Mating-Type Gene Structure and Spatial Distribution of *Didymella tanacetii* in Pyrethrum Fields

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ABSTRACT

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Tan spot of pyrethrum (*Tanacetum cinerariifolium*) is caused by the ascomycete *Didymella tanacetii*. To assess the evolutionary role of ascospores in the assumed asexual species, the structure and arrangement of mating-type (*MAT*) genes were examined. A single *MAT1-1* or *MAT1-2* idiomorph was identified in all isolates examined, indicating that the species is heterothallic. The idiomorphs were flanked upstream and downstream by regions encoding pyridoxamine phosphate oxidase-like and DNA lyase-like proteins, respectively. A multiplex *MAT*-specific polymerase chain reaction assay was developed and used to genotype

325 isolates collected within two transects in each of four fields in Tasmania, Australia. The ratio of isolates of each mating-type in each transect was consistent with a 1:1 ratio. The spatial distribution of the isolates of the two mating-types within each transect was random for all except one transect for *MAT1-1* isolates, indicating that clonal patterns of each mating-type were absent. However, evidence of a reduced selection pressure on *MAT1-1* isolates was observed, with a second haplotype of the *MAT1-1-1* gene identified in 4.4% of *MAT1-1* isolates. In vitro crosses between isolates with opposite mating-types failed to produce ascospores. Although the sexual morph could not be induced, the occurrence of both mating-types in equal frequencies suggested that a cryptic sexual mode of reproduction may occur within field populations.

Pyrethrum (*Tanacetum cinerariifolium*) is an Asteraceae crop grown for the extraction of pyrethrins from achenes on the seed (MacDonald 1995). Pyrethrins are one of the world's most commonly used botanicides (Khater 2012) and consist of six chemical esters which, together, provide rapid knockdown and kill a variety of arthropods (Crombie 1980, 1995). Australia supplies the majority of the world pyrethrin market from a highly mechanized production system. In Australia, plants are sown in austral spring and reach first harvest in the austral summer 18 months later, after which plants are harvested annually for up to 4 years (Greenhill 2007).

Tan spot has become a major factor limiting productivity and profitability of the Australian pyrethrum industry since 2009, when it was associated with a substantial increase in foliar disease severity in many fields (Hay et al. 2015). The disease was first discovered in 2001 and is caused by a fungal complex of two closely related Didymellaceae species: *Didymella tanacetii* and *D. rosea* (syn. *Microsphaeropsis tanacetii*) (Pearce et al. 2016; Pethybridge et al. 2008). Symptoms include necrotic lesions on leaves, stems, and buds, which coalesce and lead to a loss of green leaf area. This results in significant yield reductions and can lead to plant death (Hay et al. 2015; Pethybridge et al. 2008). *Didymella tanacetii* is considered the dominant pathogen associated with tan spot and is isolated more frequently from diseased leaves than *D. rosea* (Pearce et al. 2016). The life cycle of *D. tanacetii* remains cryptic. However, pycnidia are commonly found within diseased tissue (Pethybridge et al. 2008). The presence of a sexual morph is unknown but, if present, could play an important role in inoculum dispersal and increase the probability of novel gene combinations evolving (MacDonald and Linde 2002).

Mating-type (*MAT*) genes govern the ability of fungi to sexually reproduce. They control pathways that assist with fertilization, attraction of compatible mating partners, and haploid cell identity (Cassleton 2008; Debuchy and Turgeon 2006). In most filamentous ascomycetes, two idiomorphs, commonly termed *MAT1-1* and *MAT1-2*, can be found at the *MAT* locus (Metzenberg and Glass 1990; Turgeon 1998). Molecular analyses suggest they encode transcriptional regulators, with each *MAT* gene encoding specific DNA-binding motifs (Arie et al. 1997). Across all genera, the *MAT1-2* idiomorph contains a single gene (*MAT1-2-1*) encoding a high mobility group (HMG) motif protein (Coppin et al. 1997). In comparison, the *MAT1-1* idiomorph can consist of up to four genes (Groenewald et al. 2007; Kanematsu et al. 2007; Turgeon 1998) but consistently contains a gene (*MAT1-1-1*) that encodes an α domain protein (Turgeon and Yoder 2000).

Homothallic and heterothallic species have been identified within the Didymellaceae family (Chérif et al. 2006; Vaghefi et al. 2015; Woudenberg et al. 2012). For homothallic Didymellaceae species, the *MAT* genes are linked at the same chromosome location; however, the order of the two genes is variable (Vaghefi et al. 2015; Woudenberg et al. 2012; Yun et al. 2013). For some putative heterothallic Didymellaceae species, only one of the *MAT* idiomorphs has been identified within populations, suggesting that sexual reproduction in these species does not occur (Vaghefi et al. 2015; Woudenberg et al. 2012).

The increase in disease frequency of tan spot in pyrethrum has raised the question of a possible role for sexual reproduction in disease spread and incidence. Identifying the molecular potential for sexual reproduction and conditions that are conducive for production of the sexual morph would be beneficial to identify the potential impacts imposed by the asexual and sexual components of the reproductive cycle. The objectives of this study were to (i) characterize the *MAT* locus and the flanking genes in *D. tanacetii*, (ii) test for the production of ascospores in vitro, (iii) develop a screening protocol for mating-type

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identification in *D. tanacetii*, and (iv) determine the distribution of isolates of each mating-type among field populations of *D. tanacetii*.

MATERIALS AND METHODS

Isolates and DNA extraction. *Didymella tanacetii* isolates for isolation and characterization of the *MAT* idiomorphs and flanking genes were randomly selected representatives of a collection obtained from diseased pyrethrum leaves between 2004 and 2012 in Tasmania, Australia (Hay et al. 2015) (Table 1). For extraction of genomic DNA, isolates were refreshed from -80°C storage on potato dextrose agar (PDA; Amyl Media, Dandenong, Victoria, Australia) and incubated at 21°C in the dark. Mycelia were scraped from the surface of cultures, transferred into 20 ml of 2% malt extract broth (Oxoid Ltd., Hampshire, United Kingdom), and incubated at approximately 20°C on an orbital shaker at 54 rpm for 12 days. Excess moisture was removed using a sterile paper towel and the mycelium was ground in liquid nitrogen. DNA was extracted from the mycelia using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. DNA was quantified using a high-sensitivity double-stranded DNA QUBIT kit (Life Technologies, Carlsbad, CA) and diluted to 1 ng/ μl for polymerase chain reaction (PCR).

