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BREAKTHROUGHS AND RESOURCES

First molecular phylogeny of mycoparasitic species of *Sphaerellopsis* isolated from rust fungi in Australia

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Abstract
Three species, *Sphaerellopsis filum*, *S. macroconidialis*, and *S. paraphysata*, from Queensland and Victoria, Australia, were identified and characterised by multilocus sequence analyses. This study clarifies earlier reports of *Sphaerellopsis* in Australia and provides the first report of *S. filum* in Australia. We also confirm the presence of *S. macroconidialis* and *S. paraphysata* in Australia. A single-locus phylogeny based on the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) provided sufficient resolution for species-level identification and yielded a topology consistent with that of the combined dataset of ITS, large subunit of the rDNA, and the RNA polymerase II second largest subunit. The high intron variability in the translation elongation factor 1-α region among *Sphaerellopsis* spp. made it unsuitable for phylogenetic analysis. The specimens and data generated here lay the groundwork for future studies into the evolution and molecular basis of mycoparasitism in *Sphaerellopsis*.

Keywords Rust hyperparasite · *Sphaerellopsis filum* · *S. paraphysata* · *S. macroconidialis*

Introduction

The fungal genus *Sphaerellopsis* (*Leptosphaeriaceae*, *Pleosporales*) accommodates nine recognised species (https://www.indexfungorum.org/), seven of which are supported by DNA sequence data (Gómez-Zapata et al. 2024). Most *Sphaerellopsis* spp. have been found in association with rust fungi (*Pucciniales*). However, in vitro evidence of mycoparasitism has only been confirmed for *S. filum*, *S. macroconidialis*, and *S. paraphysata* (Gómez-Zapata et al. 2024). Other species, such as *S. artemisiae* and *S. isthmospora*, were considered saprobic (Phookamsak et al. 2019; Doilom et al. 2021). Further investigation is needed to understand the geographic distribution, host range, and species diversity within *Sphaerellopsis*, and to elucidate the mechanisms underlying their mycoparasitic and saprobic lifestyles, as well as their potential application in the biological control of rust fungi.

Despite the ecological importance of *Sphaerellopsis*, little is known about its diversity in Australia. To date, four species, namely, *S. filum* (Driessen et al. 2004), *S. hakeae* (Crous et al. 2016), *S. macroconidialis*, and *S. paraphysata* (Gómez-Zapata et al. 2024), have been recorded in Australia. Of these, *S. hakeae* has only been reported from Australia (Crous et al. 2016; Gómez-Zapata et al. 2024). Prior to this study, no viable cultures of *Sphaerellopsis* were maintained in Australian culture collections. Here, we report multilocus characterisation of several *Sphaerellopsis* spp. collected from different parts of Australia. Cultures of these fungi have been deposited in the Queensland Plant Pathology Herbarium (BRIP, Brisbane, Qld), Victorian Plant Pathogen Collection (VPRI, Melbourne, Australia), and Westerdijk Fungal Biodiversity Institute (CBS, Utrecht, Netherlands) to support future studies on the evolution and molecular mechanisms of mycoparasitism in this important group of fungi.

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Materials and methods

Plant samples with rust pustules (*Pucciniales*) were collected in Queensland and Victoria, Australia (Table 1). Each specimen was screened for *Sphaerellopsis*-like pycnidia in rust pustules under a stereomicroscope, and individual pycnidia were transferred to 1 ml of sterile distilled water for 5 min before streaking the supernatant onto streptomycin-amended potato dextrose agar (Amyl Media, Australia), V8 agar (10% v/v clarified, Campbell Soup Company, Australia), and water agar (LabChem, Australia). Single-conidium isolates were established as described by Driessen et al. (2004) and maintained at 24 °C under a 12-h photoperiod. DNA extraction from 4-week-old mycelia cultured in Czapek-Dox broth (Bacto Laboratories, Australia) was completed as described by Vaghefi et al. (2024).

