FINAL REPORT

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Identifying the presence of *Tolypocladium cylindrosporum* in Australian sheep growing areas

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Executive Summary

Initial analysis of existing sequence data suggested that the fungus *Tolypocladium cylindrosporum* was likely to be present in soil in Australia, particularly in Tasmania and Victoria. A diagnostic assay that can distinguish *T. cylindrosporum* from the closely related *T. inflatum* fungus was designed and optimised. A total of 28 soil samples was collected from five properties in Tasmania and two properties in Victoria. DNA extracted from these samples was analysed for the presence of *T. cylindrosporum,* however no samples returned positive sequence results that would confirm the presence of *T. cylindrosporum* at these locations. It is possible that the fungus may be present or more abundant, either at different times of the year to when we collected, or at different sites, however we did sample both during summer when the fly season was underway in Tasmania and we were able to collect adult flies, as well as in autumn in Victoria when overwintering larvae would be in the soil. We were not able to identify it on these sheep grazing properties from this preliminary study and thus it remains unclear whether this fungus is present in Australian soil and if additional sites or sampling at different times of the year would detect it.

Introduction and Hypothesis

Whilst there are several species of fungi that can infect and kill insects, A Review of Sheep Blowfly Pathogen Control (ON-00620) identified one species, *Tolypocladium cylindrosporum* (*T. cylindrosporum*), as capable of killing the Australian sheep blowfly, *Lucilia cuprina*. It acts by killing larvae and pupa in the soil and hence it could have the potential to be developed as a novel biocontrol agent. Whilst there is no record of *T. cylindrosporum* being present in Australia, an initial review of microbiome sequencing data from Australian soil samples by Biomes of Australian Soil Environments (BASE) suggests that there is a possibility it is potentially identified in both Victoria and Tasmania (Figure 1 below).

cylindrosporum TYPE STRAIN	CGCCCCGGCGCCCTCGGCGTCCCGGAACCAGGCGCCCGCCGGAGGACCCAAACTCTTGTTTAAACCATAG
*inflatum*_ TYPE STRAIN	CGCCCCGGCGCC- TCGGCGTCCCGGAACCAGGCGCCCGCCGGAGGACCCAAACTCTTGTTTAA- CCATAG
NZ F603 cylindrosporum	CGCCCCGGCGCCCTCGGCGTCCCGGAACCAGGCGCCCGCCGGAGGACCCAAACTCTTGTTTAAACCATAG
NZ F604 cylindrosporum	CGCCCCGGCGCCCTCGGCGTCCCGGAACCAGGCGCCCGCCGGAGGACCCAAACTCTTGTTTAAACCATAG
BASE_102.100.100/9466 ACT	CGCCCCGGCGCCCTCGGCGTCCCGGAACCAGGCGCCCGCCGGAGGACCCAAACTCTTGTTTAAACCATAG
BASE_102.100.100/15979 TAS_grazing	CGCCCCGGCGCCCTCGGCGTCCCGGAACCAGGCGCCCGCCGGAGGACCCAAACTCTTGTTTAAACCATAG

Figure 1. Alignment of nucleotide sequences for the *T. cylindrosporum*, *T. inflatum*, and published NZ strains of *T. cylindrosporum* (Wright et al. 2009) and Australian soil samples from BASE.

The two nucleotide differences highlighted in green hold up in comparing multiple isolates assigned to each species, so very likely this is a diagnostic difference between the two species that indicates the Tasmanian and Victorian sequences in BASE look like *T. cylindrosporum*.

Project Objectives

Soil samples were to be collected from 5 Victorian and 5 Tasmanian properties when traps are being set as part of the current 2019/2020 blowfly population collections (ON-00624). When possible, these samples will be collected from locations within paddocks that had struck sheep grazing in them in the prior year (4 samples per property). DNA would then be extracted from soil samples for testing the presence of *T. cylindrosporum.* Tests to allow detection of the *T. cylindrosporum* and *T. inflatum* strains were to be developed and used to identify the presence of *T. cylindrosporum* in the soil sample DNA. If the project had detected the presence of *T. cylindrosporum,* a further objective was to culture the sample, however this objective was not pursued given no positive samples were obtained.

Methodology

Soil was collected using either a soil core sampler or small shovel and stored in 50 ml Falcon tubes until processed. DNA was extracted from soil using the DNeasy Powerlyser Powersoil Kit (Qiagen). DNA was quantified using a Qubit broad range DNA quantification kit (Appendix 1). The samples that had fungal DNA present (not identified as *T. cylindrosporum*) will be retained in the freezer at -20°C for an additional year in case there is the potential for culturing in future.

A diagnostic assay was developed based on a commonly used region for discriminating between fungal species, the Internal transcribed Spacer (ITS) region. The ITS sequences of *T. cylindrosporum* vs *T. inflatum* and several other fungal species were aligned and this allowed us to design and then test a series of primers that might be able to specifically amplify the *T. cylindrosporum* DNA. We also ordered and cloned *T. cylindrosporum* and *T. inflatum* specific DNA fragments that could be used as positive controls to optimise the test. Multiple primer combinations and testing temperatures were used to optimise the diagnostic test that could identify the *T. cylindrosporum* or *T. inflatum* strains (Appendix 2).