PCR amplification and sequencing. All PCR assays were performed in a C1000 thermocycler (Bio-Rad Laboratories, Hercules, CA) in a total volume of 20 μl . PCR mixes contained 0.5 U of TopTaq polymerase (Qiagen), 1 $\times$ PCR buffer, 1 $\times$ CoralLoad, 1.5 mM MgCl_2 , 0.2 μM each primer, 200 μM each dNTP (Bioline, Alexandria, NSW, Australia), and 4 to 6 ng of genomic DNA, except where otherwise indicated. Conditions for amplification, except where noted, were an initial denaturation for 5 min at 94°C ; followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 2 min 20 s; followed by a final elongation at 72°C for 5 min. Amplified products were visualized via gel electrophoresis using a 1.5% agarose gel (wt/vol) (Life Technologies) prestained with GelRed (Biotium Inc., Hayward, CA) in 1 $\times$ Tris-acetate-EDTA buffer. For nucleotide sequencing, PCR products were prepared using the

UltraClean PCR-clean up kit (Mo Bio Laboratories Inc., Carlsbad, CA), according to the manufacturer's instructions. Amplicons were sequenced with the forward and reverse primers by the Australian Genome Research Facility (AGRF) (Melbourne, Australia) using Big Dye Terminator v3.1 chemistry and capillary separation on an AB 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA). Consensus sequences were obtained from pairwise alignment of the forward and reverse sequences using MAFFT v7.017 (Katoh et al. 2002) in Geneious v7.1.0 (Biomatters Ltd., Auckland, New Zealand).

Identification of the *MAT* idiomorphs and flanking genes. To provide genomic data to assist with the characterization of the *MAT* idiomorphs, the *D. tanacetii* isolate, BRIP 61988, was selected as a representative isolate for genomic sequencing (Table 1). DNA (2 μg) was sent to the AGRF for high-throughput DNA shotgun 454 sequencing using the Roche GS FLX (454) system (Rothberg and Leamon 2008). Of the 135,404 sequences generated (average length: 413 bp), only 44,825 were able to be assembled to produce 4,801 contigs (>500 bp) of 905 bp in average length. Due to the large number of unaligned reads, the raw sequence reads were also used to create a local custom nucleotide BLAST database for reference in Geneious. A search of the assembled contigs and local BLAST database of *D. tanacetii* did not identify sequences homologous to a *MAT1-1* or *MAT1-2* idiomorph. Therefore, to identify the *MAT* idiomorphs in *D. tanacetii*, isolates ($n = 7$) were screened with degenerate primers to amplify conserved regions of the two prevalent *MAT* genes (Table 1). Primers Sn-ab₁ and Sn-ab₂ (Hsiang et al. 2003) (Table 2; Fig. 1) targeted an approximately 241-bp fragment of the α domain of *MAT1-1*. HMGTPF and HMGTPR, modified versions of primers HMGF_Phoma and HMGR_Phoma (Woudenberg et al. 2012) (Table 2; Fig. 1), targeted an approximately 280-bp fragment of the HMG domain of *MAT1-2*. The PCR for Sn-ab₁/Sn-ab₂ contained 0.4 μM each primer and 2.6 mM MgCl_2 . PCR conditions of 30 cycles and an extension time of 20 s were used for both PCR.

Isolates identified as containing putative *MAT1-1* (BRIP 91993 and BRIP 61999) or *MAT1-2* (BRIP 61994) genes were used in a nested PCR to amplify the entire *MAT* genes for sequencing. The initial PCR used primers MATidio-Fd and MATidio-Rv (Table 2; Fig. 1) to amplify a 3,000-bp fragment. The PCR contained 2.0 mM MgCl_2 , with annealing at 55°C and an extension time of 3 min 30 s. The initial PCR products of BRIP 61993 and BRIP 61999 were used in subsequent PCR with the primer pairs MATidio-Fd/MAT1R2-Phoma and MAT1F2-Phoma/MATidio-Rv (Woudenberg et al. 2012) (Table 2; Fig. 1) to amplify *MAT1-1* in two overlapping fragments. The initial PCR product of BRIP 61994 was used in subsequent PCR with the primer pairs HMGTPF/MATidio-Rv and MATidio-Fd/HMGTPR (Table 2; Fig. 1) to amplify *MAT1-2* in two overlapping fragments. The PCR contained 2.0 mM MgCl_2 and 0.4 to 2 μl of PCR product as target DNA. The PCR to amplify *MAT1-1* used annealing at 57°C , and an extension time of 2 min 30 s was used for each of the four PCR. The two overlapping amplicons of each gene were sequenced and combined. They were compared with sequences within the GenBank database, using BLASTn (NCBI Resource Coordinators 2015; Benson et al. 2015), to confirm the amplification of genes homologous to *MAT1-1* or *MAT1-2*. Based on MAFFT alignment of the obtained sequences, PCR primers were designed to allow one-step amplification of each of the *MAT* genes of *D. tanacetii* using Primer3 v2.3.4 (Koressaar and Remm 2007; Untergasser et al. 2012) and Netprimer (Premier Biosoft International, Palo Alto, CA). A common reverse primer, DT-MAT-uniR (Table 2; Fig. 1), annealed in a mutual conserved region downstream of the *MAT* genes. This primer was combined with *MAT*-specific forward primers DT-MAT-1F and DT-MAT-2F (Table 2; Fig. 1) for amplification and sequencing of *MAT1-1* and *MAT1-2*, respectively. These primers were used to amplify and sequence the *MAT* genes of 13 *D. tanacetii* isolates (Table 1). Conditions for amplification included an extension time of 2 min 10 s.

TABLE 1. *Didymella tanacetii* isolates used for identification of the mating-type (*MAT*) idiomorphs and the gene regions located upstream and downstream

Isolate ^a	Year collected	Location ^b	Mating-type ^c	Accession number ^d
BRIP 61993 ^{efg}	2010	Wesley Vale	<i>MAT1-1</i> (I)	KU030827
BRIP 61992 ^{fg}	2004	Penguin	<i>MAT1-1</i> (I)	KU030826
BRIP 61999 ^{efg}	2004	Kindred	<i>MAT1-1</i> (I)	KU030830
TAS 041-0043 ^g	2004	Table Cape	<i>MAT1-1</i> (I)	...
BRIP 61990 ^{efg}	2012	Sassafras	<i>MAT1-1</i> (II)	KU030824
BRIP 61989 ^{fg}	2012	Sassafras	<i>MAT1-1</i> (II)	KU030823
BRIP 50785 ^{fg}	2004	Sassafras	<i>MAT1-1</i> (II)	KU030822
BRIP 61988 ^{fg}	2012	Sassafras	<i>MAT1-2</i>	KU342705
BRIP 61991 ^{efg}	2012	Table Cape	<i>MAT1-2</i>	KU030825
BRIP 61994 ^{efg}	2009	Penguin	<i>MAT1-2</i>	KU030828
TAS 041-0071 ^{eg}	2005	Sassafras	<i>MAT1-2</i>	...
TAS 041-0050 ^g	2004	Montumana	<i>MAT1-2</i>	...
TAS 041-0079 ^g	2004	Penguin	<i>MAT1-2</i>	...
TAS 041-0025 ^g	2010	Forth	<i>MAT1-2</i>	...
TAS 041-0064 ^g	2004	Table Cape	<i>MAT1-2</i>	...

^a BRIP = Queensland Plant Pathology herbarium collection, Brisbane, Australia and TAS = Tasmanian Institute of Agriculture fungal collection, Tasmania, Australia.

^b Location in Tasmania, Australia.

^c *MAT1-1* (I) = haplotype I of *MAT1-1* and *MAT1-1* (II) = haplotype II of *MAT1-1*.

^d GenBank accession number.

^e Isolates screened with HMGTPF/HMGTPR and Sn-ab₁/Sn-ab₂ primer sets.

^f Isolates for which the entire idiomorph and the flanking genes were sequenced.

^g Isolates for which *MAT* genes were sequenced.

