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# *Stagonosporopsis* spp. associated with ray blight disease of *Asteraceae*

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**Abstract** Ray blight disease of pyrethrum (*Tanacetum cinerariifolium*) is shown to be caused by more than one species of *Stagonosporopsis*. The Australian pathogen, previously identified as *Phoma ligulicola* var. *inoxydabilis*, represents a new species described as *Stagonosporopsis tanaceti* based on morphological characters and a five-gene phylogeny employing partial sequences of the actin, translation elongation factor 1-alpha, internal transcribed spacers and 5.8S of the nrDNA, 28S large subunit and beta-tubulin 2 gene sequences. Furthermore, the two varieties of *Stagonosporopsis ligulicola* are elevated to species level as *S. chrysanthemi* and *S. inoxydabilis* based on their DNA phylogeny and morphology.

**Keywords** *Phoma ligulicola* var. *inoxydabilis* · Ray blight · *Stagonosporopsis*

## Introduction

Ray blight disease of pyrethrum (*Tanacetum cinerariifolium* (Trev.) Schultz Bip.) is a major threat to the Australian pyrethrum industry, capable of causing complete yield loss (Pethybridge and Hay 2001; Pethybridge et al. 2007, 2008b, c). Pyrethrum, a perennial herbaceous member of the *Asteraceae*, is commercially cultivated for the extraction of pyrethrins from the achenes of its flowers (Bhat and Menary 1984; Grdiša et al. 2009). Pyrethrins have unique insecticidal properties that render them increasingly popular for pest control in both household and agricultural situations. They are biodegradable insecticides with low toxicity to mammalians and a rapid knock-down effect on insects, but also act as effective repellent at lower concentrations (Grdiša et al. 2009). The Australian pyrethrum industry supplies approximately 60 % of the global market requirements, for which production is centred in northern Tasmania and the Ballarat region of Victoria.

Ray blight disease was first described in Tasmanian fields in 1995, which led to the causal organism being identified as *Phoma ligulicola* var. *inoxydabilis* (Pethybridge and Wilson 1998). Further studies on the morphology and biology of the pathogen, however, revealed some divergence from the published descriptions of the representative strains, including the ex-type strain (CBS 425.90). Although *Phoma ligulicola* is reported to be homothallic and produces abundant ascomata in culture (Van der Aa et al. 1990; Boerema et al. 2004), the teleomorph was never found in the field, and all attempts to induce mating of the Australian isolates in vitro failed (Pethybridge et al. 2008c; Jones 2009). Moreover, *P. ligulicola* var. *inoxydabilis* is known to infect zinnia (Van der Aa et al. 1990; De Gruyter et al. 2002), which has been found to be a non-host to the Australian pathogen (Pethybridge et al. 2008a). These, along with the high level of variation found in morphological features and virulence of the pathogen population (Jones 2009) suggested the possibility that several species could be associated with the disease. These findings led to the conclusion that the taxonomic position of the Australian isolates associated with ray blight disease required further investigation.

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Ray blight was first reported on *Chrysanthemum morifolium* in the USA, and named after the most conspicuous symptom of the disease, i.e., discolouration and distortion of the flower buds and ray florets. The causal agent was originally described as *Ascochyta chrysanthemi* (Stevens 1907). The teleomorph was first described as *Mycosphaerella ligulicola* (Baker et al. 1949), which was later transferred to the genus *Didymella* (Müller and Von Arx 1962). The taxonomy of the ray blight pathogen then underwent many changes; a detailed review of which is given by Walker and Baker (1983). In 1971, Garibaldi and Gullino re-examined type specimens of *Sphaerella chrysanthemi* (Tassi 1900), previously isolated from *Chrysanthemum marginatum* in Italy. They renamed it as *Didymella chrysanthemi* (Tassi) Garibaldi and Gullino, and listed *Didymella ligulicola* as synonym. They also referred to *Ascochyta chrysanthemi* as synonym of *Phoma chrysanthemi* (Voglino 1902), a pathogen of *Chrysanthemum indicum*. These binomials were later adopted extensively in the literature as the causal agent of ray blight (Boerema and Van Kesteren 1974; Boerema and Bollen 1975; Richardson 1979; EPPO 1980, 1982; Punithalingam 1980) but were rejected by Walker and Baker (1983) after re-examination of Tassi's type specimen, which they suggested to be a species of *Mycosphaerella*. Walker and Baker (1983) could not locate Voglino's type specimen, but based on the published descriptions of *Phoma chrysanthemi,* argued that it was different from *Ascochyta chrysanthemi*, and suggested the continued use of *Ascochyta chrysanthemi* and its purported teleomorph, *Didymella ligulicola,* for the chrysanthemum ray blight pathogen.

In 1990, *Ascochyta chrysanthemi* was reclassified as *Phoma ligulicola,* and divided into two varieties, namely var. *ligulicola* and var. *inoxydabilis* (Van der Aa et al. 1990). Although morphologically similar, the two varieties were reported to vary in their host range and geographical distribution, and also reacted differentially to sodium hydroxide (NaOH) treatment (Boerema and Howeler 1967). Variety *ligulicola* produces a colourless antibiotic metabolite 'E' that upon application of NaOH oxidizes to red pigment β. The other variety does not produce metabolite 'E' and therefore no oxidization occurs upon NaOH application, hence the name *inoxydabilis* (Van der Aa et al. 1990; De Gruyter et al. 2002; Boerema et al. 2004).

Recently, in a taxonomic revision of *Phoma* and related genera, reference strains of the ray blight pathogen were transferred to *Stagonosporopsis* based on molecular data and morphological observations (Aveskamp et al. 2010), but the authors maintained the separation of the two varieties. *Stagonosporopsis* was originally separated from *Ascochyta* due to occasional formation of multi-septate (*Stagonospora*-like) conidia (Diedicke 1912). As no type material was specified by Diedicke (1912), the first species described, namely *S. actaeae*, has been interpreted as the generic type by some authors (Boerema et al. 1997, 2004). However, the combination *S. boltshauseri*, currently known as *S. hortensis* (Boerema and Verhoeven 1979; Aveskamp et al. 2010), was chosen as the lectotype by Clements and Shear (1931). *Stagonosporopsis* synanamorphs have been described for several *Phoma* species in section *Heterospora*. The characteristic of section *Heterospora* is the in vivo production of distinctly large conidia ('ascochytoid /stagonosporoid') in addition to relatively small 'phomoid' conidia. The large conidial phenotypes may be dominant in vivo and, thus, described as *Stagonosporopsis* synanamorphs (Boerema and Verhoeven 1979; Boerema et al. 1997, 2004).

In the phylogenetic reassessment of *Didymellaceae* conducted by Aveskamp et al. (2010), some of the *Phoma* species with known *Stagonosporopsis* synanamorphs were retrieved in a highly supported group with the presumed types of *Stagonosporopsis*. Aveskamp et al. (2010) recombined these taxa into *Stagonosporopsis* and proposed an emended description for the genus. Moreover, many other *Phoma* species from sections *Phoma* and *Phyllostictoides*, including both *P. ligulicola* varieties, were recovered in this clade. In fact, *P. ligulicola* had previously been reported to display conidial dimorphism; however, it was classified in section *Phyllostictoides* because the occasional 1-septate (ascochytoid) conidia were found to be the same size as the aseptate conidia (Boerema et al. 1997; De Gruyter et al. 2002). Currently, *Stagonosporopsis* accommodates more than 40 species, six of which have been recorded on different members of the *Asteraceae* family, namely *S. artemisiicola*, *S. dennisii*, *S. dorenboschii*, *S. heliopsidis*, *S. ligulicola*, and *S. rudbeckiae*. Of these, only *S. ligulicola* has been reported to cause the characteristic ray blight symptoms, i.e., deformation and necrosis of the buds and ray florets, which may extend 2–3 cm below the peduncle and cause the flower head to droop (Stevens 1907; Pethybridge et al. 2008a, c).

