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The development of a validated real-time (TaqMan) PCR for detection of *Stagonosporopsis andigena* and *S. crystalliniformis* in infected leaves of potato and tomato

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Abstract *Stagonosporopsis andigena* and *S. crystalliniformis* are serious foliage pathogens on potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicum*). As both species have been recorded only in the Andes area, *S. andigena* is listed as an A1 quarantine organism in Europe. The actin region of isolates of *Stagonosporopsis* and allied species of *Boeremia*, *Didymella*, *Peyronellaea* and *Phoma* was amplified using generic primers. DNA sequence differences of the actin gene were utilised to develop species-specific real-time (TaqMan) PCR assays for the detection of *S. andigena* and *S. crystalliniformis* in leaves of potato or tomato. The specificity of the TaqMan PCR assays was determined on genomic DNA extracted from two *S. andigena* and two *S. crystalliniformis* isolates and 16 selected isolates of *Stagonosporopsis*, *Phoma* and *Boeremia*, which are the closest relatives. The validation of the methods developed included the DNA extraction and the TaqMan PCR assays. The performance criteria specificity, analytical sensitivity, reproducibility, repeatability and robustness of the TaqMan PCR assays demonstrated the reliability of both methods for the detection of *S. andigena* and *S. crystalliniformis* in leaf material. The TaqMan PCR assays were tested on symptomatic leaves of potato and tomato that were obtained after artificial inoculation of detached leaves with both pathogens under quarantine conditions. In the artificial inoculation experiments both *S. andigena* and *S. crystalliniformis* caused leaf infections on potato and tomato.

Keywords Actin · *Boeremia* · Molecular phylogeny · *Peyronellaea* · *Phoma*

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Introduction

The molecular phylogeny of the genus *Phoma* has been studied intensively at the National Plant Protection Organization (Plantenziektenkundige Dienst, PD) and CBS-KNAW Fungal Biodiversity Centre in the Netherlands during the past few years to clarify the taxonomy of the genus and to provide tools for the development of molecular detection and identification methods. Results of these studies demonstrated that the taxonomy based on morphological characters does not reflect the molecular phylogeny, and therefore, many species described in *Phoma* have been reclassified in other genera (Aveskamp et al. 2009a, b, 2010; de Gruyter et al. 2009, 2010). Several of these recently introduced genera are closely related to *Phoma*, including *Boeremia*, *Peyronellaea* and *Stagonosporopsis* (Aveskamp et al. 2009a, b, 2010). These represent many destructive pathogens that occur in economically important crops. Several species that pose a threat in important food crops have been recorded only in restricted regions and are therefore listed on quarantine lists of countries in other parts of the world. However, other *Phoma*-like pathogens have already been spread more widely, such as *Boeremia foveata*, syn. *Phoma foveata*, the cause of gangrene on potatoes. This species probably originates from the Andes region (Otazú et al. 1979), and it is likely that this pathogen was spread by infected seed potatoes in the past. The international trade of plant material continuously extends, and to reduce the risk of introducing harmful pathogens, fast, reliable and validated molecular detection and identification methods are needed.

This paper deals with two *Stagonosporopsis* species that have been recently excluded from *Phoma*: *S. andigena* and *S. crystalliniformis* (Aveskamp et al. 2010). Both species have been respectively recorded as serious pathogens on potato and tomato in the Andes region. *Stagonosporopsis andigena* was originally described as *Phoma andina* (Turkensteen 1978), as the cause of 'black potato blight' and leaf spots on potato plants in Peru and Bolivia. *Stagonosporopsis crystalliniformis* was initially found as a new disease, locally known as 'carate' on tomato in Colombia (Navarro and Puerta 1981). The pathogen causes necrotic spots on all aerial parts of the plant and was reported as very destructive. A total plant necrosis and mummification of the fruits may occur. Later, the fungus was found on dying potato stems in Venezuela in 1980, and isolates were frequently obtained from potato leaf spots in Colombia (Loerakker et al. 1986). The pathogen was originally described as a variety of *Phoma andina,* namely *P. andina* var. *crystalliniformis* (Loerakker et al. 1986). In a morphological study dealing with *Phoma* species that produce characteristic dendrite crystals in pure culture, it was concluded that both *P. andina* varieties could be recognised at species level as *P. andina* and *P. crystalliniformis* (Noordeloos et al. 1993). However, the name *P. andina* appeared to be a homonym and therefore, *P. andina* was renamed as *P. andigena* (Boerema et al. 1995). Both species have not been reported outside the Andes region so far. The European Plant Protection Organization (OEPP/EPPO) lists *S. andigena* as an A1 quarantine organism (OEPP/EPPO 1984). The cultivation of potatoes and tomatoes is of significant importance in the Netherlands and, therefore, we developed a specific real-time PCR assay based on TaqMan® technology for the detection of both organisms to be applied in case suspected samples need to be investigated.

Phylogenetic studies in *Phoma* revealed that actin sequences provide a higher resolution for the delimitation of allied species compared to ITS sequences (Aveskamp et al. 2009b). Actin has also been used for the development of PCR methods for detection of pathogens that belong to other important genera, such as *Mycosphaerella* (Arzanlou et al. 2009) and *Cercospora* (Lartey et al. 2003).

The aim of this study was to develop TaqMan PCR assays for the direct detection of *Stagonosporopsis andigena* and *S. crystalliniformis* in leaves of potato or tomato. Both assays developed were based on DNA sequence differences of the actin gene. The validation of both assays developed included DNA extraction and TaqMan PCR of spiked samples. The results of the performance criteria specificity, analytical sensitivity, reproducibility, repeatability and robustness are provided. Both PCR assays were tested on symptomatic leaves of potato and tomato, which were obtained after artificial inoculation of detached leaves with both pathogens under quarantine conditions.