The coding sequences (CDS) of *MAT1-1-1* and *MAT1-2-1* were inferred following alignment of the obtained sequences to *MAT1-1-1* and *MAT1-2-1* of *Peyronella pinodella* strains CBS 110.32 (GenBank accession: JF815529) and CBS 108.31 (JF815531), respectively. MAFFT alignment was used to identify variation between and within each *MAT* gene and restriction enzymes selected to target identified polymorphisms.

To identify the putative genes upstream of the *MAT* genes, the GTPase activator-like protein (*GAP*) and protein with a pyridoxamine 5'-phosphate oxidase (PNP) domain (*ORF1*) genes of *Stagonosporopsis tanacetii* CBS 131484 (KJ139680 and KJ139678), which resided upstream of the *MAT* gene in *S. tanacetii* (Vaghefi et al. 2015), were used to search the local BLAST database of *D. tanacetii* for homologous sequences. MAFFT alignment of raw sequence reads with high similarity provided partial coverage of each of the *GAP* and *ORF1* genes. To obtain the nucleotide sequence spanning the partial sequences, a forward (DT-GAP1F) and reverse (DT-GAP2R) primer were designed to target the partial sequences of *GAP* and *ORF1*, respectively (Table 2; Fig. 1). The primers were used to amplify and sequence the nucleotides spanning the partial sequences of the *GAP* and *ORF1* in *D. tanacetii* isolate BRIP 61988. The PCR included 30 cycles with annealing at 55°C and an extension time of 3 min. In addition to

DT-GAP1F and DT-GAP2R, the primers DT-GAP3F and DT-GAP3R (Table 2; Fig. 1) were used for sequencing to obtain sufficient coverage. The obtained consensus sequence was aligned with KJ139680 and KJ139678 to identify the location of the intergenic region between the *GAP* and *ORF1* genes. To sequence the complete *ORF1* of *MAT1-1* and *MAT1-2* isolates of *D. tanacetii* and confirm its location upstream of the *MAT* idiomorphs, the forward primer ORF-int-F (Table 2; Fig. 1) was subsequently designed to anneal within the identified intergenic region between *GAP* and *ORF1*. ORF-int-F was used in combination with the reverse primers HMGTPR or DT-Sn-ab₁ (modified version of Sn-ab₁ based on *D. tanacetii* sequence data) (Table 2; Fig. 1), which annealed in the *MAT* CDS of *MAT1-1* and *MAT1-2* isolates, respectively. Using these primers, *ORF1* was amplified and sequenced for *MAT1-1* (*n* = 6) and *MAT1-2* (*n* = 3) isolates of *D. tanacetii* (Table 1). The PCR used an annealing temperature of 57°C. The CDS of *ORF1* was inferred following alignment of the obtained consensus sequences to KJ139678.

To identify the putative gene downstream of the *MAT* genes, the DNA lyase-like protein (*DLP*) gene of *S. tanacetii* CBS 131484 (KJ139679), which resided downstream of the *MAT* gene in *S. tanacetii* (Vaghefi et al. 2015), was used to search the local BLAST database of *D. tanacetii* for homologous sequences. Multiple raw reads

TABLE 2. List of primers used for polymerase chain reactions and DNA sequencing in this study

Primer name	Sequence 5' to 3'	Reference	Binding location ^a
HMGTPR	CRGGYTTDCGRGGRCWRTACTT	This study	Downstream <i>MAT1-2-1</i> HMG domain
HMGTPF	CGYCCVVRTGAAYTGCTGGAT	This study	Upstream <i>MAT1-2-1</i> HMG domain
Sn-ab ₁	AARGSNYTNAAYGCNTTYGTNGG	Hsiang et al. 2003	Upstream <i>MAT1-1-1</i> α domain
Sn-ab ₂	TCYTTNCCDAYYTGRTCNCGDAT	Hsiang et al. 2003	Downstream <i>MAT1-1-1</i> α domain
MATidio-Fd	GGRAGRATIGCIGAYTGGAARGG	Woudenberg et al. 2012	Upstream <i>MAT</i> genes
MATidio-Rv	TGGIGITGYGGIACKTTYATYTGG	Woudenberg et al. 2012	Downstream <i>MAT</i> genes
MAT1F2-Phoma	CTGGAATIGCWGRCATGGC	Woudenberg et al. 2012	Internal <i>MAT1-1-1</i> gene
MAT1R2-Phoma	TGTCGCTTYGYICGTCGC	Woudenberg et al. 2012	Internal <i>MAT1-1-1</i> gene
DT-MAT-UniR	GGAAYAGCGACAGTTAGATGCG	This study	Downstream <i>MAT</i> genes
DT-MAT-1F	GAAACAATACTTGCCGACACG	This study	<i>D. tanacetii</i> -specific upstream <i>MAT1-1-1</i>
DT-MAT-2F	CCACTACTCCCTTCAGACC	This study	<i>D. tanacetii</i> -specific upstream <i>MAT1-2-1</i>
DNL-6169F	CATTGACTTCCTGCATACTTGTG	This study	Upstream <i>DLP</i> gene
DNL-7883R	GAAGGAACAATAATGCCGTGAAG	This study	Downstream <i>DLP</i> gene
DT-MAT1-Spec-F	TGCGCCTTACCTCAACATCC	This study	Internal <i>D. tanacetii</i> <i>MAT1-1-1</i> gene
DT-MAT2-Spec-F	TTCATCTACCAGAGTCCAATCG	This study	Internal <i>D. tanacetii</i> <i>MAT1-2-1</i> gene
DNL-int-F	GGTTCATGATGTCGAGGATGTAG	This study	Internal <i>DNA lyase-like protein</i> gene
DNL-int-R	CTGTGTGCGGAGTTCCAC	This study	Internal <i>DNA lyase-like protein</i> gene
DT-GAP1F	GTAATGATGACTCGAGCCTGAAG	This study	Internal <i>GAP</i> gene
DT-GAP2R	TTCATACCAAGAAGAAGCTCGG	This study	Internal <i>ORF1</i> gene
DT-GAP3F	TGTTGTTGCTGTTGTTGCTG	This study	Internal <i>GAP</i> gene
DT-GAP3R	AGTCGAATATGGCGTGCTG	This study	Internal <i>ORF1</i> gene
ORF-int-F	GGTTGGCTGTCGTTACTCG	This study	Upstream <i>ORF1</i> gene
DT-Sn-ab ₂	TCTTTACCAATCTGGTCGC	This study	Downstream <i>MAT1-1-1</i> α domain
MAT2TP-F	ACTCGTTGCTCACAGATATGG	This study	Internal <i>MAT1-2-1</i> gene
MAT2TP-R	GGACTCTGGTAGATTGAAGATGG	This study	Internal <i>MAT1-2-1</i> gene
MAT1TP-F	CTGGAATCGCAGACATGAC	This study	Internal <i>MAT1-1-1</i> gene
MAT1TP-R	TGACGTTTTGCTCGTCGC	This study	Internal <i>MAT1-1-1</i> gene

^a HMG = high mobility group, *DLP* = DNA lyase-like protein, *GAP* = GTPase activator-like protein, and *ORF1* = protein with a pyridoxamine 5'-phosphate oxidase domain.