The aim of this study was to elucidate the taxonomy of the pyrethrum ray blight pathogen in Australia through morphological studies and phylogenetic analysis. To this end, a collection of cultures obtained from ray blight symptoms of pyrethrum in Australia as well as overseas isolates associated with chrysanthemum were used to construct a multi-locus phylogeny. Additional strains of other *Stagonosporopsis* species were included and the evolutionary basis for separation of the two varieties of the ray blight pathogen was clarified.

## Materials and methods

### Isolates

Ten isolates associated with ray blight symptoms on pyrethrum, previously identified as *Phoma ligulicola* var.

*inoxydabilis*, were selected from different geographical locations in Tasmania. This included nine isolates collected by S.J. Pethybridge and one isolate sourced from M.J. Priest, New South Wales Agriculture, Australia (DAR 70020). In addition, five ray blight isolates associated with *Chrysanthemum* spp., previously identified as *P. ligulicola* var. *ligulicola,* were sourced from the American Type Culture Collection (ATCC), the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), and the International Collection of Microorganisms from Plants (ICMP). Reference specimens representing the two varieties of the ray blight pathogen (CBS 425.90; ex-holotype, and CBS 500.63; reference strain according to Boerema et al. (2004)) were obtained from culture collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands (Table 1).

## Morphological analysis

Isolates were inoculated onto oatmeal agar (OA), malt extract agar (MEA) and cherry decoction agar (CHA) (Crous et al. 2009) and incubated as described by Boerema et al. (2004). Colony growth was measured after 1 week, and colony colours were rated after 2 weeks using the colour charts of Rayner (1970). Microscopic examinations were conducted after maturation of pycnidia on OA. Structures were mounted in lactic acid, and the size of pycnidia and pycnidiospores were determined by calculating 95 % confidence intervals for 30 measurements. The extreme sizes are given in parentheses. The production of metabolite 'E' was determined by application of a droplet of 1N NaOH to cultures growing on OA (Boerema et al. 2004).

## PCR, sequencing and phylogenetic analysis

DNA extraction was performed using a QIAGEN DNeasy Plant Mini Kit (QIAGEN, Australia), according to the manufacturer's instruction. All DNA extracts were diluted to 2 ng/μL and stored at −20 °C before use. The 5.8S nuclear ribosomal RNA gene and the flanking internal transcribed spacers (ITS), and partial sequences of the 28S nuclear ribosomal RNA gene (LSU), partial actin (ACT), translation elongation factor 1-α (EF1-α) and β-tubulin (TUB2) genes were amplified using the primer pairs V9G (de Hoog and Gerrits Van den Ende 1998) and ITS4 (White et al. 1990), LR0R (Rehner and Samuels 1994) and LR7 (Vilgalys and Hester 1990), ACT-512F and ACT-783R (Carbone and Kohn 1999), EF1-983F and EF1-1567R (http://www.aftol.org/pdfs/EF1primer.pdf), BT2Fd and BT4R (Woudenberg et al. 2009), respectively. The PCR reactions were performed in a BioRad Thermal Cycler (BioRad, Australia) in a total volume of 25 μL. The LSU PCR mixture contained 10 ng genomic DNA, Hi-Fi reaction buffer (Bioline, Australia) containing 2 mM $MgCl_2$, 0.2 μM of each primer, 0.2 mM of each dNTP and 0.5 U Velocity DNA polymerase (Bioline, Australia). PCR conditions included an initial denaturation step of 5 min at 98 °C, followed by 35 cycles of 45 s at 98 °C, 45 s at 58 °C and 2 min at 72 °C, and a final denaturation step of 7 min at 72 °C. TUB2 and EF1-α PCR mixtures contained 10 ng genomic DNA, Mango*Taq* reaction buffer (Bioline, Australia), 2 mM $MgCl_2$, 0.2 μM of each primer, 0.1 mM of each dNTP and 0.5 U Mango*Taq* DNA polymerase (Bioline, Australia). Conditions for amplification were an initial denaturation step of 5 min at 94 °C, followed by 40 cycles of 30 s at 94 °C, 30 s at $T_a$ (62 °C and 50 °C for TUB2 and EF1-α, respectively), and 30 s at 72 °C, and a final denaturation step of 7 min at 72 °C. The ITS and ACT amplifications were conducted as described by Aveskamp et al. (2009a). The PCR products were sequenced twice at both forward and reverse directions at the Australian Genome Research Facility (AGRF, Melbourne). The same primer combinations as the PCR were used for sequencing, except for LSU, where the primer LR5 (White et al. 1990) was used to ensure complete coverage of the locus (Aveskamp et al. 2009a). The consensus sequences were obtained by alignment of forward and reverse sequences using Geneious Pro v. 4.8.5 (Drummond et al. 2011). The consensus sequences were deposited in GenBank (Table 1), and taxonomic novelties were deposited in MycoBank (Crous et al. 2004).

BLAST searches in GenBank using the obtained LSU and ITS sequences revealed highest similarity with multiple *Stagonosporopsis* spp., recently classified in *Didymellaceae* by Aveskamp et al. (2010). Therefore, to study the taxonomic placement of the isolates within the *Stagonosporopsis* spp., sequences of *Stagonosporopsis* type strains available from GenBank were retrieved for inclusion in the phylogenetic analyses. Where sequences of the type strains were not available, those of reference strains according to Aveskamp et al. (2010) were used. Since sequences of translation elongation factor 1-alpha were not available for the GenBank retrievals, the phylogenetic analysis was conducted in two parts: first, the sequences were combined with the *Stagonosporopsis* reference sequences to construct a four-gene phylogeny showing the position of the ray blight isolates within the genus *Stagonosporopsis*. In the second analysis, the sequences of five loci obtained from the Australian and overseas ray blight isolates along with the reference strains of the ray blight pathogen were used to obtain a five-gene phylogeny depicting the phylogenetic relation of the ray blight isolates. All the isolates used in the phylogenetic analyses are shown in Table 1.