Four loci were amplified and sequenced for the identification of *Sphaerellopsis* spp. (Gómez-Zapata et al. 2024). These included the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (nrDNA), partial sequences of the large subunit of rDNA (28S), RNA polymerase II second largest subunit (*RPB2*), and translation elongation factor 1-α (*TEF-1α*). PCR reactions consisted of Hot Start Taq Master Mix (New England Biolabs Inc, USA), 0.4 µM of forward and reverse primers, and 10 ng of the target DNA. PCR protocols included an initial denaturation step for 30 s at 95 °C, followed by 30 cycles (35 for *TEF-1α*) of denaturation at 95 °C for 30 s, annealing for 30 s at 55 °C for ITS, *TEF-1α* and *RPB2*, and 45 s at 54 °C for LSU, and elongation at 68 °C for 60 s, and a final extension for 5 min at 68 °C. Sanger sequencing was conducted by Macrogen Inc. (Seoul, South Korea) using the PCR amplification primers.

Consensus sequences were produced in Geneious Prime v.2025.1.2 (Biomatters Inc., New Zealand) and deposited in the NCBI GenBank database (Table 1). Reference *Sphaerellopsis* sequences were obtained from Trakunyingcharoen et al. (2014), Crous et al. (2016), Phookamsak et al. (2019), Doilom et al. (2021), and Gómez-Zapata et al. (2024). The ITS sequence of WAC 11350 recorded as *S. filum* in Western Australia was obtained from Driessen et al. (2004). *RPB2* sequences for strains CBS 234.51 and CBS 235.51 were extracted from their genome (D'Angelo et al. 2025). Alignments and Maximum Likelihood phylogenetic trees were constructed as described by Vaghefi et al. (2020).

Results

Seven isolates of *Sphaerellopsis* species were recovered from rust pustules collected in Queensland and Victoria (Table 1). Three isolates were identified as *S. filum* from *Puccinia* spp. infecting *Holcus* and *Poa* hosts in Victoria, while two isolates from *Puccinia allii* on *Allium fistulosum* in the Queensland were identified as *S. macroconidialis*. In addition, two isolates of *S. paraphysata* were recovered from *Puccinia clemensiae* infecting *Smilax australis* in Queensland. Voucher cultures of all isolates were deposited in Queensland Plant Pathology Herbarium, Victorian Plant Pathogen Collection, and Westerdijk Fungal Biodiversity Institute to support future research.

Attempts to use the *TEF-1α* region for phylogenetic analyses were unsuccessful due to the high intron variability across species (only 18.8% conserved sites) (Fig. 1A and B), resulting in poor sequence alignment quality. Therefore, *TEF-1α* was excluded from further analyses. Phylogenetic analyses based on the ITS region yielded a well-resolved, single-locus phylogeny consistent with the topology of the multi-locus phylogeny constructed using concatenated sequences of ITS, 28S, and *RPB2* regions (Fig. 1C and D). An isolate originally reported as *S. filum* on *Puccinia boroniae* in Western Australia (WAC 11350; Driessen et al. 2004) clustered with *S. macroconidialis*.

Discussion

This study provides the first report of *S. filum* in Australia and confirms the presence of *S. macroconidialis* and *S. paraphysata* in eastern Australia, extending earlier records by Gómez-Zapata et al. (2024) based on herbarium specimens. The detection of *S. macroconidialis* on *Puccinia allii*, along with the re-identification of isolate WAC 11350 (Driessen et al. 2004), indicates a broader distribution of this species in Australia than previously recognised. In contrast, *S. hakeae*, so far reported only from Australia (Crous et al. 2016; Gómez-Zapata et al. 2024), and *S. artemisiae* and *S. isthmospora*, known only from China (Doilom et al. 2021; Phookamsak et al. 2019), were not found in this study.

The *TEF-1α* primers recommended by Gómez-Zapata et al. (2024) (EF1-728F and EF1-986R; Carbone and Kohn 1999) amplified a ~ 300-bp intron-rich region that is conserved within species, but highly variable among *Sphaerellopsis* spp., making alignment highly ambiguous and unreliable. For future studies, it may be preferable to use the primers listed by Trakunyingcharoen et al. (2014), which amplify a region spanning several exons that are more conserved among *Sphaerellopsis* species.