Fig. 1. Location of primers used in this study. Genes and direction are represented by white blocked arrows. Intergenic regions are represented by thin black lines. *GAP* = GTPase activator-like protein and *ORF1* = protein with a pyridoxamine 5'-phosphate oxidase domain. Small black arrows indicate the direction of primers. Primers 1 = DT-GAP1F, 2 = DT-GAP3F, 3 = ORF-int-F, 4 = DT-GAP3R, 5 = DT-GAP2R, 6 = MATidio-Fd, 7 = DT-MAT-1F, 8 = Sn-ab₁, 9 = Sn-ab₂, 10 = DT-Sn-ab₂, 11 = DT-MAT1-Spec-F, 12 = MAT1F2-Phoma, 13 = MAT1TP-F, 14 = MAT1R2-Phoma, 15 = MAT1TP-R, 16 = DT-MAT-2F, 17 = HMGTPF, 18 = MAT2TP-F, 19 = HMGTPR, 20 = MAT2TP-R, 21 = DT-MAT2-Spec-F, 22 = DNL-6169F, 23 = DT-MAT-UniR, 24 = MATidio-Rv, 25 = DNL-int-F, 26 = DNL-int-R, and 27 = DNL-7883R. Figure is not to scale.

were homologous to the 3' end of the *DLP* gene of *S. tanacetii*. The reads were aligned and the reverse primer DN-7883R (Table 2; Fig. 1) was designed to anneal downstream of the putative *DLP* gene. To sequence the entire *DLP* of *D. tanacetii* and confirm its location downstream from the *MAT* genes, DN-7883R was used in combination with the forward primer DNL-6169F (Table 2; Fig. 1), designed to anneal in the mutual conserved region downstream of the *MAT* genes. Amplification and sequencing of the entire *DLP* of *MAT1-2* isolates ($n = 3$) was performed using an annealing temperature of 55°C. Because insufficient coverage was obtained from the initial sequencing reaction, the internal primers DNL-int-F and DNL-int-R (Table 2; Fig. 1) were designed, based on alignment of the obtained sequence data, to obtain complete sequence coverage. Amplification and sequencing of the entire *DLP* of *MAT1-1* isolates ($n = 6$) required two separate PCR and sequencing reactions with the primer combinations DNL-int-F/DNL-7883R and DNL-6169F/DNL-int-R to produce two overlapping fragments. The PCR used an annealing temperature of 55°C and an extension time of 2 min. The CDS of *DLP* was inferred following alignment of the consensus sequences to KJ139679.

The obtained *MAT* idiomorph and flanking gene sequences were combined for nine *D. tanacetii* isolates using de novo assembly to produce a continuous nucleotide sequence. The *MAT*, *DLP*, and *ORF1* genes and the intergenic regions between these genes of the *D. tanacetii* isolates were aligned using MAFFT to identify sequence similarity within and between isolates of each mating-

type. In addition, the sequences were compared with other fungal species in GenBank using BLASTn. Sequences were deposited in GenBank (Table 1).

Distribution of mating-types of *D. tanacetii* within pyrethrum fields. Isolates of *D. tanacetii* were collected from four fields in July or August 2012 for population analysis of the frequency and distribution of mating-types. Sites were selected to allow comparison at regional and field levels, with two fields in each of Sassafras, Tasmania, Australia (41°17' South, 146°30' East) (S1 and S2), and Table Cape, Tasmania, Australia (40°56' South, 145°43' East) (TC1 and TC2). The fields were sown between August and October 2011 with seed harvested from each of four different pyrethrum seed crops. Field S1 was sown with a different variety than S2, TC1, and TC2. Within each field, two parallel 50-m transects were sampled at 0.5-m intervals. At each sample point, the nearest pyrethrum plant was identified and several diseased leaves were removed. Leaves were surface sterilized via immersion in bleach (1% available chlorine) for 1 min, followed by three 30-s rinses in sterile, deionized water. For each sampling unit, four leaves were selected and small tissue fragments were sectioned from lesion margins and plated onto 2% water agar (WA). Plates were incubated in the dark at 21°C for 5 to 7 days and examined at ×63 magnification. A small section of singular hyphae of each putative *D. tanacetii* isolate, identified by distinct hyphal characteristics, was transferred to PDA and incubated at 21°C in the dark for 5 days, before transfer to a light box under a system of 14 h of light and 10 h of darkness at 21 to 24°C for 5 days for further confirmation of the pathogen as *D. tanacetii* (Pearce et al. 2016). The number of sampling units at which *D. tanacetii* occurred was also recorded based on characteristics of mycelium morphology. For sampling units for which multiple *D. tanacetii* individuals were identified, a single culture was arbitrarily selected and stored at -80°C (Hay et al. 2015). Total genomic DNA was extracted from the isolates, quantified, and diluted to 1 ng/μl, as described previously.

To allow rapid identification of the mating-type of *D. tanacetii* isolates, a multiplex *MAT*-specific PCR assay, consisting of a single reverse primer and two forward primers, was designed. The reverse primer DT-MAT-UniR was combined with the *MAT*-specific forward primers DT-MAT1-Spec-F and DT-MAT2-Spec-F (Table 2; Fig. 1). The forward primers were designed to anneal within the CDS of each of the *MAT* genes to produce differentiating amplicons of 1,452 and 1,100 bp for *MAT1-1* and *MAT1-2* individuals, respectively (Fig. 2A). The primers were tested as pairs and as a multiplex against a subset of *D. tanacetii* isolates ($n = 10$) of known mating-type to confirm their ability to differentiate the mating-type (Fig. 2B). The PCR contained 0.15 μM each primer and consisted of 30 cycles and an extension time of 75 s. The multiplex PCR was used to identify the mating-type of isolates within the field populations of *D. tanacetii*. Amplified products were visualized as previously described and the mating-type of isolates identified based on the PCR product length.

The populations were further characterized by identifying the proportion of *MAT1-1* isolates that contained polymorphisms in the *MAT* CDS, identified from the nucleotide alignment of the *MAT1-1-1* gene (referred to as a second haplotype of *MAT1-1*; haplotype II). The multiplex PCR products of isolates identified as *MAT1-1* were digested with *EcoRV*-HF (New England BioLabs, Ipswich, MA), which targets the nucleotide motif GAT^AATC and cleaved the *MAT1-1-1* of the second haplotype at codon 123, where a single-nucleotide polymorphism (SNP) (A/C) resided that differentiated the two *MAT1-1* haplotypes. Restriction digests were undertaken with 1× CutSmart buffer, 5 U of the *EcoRV*-HF enzyme, and approximately 250 ng of PCR product in a 10-μl reaction. Tubes were incubated at 37°C for 15 min followed by 65°C for 20 min. Digested products (5 μl) were visualized as previously, and isolates with a digestion pattern consisting of a 1,238- and 214-bp fragment identified as the second haplotype of *MAT1-1*. The *MAT1-1-1* gene of isolates with a positive digest was amplified and sequenced with