The sequences were aligned by ClustalW v. 2.0 (Larkin et al. 2007) and edited manually where necessary using Geneious Pro v. 4.8.5 (Drummond et al. 2011). For the first

**Table 1** Isolates used for DNA analysis. Where available, GenBank accession numbers are given. The accession numbers in bold were obtained from other studies. Sequences coded as QBank were not available in GenBank and were obtained from the Qbank website (www.q-bank.eu)

| Strain no. [a] | Holomorph[b] | Substrate | Country | GenBank Accession number | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | LSU | ITS | TUB2 | ACT | EF1-α |
| CBS 431.74; PD 74/2447 | *Boeremia exigua* var. *exigua* B | *Solanum tuberosum* | Netherlands | **EU754183** | **FJ427001** | **FJ427112** | **EU880854** | **GU349080** |
| CBS 106.96; PD 94/1318 | *Stagonosporopsis actaeae* T | *Actaea spicata* | Netherlands | **GU238166** | **GU237734** | **GU237671** | **QBank** | – |
| CBS 177.93; PD 90/115 | *S. ajacis* T | *Delphinium* sp. | Kenya | **GU238168** | **GU237791** | **GU237673** | **QBank** | – |
| CBS 101.80; PD 75/909; IMI 386090 | *S. andigena* B | *Solanum* sp. | Peru | **GU238169** | **GU237714** | **GU237674** | **QBank** | – |
| CBS 102636; PD 73/1409 | *S. artemisiicola* B | *Artemisia dracunculus* | France | **GU238171** | **GU237728** | **GU237676** | **QBank** | – |
| CBS 178.25; MUCL 9915 | *S. astragali* B | *Astragalus* sp. | Unknown | **GU238172** | **GU237792** | **GU237677** | **QBank** | – |
| CBS 248.90 | *S. caricae* | *Carica papaya* | Chile | **GU238175** | **GU237807** | **GU237680** | **QBank** | – |
| ATCC 10748 | *S. chrysanthemi* | *Chrysanthemum morifolium* | USA | JQ897460 | JQ897484 | JQ897504 | JQ897508 | JQ897521 |
| CBS 137.96 | *S. chrysanthemi* | *Chrysanthemum morifolium* | Netherlands | **GU238191** | **GU237783** | **GU237696** | **QBank** | JQ897525 |
| CBS 500.63; MUCL 8090 | *S. chrysanthemi* B | *Chrysanthemum indicum* | Germany | **GU238190** | **GU237871** | **GU237695** | **QBank** | JQ897537 |
| DSMZ 62547 | *S. chrysanthemi* | *Chrysanthemum* sp. | Germany | JQ897464 | JQ897482 | JQ897501 | JQ897505 | JQ897529 |
| DSMZ 63133 | *S. chrysanthemi* | *Chrysanthemum indicum* | Germany | JQ897465 | JQ897486 | JQ897492 | JQ897509 | JQ897535 |
| ICMP 10673 | *S. chrysanthemi* | *Chrysanthemum* sp. | New Zealand | JQ897466 | JQ897485 | JQ897497 | JQ897514 | JQ897524 |
| ICMP 2287 | *S. chrysanthemi* | *Chrysanthemum* sp. | New Zealand | JQ897467 | JQ897480 | JQ897494 | JQ897506 | JQ897522 |
| CBS 713.85; ATCC 76027; PD 83/826 | *S. crystalliniformis* T | *Lycopersicon esculentum* | Colombia | **GU238178** | **GU237903** | **GU237683** | **QBank** | – |
| CBS 133.96; PD 79/127 | *S. cucurbitacearum* | *Cucurbita* sp. | New Zealand | **GU238181** | **GU237780** | **GU237686** | **QBank** | – |
| CBS 631.68; PD 68/147 | *S. dennisii* B | *Solidago floribunda* | Netherlands | **GU238182** | **GU237899** | **GU237687** | **QBank** | – |
| CBS 426.90; IMI 386093; PD 86/551 | *S. dorenboschii* B | *Physostegia virginiana* | Netherlands | **GU238185** | **GU237862** | **GU237690** | **QBank** | – |
| CBS 109182; PD 74/231 | *S. heliopsidis* B | *Heliopsis patula* | Netherlands | **GU238186** | **GU237747** | **GU237691** | **QBank** | – |
| CBS 104.42 | *S. hortensis* B | Unknown | Netherlands | **GU238198** | **GU237730** | **GU237703** | **QBank** | – |
| CBS 562.81; ICMP 6884 | *S. loticola* T | *Lotus pedunculatus* | New Zealand | **GU238192** | **GU237890** | **GU237697** | **QBank** | – |
| CBS 101494; PD 98/5247 | *S. lupini* B | *Lupinus albus* | UK | **GU238194** | **GU237724** | **GU237699** | **QBank** | – |
| CBS 634.92; IMI 193307 | *S. oculo-hominis* T | Human | USA | **GU238196** | **GU237901** | **GU237701** | **QBank** | – |
| CBS 425.90; PD 81/520 | *S. inoxydabilis* T | *Chrysanthemum parthenii* | Netherlands | **GU238188** | **GU237861** | **GU237693** | **QBank** | JQ897527 |
| PD 85/259 | *S. inoxydabilis* | *Matricaria* sp. | Netherlands | **GU238189** | **GU237920** | **GU237694** | **QBank** | JQ897526 |
| CBS 109180; PD 79/175 | *S. rudbeckiae* B | *Rudbeckia bicolor* | Netherlands | **GU238197** | **GU237745** | **GU237702** | **QBank** | – |
| BRIP 57320; CBS 131484; TAS 1 | *S. tanaceti* T | *Tanacetum cinerariifolium* | Australia, Tas. | JQ897461 | JQ897481 | JQ897496 | JQ897512 | JQ897536 |
| BRIP 57321; CBS 131485; TAS 55504 | *S. tanaceti* | *T. cinerariifolium* | Australia, Tas. | JQ897462 | JQ897487 | JQ897502 | JQ897516 | JQ897520 |
| BRIP 57322; TAS 3 | *S. tanaceti* | *T. cinerariifolium* | Australia, Tas. | JQ897470 | JQ897476 | JQ897500 | JQ897517 | JQ897530 |
| BRIP 57323; TAS 55503 | *S. tanaceti* | *T. cinerariifolium* | Australia, Tas. | JQ897472 | JQ897477 | JQ897503 | JQ897510 | JQ897523 |
| DAR 70020 | *S. tanaceti* | *T. cinerariifolium* | Australia, Tas. | JQ897463 | JQ897488 | JQ897495 | JQ897513 | JQ897532 |

**Table 1** (continued)

| Strain no. [a] | Holomorph [b] | Substrate | Country | GenBank Accession number | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | LSU | ITS | TUB2 | ACT | EF1-α |
| TAS 4 | *S. tanaceti* | *T. cinerariifolium* | Australia, Tas. | JQ897471 | JQ897475 | JQ897493 | JQ897511 | JQ897528 |
| TAS 7 | *S. tanaceti* | *T. cinerariifolium* | Australia, Tas. | JQ897474 | JQ897478 | JQ897491 | JQ897507 | JQ897533 |
| TAS 18 | *S. tanaceti* | *T. cinerariifolium* | Australia, Tas. | JQ897468 | JQ897483 | JQ897490 | JQ897519 | JQ897538 |
| TAS 20 | *S. tanaceti* | *T. cinerariifolium* | Australia, Tas. | JQ897469 | JQ897489 | JQ897499 | JQ897518 | JQ897531 |
| TAS 58905 | *S. tanaceti* | *T. cinerariifolium* | Australia, Tas. | JQ897473 | JQ897479 | JQ897498 | JQ897515 | JQ897534 |
| CBS 379.91; PD 77/675 | *S. trachelii* B | *Campanula isophylla* | Netherlands | **GU238173** | **GU237850** | **GU237678** | **QBank** | – |
| CBS 329.67; PD 66/302 | *S. valerianellae* B | *Valerianella locusta* var. *oleracea* | Netherlands | **GU238201** | **GU237832** | **GU237706** | **QBank** | – |

[a] *ATCC* American Type Culture Collection, Virginia, U.S.A.; *CBS* Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; *DSMZ* Deutsche Sammlung von Mikroorganismen und Zellkulturen; *ICMP* International Collection of Micro-organisms from Plants; *IMI* International Mycological Institute, CABI-Bioscience, Egham, Bakeham Lane, U.K.; *MUCL* Mycotheque de l'Universite catholique de Louvain, Louvain-la-Neuve, Belgium; *PD* Plant Protection Service, Wageningen, the Netherlands; *TAS* Tasmanian Institute of Agriculture Fungal Collection

[b] *T* Ex-type strain; *B* Reference strain according to Boerema et al. (2004)

analysis including a four-locus phylogeny of *Stagonosporopsis* species, an unambiguous alignment of the ACT region was not possible due to a high level of polymorphism. Therefore, ambiguously aligned positions from intronic regions of ACT sequence were delimited manually, as described by Lutzoni et al. (2000), and excluded from the analysis. The alignments were deposited in TreeBASE (www.treebase.org/treebase-web/home.html).