Success in Achieving Objectives

Five properties were samples in Tasmania, from a set of different sites including near the flytraps, under sheep carcasses and from rubbish heaps (where present and accessible).
Soil samples from two Victorian properties (Shepparton and Beaufort regions), however due to the state of emergency and restricted movement during the COVID outbreak the remaining sites were not sampled.

DNA was successfully extracted from all samples, with varying concentrations extracted from the different soil samples (Appendix 1).

Given no samples have been detected with the presence of *T. cylindrosporum* and the restrictions on use of University facilities the final objective of culturing the samples from the soil has not been required, nor has work to do this proceeded and the milestones and project cost have been adjusted to reflect this.

Results and Discussion

DNA samples were extracted from the soil and DNA quantified as per the table (Appendix 1)
The PCR assay was optimised allowing us to differentiate between the presence of *T. inflatum* or *T. cylindrosporum* (Appendix 2) and we can see the presence of the correct sized *T. cylindrosporum* DNA band in the Tc_+ve lane which is the control DNA sequence we ordered to initially optimise the assay while this band is not present in the Ti_+ve lane as would be expected. Sequencing of any PCR products that were potentially the correct diagnostic size (100 bp) showed that these were not *T. cylindrosporum* (nor *T. inflatum*) (Figure 2 and Figure 3).

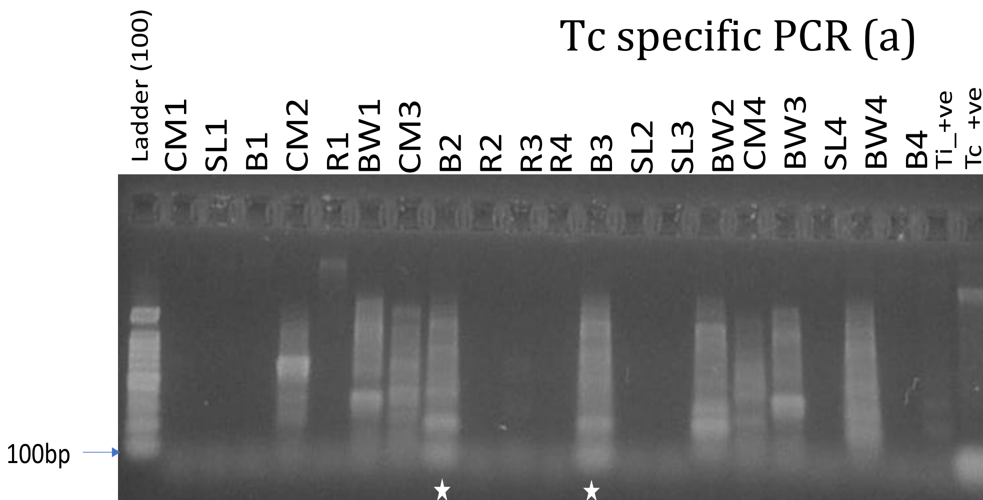

Figure 2. Agarose gel for analysis of Tasmanian properties using the *T. cylindrosporum* specific assay. Those with a white star were investigated further as potential correct sized fragments, however while the B2 and B3 were cloned and sequenced, neither product was *T. cylindrosporum* or *T. inflatum*. Instead the sequence fragments were unidentified based on searches of fungal sequence databases.