Fig. 2. Multiplex mating type (*MAT*) gene-specific polymerase chain reaction (PCR) and restriction digest for detection of the mating-type of *Didymella tanacetii* isolates. **A**, Binding position of primers for the multiplex PCR for identification of *MAT1-1* and *MAT1-2* isolates. Blocked white arrows indicate *MAT* genes. Black boxes within the blocked white arrows indicate introns. Dark gray regions indicate unconserved regions and light gray regions indicate highly conserved regions. Arrows indicate the position and direction of primers. **B**, Electrophoresis gel of *MAT*-specific multiplex PCR products for identification of *MAT1-1* and *MAT1-2* isolates and products from *EcoRV* digestion of the *MAT1-1* products. Ladder in lane 1 is Bioline hyperladder II. Lanes 2 and 3 contain the PCR product amplified in *MAT1-1* haplotype I BRIP 61993 and haplotype II BRIP 61990 isolates, respectively. Lane 4 contains the PCR product amplified in *MAT1-2* isolate BRIP 61988. Lane 5 contains the nontemplate control (NTC). Lanes 6 and 7 contain the *EcoRV*-digested PCR product of *MAT1-1* haplotype II and I, respectively.

the primers DT-MAT1F and DT-MAT-UniR to confirm the presence of additional SNP that co-occurred with the restriction-digest-targeted SNP.

To detect isolates within the population which contained both *MAT1-1* and *MAT1-2-1* (i.e., homothallic isolates), 10% of isolates ($n = 34$) (50:50 split of each mating-type, identified from the multiplex PCR) were randomly selected. The isolates were screened with primers designed to target a section of the *MAT* gene CDS of the opposite mating-type to which they were identified. *MAT1-1* isolates were screened with the primer pair MAT2TP-F/MAT2TP-R and *MAT1-2* isolates with MAT1TP-F/MAT1TP-R (Table 2; Fig. 1). The PCR involved 30 cycles and an extension time of 20 s. Products were visualized as previously and compared with the respective positive and negative controls.

Ordinary runs analysis was used to assess spatial patterns of pathogen incidence and of each mating-type within the two transects in each field. The analysis was conducted four times to assess the presence or absence in each transect of each of (i) *D. tanacetii*, (ii) *MAT1-1* isolates (without differentiation based on haplotype), (iii) *MAT1-1* isolates of the dominant haplotype only (haplotype I), and (iv) *MAT1-2* isolates. For each analysis, sampling units with a presence were assigned a value of 1 and the remainder 0. For each analysis, the number of runs (defined as a succession of like events) was calculated and a Z statistic used to determine whether the observed number of runs significantly differed from the expected number of runs, under a null hypothesis of randomness (Madden et al. 1982). A χ^2 analysis was used to determine the goodness of fit of a 1:1 ratio of *MAT1-1* to *MAT1-2* isolates at an overall, regional, field, and transect population level. Data analysis was undertaken in R v3.0.1 (R Core Team 2014).

In vitro production of the sexual morph. *Didymella tanacetii* isolates of known mating-type were plated onto PDA and incubated in the dark at 21°C for 3 days, before transfer to a light box under a system of 14 h of light and 10 h of darkness at 21 to 24°C for 14 days. Conidia were collected by flooding plates with sterile deionized H₂O and the spore concentration was adjusted using a hemocytometer to 10⁵ spores/ml. An aliquot (100 µl) of each conidial suspension was plated onto 2% WA and incubated in the dark (21°C) for 24 h to quantify germination efficiency. Germination was quantified by examining 100 conidia at ×400 magnification. In all, 10 conidia were selected in each of 10 fields of view and the percentage of germinated conidia determined. Conidia were considered germinated when the germ tube was greater than or equal to the length of the conidium.

Conidial suspension mixtures (1:1 ratio) were prepared of crosses BRIP 61993 (*MAT1-1* haplotype I) × BRIP 61988 (*MAT1-2*) and BRIP 50785 (*MAT1-1* haplotype II) × BRIP 61988 (*MAT1-2*), along with single-isolate suspensions of each isolate (as controls) and a combination of *MAT1-1* and *MAT1-2* isolates from the 2012 field populations ($n = 17$) (including one representative of *MAT1-1* haplotype II). To overcome any issues with vegetative incompatibility between *D. tanacetii* isolates, an aliquot (100 µl) of each conidial mixture was plated onto 2% WA to confirm that the selected inoculation method would result in the formation of an interwoven mycelial matrix from the hyphal strands of germinated conidia of different isolates. To increase the likelihood of pseudothecia production, each of the conidial suspensions was inoculated onto three media and incubated under four environmental conditions. The media and inoculation methods were (i) half-strength oatmeal agar (Boerema et al. 2004) plates with 1 ml of conidial suspension spread evenly across the surface; (ii) field-collected pyrethrum stems autoclaved twice (121°C for 15 min), immersed in the conidial suspensions for 15 min, and placed on 2% WA; and (iii) 3-month-old pyrethrum seedlings grown under sterile conditions (three fully expanded leaves, 5 to 7 cm in length), immersed in the conidial suspensions for 15 min, and placed into glass petri dishes containing sterile moistened filter paper (Chilvers et al. 2009). Each treatment plate was sealed with commercial film wrap. Inoculated media were incubated at 21°C in the dark for 36 h

prior to placement under different temperature conditions: (i) darkness at 5°C; (ii) darkness at 10°C; (iii) cycle of 12 h of light and 12 h of darkness with fluorescent tubes at 10°C; and (iv) growth cabinet under a cycle of system of 14 h of light and 10 h of darkness with fluora tubular fluorescent tubes (OSRAM, Munich, Germany), fluctuating temperatures of 18 and 10°C, and 50% relative humidity. Plates were examined biweekly for 16 weeks for the production of pseudothecia. Sections containing putative pseudothecia were excised, mounted in water, and observed by light microscopy at ×100 to ×400 magnification to detect ascospores or asci. Three replicates were included for each conidial mixture-media-treatment. At the end of the 16-week period, each conidial mixture-media-treatment was scored for the presence of asci or ascospores.

RESULTS

Identification and arrangement of the *MAT* idiomorphs.

Using the stepwise amplification approach, the *MAT* idiomorphs and the flanking genes were identified in nine *D. tanacetii* isolates. The *MAT* genes were successfully amplified in all *D. tanacetii* isolates tested with the primers DT-MAT-UniR and DT-MAT-1F/DT-MAT1-2F and sequenced for 20 isolates. Two variants in the *MAT* gene structure were identified. All isolates contained a *MAT* gene with a structure homologous to either the *MAT1-1-1* of *P. pinodella* CBS 110.32 or the *MAT1-2-1* of *P. pinodella* CBS 108.31. Alignment of the *MAT* idiomorphs and flanking genes resulted in sequences of 5.87 kb for *MAT1-1* isolates and 6.3 kb for *MAT1-2* isolates (Fig. 3). The size of the idiomorph, determined by the length of dissimilar nucleotides, was 2.1 and 2.5 kb for *MAT1-1* and *MAT1-2* isolates, respectively (Fig. 3).