Each phylogenetic analysis was conducted using two methods; Bayesian Inference (BI) and Maximum likelihood (ML). For BI analysis, the best nucleotide substitution model for each locus was determined by MrModeltest v. 2.3 (Nylander 2004) to be (SYM+I) for ITS; (GTR+I+G) for LSU and TUB2; and (GTR+I) for ACT and EF1-α. The congruency of the datasets was tested through Incongruence-Length Difference (ILD) partition homogeneity test (Farris et al. 1995) using PAUP v.4.0b10 (Swofford 2003). The sequence datasets did not show any conflict in tree topology; $p$ value=0.06 for the four-gene phylogeny, and 0.78 for the five-gene phylogeny. Therefore, the analyses of two MCMC chains were run for the concatenated sequence datasets using MrBayes v. 3.1.2 (Ronquist and Huelsenbeck 2003). One tree was saved per 100 generation and the run was ended automatically when the standard deviation of split frequencies reached below 0.01. To avoid suboptimal trees being taken into account in the consensus tree, a 25 % burn-in of the saved trees was used. The resulting 50 % majority rule consensus tree was viewed in TreeView v. 1.6.6. (Page 1996) and lodged with the TreeBASE (www.treebase.org/treebase-web/home.html). A second measure of branch support was obtained through ML analysis using the GTRGAMMA model applied to the individual partitions with 1,000 pseudoreplicates using RAxML (Randomised Axelerated Maximum Likelihood) v. 7.2.6 (Stamatakis 2006).

## Results

### Phylogeny

In the first analysis, consisting of 38 taxa in a four-locus phylogeny, the aligned matrix had a total length of 2 311 characters (LSU: 1 327, ITS: 493, TUB2: 342, ACT: 149), of which 2 135 (LSU: 1 305, ITS: 460, TUB2: 236, ACT: 134) were constant and 176 (LSU: 22, ITS: 33, TUB2: 106, ACT: 15) were variable. The consensus tree obtained from RAxML supported the tree topology obtained with BI. The analysis resulted in detection of the five *Stagonosporopsis* clades previously described by Aveskamp et al. (2010) (Fig. 1). The pyrethrum ray blight isolates from Australia clustered in a well-supported monophyletic group in clade S1, separate from the two previously described ray blight

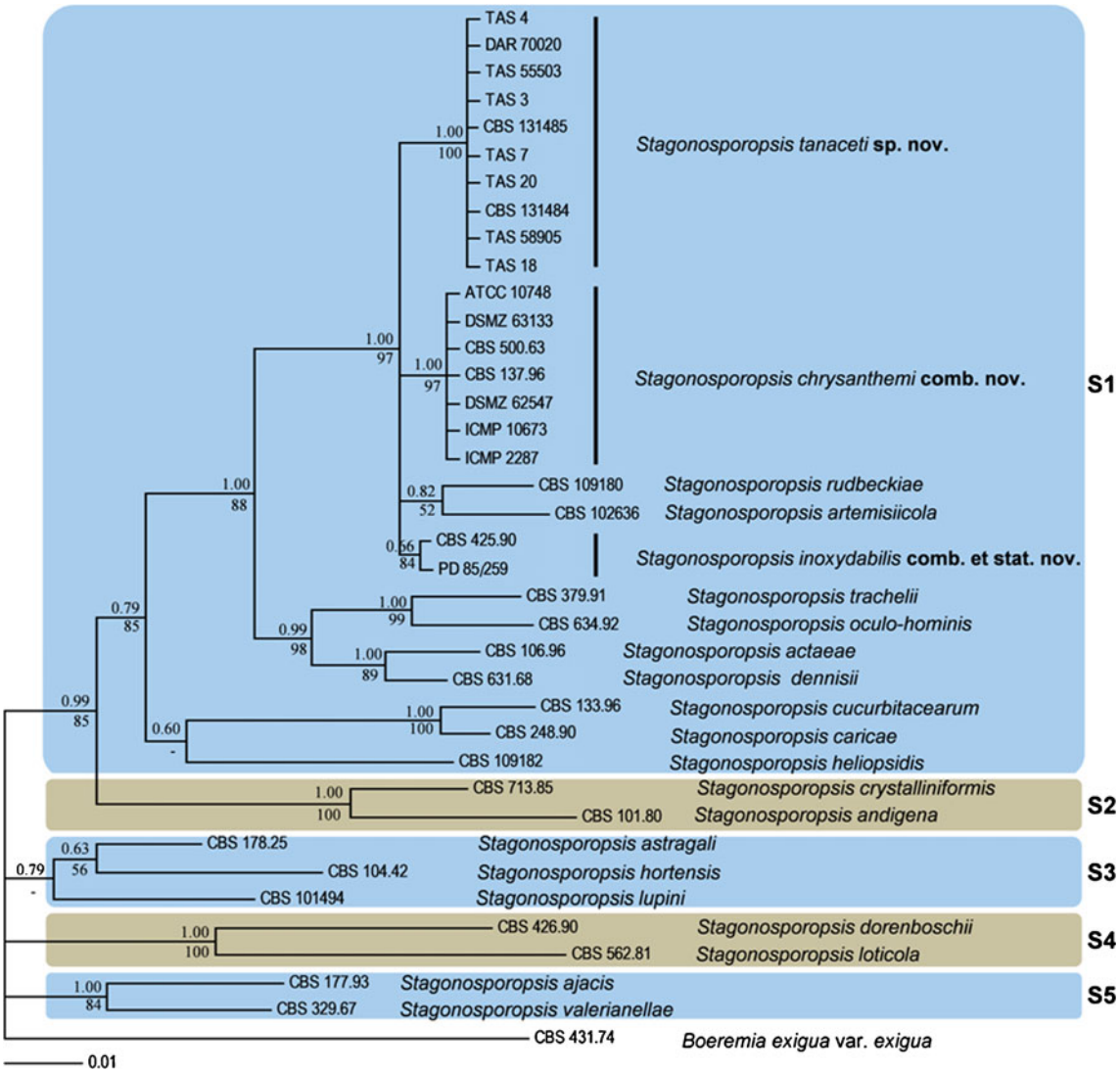


**Fig. 1** Reconstructed phylogeny of *Stagonosporopsis* spp. based on a 50 % majority rule consensus tree using BI analysis of the combined LSU, ITS, TUB2 and ACT alignment. The BI Posterior Probabilities are presented above the branch and bootstrap support values of the ML analysis are given below the branch. Branches with less than 50 % support in the ML analysis are shown with a *hyphen*. The *bar* indicates the number of substitutions per site. The tree is rooted with *Boeremia exigua* var. *exigua* (CBS 431.74). S1 to S5 represent the *Stagonosporopsis* clades defined by Aveskamp et al. (2010)

pathogens. In addition, isolates belonging to these two varieties clustered in separate monophyletic groups in S1. Other *Stagonosporopsis* species associated with other members of *Asteraceae* family were retrieved in S1 and S4 clades.