Material and methods

Fungal isolates and plant material

The *Stagonosporopsis* isolates and additional isolates of *Boeremia*, *Didymella*, *Peyronellaea* and *Phoma* species that were included in this study were obtained

from the culture collections of CBS, DAOM and PD (Table 1). The isolates were selected as being the most closely related species to *S. andigena* and *S. crystalliniformis* based on a multigene analysis of parts of 28S nrDNA (Large Subunit—LSU), ITS and β-tubulin (Aveskamp et al. 2010). Additional isolates, which were formerly classified in *Phoma* and obtained from potato or tomato were added as well as some related species, which had been isolated from other crops in the Andes region in the past. The freeze-dried isolates were revived overnight in 2 ml malt/peptone (50 %/50 %) liquid medium and subsequently transferred and maintained on oatmeal agar (OA) (Crous et al. 2009). The isolates which were stored at −196 °C were directly transferred on OA. The plants used in this study were the potato cultivars 'Bintje', 'Bionica' and 'Berthaultii' and the tomato cultivars 'Moneymaker', 'Microtom' and 'Heinz'.

DNA extractions

Genomic DNA isolation from fungal cultures was performed using the Ultraclean Microbial DNA isolation kit (Mo Bio Laboratories, Carlsbad, California, USA) according to the instructions of the manufacturer. All DNA extracts were diluted 10× in milliQ water and stored at 4 °C before use. Leaf plugs, 0.9 cm diam, which were taken from approximate 6 weeks old plants, were transferred to 1.5 ml microcentrifuge tubes with caps (QIAGEN Benelux bv, Venlo, The Netherlands), one per tube, and stored at −20 °C. For DNA extraction, two stainless steel beads (3.2 mm diam) and 300 µl lysis buffer with 0.2 µl RNase (8 mg/ml) (Sbeadex® maxi plant kit, LGC Genomics, Berlin, Germany) were added. The tubes were shaken in a 96 rack in a beat mill (Mixer Mill MM300; Retsch GmbH, Haan, Germany) for 30 s on 30 r/s speed. The orientation of the rack was changed and the mixer mill was switched on a second time. After 30 min incubation at 65 °C in a water bath the tubes were centrifuged for 5 min at 3,000×*g*. The DNA isolation was performed on 50 µl supernatant using the KingFisher 96 magnetic particle processor (Thermo Electron Corporation, Breda, The Netherlands) with the reagents of the Sbeadex® maxi plant kit according to the manufacturer's instructions. A negative control, which consisted of healthy leaf material, was included. A 0.9 cm diam leaf disk was spiked with mycelium scraped from one 0.9 cm diam agar plug to make spiked samples.

Actin amplifications and sequencing

The actin region was amplified using the primer pair ACT-512F/ACT-783R designed by Carbone and Kohn (1999) (Table 2). The amplification reactions were performed as described by Aveskamp et al. (2009b). Consensus sequences were computed from forward and reverse sequences using the BioNumerics v4.60 software package (Applied Maths, Sint-Martens-Latem, Belgium) and were deposited in GenBank (Table 1).

Phylogenetic analyses

The obtained sequence data were aligned using the MAFFT multiple sequence alignment programme (Katoh et al. 2009). The phylogeny was rooted to *Phoma dimorphospora*, strain CBS 345.78. A Bayesian analysis was conducted with the MrBayes v3.1.2 programme (Huelsenbeck and Ronqvist 2001) using the default settings but with the following adjustments: GTR model with gamma-distributed rate variation in two parallel runs, model selected using Findmodel (http://hcv.lanl.gov/content/hcvdb/findmodel/findmodel.html), and an MCMC heated chain with a "temperature" value of 0.05. The number of generations, sample frequencies and burn-in ratio were set at 5 M, 10 and 0.05, respectively and the run was automatically stopped as soon as the average standard deviation of split frequencies equalled 0.01. The resulting tree was printed with TreeView v1.6.6 (Page 1996) and alignments and the tree was deposited in TreeBASE (www.treebase.org).

Design of the PCR primers and probes to detect *Stagonosporopsis andigena* and *S. crystalliniformis*

The actin sequences included in the phylogenetic study were aligned with MegAlign Software (DNA Star Inc., Madison, WI, USA). The primer pairs and probes were designed using Primer Express® software v3.0 (Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands). The primer-probe combinations were blasted against the NCBI GenBank to minimise the likelihood of non-specific detection. The probes were 5′-end labelled with FAM (6-carboxy-flourescein) as the fluorescent reporter dye, and the 3′-end was modified with the non-fluorescence quencher dye Black Hole Quencher® 1 (BHQ-1). The primers and