The predicted *MAT1-1-1* of *D. tanacetii* was 1,127 bp, with a single intron of 53 bp located within the α domain (nucleotide position 245 to 297), and encoded a protein of 357 amino acids (aa) (Fig. 3). Alignment of the *MAT1-1-1* sequences identified a second haplotype (haplotype II) attributed to five SNP within the *MAT1-1-1* CDS (99.56% sequence homology) (Table 3; Fig. 3), resulting in three nonsynonymous amino acid changes (S177F, L215I, and V272I). BLASTn analysis of *MAT1-1-1* identified the highest similarity to the *MAT1-1-1* of homothallic *P. zeae-maydis* isolate 3018 (KF443802; 79.24% sequence homology) and heterothallic *P. pinodella* CBS 110.32 (JF815529; 77.73% sequence homology). The predicted *MAT1-2-1* was 1,109 bp, with a single intron of 56 bp located within the HMG domain (nucleotide position 488 to 543), and encoded a protein of 351 aa (Fig. 3). BLASTn analysis of *MAT1-2-1* identified the highest similarity to the *MAT1-2-1* of *P. zeae-maydis* isolate 3018 (77.73% sequence homology) and *P. pinodella* CBS 108.31 (JF815531; 75.56% sequence homology).

No mutual conserved regions were found upstream of the start codons of *MAT1-1-1* and *MAT1-2-1*. However, a gene (herein referred to as *ORF1*) encoding a protein homologous to the pyridoxamine phosphate oxidase-like protein of *S. tanacetii* (AHY81342; 86.8% sequence homology) and *ORF1* proteins of *Phoma clematidina* (AER26925; 83.9% sequence homology) and *Cochliobolus heterostrophus* (AAB82944; 72.4% sequence homology) was identified. Comparison of the region between the *MAT* gene and *ORF1* for the three *MAT1-1* haplotype I isolates identified an SNP in one isolate (Fig. 3). Comparisons of the three *MAT1-2* isolates for the same region identified three insertion/deletions and two SNP (Fig. 3). In *D. tanacetii*, the *ORF1* resided further upstream from the *MAT* gene start codon in *MAT1-2* isolates (1,005 bp) than in *MAT1-1* isolates (556 or 557 bp) (Fig. 3). Furthermore, differences in intron length (256 or 265 bp), number of amino acids (238 or 241), and stop codon (TAG or GTA) were identified between the *ORF1* of *D. tanacetii* *MAT1-1* and *MAT1-2* isolates. A highly conserved mutual region was found downstream of the two *MAT* idiomorphs. The conserved region resided 436 and 437 bp from the stop codon of the *MAT* genes and varied in length from 585 to 589 bp for *MAT1-1* and *MAT1-2* isolates, respectively. The region was

more highly conserved (99.15%) between *MAT1-1* haplotype I isolates and *MAT1-2* isolates than between isolates of the two haplotypes of *MAT1-1* (98.63%) (Table 3). Eight SNP were identified in this region in *MAT1-1* haplotype II isolates which were absent in either *MAT1-1* haplotype I isolates or *MAT1-2* isolates. The conserved region terminated at the stop codon of an open reading frame which encoded a protein homologous to the DLP of *S. tanacetii* (AHY81343; 81.9% aa sequence homology) and the apurinic/apyrimidinic endonuclease 2 protein of *Aureobasidium melanogenum* (KEQ66557; 54.0% aa sequence homology). The DLP of *D. tanacetii* constituted 2,201 bp, arranged into three exons, and encoded a protein of 645 aa (Fig. 3). The encoded DLP protein sequence was identical for *MAT1-1* haplotype I and *MAT1-2* isolates. In comparison, the *MAT1-1* haplotype II isolates shared 97.67% amino acid homology within this region with the *MAT1-1* haplotype I and *MAT1-2* isolates (Table 3; Fig. 3). Within the three sequenced *MAT1-2* isolates, two synonymous mutations were identified in the CDS of the DLP (Table 3; Fig. 3).

Distribution of mating-type of *D. tanacetii* within pyrethrum fields. *D. tanacetii* was identified in all fields from 20 to 67% of sampling units per transect (Table 4). Pure cultures could not be obtained for all sampling units at which *D. tanacetii* was identified; however, in total, 325 isolates were obtained (S1 = 112, S2 = 68, TC1 = 102, and TC2 = 43) (Table 4). Ordinary runs analysis of pathogen incidence of *D. tanacetii* within transects identified a significant spatial aggregation of *D. tanacetii* isolates only in transect 2 of field TC2 ($Z = -1.77$, $P = 0.04$) at Table Cape.

The multiplex *MAT*-specific PCR produced single-amplification bands in all *D. tanacetii* isolates, with amplicons of approximately 1,450 and 1,150 bp amplified in the known *MAT1-1* and *MAT1-2* individuals, respectively (Fig. 2). No amplicons were produced when a random subset of these isolates was screened with PCR primers specific for the opposite mating-type to detect homothallic individuals. Overall, 48.9% of isolates were identified as *MAT1-1* and 51.1% as *MAT1-2* (Table 4). The frequency varied at a field level from 38.2 to 57.4% and 42.6 to 61.8% for *MAT1-1* and *MAT1-2*, respectively, and at a transect level from 34.6 to 68.2% and 31.8 to 65.4% for *MAT1-1* and *MAT1-2*, respectively. Restriction enzyme digest of the *MAT1-1* gene identified seven *MAT1-1* haplotype II isolates. Sequencing the *MAT1-1-1* in the *MAT1-1* haplotype II isolates confirmed the presence of all five SNP characteristic of the second haplotype. The *MAT1-1* haplotype II isolates were distributed within transects at Sassafras, with none detected at Table Cape.

Ordinary runs analysis identified no spatial aggregation of *MAT1-2* isolates within transects ($Z \geq -0.94$, $P \geq 0.17$) (Table 4). Spatial aggregation of *MAT1-1* isolates was only detected in the second transect of S2 at Sassafras (S2-2) ($Z_{S2-2} = -1.97$, $P_{S2-2} = 0.03$, $Z_{rest} \geq -1.30$, $P_{rest} \geq 0.09$) (Table 4). The exclusion of *MAT1-1* haplotype II isolates did not alter these observations ($Z_{S2-2} = -2.29$, $P_{S2-2} = 0.01$, $Z_{rest} \geq -1.15$, $P_{rest} \geq 0.13$) (Table 4). The ratio of *MAT1-1* to *MAT1-2* isolates was higher at Sassafras than Table Cape. However, it was not significantly different from a 1:1 ratio at an overall level for all fields and transects at Sassafras and for field TC2 or the transects located in it at Table Cape ($0.09 \leq \chi^2 \leq 3.2$,

$df = 1$, $0.07 \leq P \leq 0.76$) (Table 4). Within field TC1, the ratio was not consistent with a 1:1 ratio ($\chi^2 = 5.65$, $df = 1$, $P = 0.017$) (Table 4), which contributed to the same result observed overall at Table Cape ($\chi^2 = 6.63$, $df = 1$, $P = 0.01$) (Table 4). However, within each of the two transects in TC1, the ratio was consistent with a 1:1 ratio ($\chi^2 \leq 3.07$, $df = 1$, $P \geq 0.08$) (Table 4).

In vitro production of the sexual morph. Conidial germination efficiency in all isolates was $\geq 95\%$ and the germ tubes and subsequent mycelia produced a continuous hyphae matrix when conidial mixtures of isolates were inoculated and incubated on WA. Asci or ascospores were not detected under any experimental conditions during or at the conclusion of the 16-week incubation period.