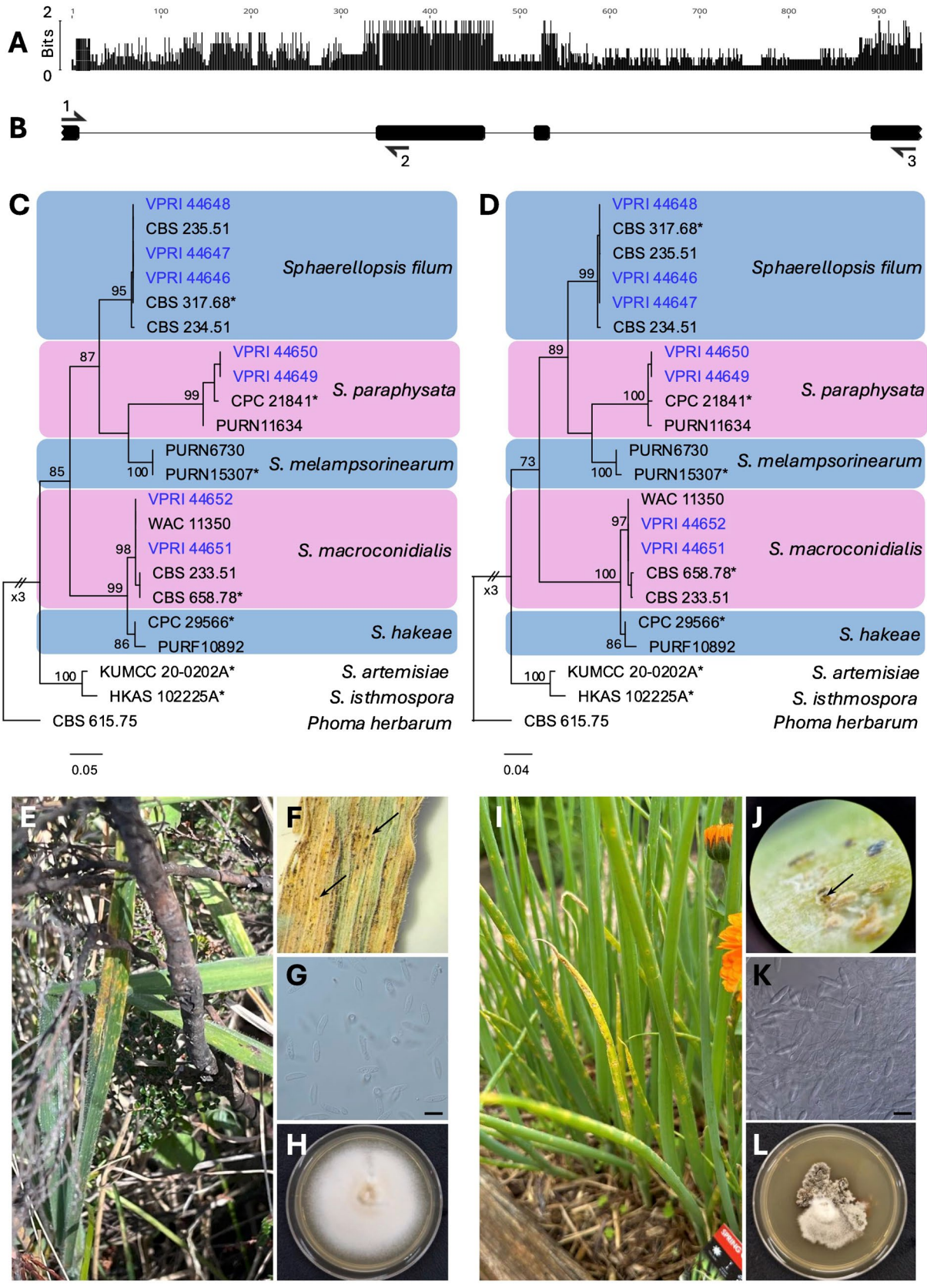
The global biogeography of *Sphaerellopsis* is poorly documented, but a recent study suggested no strong host-specificity for *Sphaerellopsis* species (Gómez-Zapata et al. 2024). This pattern is consistent with the ecological