In the second analysis including only the ray blight isolates, a total of 2 914 characters were analysed (LSU: 1 327, ITS: 489, EF1-α: 470, TUB2: 333, ACT: 295), of which 133 sites were variable (LSU: 9, ITS: 18, EF1-α: 31, TUB2: 28, ACT: 47). The addition of the intron regions of ACT gene and EF1-α gene sequences to the analysis further supported the separation of the three ray blight pathogens into well-defined monophyletic clades (Fig. 2).

## Taxonomy

Morphological observations and phylogenetic analyses clearly showed that the Australian ray blight isolates represented a new species, described below. Furthermore, the two previously known varieties of the ray blight pathogen were elevated to species level based on the phylogenetic analyses as well as morphological and ecological information from previous studies. A list of other *Stagonosporopsis* spp. occurring on *Asteraceae* and the available information on their pathogenicity is also provided.

***Stagonosporopsis artemisiicola*** (Hollós) Aveskamp, Gruyter & Verkley, *Stud. Mycol.* 65: 44 (2010)

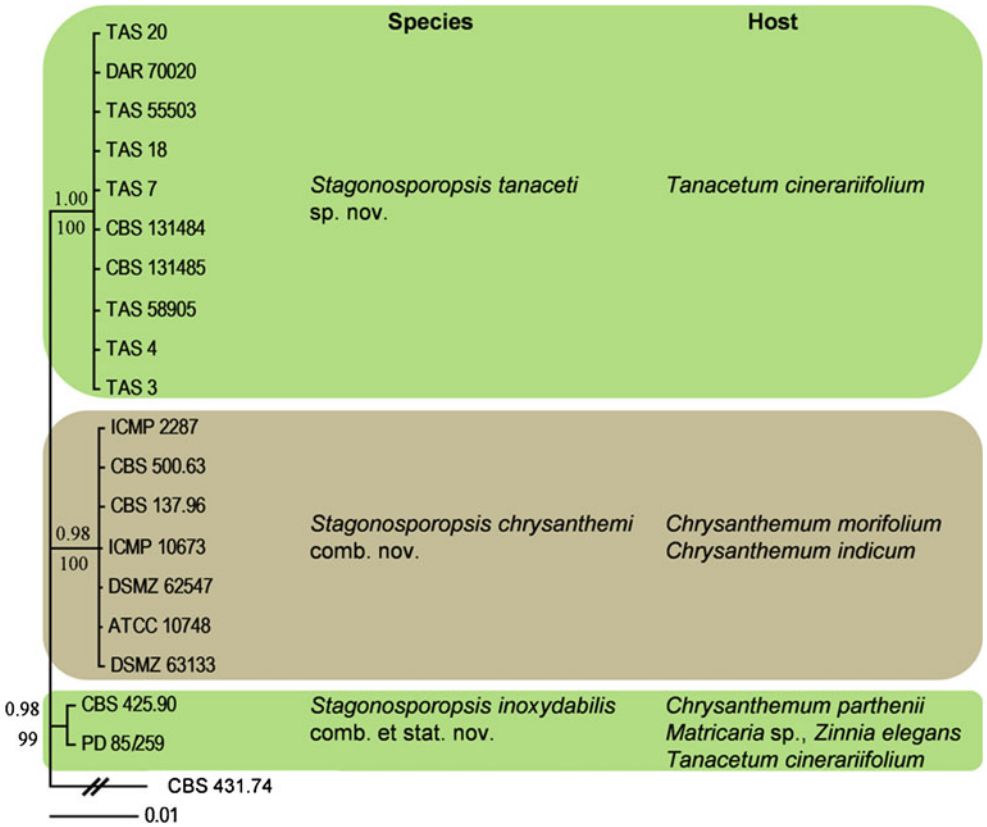


**Fig. 2** Bayesian phylogeny based on a 50 % majority rule consensus tree of the combined LSU, ITS, EF1-α, TUB2 and ACT alignment of the ray blight isolates and their *Asteraceae* hosts. The BI Posterior Probabilities are presented above the branch and bootstrap support values of the ML analysis are given below the branch. The *bar* indicates the number of substitutions per site. The tree is rooted with *Boeremia exigua* var. *exigua* (CBS 431.74)

*Basionym*: *Phoma artemisiicola* Hollós, *Mat. Természettud. Közlem.* 35: 40 (1926) (as '*artemisaecola*').

*Note*: Recorded on dead stems of wild *Artemisia vulgaris* in Hungary and cultivated plants of *A. dracunculus* in France. In the latter case the fungus was thought to be the cause of premature plant death (De Gruyter et al. 2002; Boerema et al. 2004).

***Stagonosporopsis chrysanthemi*** (F. Stevens) Crous, Vaghefi & P.W.J. Taylor, comb. nov. (Fig. 3).

MycoBank: MB 800037

*Basionym*. *Ascochyta chrysanthemi* F. Stevens, *Bot. Gaz.* 44(4): 246 (1907)

= *Mycosphaerella ligulicola*, K.F. Baker, Dimock & L.H. Davis, *Phytopathology* 39: 799 (1949)

≡ *Didymella ligulicola* (K.F. Baker, Dimock & L.H. Davis) Arx, *Beitr. Kryptfl. Schweiz* 11(2): 364 (1962)

≡ *Didymella ligulicola* (K.F. Baker, Dimock & L.H. Davis) Arx var. *ligulicola, Stud. Mycol.* 32: 9 (1990)