DISCUSSION

This study, to the best of our knowledge, was the first to examine the mating-type structure of *D. tanacetii* and the frequency and distribution of mating-types of *D. tanacetii* within Australian pyrethrum fields. The *MAT* loci identified exhibited the typical characteristics and organization of a heterothallic Dothideomycete species. This included a single gene per idiomorph, the location of conserved introns in the HMG and α domain motifs (Groenewald et al. 2006; Turgeon 1998; Woudenberg et al. 2012), and open reading frames flanking the idiomorphs encoding *ORF1* and DLP genes (Vaghefi et al. 2015; Woudenberg et al. 2012). The occurrence of homologous gene sequences flanking both the *MAT* idiomorphs indicated that the *MAT* genes of *D. tanacetii* resided at the same

TABLE 3. Pairwise comparison of genome regions of *Didymella tanacetii* isolates of each mating-type, including the two identified haplotypes of *MAT1-1*

Genome region ^b	Mating-type ^a		
	<i>MAT1-1</i> haplotype I	<i>MAT1-1</i> haplotype II	<i>MAT1-2</i>
<i>MAT</i> gene			
<i>MAT1-1</i> haplotype I	100/100	99.56/99.16	NA
<i>MAT1-1</i> haplotype II	99.56/99.16	100/100	NA
<i>MAT1-2</i>	NA	NA	100/100
Conserved region 5' of <i>MAT</i> idiomorphs			
<i>MAT1-1</i> haplotype I	100/NA	98.63/NA	99.15/NA
<i>MAT1-1</i> haplotype II	98.63/NA	100/NA	97.79/NA
<i>MAT1-2</i>	99.15/NA	97.79/NA	100/NA
<i>ORF1</i> gene			
<i>MAT1-1</i> haplotype I	100/100	99.08/99.58	83.77/88.70
<i>MAT1-1</i> haplotype II	99.08/99.58	100/100	83.77/88.29
<i>MAT1-2</i>	83.77/88.70	83.77/88.29	100/100
DLP gene			
<i>MAT1-1</i> haplotype I	100/100	98.64/97.67	99.91–100/100
<i>MAT1-1</i> haplotype II	98.64/97.67	100/100	98.55–98.64/97.67
<i>MAT1-2</i>	99.91–100/100	98.55–98.64/97.67	99.91/100

^a Nucleotide identity/amino acid identity shown for *MAT1-1* haplotype I (KU030826, KU030827, and KU030830), *MAT1-1* haplotype II (KU030822, KU030823, and KU030824), and *MAT1-2* (KU030825, KU030828, and KU030829) isolates; NA = not applicable.

^b *ORF1* = Protein with a pyridoxamine 5'-phosphate oxidase domain and DLP = DNA lyase-like protein.

Fig. 3. Structural arrangement of mating type (*MAT*) idiomorphs and flanking genes of *MAT1-1* and *MAT1-2* isolates of *Didymella tanacetii*. Black, boxed arrows represent the direction of genes. White boxes within genes indicate introns. Thin black lines connecting the boxed arrows represent intergenic regions. Thick lines under the *MAT* genes identify the α domain and high mobility group domain of *MAT1-1-1* and *MAT1-2-1*, respectively. The boxed region indicates the highly conserved region of the *MAT* idiomorphs. Open triangles above the *MAT1-1* and *MAT1-2* diagrams indicate synonymous mutations within genes or introns between the two *MAT1-1* haplotypes and between isolates of *MAT1-2* of *D. tanacetii*, respectively. The asterisk above the open triangle in the *MAT1-1* diagram identifies the single-nucleotide polymorphism identified between the three *MAT1-1* haplotype I isolates. Closed triangles below the *MAT1-1* diagram indicate nonsynonymous mutations in gene regions between the two *MAT1-1* haplotypes of *D. tanacetii*. Figure is to scale.

chromosome location. In addition, *D. tanacetii* isolates containing both mating-types were not found, providing further evidence for heterothallism.

The dissimilar sequence length and identity upstream of the *MAT1-1-I* and *MAT1-2-I* genes identified in *D. tanacetii* has also been reported between the *MAT* idiomorphs of many heterothallic Didymellaceae species (Chérif et al. 2006; Cozijnsen and Howlett 2003; Woudenberg et al. 2012). This is not observed in other well studied species, including *C. heterostrophus* and *Alternaria alternata* (Barve et al. 2003; Turgeon et al. 1993). In addition, the differences observed between the idiomorphs in the length, amino acid sequence, and protein stop codons of *ORF1*, which resided upstream of the *MAT* idiomorphs of *D. tanacetii*, are also common for Didymellaceae species. Deletion of *ORF1* in *C. heterostrophus* did not affect the ability of compatible isolates to reproduce (Wirsal et al. 1998). However, the *ORF1* of *C. heterostrophus* is smaller than the *ORF1* of *D. tanacetii* and has no nucleotide polymorphisms between the *MAT* idiomorphs. Disruption of this gene in sexual heterothallic Didymellaceae species has not been conducted.

The identification of two haplotypes of *MAT1-I* in *D. tanacetii* (haplotype I and II), which differed in the nucleotide sequence of *MAT1-1-I*, conserved region downstream of *MAT1-1-I*, and the flanking genes, questioned the functionality of the encoded proteins and the evolutionary relationship between the haplotypes. The effect of the amino acid changes on the function of the *MAT1-1-I* protein in the second haplotype are hypothesized to be minimal, because the polymorphisms do not occur in the α domain. Furthermore, the amino acid substitutions that occurred in the *MAT1-1-I* protein of haplotype II isolates either occurred also in closely related species (Barve et al. 2003; Woudenberg et al. 2012; Yun et al. 2013), at amino acid sites with low amino acid conservation, or at amino acid sites which were absent in known sexually reproducing species (Zhong and Steffenson 2001). However, without successful crosses, the impact of the mutations on gene activity could not be fully assessed. Nonsynonymous amino acid changes have been identified in *MAT* genes of geographically diverse isolates (different countries) of *C. heterostrophus* (Turgeon 1998), *Phaeosphaeria nodorum* (Bennett et al. 2003), and *Cladosporium fulvum* (Stergiopoulos et al. 2007) and within field

populations of *Cercospora beticola* (Bolton et al. 2012). Phylogenetically, the two haplotypes of *MAT1-I* (represented by BRIP 61992, BRIP 61990, and BRIP 50785) are the same species and were identical for the nucleotide sequences of six barcode genes (Pearce et al. 2016). This indicated that the observed polymorphisms most likely occurred through genetic drift because the *MAT* genes evolve at a faster rate than these barcode genes (Turgeon 1998). In recombining populations, selection is expected to maintain homogeneity of the *MAT* genes (Lee et al. 2010). Thus, the occurrence of the second *MAT1-I* haplotype indicated a decreased selection pressure on the *D. tanacetii* population at Sassafras. Furthermore, one of the *MAT1-I* haplotype II isolates identified in the initial *MAT* sequencing, BRIP 50785, was collected in 2004. This indicated that the second haplotype has been present within the population for over a decade. Because *D. tanacetii* is seedborne in pyrethrum (Pethybridge et al. 2006), it is difficult to determine whether the second haplotype evolved within the current geographical region or was introduced from seed. Interestingly, the two fields at Sassafras were sown with different pyrethrum varieties and the seed used to sow the fields was harvested in two separate years (2006 or 2011).