Table 1 *Sphaerellopsis* isolates collected and sequenced in this study

Species	Strain[a]	Source	Location	Date	Collector	GenBank accession number[b]			
						28S	ITS	*RPB2*	*TEF-1α*
Sphaerellopsis filum	UOM 24108; VPRI 44648	*Puccinia* sp. ex *Poa* sp.	Macedon Ranges, Victoria	03/06/2024	J. Risteski	PV658825	PV658818	PV698344	PV679997
	UOM 24110; VPRI 44647	*Puccinia* sp. ex *Holcus* sp.	Mt Buffalo, Victoria	01/05/2024	J. Risteski	PV658824	PV658817	PV698343	PV679996
	UOM 24111; VPRI 44646	*Puccinia* sp. ex *Holcus* sp.	Mt Buffalo, Victoria	01/05/2024	J. Risteski	PV658823	PV658816	PV698342	PV679995
S. macroconidialis	UOM 25055; VPRI 44651	*Puccinia allii* ex *Alliumfistulosum*	Toowoomba, Queensland	29/09/2024	L. Kiss	PV658828	PV658821	PV698347	PV680000
	UOM 25054; VPRI 44652	*P. allii* ex *A.fistulosum*	Toowoomba, Queensland	29/09/2024	L. Kiss	PV658829	PV658822	PV698348	PV680001
S. paraphysata	UOM 25060; VPRI 44649	*Puccinia clemensiae* ex *Smilax australis*	Eliot Creek, Queensland	08/08/2024	R.G. & M.D.E Shivas	PV658826	PV658819	PV698345	PV679998
	UOM 25058; VPRI 44650	*P. clemensiae* ex *S. australis*	Eliot Creek, Queensland	08/08/2024	R.G. & M.D.E Shivas	PV658827	PV658820	PV698346	PV679999

[a]UOM, Plant pathology culture collection of the University of Melbourne; VPRI, Victorian Plant Pathogen Herbarium, Melbourne, Victoria, Australia

[b]28S, large subunit of nuclear ribosomal DNA (rDNA); ITS, internal transcribed spacer (ITS) region of the rDNA; *TEF-1α*, translation elongation factor 1-α; *RPB2*, RNA polymerase II second largest subunit