Table 1 List of isolates included in this study

Species name	Isolate Nr.	GenBank accession	Host, substrate	Country
Boeremia exigua var. *exigua*	CBS 431.74; PD 74/2447	EU880854	*Solanum tuberosum*	Netherlands
Boeremia exigua var. *gilvescens*	CBS 101156, PD 90/731	EU880848	*Solanum tuberosum*	Philippines
Boeremia foveata	CBS 341.67; CECT 20055; IMI 331912	EU880894	*Solanum tuberosum*	U.K
Boeremia lycopersici	CBS 378.67; PD 76/276	EU880898	*Solanum lycopersicum*	Netherlands
Boeremia noackiana	CBS 101203; PD 79/1114	EU880882	*Phaseolus vulgaris*	Colombia
Paraphoma chrysanthemicola	CBS 522.66	**JN251989**	*Chrysanthemum morifolium*	U.K.
Peyronellaea anserina	CBS 360.84	**JN251981**	Potato flour	Netherlands
Peyronellaea glomerata	CBS 528.66; PD 63/590	FJ426905	*Chrysanthemum* sp.	Netherlands
Peyronellaea pomorum var. *pomorum*	CBS 539.66; ATCC 16791 IMI 122266; PD 64/914	FJ426946	*Polygonum tataricum*	Netherlands
Peyronellaea subglomerata	CBS 110.92; PD 76/1010	FJ426966	*Triticum* sp.	U.S.A.
Phoma chenopodiicola	CBS 128.93; PD 79/140	**JN251985**	*Chenopodium quinoa*	Peru
Phoma destructiva var. *destructiva*	CBS 133.93; PD 88/961; IMI 173142	**JN251987**	*Solanum lycopersicum*	Guadeloupe
Phoma destructiva var. *diversispora*	CBS 162.78; PD 77/725	**JN251988**	*Solanum lycopersicum*	Netherlands
Phoma eupyrena	CBS 374.91; PD 78/391	FJ426892	*Solanum tuberosum*	Netherlands
Phoma herbarum	CBS 615.75; PD 73/665; IMI 199779	EU880896	*Rosa multiflora*	Netherlands
Phoma huancayensis	CBS 105.80; PD 75/908	**JN251986**	*Solanum* sp.	Peru
Phoma labilis	CBS 124.93; PD 87/269	**JN251979**	*Solanum lycopersicum*	Netherlands
Phoma macrostoma var. *incolorata*	CBS 109173; PD 83/908	**JN251984**	*Malus sylvestris*	Netherlands
Phoma macrostoma var. *macrostoma*	CBS 482.95	**JN251983**	*Larix decidua*	Germany
Phoma nemophilae	CBS 249.38	**JN251964**	*Nemophila insignis*	Denmark
Phoma subherbarum	CBS 249.92; PD 78/1088	**JN251982**	*Solanum* sp.	Peru
Stagonosporopsis actaeae	CBS 106.96; PD 94/1318	**JN251974**	*Actaea spicata*	Netherlands
Stagonosporopsis ajacis	CBS 177.93; PD 90/115	**JN251962**	*Delphinium* sp.	Kenya
Stagonosporopsis andigena	CBS 101.80; PD 75/909; IMI 386090	**JN251958**	*Solanum* sp.	Peru
Stagonosporopsis andigena	CBS 269.80; PD 75/914	**JN251959**	*Solanum* sp.	Peru
Stagonosporopsis artemisiicola	CBS 102636; PD 73/1409	**JN251971**	*Artemisia dracunculus*	France
Stagonosporopsis astragali	CBS 178.25; MUCL 9915	**JN251963**	*Astragalus* sp.	Unknown
Stagonosporopsis caricae	CBS 248.90	**JN251969**	*Carica papaya*	Chile
Stagonosporopsis crystalliniformis	CBS 713.85; ATCC 76027; PD 83/826	**JN251960**	*Solanum lycopersicum*	Colombia
Stagonosporopsis crystalliniformis	CBS 771.85; IMI 386091; PD 85/772	**JN251961**	*Solanum tuberosum*	Colombia
Stagonosporopsis cucurbitacearum	CBS 133.96; PD 79/127	**JN251968**	*Cucurbita* sp.	New Zealand
Stagonosporopsis dennisii	CBS 135.96; IMI 19337; PD 95/4756	**JN251975**	*Solidago canadensis*	Canada
Stagonosporopsis dorenboschii	CBS 426.90; IMI 386093; PD 86/551	**JN251980**	*Physostegia virginiana*	Netherlands
Stagonosporopsis heliopsidis	DAOM 221138; PD 95/6189;	**JN251970**	*Ambrosia artemisiifolia*	Canada
Stagonosporopsis hortensis	CBS 572.85; PD 79/269	**JN251966**	*Phaseolus vulgaris*	Netherlands
Stagonosporopsis ligulicola var. *inoxydabilis*	CBS 425.90; PD 81/520	**JN251972**	*Chrysanthemum parthenii*	Netherlands
Stagonosporopsis ligulicola var. *ligulicola*	CBS 500.63; MUCL 8090	**JN251973**	*Chrysanthemum indicum*	Germany

Table 1 (continued)

Species name	Isolate Nr.	GenBank accession	Host, substrate	Country
Stagonosporopsis loticola	CBS 562.81; PDDCC 6884	**JN251978**	*Lotus pedunculatus*	New Zealand
Stagonosporopsis lupini	CBS 375.84; PD 80/1250	**JN251967**	*Lupinus mutabilis*	Peru
Stagonosporopsis oculo-hominis	CBS 634.92; IMI 193307	**JN251976**	Human	U.S.A.
Stagonosporopsis trachelii	CBS 379.91; PD 77/675	**JN251977**	*Campanula isophylla*	Netherlands
Stagonosporopsis valerianellae	CBS 329.67; PD 66/302	**JN251965**	*Valerianella locusta* var. *oleracea*	Netherlands
Outgroup				
Phoma dimorphospora	CBS 345.78; PD 76/1015	**JN251990**	*Chenopodium quinoa*	Peru

Newly generated sequences are indicated in bold

probes were manufactured by Biolegio bv, Nijmegen, The Netherlands.

TaqMan PCR amplification

PCR amplifications of genomic DNA were performed in PCR tubes in 96-well-plates (Bioplastics, Landgraaf, The Netherlands) in a total volume of 30 μl containing the following reaction mixture: 1× TaqMan® Universal PCR Master Mix (Applied Biosystems), 250 nM of each primer, 83.3 nM probe, and 1 μl of genomic DNA. The cycle parameters were 10 min at 95 °C to activate the hot start Taq DNA polymerase, followed by 40 cycles of a 2-step amplification (15 s 95 °C; 1 min 60 °C). The TaqMan PCR was carried out in an ABI PRISM 7500 Sequence detector (Applied Biosystems). Each series of amplification reactions included sterile MQ water as an external negative control to test for contamination with DNA as well as the DNA from the reference strains *S. andigena* (CBS 101.80) and *S. crystalliniformis* (CBS 713.85) as a positive control.