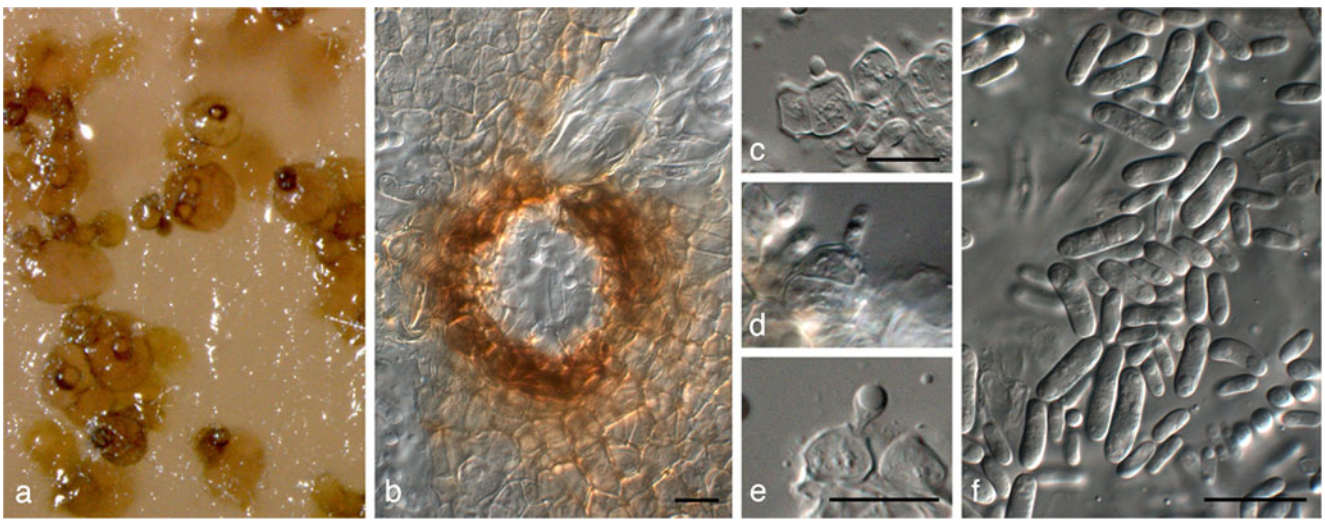


**Fig. 3** *Stagonosporopsis chrysanthemi* (CBS 500.63). **a** Colony sporulating on OA; **b** close-up of darkened ostiolar area; **c**–**e** conidiogenous cells; **f** conidia. (Scale bars = 10 μm)

≡ *Stagonosporopsis ligulicola* (K.F. Baker, Dimock & L.H. Davis) Aveskamp, Gruyter & Verkley var. *ligulicola*, *Stud. Mycol.* 65: 46 (2010)

= *Phoma ligulicola* Boerema var. *ligulicola*, *Stud. Mycol.* 32: 9 (1990)

Not to be confused with:

*Mycosphaerella chrysanthemi* (Tassi) Tomilin, Opredelitel' Gribov roda Mycosphaerella Johans: 60 (1979)

*Basionym*. *Sphaerella chrysanthemi* Tassi, Bulletin Labor. Orto Bot. de R. Univ. Siena 3: 117 (1900)

≡ *Didymella chrysanthemi* (Tassi) Garib. & Gullino, *L'Agricoltura Italiana*, Pisa 71: 286 (1971)

*Phomopsis chrysanthemi* (Voglino) M.E.A. Costa & Sousa da Câmara, Portug. *Acta Biol.* 3: 301 (1952)

*Basionym. Phoma chrysanthemi* Voglino, *Malpighia* 16: 329 (1902)

*Specimen examined*: Germany, Berlin, from *Chrysanthemum indicum*, 1963, *R. Schneider*, CBS H-11952, culture CBS 500.63=MUCL 8090.

*Notes*: Aveskamp et al. (2010) proposed the name *Stagonosporopsis ligulicola* var. *ligulicola*, and cited *Mycosphaerella ligulicola* (Baker et al. 1949) as the basionym. Here, we argue that the correct basionym is *Ascochyta chrysanthemi* (Stevens 1907). After describing the teleomorph in *Mycosphaerella*, Baker et al. (1949) introduced the epithet *ligulicola* (from the Latin *ligua*; ray flower, and *colo*; to inhabit) to avoid confusion with *Sphaerella chrysanthemi* (Tassi 1900). Later, when the anamorph was transferred to the genus *Phoma*, Van der Aa et al. (1990) maintained the epithet *ligulicola* to avoid homonymy with *Phoma chrysanthemi* (Voglino 1902). Therefore, after transferring the species to *Stagonosporopsis*, the older epithet, *i.e.*, *chrysanthemi* has preference over *ligulicola*, thus a combination based on the oldest epithet is proposed.

For detailed in vivo and in vitro descriptions as well as ecological information refer to Van der Aa et al. (1990) and De Gruyter et al. (2002). Host range of the ray blight pathogen of chrysanthemum has been studied (Chesters and Blakeman 1967; Peregrine and Watson 1964). However, this was before the separation of the species into different varieties; therefore, it is not clear which fungus was tested for pathogenicity.

***Stagonosporopsis dennisii*** Boerema, Gruyter & Noordel., *Persoonia* 16(3): 350 (1997)

*Note*: Recorded on dead stems of *Solidago* spp. No data on pathogenicity (Boerema 1976; Boerema et al. 2004).

***Stagonosporopsis dorenboschii*** (Noordel. & Gruyter) Aveskamp, Gruyter & Verkley, *Stud. Mycol.* 65: 45 (2010)

*Basionym*: *Phoma dorenboschii* Noordel. & Gruyter, *Persoonia* 15(1): 83 (1992)

*Note*: Isolated from leaves and stems of different plant families including *Asteraceae* (on *Callistephus* sp.), causing leaf spots and anthracnose. It seems to be a plurivorous opportunistic pathogen (Boerema and Noordeloos 1992; Boerema et al. 2004).

***Stagonosporopsis heliopsidis*** (H. C. Greene) Aveskamp, Gruyter & Verkley, *Stud. Mycol.* 65: 45 (2010)

*Basionym*: *Phyllosticta heliopsidis* H.C. Greene, *Trans. Wisconsin Acad. Sci.* 50: 158 (1961)

*Note*: Recorded on *Heliopsis* spp. in the USA and *Ambrosia artemisiifolia* in Canada. Mostly affecting leaves but also stems and inflorescences (Greene 1961; De Gruyter et al. 2002; Boerema et al. 2004).

***Stagonosporopsis inoxydabilis*** (Boerema) Crous, Vaghefi & P.W.J. Taylor, comb. et stat. nov. (Fig. 4).

MycoBank: MB 800045

*Basionym*. *Phoma ligulicola* var. *inoxydabilis* Boerema, *Stud. Mycol.* 32: 9 (1990)

≡ *Didymella ligulicola* var. *inoxydabilis* Boerema, *Stud. Mycol.* 32: 9 (1990)

≡ *Stagonosporopsis ligulicola* var. *inoxydabilis* (Boerema) Aveskamp, Gruyter & Verkley, *Stud. Mycol.* 65: 45 (2010)

*Specimen examined*: The Netherlands, from *Chrysanthemum parthenii*, 1981, *G.H. Boerema*, (holotype specimen CBS H-7611, culture ex-holotype CBS 425.90= PD 81/520).

*Notes*: Differs from *Stagonosporopsis chrysanthemi* by frequent production of pseudothecial ascomata in culture, slower growth rate, less conidial variability and no production of pigments upon NaOH application. For detailed in vivo and in vitro description as well as ecological information refer to Van der Aa et al. (1990) and De Gruyter et al. (2002).

Van der Aa et al. (1990) considered it likely that *Phoma chrysanthemi* (Voglino 1902) belonged to this species. This observation was based on published descriptions, as Boerema and Van Kesteren (1974) had previously discussed the similarity of Voglino's type material to *Ascochyta chrysanthemi* (Boerema et al. 2004). This synonymy, however, could not be established here as Voglino's type specimen was not studied.

***Stagonosporopsis rudbeckiae*** (Fairm.) Aveskamp, Gruyter & Verkley, *Stud. Mycol.* 65: 45 (2010)

*Basionym*. *Phoma rudbeckiae* Fairm., *Proc. Rochester Acad. Sci.* 1: 51 (1890)

*Note*: Specific pathogen of *Rudbeckia* spp., causing leaf spots; large opaque-blackish lesions with a clearly defined outline (De Gruyter et al. 2002; Boerema et al. 2004).