A
Bits
B
C
VPRI 44648
CBS 235.51
VPRI 44647
VPRI 44646
CBS 317.68*
CBS 234.51
Sphaerellopsis filum
VPRI 44650
VPRI 44649
CPC 21841*
PURN11634
S. paraphysata
PURN6730
PURN15307*
S. melampsorinearum
VPRI 44652
WAC 11350
VPRI 44651
CBS 233.51
CBS 658.78*
S. macroconidialis
CPC 29566*
PURF10892
S. hakeae
KUMCC 20-0202A*
HKAS 102225A*
S. artemisiae
S. isthmospora
CBS 615.75
Phoma herbarum
0.05
D
VPRI 44648
CBS 317.68*
CBS 235.51
VPRI 44646
VPRI 44647
CBS 234.51
Sphaerellopsis filum
VPRI 44650
VPRI 44649
CPC 21841*
PURN11634
S. paraphysata
PURN6730
PURN15307*
S. melampsorinearum
WAC 11350
VPRI 44652
VPRI 44651
CBS 658.78*
CBS 233.51
S. macroconidialis
CPC 29566*
PURF10892
S. hakeae
KUMCC 20-0202A*
HKAS 102225A*
S. artemisiae
S. isthmospora
CBS 615.75
Phoma herbarum
0.04
E
F
G
H
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◂**Fig. 1** **A** Distribution of nucleotide positions across the partial *TEF-1α* exons and introns for *Sphaerellopsis* isolates with available sequence data. *TEF-1α* sequences for CBS 234.51 and CBS 235.51 were extracted from their genome (D'Angelo et al. 2025). Each bar represents one nucleotide position, and the height of the bar is proportional to the information content of that position. **B** Map of the partial *TEF-1α* gene showing exons (blocks) and introns (lines). Numbered half-arrows show the position of primers (1: EF1-728: CAT CGA GAA GTT CGA GAA GG, 2: EF1-986R TAC TTG AAG GAA CCC TTA CC (Carbone, Kohn 1999), 3: EF-2: GGA(R)GTA CCAGT(S)ATCATGTT (O'Donnell et al. 1998)). **C** Single-locus phylogeny of *Sphaerellopsis* species inferred using Maximum Likelihood (ML) analysis of the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA). **D** Multilocus phylogeny of *Sphaerellopsis* species inferred using ML analysis of concatenated alignment of the ITS region, partial large subunit of the rDNA, and partial RNA polymerase II second largest subunit. Ex-type strains are denoted by an asterisk. ML bootstrap values based on 1000 pseudoreplicates are shown at the nodes. The trees were rooted to *Phoma herbarum* (CBS 615.75). The scale bar shows nucleotide substitutions per site. (**E**) Symptoms of *Puccinia* sp. infected with *S. filum* on *Holcus* sp. plant. **F** Pycnidia of *S. filum* within *Puccinia* sp. uredinia on a *Holcus* sp. leaf (arrows). **G** Pycnidiospores of *S. filum* VPRI 44647. **H** Colony of *S. filum* VPRI 44647 on saccharose-free Czapek-Dox medium supplemented with 2% malt extract (MCzDA) (Szentiványi et al. 2005). **I** Symptoms of *Puccinia allii* infected with *S. macroconidialis* on *Allium fistulosum*. **J** Pycnidia of *S. macroconidialis* within uredinia of *Puccinia allii* (arrow). **K** Pycnidiospores of *S. macroconidialis* VPRI 44652. **L** Colony of *S. macroconidialis* VPRI 44652 on MCzDA. Scale bars: 10 µm

association between *Sphaerellopsis* and *Pucciniales*, which they parasitise. *Sphaerellopsis macroconidialis*, *S. paraphysata*, *S. filum*, and *S. melampsorinearum* were reported to have a cosmopolitan distribution, although *S. paraphysata* was more abundant in the tropics (Gómez-Zapata et al. 2024), which is consistent with our finding of this species in Queensland.

To the best of our knowledge, only one living culture of *S. hakeae* and one of *S. paraphysata* from Australia are held in the Westerdijk Culture Collection (CBS), along with herbarium specimens of *S. hakeae* and *S. macroconidialis* in the Purdue University Fungal Reference Collection (PUR). No living cultures of any *Sphaerellopsis* species were previously available in Australian culture collections. Our study contributes additional isolates of *S. paraphysata* to public collections, as well as the first *S. filum* and *S. macroconidialis* isolates recorded in Australia. The preservation of viable cultures of these isolates in public collections will enable research into their biology and potential use as biocontrol agents against rust fungi. Given the ecological importance of *Sphaerellopsis* as mycoparasites of rusts, understanding their evolutionary history, distribution, and molecular basis of mycoparasitism will be critical for exploring their application in integrated rust management programmes.

Author contribution Niloofar Vaghefi, Levente Kiss, and Alexander Idnurm contributed to the study conception and design. Material preparation, data collection, and analyses were performed by Jason Risteski, Levente Kiss, Roger Shivas, Joshua Sun, Yu Pei Tan, and Niloofar Vaghefi. The first draft of the manuscript was written by Jason Risteski and Niloofar Vaghefi, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability All sequences produced in this study are publicly available in NCBI GenBank database. Sequence alignments and phylogenetic trees are available at https://zenodo.org/records/15779777.

Declarations

Ethical approval Not applicable.

Consent to participate Not applicable.

Consent to publish Not applicable.

Competing interests The authors declare no competing interests.

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