A generic COX TaqMan PCR, to amplify a conserved region in the plant cytochrome oxidase (COX) gene was included to follow PCR inhibitors and potential inefficiencies of the DNA extraction if plant material was involved. The PCR performed included a

Table 2 Characteristics of primers and TaqMan probes designed for the detection of *S. andigena* and *S. crystalliniformis*, potato cytochrome oxidase DNA and conventional primers used for amplification of ITS1-ITS4 and actin regions

	Sequence (5′-3′)	Dye	Amplification
	TaqMan primers/probes		
S.andF2	TCT TCC GTA AGT CCT CCA AT C		Actin, *S. andigena*
S.andR1	GTG TTG TCA GTG GGA GGT TCA C		
S.andP1 probe	ACC TGG CAG CAG CAG CGT TCC T	5′ end FAM; 3′ end BHQ-1	
S.crysF2	GCA GTC TT CCGT AAG TCC C		Actin, *S. crystalliniformis*
S.crysR1	TCG CGG GCG TT TGCT		
S.crysP1 probe	CTG GCA GCA ACA GCA GCA GCG TTA CT	5′ end FAM; 3′ end BHQ-1	
COX-F	CGT CGC ATT CCA GAT TAT CCA		Cox, universal
COX-RW	CAA CTA CGG ATA TAT AAG RRC CRR AAC TG		
COXSOL1511T probe	AGG GCA TTC CAT CCA GCG TAA GCA	5′ end YY; 3′ end BHQ-1	
	Conventional primers		
ACT-512F	ATG TGC AAG GCC GGT TTC GC		Actin, universal
ACT-783R	TAC GAG TCC TTC TGG CCC AT		
ITS1	TCC GTA GGT GAA CCT GCG G		ITS, universal
ITS4	TCC TCC GCT TAT TGA TAT GC		

30 μl reaction mixture which contained 15 μl Premix Ex Taq™ (Takara BIO Europe SAS, Saint-Germain-en-Laye, France), 0.5 μl ROXII (Takara), 200 nM of each primer COX-F (Weller et al. 2000) and COX-RW (Mumford et al. 2004), 100 nM COXSOL1511T probe (Mumford et al. 2004) and 1 μl of DNA obtained from a spiked sample or artificially infected leaf material. The probe was 5′-end labelled with the reporter dye Yakima Yellow® (YY) and the 3′-end was modified with the non-fluorescence quencher dye BHQ-1 (Table 2).

The cycle parameters were similar to those described above. The Ct-value was automatically calculated for each PCR by the algorithm ABI PRISM system software. Genomic DNA of the isolates as well as healthy plant material was included as positive and negative controls.

ITS amplifications

In case the results of the specific Taqman PCR were negative, an ITS-PCR was performed to demonstrate the PCR-ability of the isolated DNA. PCR amplifications were performed in duplicate in PCR tubes, total volume of 25 μl, which contained the following reaction mixture: 2.5 μl 10× buffer (Roche, Almere, The Nederlands), 60 μM dNTP's , 0.625 U Taq polymerase, 200 nM of the primers ITS1 and ITS4 (White et al. 1990) (Table 2), and 1 μl of genomic DNA. The cycle parameters were 2 min at 95 °C, 35 cycles of a 3-step amplification (30 s 95 °C; 30 s 57 °C; 1 min 72 °C), finally 10 min 72 °C. The ITS PCR was performed in a DNA Engine (PTC-200) instrument (BioRad, Veenendaal, Nederland) in 96-wells-plates. A negative control, Milli-Q water, was included in each run. The PCR products were separated on a 1 % agarose gel and stained with GelRed (Biotium Inc, Hayward, CA, USA).

Assessment of TaqMan assay performance criteria

The TaqMan PCR methods for the detection of *Stagonosporopsis andigena* and *S. crystalliniformis* were validated for several performance characteristics. The specificity of the assays was tested with the TaqMan PCR on 1 μl genomic DNA extracted from the two *S. andigena* and two *S. crystalliniformis* isolates and 16 additional isolates of the allied *Stagonosporopsis*, *Phoma* and *Boeremia* species (Table 3). The analytical sensitivity (detection limit) of both TaqMan PCR assays was determined in duplicate in a 10-fold serial dilution of the genomic DNA in sterile MQ water in a range of approximately 1 ng–1 fg obtained from pure cultures of *S. andigena* (CBS 101.80) and *S. crystalliniformis* (CBS 713.85). In addition, the analytical sensitivity was determined in duplicate on DNA extracted from leaf material of the potato cv 'Bintje' or tomato cv 'Moneymaker' spiked with mycelium of isolate CBS 101.80 or CBS 713.85 in a serial dilution with DNA extracted from healthy leaves. A 0.9 cm diam leaf disk was spiked with mycelium scraped from one 0.9 cm diam agar plate. After the DNA extraction, a serial dilution 1:0, 1:1, 1:5, 1:20 and 0:1 in a total volume of 20 μl was made of the DNA of the spiked samples with DNA extracted from healthy leaves. One μl of the mixtures was tested in duplicate with the TaqMan PCR assays. The effect of host plant material on the amplification of DNA of the target organisms was tested in duplicated with spiked samples consisting of DNA of *S. andigena* (CBS 101.80) or *S. crystalliniformis* (CBS 713.85) with leaf material of potato 'Bintje', 'Bionica', 'Bethaultii' and tomato 'Moneymaker', 'Microtom' and 'Heinz' respectively. The robustness of the assays was assessed by changing two important parameters, the PCR machine and the Taq polymerase kit. The TaqMan PCR assays were repeated on another AB7500 PCR machine using the same samples of the serial dilution of the *S. andigena* and *S. crystalliniformis* isolates as described above, and the Taq polymerase kit was altered by use of the Premix Ex Taq™ (Takara).

