrysanthemi* over a range of annealing temperatures from 58 to 63 °C. Primers StagFd1 and SiSR3 amplified a band of ~630 bp in *S. inoxydabilis* and no band in either *S. chrysanthemi* or *S. tanaceti* over a range of annealing temperatures from 58 to 63 °C. Primers StagFd1 and ScSR2 resulted in a ~ 560 bp amplicon in *S. chrysanthemi* and no amplification in either *S. inoxydabilis* or *S. tanaceti* at 60 to 63 °C (Supplementary Fig. 1). The multiplex PCR assay produced single intense species-specific amplicons when DNA of only one species was included in the reaction; ~ 560 in *S. chrysanthemi*, ~ 630 bp in *S. inoxydabilis* and ~400 bp in *S. tanaceti*. All three amplicons were amplified when DNA of the three species were used in one reaction (Fig. 4).

Application of the multiplex PCR assay to screen 407 *Stagonosporopsis* isolates collected from pyrethrum fields in Tasmania detected a single ~400 bp amplicon for all the isolates, verifying their identity as *S. tanaceti*, and confirming lack of intra-specific length variation (*data not shown*). No intra-specific length variation was found among the amplicons from the six *S. chrysanthemi* or the three *S. inoxydabilis* isolates tested (Figs. 5 and 6).

Validation of the species-specific PCR assays

All PCR assays were specific to their target species. ITS amplification from the gDNA of the 14 fungal species

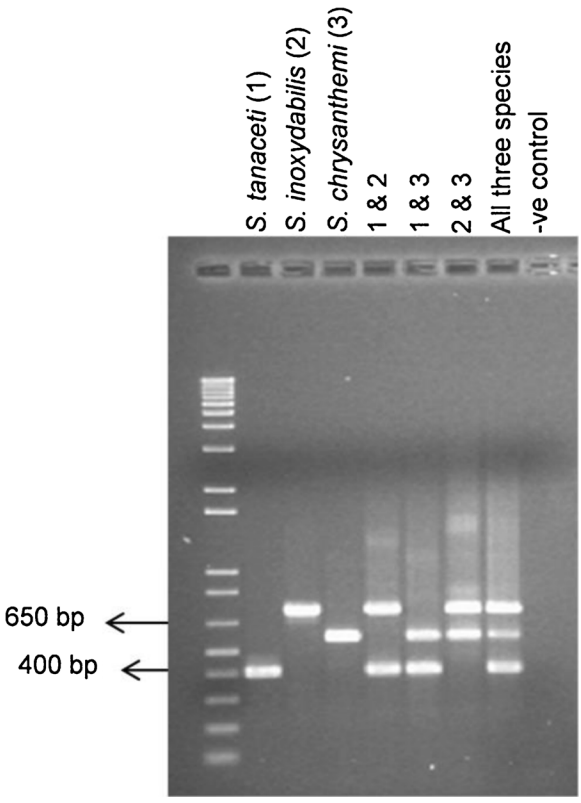

Fig. 4 Multiplex PCR assay enabling identification and differentiation of *Stagonosporopsis tanaceti*, *S. chrysanthemi* and *S. inoxydabilis* in one reaction. The first lane represents the 1Kb Plus DNA ladder (Life Technologies, Australia)

associated with pyrethrum, as well as the imported DNA samples of the *Stagonosporopsis* spp., demonstrated that all the DNA samples were PCR-amplifiable (*data not shown*). No amplification resulted from the same DNA samples when used in the uniplex and multiplex species-specific assays (Figs. 5 and 6; Supplementary Fig. 2). Species-specific PCR assays using 2-fold dilution series of DNA samples from the target species detected DNA quantities as low as 4 fg (Supplementary Fig. 3). All assays were repeatable and reproducible, and the same results were obtained when repeating the assays under the same conditions, and when performing the assays in a different laboratory with changed PCR reagents, thermocycler and oligonucleotide supplier.