***Stagonosporopsis tanaceti*** Vaghefi, S.J. Pethybridge, Crous & P.W.J. Taylor, sp. nov. (Fig. 5).

MycoBank: MB 800025

*Etymology*: Named after the host genus on which it occurs, *Tanacetum.*

*Conidiomata* pycnidial, unilocular, solitary or confluent, on the agar surface and immersed, globose to subglobose,

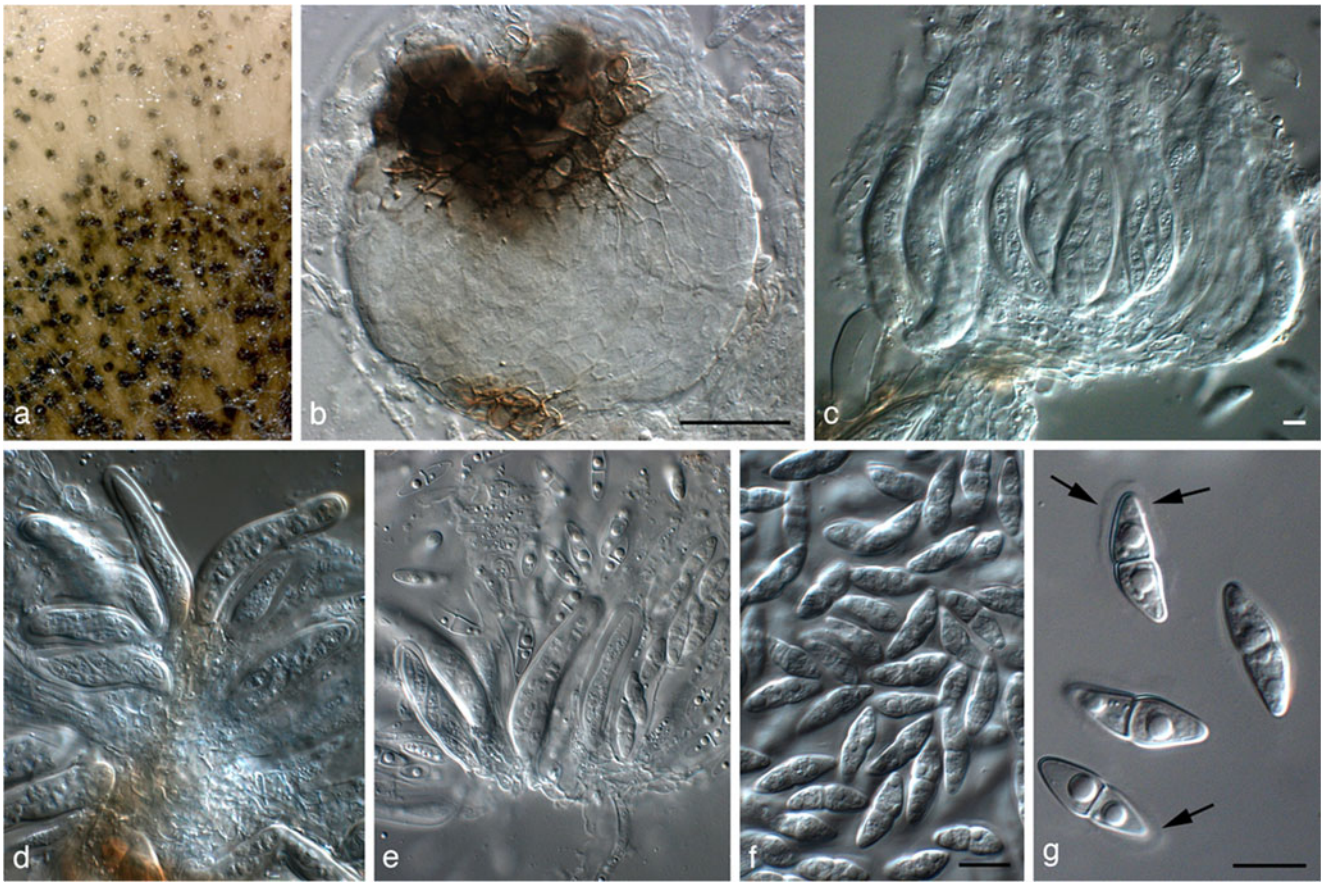


**Fig. 4** *Stagonosporopsis inoxydabilis* (CBS 425.90). **a** Colony sporulating on OA; **b** close-up of ascoma with darkened ostiolar area; **c**–**e** Stipitate, bitunicate asci; **f**, **g** Ascospores (*arrows* denote sheath) (Scale bars: b = 35 μm, all others = 10 μm)

glabrous or with mycelial outgrowth, with 1–3 ostioles, non- to slightly papillate, 90–450 μm diam. *Conidiogenous cells* lining the inner cavity, hyaline, smooth, subglobose to ampulliform, 6–8×4–6 μm. *Conidia* ellipsoid to oblong, thin-walled, hyaline, with two polar guttules, mainly aseptate, rarely 1-euseptate, (4.5–)7–9(−15)×(2.5–)3–3.5(−4.5) μm, but occasionally distinctly large, up to 20×4.5 μm. *Chlamydospores* aseptate, globose, brown, intercalary, in chains or clusters, present in older cultures. Description based on OA.

Sexual state unknown.

Culture characteristics: On OA: colonies reaching 43–60 mm diam after 7 day, margin regular or slightly irregular. Aerial mycelium floccose and white. Colony variable from white/cream to grey olivaceous and olivaceous black in centre. Reverse similar. Pycnidia rare to abundant, scattered on the surface or submerged in agar. No colour change upon application of NaOH; On MEA: Growth rate 45–70 mm diam after 7 day, margin regular or slightly irregular. Aerial mycelium floccose to felty, white/cream to pale buff and pale olivaceous grey. Colony buff to pale olivaceous grey. Reverse buff to rosy buff darkening to black towards the centre; On CHA: Growth rate 40–50 mm diam after 7 day, surface olivaceous grey to olivaceous black, partly to completely covered by woolly smoke-grey to pale olivaceous grey aerial mycelium. Reverse similar.

*Holotype*: Australia, northern Tasmania, Scottsdale, from *Tanacetum cinerariifolium,* 2004, *S.J. Pethybridge*, (CBS H-20947, culture ex-type CBS 131484, ex-isotype culture BRIP 57320 = TAS 1).

*Note*: Differs from *Stagonosporopsis chrysanthemi* by absence of ascomata in culture, slower growth rate, wider and less variable conidia and no production of pigments upon NaOH application. *Stagonosporopsis tanaceti* shows some resemblance to *S. inoxydabilis* but can be differentiated by its faster growth rate, larger conidia, presence of chlamydospores, and more importantly, lack of ascomata, which develop intermingled with pycnidia in fresh cultures of *S. inoxydabilis*. *Stagonosporopsis tanaceti* has been found to infect other *Asteraceae* species, namely *Tagetes patula* and *Chrysanthemum carinatum*, after artificial inoculation of plants in glasshouse but, contrary to *S. inoxydabilis*, is unable to infect *Zinnia elegans* (Pethybridge et al. 2008a).