The repeatability and reproducibility were determined following the Dutch guideline for the validation of detection and identification methods for plant pathogens and pests (2010) and the OEPP/EPPO guideline PM 7/98 (1) (2010), to obtain information about the sensitivity of the analysis for small variation in the execution under routine-like circumstances. At eight different times, 0.9 cm diam leaf disks of potato cv. 'Bintje' and tomato cv 'Moneymaker' were spiked with mycelium scraped from 0.45 to 1.8 cm diam plugs taken from colonies of the isolates *S. andigena* (CBS 101.80) and *S. crystalliniformis* (CBS 713.85) in eight replicates. DNA was extracted as described above in duplicate by two different technicians (A, B). One μl of the DNA extract was tested with the TaqMan PCR assays which were performed once (A) or in duplicate (B) under the same laboratory

Table 3 Results of the specificity test with the primer pairs designed for *Stagonosporopsis andigena* and *S. crystalliniformis*

Isolate		Strain nr.	TaqMan PCR, Ct values	
			S. andigena	*S. crystalliniformis*
1	*Stagonosporopsis andigena*	CBS 269.80	22.82	nd[a]
2	*Stagonosporopsis crystalliniformis*	CBS 713.85	nd	22.21
3	*Boeremia exigua* var. *exigua*	CBS 431.74	nd	nd
4	*Boeremia foveata*	CBS 341.67	nd	nd
5	*Boeremia lycopersici*	CBS 378.67	nd	nd
6	*Boeremia noackiana*	CBS 101203	nd	nd
7	*Stagonosporopsis valerianellae*	CBS 329.67	nd	nd
8	*Peyronellaea anserina*	CBS 360.84	nd	nd
9	*Stagonosporopsis andigena*	CBS 101.80	23.84	nd
10	*Stagonosporopsis crystalliniformis*	CBS 771.85	nd	22.23
11	*Stagonosporopsis cucurbitacearum*	CBS 133.96	nd	nd
12	*Stagonosporopsis lupini*	CBS 375.84	nd	nd
13	*Peyronellaea glomerata*	CBS 528.66	nd	nd
14	*Peyronellaea pomorum* var. *pomorum*	CBS 539.66	nd	nd
15	*Peyronellaea subglomerata*	CBS 110.92	nd	nd
16	*Phoma chrysanthemicola*	CBS 522.66	nd	nd
17	*Phoma eupyrena*	CBS 374.91	nd	nd
18	*Phoma herbarum*	CBS 615.75	nd	nd
19	*Phoma huancayensis*	CBS 105.80	nd	nd
20	*Phoma labilis*	CBS 124.93	nd	nd

[a]nd: not detected

conditions. A positive control, genomic DNA of both strains, and sterile MQ water as a negative control were included.

Testing the TaqMan PCR assays on artificially inoculated potato and tomato leaves

Detached leaves from 5 weeks old plants of potato 'Bintje' and tomato 'Moneymaker' were transferred into 18×14×5.5-cm transparent plastic boxes, one leaf per box. Each leaf was inoculated on the upper side with three to four 6 mm diam mycelium bearing agar plugs of one of the isolates *S. andigena* (CBS 101.80, 269.80) or *S. crystalliniformis* (CBS 713.85, 771.85) taken from a 10 days old culture grown on OA. The boxes were incubated during 2 days at 10 °C in darkness, followed by 5 days at 20 °C in daylight. At 7 days after inoculation, 0.9 cm diam. plugs were taken from the infected leaf tissue at the margin of the lesion and collected into a 1.5 ml Qiagen microtube, and stored at −20 °C until DNA extraction. After DNA extraction, both TaqMan PCR assays developed for *S. andigena* and *S. crystalliniformis* were performed in duplicate as described above.

Results

DNA phylogeny of the actin gene

The aligned sequence length obtained for the actin region was 314 nucleotides long. Of these 314 characters, 191 unique site patterns were present. The analysis run in MrBayes resulted in 46,002 trees after 230,000 generations, from which the burn-in was discarded and the consensus tree and posterior probabilities were calculated based on 25,819 trees. A main clade which represents the genus *Stagonosporopsis* was found (support 96 %, Fig. 1) in agreement with a multigene analysis based on LSU, ITS and β-tubulin (Aveskamp et al 2010). In this clade, *Stagonosporopsis andigena* and *S. crystalliniformis* could be recognised in a well-supported subclade demonstrating that both species are closely allied. The clade representing