Discussion

Stagonosporopsis chrysanthemi and *S. inoxydabilis* were able to infect and cause disease on pyrethrum, with

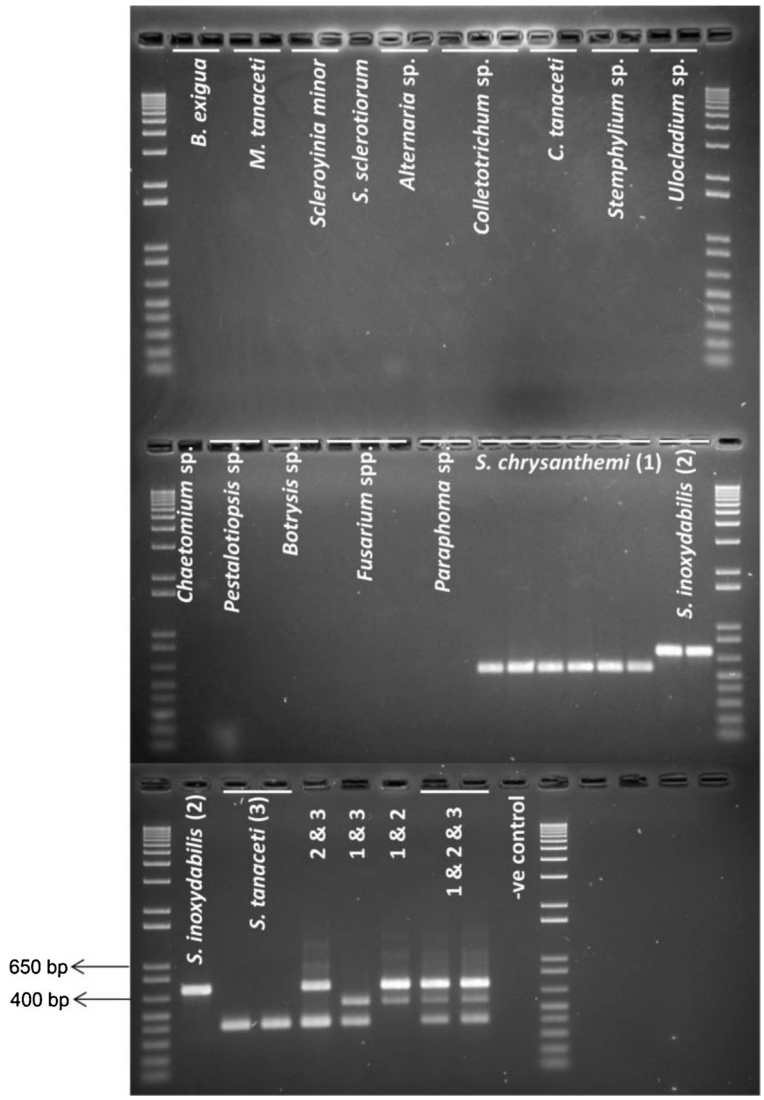

Fig. 5 Specificity test of the multiplex PCR assay using the DNA samples from pure cultures of 14 fungal species associated with pyrethrum in Australia. The first and last lanes in each gel are the 1Kb Plus DNA ladder (Life Technologies, Australia)

similar symptoms to those caused by *S. tanaceti*, resulting in plant death in only three weeks. This is the first report of *S. chrysanthemi* causing disease on pyrethrum. Due to quarantine restrictions, the pathogenicity trial was conducted in closed plastic containers in an incubator, creating a humid environment highly conducive to disease development, which may have accelerated the rate of symptom development and plant death. The timing of infection and severity of the symptoms may be different in a glasshouse assay or under field conditions. Further trials may provide more information on the timing and severity of infection in different hosts and environments.