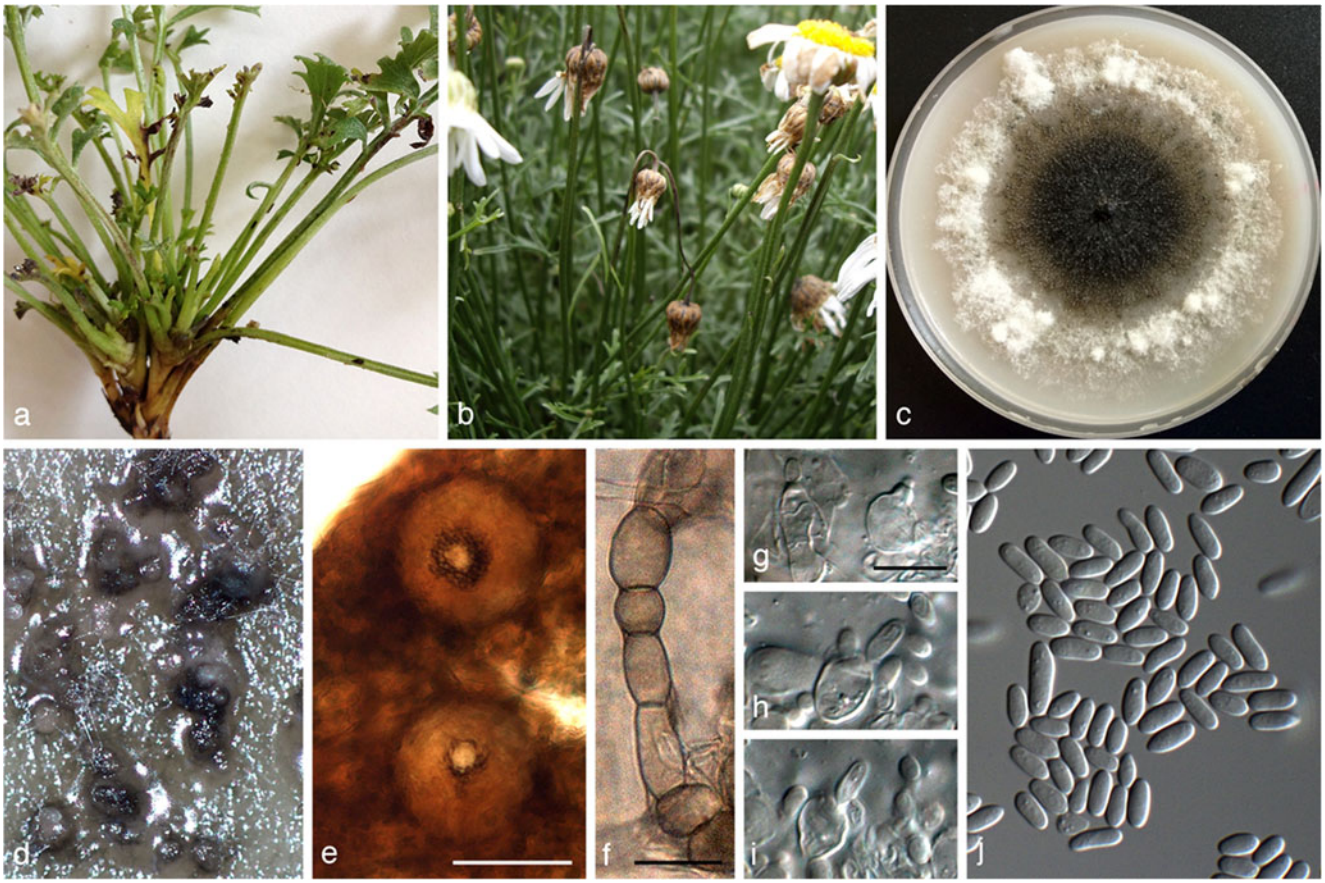


**Fig. 5** *Stagonosporopsis tanaceti* (CBS 131484). **a** Leaf necrosis; **b** drooping flower heads; **c** colony on OA; **d**, **e** close-up of pycnidia, showing darkened ostiolar area; **f** chain of chlamydospores; **g**–**i** conidiogenous cells; **j** conidia. (Scale bars: e = 150 μm, all others = 10 μm)

## Discussion

The taxonomy of the pathogens associated with ray blight of *Asteraceae* was investigated, which resulted in the recognition of three species, namely *Stagonosporopsis tanaceti*, *S. inoxydabilis* and *S. chrysanthemi*, based on morphological features and phylogenetic analyses.

A previous phylogenetic study on the ray blight pathogens was based solely on ITS sequence data, which was unable to distinguish the different species associated with ray blight of *Asteraceae* (Pethybridge et al. 2004). The present study also found the ITS sequence to be of low discriminatory value at species level, as only a single nucleotide change was found between the species. This is in line with other studies that showed the ITS sequence to be insufficient for species recognition within *Phoma*-like genera (Abeln et al. 2002; Cullen et al. 2007). Variation found in the partial TUB2, EF1-α and ACT sequences in the current study proved useful in distinguishing the three ray blight pathogens, with the ACT sequence showing the highest degree of polymorphism. The high discriminatory power of ACT sequence data and its potential for development of DNA barcodes has been suggested before (Aveskamp et al. 2009b, 2010). Aveskamp et al. (2010) tested partial EF1-α and calmodulin (CAL) gene sequences for species recognition within the *Didymellaceae,* but found that the primer combinations developed by Carbone and Kohn (1999) failed to amplify the targeted loci in some strains. In the current study, the EF1-α locus was successfully amplified using the primer pair EF1-983F and EF1-1567R (http://www.aftol.org/pdfs/EF1primer.pdf). However, the attempts to amplify the CAL locus using primers CAL-228F, CAL-737R (Carbone and Kohn 1999) as well as the reverse primer CAL2Rd (Quaedvlieg et al. 2011) failed to produce a product for the *S. tanaceti* strains.

The nomenclature of the ray blight pathogen has been controversial and has undergone many changes, reviewed by Walker and Baker (1983). The controversy was due to confusion with *Mycosphaerella chrysanthemi* (Tassi) Tomilin and *Phoma chrysanthemi* Voglino, which were suggested as replacement names for the ray blight pathogen by Garibaldi and Gullino (1971). Since Tassi's and Voglino's type material were not studied here, it was not possible to accept or reject their synonymy with the ray blight pathogens

recognised here. Considering the well-established evidence that classification of *Phoma*-like organisms based on morphological features alone can be misleading (Aveskamp et al. 2008, 2009a, 2010; De Gruyter et al. 2009, 2010, 2012), it is suggested that further studies using molecular analysis be conducted on herbarium type specimens of the aforementioned binomials, before establishing any synonymy to the ray blight species described here.

*Stagonosporopsis tanaceti* was newly described as the causal agent of ray blight of pyrethrum in Australia. Ray blight has previously been reported to occur on chrysanthemum in Australia, with the causal agent identified as *Ascochyta chrysanthemi* at the time (Oxenham 1963; Simmonds 1996). However, since we were not able to locate the associated isolates, it was not possible to validate their identification as either *S. chrysanthemi* or *S. tanaceti* as both species have been found to infect *Chrysanthemum* spp. (Van der Aa et al. 1990; Boerema et al. 2004; Pethybridge et al. 2008a). *Stagonosporopsis inoxydabilis*, reported to infect pyrethrum plants in Europe (Van der Aa et al. 1990), is not yet known to occur in Australia. This has important quarantine implications in terms of preventing the introduction of the species into Australia. Moreover, it highlights the need for the development of taxon-specific DNA-based markers with the ability to differentiate the three ray blight species recognised here.

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