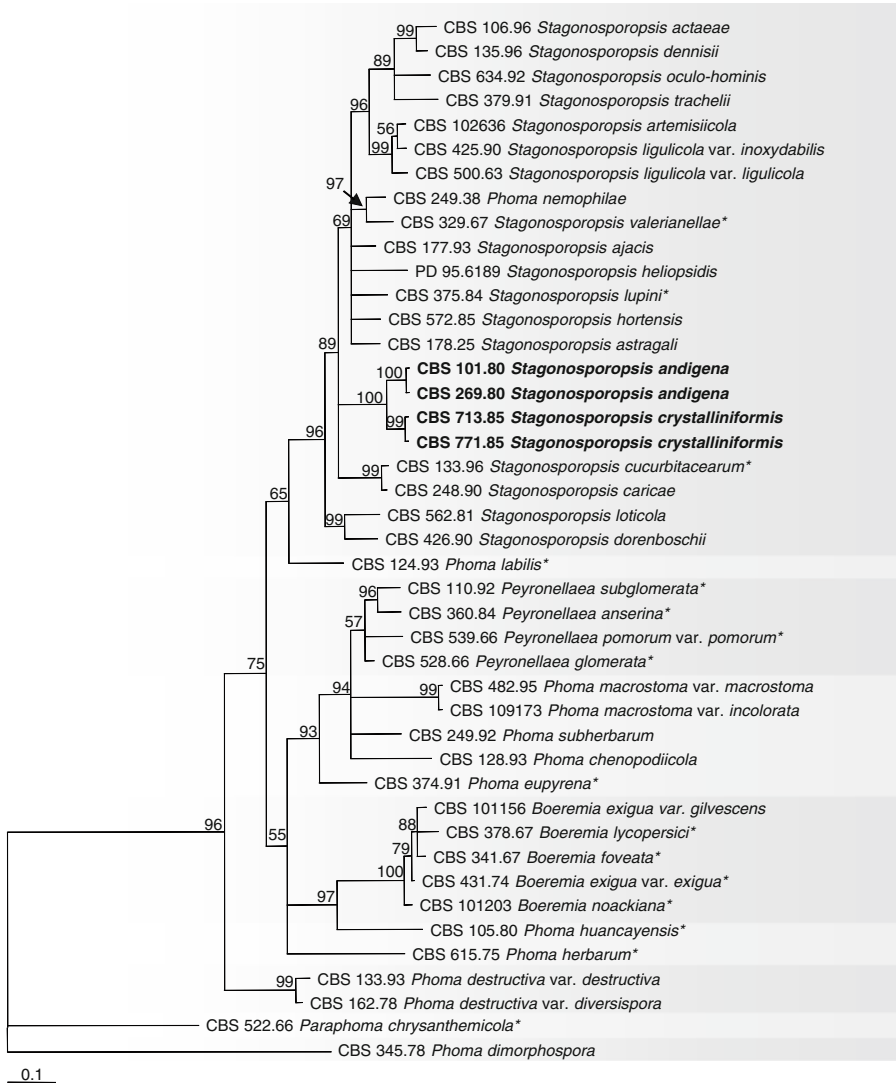

Fig. 1 The phylogenetic relationships of *Stagonosporopsis andigena* and *S. crystalliniformis* and species classified in the genera *Stagonosporopsis*, *Boeremia*, *Phoma*, and *Peyronellaea*, based on the strict consensus tree from a Bayesian analysis of 43 actin sequences. The Bayesian posterior probabilities are given at the nodes. The tree was rooted with *Phoma dimorphospora* (CBS 345.78). *Strains included in the specificity test (Table 3)

Stagonosporopsis also includes *Phoma nemophilae* CBS 249.38. *Boeremia* represents a well-supported monophyletic clade, while the subclade that represents *Peyronellaea* is poorly supported.

Selection of the Primer/probe combinations

The primer/probe combinations that were selected using actin sequence differences between *Stagonosporopsis andigena*, *S. crystalliniformis* and their related species are given in Table 2. The primer pairs produce fragments of the actin gene of 97 and 89 base pairs respectively. The partial sequence alignments of the forward and reverse primers of *Stagonosporopsis andigena* and *S. crystalliniformis* contained one SNP and two SNP's difference respectively, however, the probes developed contained seven different SNP's. The blast results with the primers and probes in GenBank demonstrated no homology with sequences of organisms present in the database.

Assessment of TaqMan assay performance criteria

The Ct values of the TaqMan PCR assays performed on DNA extracts obtained from pure cultures of *S. andigena* and *S. crystalliniformis*, as well as those of the related species of *Stagonosporopsis* included in this study, are given in Table 3. The Ct values for the *S. andigena* isolates CBS 101.80 and CBS 269.80 were 23.84 and 22.82, and the Ct values for the *S.*

Table 4 Ct values resulting from TaqMan PCR on genomic DNA in duplicate in a 10-fold serial dilution of pure cultures of *Stagonosporopsis andigena* (CBS 101.80) and *S. crystalliniformis* (CBS 713.85) to detemine the analytical sensitivity and robustness

a	*S. andigena*		*S. crystalliniformis*		b	*S. andigena*		*S. crystalliniformis*	
	CBS 101.80		CBS 713.85			CBS 101.80		CBS 713.85	
1 ng	24.15	24.09	21.89	22.20	1 ng	23.84	25.02	22.21	23.83
100 pg	27.91	27.90	25.81	25.95	100 pg	28.57	nt[a]	25.56	nt
10 pg	31.37	31.79	29.84	28.96	10 pg	31.84	nt	28.71	nt
1 pg	37.45	34.92	32.93	32.78	1 pg	37.58	nt	32.94	nt
100 fg	37.02	36.06	35.15	35.54	100 fg	37.97	nt	35.15	nt
10 fg	nd[b]	nd	nd	36.27	MQ	nd	nd	nd	nd
1 fg	nd	nd	nd	nd					
MQ	nd	nd	nd	nd					

a. AB7500 machine 1, TaqMan® Universal PCR Master Mix Taq polymerase kit; b. AB7500 machine 2, Premix Ex Taq™ Taq polymerase kit

[a] nt: not tested

[b] nd: not detected

crystalliniformis isolates CBS 713.85 and CBS 771.85 were 22.21 and 22.23 respectively. All other isolates tested showed negative results, while correct PCR products were obtained with the ITS PCR demonstrating the amplification of DNA (data not shown).

The results of the TaqMan PCR assays on a 10-fold serial dilution of *S. andigena* and *S. crystalliniformis* DNA in sterile MQ water are provided in Table 4. The PCR efficiency was estimated through the linear regression of the calculated calibration curve. The Ct values obtained with the target DNA of 100 fg for *S. andigena* were discarded because the average value was out of the linear phase. The coefficients were R^2=0.998 and R^2=0.993, the amplification efficiency 92.4 % and 99.1 % for the TaqMan PCR assays for *S. andigena* and *S. crystalliniformis* respectively. The Ct threshold values 37.5 and 35.5 were determined qualitatively as the maximum values for a positive reaction for *S. andigena* and *S. crystalliniformis* respectively. These values correspond with the maximum Ct values found in the linear phases. The effect of host plant material on the amplification of DNA of the target organisms was tested with spiked samples consisting of DNA of *S. andigena* (CBS 101.80) or *S. crystalliniformis* (CBS 713.85) with leaf material of different cultivars of potato and tomato respectively (Table 5). The Ct values for *S. andigena* (29.48–30.96) and *S. crystalliniformis* (26.61–28.69) showed that it was possible to extract and amplify target DNA in the presence of plant material of the different cultivars. The TaqMan PCR assays on spiked samples of *S. andigena* and *S. crystalliniformis* with leaf of potato cv. 'Moneymaker'

Table 5 Ct values of TaqMan PCR determined in duplicate on spiked samples of *Stagonosporopsis andigena* and *S. crystalliniformis* with leaf plugs of *Solanum tuberosum*, cvs 'Bintje', 'Bionica' and 'Bethaultii', and *S. lycopersicum*, cv 'Moneymaker', 'Microtom' and 'Heinz'

	Cultivar	*S. andigena* (CBS 101.80)		*S. crystalliniformis* (CBS 713.85)	
S. tuberosum	'Bintje'	29.48	29.68	28.54	27.91
	'Bionica'	30.00	30.03	27.75	27.80
	'Berthaultii'	30.30	29.77	28.69	28.50
S. lycopersicum	'Moneymaker'	30.56	30.79	26.61	26.74
	'Microtom'	30.66	30.50	27.98	27.89
	'Heinz'	30.96	30.29	28.53	28.41
	MQ	nd[a]	nd	nd	nd