A highly specific and sensitive multiplex PCR assay for the rapid and accurate detection and identification of the three quarantine *Stagonosporopsis* spp.; S. *tanaceti*, *S. inoxydabilis* and *S. chrysanthemi*, was developed from the IGS region of the nrDNA gene complex. Identification of *Stagonosporopsis* spp. based on morphological characteristics is time consuming, requires a high level of experience and expertise, and may not even be possible due to the high similarity of some phylogenetically-close *Stagonosporopsis* species as well as variability of morphological characters in vitro (EFSA PLH Panel 2013; Stewart et al. 2015; Vaghefi et al. 2012). This has resulted in the recent development of molecular methods for detection and identification of several important phytopathogenic *Stagonosporopsis* spp., using microsatellite loci and ITS and actin sequences (Brewer et al. 2015; De Gruyter et al. 2012;

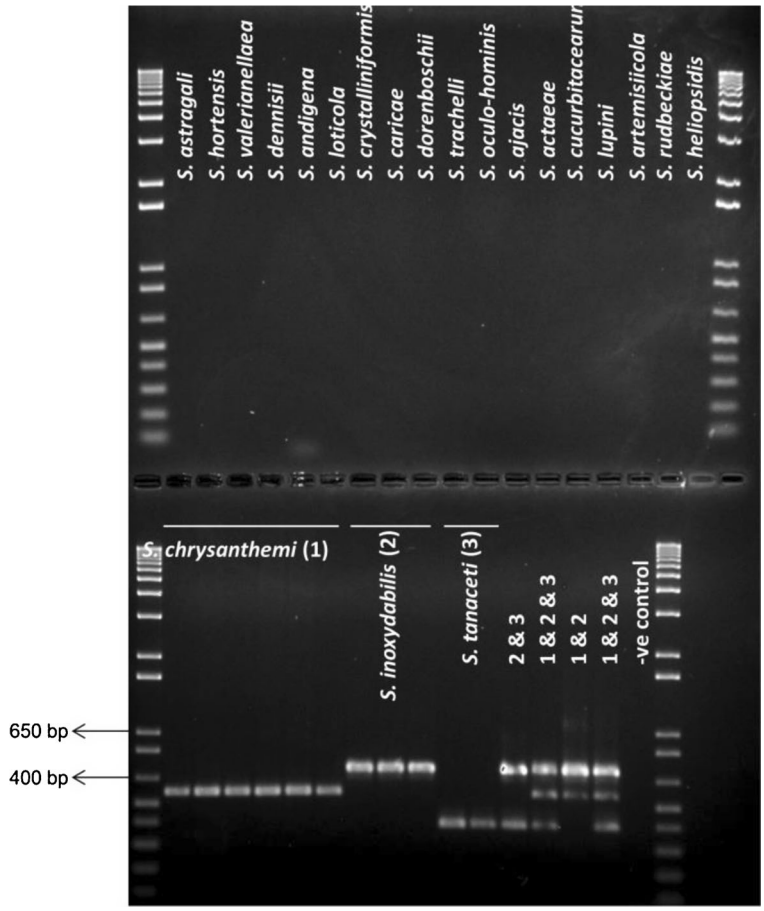

Fig. 6 Specificity test of the multiplex PCR assay against the DNA samples from pure cultures of *Stagonosporopsis* spp. The first and last lanes in each gel represent the 1Kb Plus DNA ladder (Life Technologies, Australia)

Pethybridge et al. 2004). The actin sequence could not be used for PCR-based differentiation of the three *Stagonosporopsis* spp. studied here due to the limited number of consecutive nucleotide differences, which resulted in cross-amplification at lower temperatures (*data not shown*). We targeted the IGS region of the nrDNA gene complex for species-specific marker development due to its high level of variability as well as high copy number, which enables development of highly specific and sensitive diagnostic assays (Chilvers et al. 2007; Liew et al. 1998; Muller et al. 2013; Sampietro et al. 2010; Suarez et al. 2005).