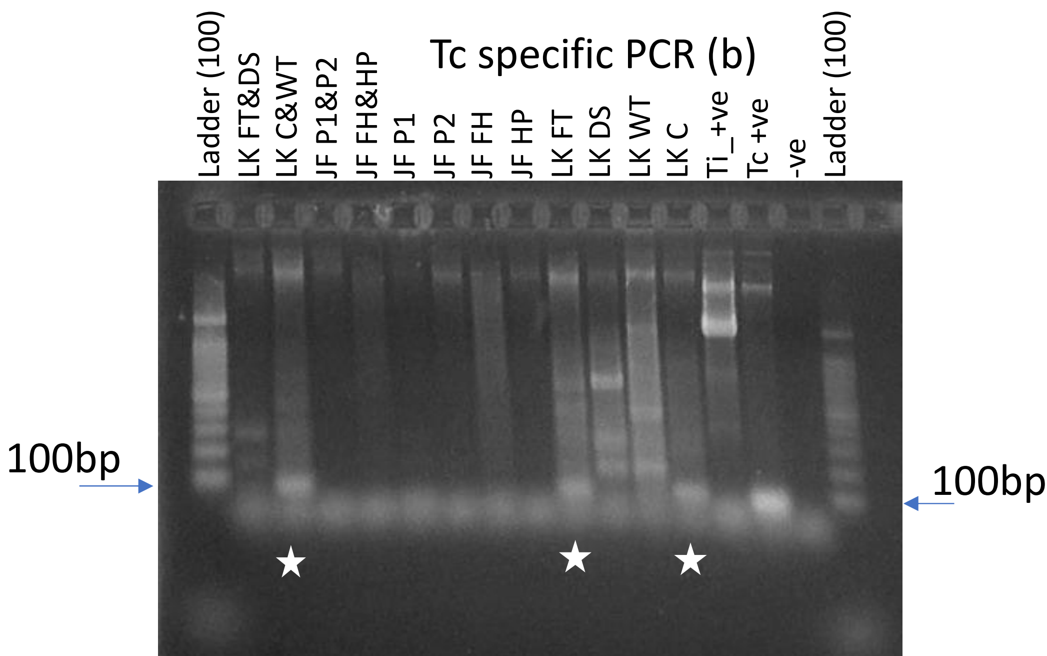

Figure 3. Agarose gel for analysis of Victorian properties using the *T. cylindrosporum* specific assay. Those with a white star were investigated further as potential correct sized fragments, however as found for the Tasmanian samples, sequencing of these showed they were also not *T. cylindrosporum* (or *T. inflatum).*

In summary, based on the sampling from these six sheep growing properties, we did not find evidence that would support the *T. cylindrosporum* strain being present in Australia. Unfortunately, this does not allow us to rule out that this strain is present elsewhere in the states or in Australia and our initial expectations were that this strain would be detected given the multiple samples that were potentially *T. cylindrosporum* from the BASE soil survey data. Wider sampling and collecting during different seasons would provide more confidence in ascertaining whether the strain is present or not.

Impact on the Wool Industry – Now and in Five Years' Time

While *T. cylindrosporum* was not identified in the samples collected in this study, it cannot be ruled out that this strain is present in Australia. There is obviously the potential for this to be developed as a new biocontrol option for supressing flystrike through reducing the blowfly population and if so it could provide additional tool to combat flystrike.

Conclusions and Recommendations

If there is the potential for *T. cylindrosporum* to be developed as a blowfly control option, future sample collections should be conducted over a greater range of properties, site types and also across the year to increase the likelihood of detecting the presence of this fungus. A fungal sample from Australia deposited at an international fungal bank has been tentatively identified as *T. cylindrosporum* and it would potentially be an avenue to determining whether this fungus is present and may allow comparisons to be made to the NZ *T. cylindrosporum* strains if testing the impact on blowfly larval survival was to be conducted at a later point in time. If non-native strains are being examined for this purpose, it would also be advisable to test the efficacy against Australian blowfly samples.

Bibliography

D. A. Wright, N. J. Cummings, N. A. Haack & T. A. Jackson (2009) *Tolypocladium cylindrosporum*, a novel pathogen for sheep blowflies, *New Zealand Journal of Agricultural Research*, 52:3, 315-321, DOI: 10.1080/00288230909510516

List of Abbreviations and/or Glossary

PCR – polymerase chain reaction
Tc - *Tolypocladium cylindrosporum*
Ti - *Tolypocladium inflatum*

Appendices

Appendix 1 – DNA quantification

State	Site code	DNA concentration (ng/ul)
Tasmania	CM1	5.54
Tasmania	SL1	8.9
Tasmania	B1	11.4
Tasmania	CM2	4.5
Tasmania	R1	17.3
Tasmania	BW1	2
Tasmania	CM3	6.11
Tasmania	B2	14
Tasmania	R2	2.82
Tasmania	R3	3.86
Tasmania	R4	3.56
Tasmania	B3	11.5
Tasmania	SL2	3.79
Tasmania	SL3	1.76
Tasmania	BW2	6.04
Tasmania	CM4	9.86
Tasmania	BW3	3.32
Tasmania	SL4	5.85
Tasmania	BW4	7.21
Tasmania	B4	13.9
Victoria (combined)	LK FT&DS	29.9
Victoria (combined)	LK C&WT	70.1
Victoria (combined)	JF P1&P2	23
Victoria (combined)	JF FH&HP	36.4
Victoria	JF P1	8.2
Victoria	JF P2	30.2
Victoria	JF FH	123
Victoria	JF HP	21.3
Victoria	LK FT	63.5
Victoria	LK DS	20.3
Victoria	LK WT	50
Victoria	LK C	29.8

Appendix 2 – PCR assay conditions and primer sequences

Tc specific PCR	Ti specific PCR
PCR conditions Initial cycle of 94°C (2 min) 35 cycles of 94°C (20 sec) 48°C (20 sec) 72°C (1 min) Final cycle of 72°C (5 min)	PCR conditions Initial cycle of 94°C (2 min) 35 cycles of 94°C (20 sec) 59°C (20 sec) 72°C (1 min) Final cycle of 72°C (5 min)
Tc_AS_3_Fwd CCTCAAGCCCCAGCC	TcTi_Fc CCTGTGAACATACCCA
Tc_ITS_R2 CGAGGTCGCCACTGCATTTG	Ti_AS_3_Rev CCAACACCAAGCCGC
Product size 100 bp	Product size 358 bp