[a]nd: not detected

Table 6 Ct values of TaqMan PCR on genomic DNA determined in duplicate in a serial dilution of spiked samples of pure cultures of *Stagonosporopsis* (*S*); *S. andigena* CBS 101.80 (*S.a.*) and *S. crystalliniformis* 713.85 (*S.c.*) with leaf material of *Solanum tuberosum* 'Bintje' (B) and *S. lycopersicum* 'Moneymaker' (M) to determine the analytical sensitivity

S. + B : B	S. a.	S. a.	S. c.	S. c.	*S.* + M : M	S. a.	S. a.	S. c.	S. c.
1:0	31.71	31.56	30.02	29.80	1:0	32.05	31.98	27.87	28.17
1:1	32.58	32.42	30.90	30.97	1:1	32.42	32.71	28.58	29.01
1:5	33.63	34.34	32.36	32.35	1:5	33.85	34.33	30.20	30.42
1:20	34.24	35.18	33.14	33.09	1:20	35.95	35.17	32.29	31.92
0:1	nd[a]	nd	nd	nd	0:1	nd	nd	nd	nd
MQ	nd	nd	nd	nd	MQ	nd	nd	nd	nd
		COX detection					COX detection		
1:0	25.60	25.65	26.46	26.56	1:0	24.91	24.84	25.95	25.89
1:1	25.95	26.01	26.55	26.52	1:1	25.25	25.20	25.94	25.92
1:5	26.09	26.22	26.45	26.52	1:5	25.56	25.17	25.96	26.03
1:20	26.46	26.53	26.54	26.57	1:20	25.87	25.83	25.92	25.96
0:1	26.67	26.45	26.64	26.71	0:1	26.02	26.02	26.02	25.93
MQ	nd	nd	nd	nd	MQ	nd	nd	nd	nd

[a] nd: not detected

and tomato cv. 'Bintje' in a serial dilution demonstrated that a 20× dilution of the DNA extract still revealed a positive result (Table 6).

Similar results of the specific TaqMan PCR assays of *S. andigena* and *S. crystalliniformis* were obtained after changing the TaqMan polymerase or performing the PCR in another AB7500 PCR machine (Table 4). The results of the repeated experiments conducted over time and performed by two persons (A, B) were comparable (Table 7). Both species were detected in all spiked samples tested by both persons at the different times. The Ct values obtained in time with both PCR assays were similar. The 64 Ct values (eight time points with eight replicates) of the TaqMan PCR developed for *S. andigena* and applied on *S. andigena* mycelium spiked with potato leaf and for *S. crystalliniformis* on

Table 7 Average Ct values of 8 replicates of real-time TaqMan PCR *Stagonosporopsis andigena* (CBS 101.80) and *S. crystalliniformis* (713.85), spiked with leaf disks of *L. esculentum* 'Moneymaker' and *S. tuberosum* 'Bintje', performed by 2 persons (A, single and B in duplicate) at 8 different times[a]

Time	*S. andigena* (CBS 101.80)								*S. crystalliniformis* (CBS 713.85)							
	Potato			Tomato			Pc[b]		Potato			Tomato			Pc	
	A	B	B	A	B	B	A	B	A	B	B	A	B	B	A	B
1	34.16	34.26	34.41	33.36	33.39	33.00	26.85	26.40	31.76	32.71	32.41	31.90	32.32	31.88	24.76	24.06
2	32.86	33.44	33.61	32.82	33.34	33.11	26.49	26.45	31.00	32.71	32.77	31.99	32.23	31.76	25.59	26.45
3	33.96	33.50	33.52	33.36	32.51	30.48	26.34	25.91	31.76	32.93	33.07	31.90	30.97	30.07	23.23	23.75
4	32.86	33.86	33.53	32.82	31.25	32.00	25.64	25.84	31.00	32.06	32.41	31.99	32.35	30.28	23.66	23.98
5	31.89	32.43	32.88	32.11	31.65	30.78	25.51	24.75	31.54	31.48	32.22	31.66	30.10	28.95	23.44	22.86
6	32.53	34.09	33.64	32.71	32.58	31.67	25.94	25.96	28.73	30.98	30.77	29.67	30.63	29.48	23.31	24.16
7	32.69	32.46	32.8	32.41	31.74	31.22	25.99	25.49	30.40	30.50	30.51	30.15	28.71	29.17	23.73	23.70
8	34.41	34.00	34.15	33.94	33.78	31.82	26.99	26.79	33.18	33.05	33.28	32.71	32.3	31.44	24.73	24.96

[a] The MQ water controls were negative

[b] Pc: Positive control, genomic DNA

tomato were all below the Ct threshold values 37.5 and 35.5, respectively.

Detection of *S. andigena* and *S. crystalliniformis* in artificial infected potato and tomato leaves

The first symptoms on the detached leaves were obtained 3 days after the artificial inoculation of *S. andigena* and *S. crystalliniformis*. In the inoculation experiments on the detached leaves it became obvious that a 10 °C initial incubation period is essential for infection. Both species caused infections on potato and tomato leaves. The symptoms caused by *Stagonosporopsis andigena* on tomato was a brown necrosis around the inoculum and developing along the veins with a chlorotic margin, comparable with those caused by *S. crystalliniformis*. In addition, *S. crystalliniformis* caused infections on potato leaves as stated by Loerakker et al. (1986).

The TaqMan PCR assays applied on infected leaf plugs demonstrated that both *Stagonosporopsis* species could be detected in infected potato and tomato leaf material. In one PCR test with the PCR developed for *S. andigena*, there was a positive PCR reaction on potato leaf infected with *S. crystalliniformis*, possibly due to a contamination (Table 8). The Ct values obtained for *S. andigena*, 26.43–28.37, were below the values obtained with spiked samples in a previous experiment (Table 5). Both reference cultures included showed negative or positive results for both PCR assays as expected, ITS amplicons were obtained with the ITS primers ITS1 and ITS4 that demonstrates the PCR-ability. Negative results with both PCR assays were obtained with non-infected leaf material and sterile MQ water.