Although multiple copies of the IGS are known to evolve in a homogenous manner through concerted evolution (Nei and Rooney 2005), in some fungal species, intra-specific variation of the IGS has been reported (Appel and Gordon 1996; Dissanayake et al. 2009; Kawabe et al. 2005; Latha et al. 2003; Srinivasan et al. 2011). Sequencing the entire IGS region of seven *S. tanaceti* isolates and two *S. chrysanthemi* isolates detected no intra-specific nucleotide variation in these species. Another characteristic of the IGS sequence in Eukaryotes is the presence of repetitive elements (sub-repeat units) (Mirete et al. 2013; Pantou et al. 2003; Wang et al. 2012), which may differ in the copy number, resulting in the IGS size variability at an intra-specific (Jackson et al. 2000) or intra-genomic (Chang et al. 2008; Ganley and Scott 1998) level. Although the IGS sequences of *S. chrysanthemi*, *S. inoxydabilis* and *S. tanaceti* had a repetitive structure (Fig. 1), no intra-specific length variation was detected among the PCR amplicons from the six *S. chrysanthemi* isolates, three *S. inoxydabilis* isolates, or 407 *S. tanaceti* isolates tested in this study. This verified the suitability of the selected IGS region as a PCR target for detection and differentiation of the three ray blight pathogens.

The species-specific diagnostic PCR assays were highly specific, sensitive, repeatable and reproducible when tested against the DNA of pure fungal cultures. The multiplex PCR assay will provide a valuable tool

for disease surveillance in Australian pyrethrum field, and may be used for screening of the *Stagonosporopsis* isolates collected from pyrethrum fields to enable timely detection of possible *S. chrysanthemi* or *S.inoxydabilis* incursions. Moreover, the *S. tanaceti*-specific assay may provide a rapid, reliable and sensitive method for pathogen detection in pyrethrum seed as infected seed is known to be a source of primary inoculum for ray blight epidemics in pyrethrum fields in Australia (Pethybridge et al. 2006). This application, however, requires *in planta* validation of the assay.

Once validated *in planta*, the developed assays may be further utilised for quarantine purposes in Australia, where *S. chrysanthemi* and *S. inoxydabilis* are not known to occur, or in Europe, where the three *Stagonosporopsis* spp. associated with ray blight of *Asteraceae* are listed as Level 2 quarantine pathogens (EPPO 2015). Current quarantine measures for importation of chrysanthemum propagative material to Australia and Europe are solely based on phytosanitary certificates, visual inspection of plant material and cultural and morphological identification of pathogen cultures (BICON 2016; EFSA PLH Panel 2013). The diagnostics assays may be incorporated into the AQIS or EPPO biosecurity processes to provide standard guidelines for rapid and accurate pathogen identification. This is of special significance for *in planta* detection of *S. chrysanthemi* or possible soil tests, as *S. chrysanthemi* is reported to be asymptomatically present in chrysanthemum cuttings (Baker et al. 1961; Chesters and Blakeman 1966; EFSA PLH Panel 2013) or as sclerotia in soil (Blakeman and Hornby 1966). Seedborne *S. tanaceti* inoculum infecting the germinating hypocotyls may also remain latent in pyrethrum crown tissue for weeks prior to the appearance of symptoms (P.W.J. Taylor, unpublished data). Sensitivity tests of the developed PCR assays for pathogen detection in symptomatic and asymptomatic plant tissue as well as infested seed or soil will further expand applications for biosecurity and quarantine purposes.

Acknowledgments We would like to thank Dr. Marin Talbot Brewer, University of Georgia, USA, for kindly providing DNA of *Stagonosporopsis citrulli*. We are also grateful to Ms. Azin Moslemi, University of Melbourne, Australia, for providing cultures of *Fusarium* spp. This project was supported by Botanical Resources Australia-Agricultural Services Pty. Ltd. The first author gratefully acknowledges the financial support from the Melbourne International Research Scholarship (MIRS) and Melbourne International Fee Remission Scholarship (MIFRS) awarded by the University of Melbourne.

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