Discussion

Stagonosporopsis andigena and *S. crystalliniformis* are foliage pathogens that have presently not been reported from outside the Andes region. The isolates included in the present study were obtained in 1975 and 1985 respectively, and recent isolates could not be obtained. Both species are distinct from *Boeremia foveata*, a pathogen of potato tubers that also originates from the Andes region, but with a wider known distribution. *Stagonosporopsis andigena* has been recorded on wild and cultivated species of potato. In terms of quarantine, the host range is important and the statement that *S. andigena* also occurs on tomato and other solanaceous weeds (OEPP/EPPO 1984) could not be confirmed by any of the citations listed, as was also noted recently (Cline 2011). However, the results of our artificial inoculations with mycelial plugs on detached leaves to obtain infected leaves for PCR assays demonstrated that *S. andigena* might also infect tomato leaves. Although we tried to infect potato tubers, no disease symptoms could be obtained in congruence with previous data for *S. andigena* (Turkensteen 1978).

The molecular phylogeny utilising actin sequence data demonstrates that *S. andigena* and *S. crystalliniformis* are closely related species of *Stagonosporopsis*. This result is in agreement with the molecular phylogeny based on LSU, ITS and β-tubulin sequences

Table 8 Ct values of Taqman PCR amplification performed on DNA extracts after duplicate inoculation and extraction from leaves of *Solanum tuberosum* 'Bintje' (B) and *S. lycopersicum* 'Moneymaker' (M) infected by *Stagonosporopsis andigena* and *S. crystalliniformis*

Target	CBS nr.	*S. andigena*		*S. crystalliniformis*	
Infected plant material					
M + *S. andigena*	101.80	26.78	26.60	nd[a]	nd
B + *S. andigena*	101.80	28.35	28.14	nd	nd
M + *S. andigena*	269.80	27.48	26.43	nd	nd
B + *S. andigena*	269.80	27.12	28.37	nd	nd
M + *S. crystalliniformis*	713.85	nd	nd	27.82	25.29
B + *S. crystalliniformis*	713.85	nd	nd	26.43	26.22
M + *S. crystalliniformis*	771.85	nd	nd	25.03	25.45
B + *S. crystalliniformis*	771.85	36.04	nd	30.22	28.72
Reference cultures					
S. andigena	101.80	24.18		nd	
S. crystalliniformis	713.85	nd		23.31	

[a]nd: not detected

(Aveskamp et al. 2010). Both isolates of each species showed a high genetic homogeneity. No sexual state has thus far been recorded for either species. *Stagonosporopsis andigena* and *S. crystalliniformis* were previously classified in *Phoma* section *Heterospora* according to their morphological characters (Boerema et al. 1997). This *Phoma* section is characterised by the formation of dimorphic conidia, namely small 'phomoid' and distinctly larger 'ascochytoid-stago nosporoid' conidia, the latter also described as synanamorph in *Stagonosporopsis* (Boerema et al. 1997, 2004). Molecular studies demonstrated that *Phoma* section *Heterospora* is polyphyletic, and that only part of the species of this section grouped in a monophyletic clade, and have been described in the amended holomorph *Stagonosporopsis* (Aveskamp et al. 2010). *Stagonosporopsis cucurbitacearum* proved to be one of the most closely related species based on the alignments of actin sequences compared in this study. *Stagonosporopsis cucurbitacearum* is regarded worldwide as an important seed-borne pathogen on *Cucurbitaceae*, esp. *Cucumis*, *Cucurbita* and *Citrullus,* but has also been recorded associated with infected leaves of potato in Peru (Turkensteen and Pinedo 1982). This finding supports the close relation of these species, and suggests a geographic origin in the Andes region.

The identification of *Phoma*-like isolates requires a high level of experience, it is time consuming, and the *in vitro* characters are often variable (Aveskamp et al. 2008), which may lead to misidentifications. Several molecular methods for the detection and identification of important pathogenic *Phoma* species have been developed during the last decade. A PCR test was developed to distinguish *Boeremia foveata* (syn. *Phoma foveata*) from *B. exigua* (syn. *P. exigua*) and its varieties, using primers that were derived from a RAPD product (Macdonald et al. 2000). A PCR-ELISA method was developed for the detection of *Stagonosporopsis cucurbitacearum* (syn. *Didymella bryoniae*, *Phoma cucurbitacearum*) in fruit crops that belong to *Cucurbitaceae* (Somai et al. 2002). Primers derived from the Internal Transcribed Spacer regions 1 & 2 (ITS) and 5.8S rDNA were developed for the detection of *D. bryoniae* and *P. foveata* (Koch and Utkhede 2004; Cullen et al. 2007). In addition, PCR assays based on the ITS region were developed for the detection and identification of *P. tracheiphila* in citrus fruits (Balmas et al. 2005; Licciardella et al. 2006; Demontis et al. 2008). Actin sequence data provided tools for the development of TaqMan PCR assays for *S. andigena* and *S. crystalliniformis*. The performance characteristics were determined, and demonstrated that the DNA extraction and PCR methods are suitable for the detection and identification of *S. andigena* an *S. crystalliniformis* in leaves of potato and tomato. The allied *Stagonosporopsis* species and more distantly related species of *Boeremia*, *Peyronellaea* and *Phoma* were not detected. Both TaqMan PCR methods were sensitive, and reliability and repeatability could be demonstrated. Extraction and amplification of target DNA occurred in the presence of plant material of the cultivars of potato 'Bintje', 'Bionica', 'Bethaultii' and tomato 'Moneymaker', 'Microtom' and 'Heinz'. The Ct values obtained with artificially inoculated leaves of potato 'Bintje' and tomato 'Moneymaker' were all below or comparable with those obtained with spiked samples.

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