Analysis of fields identified a high incidence of *D. tanacetii*. Furthermore, the occurrence of significant spatial aggregation of *D. tanacetii* isolates in only one transect indicated that the majority of fields did not contain spatial clusters of *D. tanacetii*. Spatial clustering would be expected when asexual reproduction was the sole reproduction method, resulting in localized plant-to-plant spread of individuals. Additionally, the occurrence of both mating-types at a 1:1 ratio overall and within each of the eight transects, which represented the smallest spatial hierarchical population examined, suggested that, when conditions are favorable, the species may undergo sexual reproduction. A 1:1 distribution of *MAT* isolates is consistent with reproduction occurring in previously presumed asexual heterothallic species, including *Rhynchosporium secalis* (Linde et al. 2003), *Cercospora* spp. (Groenewald et al. 2008), and *Cladosporium fulvum* (Stergiopoulos et al. 2007). For sexual reproduction to occur in heterothallic species, individuals of each mating-type need to be within a suitable spatial distance from each other to allow interaction. Significant spatial aggregation of isolates of each mating-type was

TABLE 4. Isolation frequency of *Didymella tanacetii*, ratio of mating-type (*MAT*) idiomorphs, and their spatial distribution, in commercial pyrethrum fields in Tasmania, Australia

Populations ^d	Sampling units ^a		Mating-type analysis ^b					Ordinary runs analysis ^c			
	Total	<i>D. tanacetii</i>	<i>D. tanacetii</i>	<i>MAT1-1</i>	<i>MAT1-2</i>	Ratio	χ^2 (P)	<i>D. tanacetii</i>	<i>MAT1-1</i> (I)	<i>MAT1-1</i> (I & II)	<i>MAT1-2</i>
Sassafras	400	196	180	95/7	78	1.31	3.20 (0.07)	...	...	...	...
S1	200	120	112	59/4	49	1.29	1.75 (0.19)	...	...	...	...
1	100	67	63	34/2	27	1.33	1.29 (0.26)	0.09 (0.53)	-0.62 (0.27)	-1.00 (0.15)	-0.27 (0.39)
2	100	53	49	25/2	22	1.23	0.51 (0.48)	-1.18 (0.12)	-1.30 (0.10)	-0.30 (0.38)	0.03 (0.51)
S2	200	76	68	36/3	29	1.34	1.47 (0.23)	...	...	...	...
1	100	25	22	13/2	7	2.14	2.91 (0.09)	-1.08 (0.14)	-1.22 (0.11)	-0.97 (0.17)	1.17 (0.88)
2	100	51	46	23/1	22	1.09	0.09 (0.77)	-1.60 (0.05)	-1.97 (0.03)	-2.29 (0.01)	-0.86 (0.20)
Table Cape	400	152	145	57/0	88	0.65	6.63 (0.01)	...	...	...	...
TC1	200	104	102	39/0	63	0.62	5.65 (0.02)	...	...	...	...
1	100	57	55	21/0	34	0.62	3.07 (0.08)	2.20 (0.98)	-1.15 (0.13)	...	0.54 (0.70)
2	100	47	47	18/0	29	0.62	2.58 (0.11)	-0.96 (0.17)	-0.37 (0.36)	...	-0.94 (0.17)
TC2	200	48	43	18/0	25	0.72	1.14 (0.29)	...	...	...	...
1	100	28	26	9/0	17	0.53	2.46 (0.12)	0.76 (0.77)	0.70 (0.76)	...	0.80 (0.79)
2	100	20	17	9/0	8	1.13	0.06 (0.81)	-1.76 (0.04)	0.07 (0.53)	...	1.24 (0.89)
Overall	800	348	325	152/7	166	0.96	0.15 (0.70)	...	...	...	...

^a Total = total number of sampling units and *D. tanacetii* = number of sampling units within which *D. tanacetii* was identified.

^b *D. tanacetii* = number of *D. tanacetii* isolates recovered for mating-type analysis; *MAT1-1* = number of *MAT1-1* (haplotype I)/*MAT1-1* (haplotype II) isolates identified; *MAT1-2* = number of *MAT1-2* isolates identified; ratio = ratio of *MAT1-1* (I and II):*MAT1-2* isolates; and $\chi^2 = \chi^2$ value for the goodness of fit test for deviations from an expected 1:1 ratio of mating-types; values in parentheses indicate significance.

^c Z-value estimates from ordinary runs analysis of spatial distribution of individuals within transects; values in parenthesis indicate probability of estimates. *D. tanacetii* = analysis of identified *D. tanacetii* isolates; *MAT1-1* (I) = analysis of only *MAT1-1* (I) isolates; *MAT1-1* (I & II) = analysis of *MAT1-1* isolates without differentiation based on haplotype; and *MAT1-2* = analysis of *MAT1-2* isolates.

^d Hierarchical populations: regional populations (Sassafras and Table Cape) in Tasmania, Australia; field populations, where S1 and S2 were located in Sassafras, TC1 and TC2 were located in Table Cape; and transect population from 50-m transects (1 and 2) in each field.

also only detected for *MAT1-2* isolates in S2-2. However, within this transect, *MAT1-1* isolates were not spatially aggregated. Because the sampling scale was on a plant level, this indicated that, for the majority of transects, individuals of each mating-type were randomly spread within adjacent plants. Sampling at a smaller spatial scale, including within the same plant or lesion, may give further insight into the potential occurrence of sexual recombination by suggesting that close physical proximity of the two mating-types is common in pyrethrum fields (Bolton et al. 2012; Sommerhalder et al. 2006; Zhan et al. 2002).

Despite field populations displaying characteristics reminiscent of sexual recombination, crosses of genetically compatible *D. tanacetii* isolates in vitro failed to produce asci or ascospores. Microscopic observations of compatible isolates on WA indicated that the hyphae of individuals of opposite mating-types interwove and produced a coherent hyphal matrix; thus, a lack of physical association of opposite mating-types was discounted as a reason for the absence of pseudothecia. Furthermore, conditions of incubation covered a range of conditions used to produce pseudothecia in closely related species (Chilvers et al. 2009; Houbraken and Dyer 2014). Thus, the sexual morph is proposed to be induced under highly specific environmental conditions. The exact nature of these conditions requires further study.

Didymella tanacetii is the dominant pathogen associated with tan spot; therefore, sexual reproduction in this species would have a greater impact on spatial and temporal characteristics of epidemics by providing a mechanism for long-distance dispersal. Although this study was not able to confirm the occurrence of a sexual morph, it indicated that a cryptic reproductive cycle may occur within field populations. A further consequence of sexually reproducing heterothallic species is genetic recombination. Fungal populations that reproduce sexually are likely to be genetically more diverse and have a higher adaptive potential than asexually reproducing populations (McDonald and Linde 2002). A high adaptive ability has been suggested in field isolates of *D. tanacetii* with respect to the development of fungicide resistance to the succinate-dehydrogenase-inhibiting fungicide boscalid (Hay et al. 2015). In comparison, *S. tanacetii*, which causes ray blight of pyrethrum and undergoes only asexual reproduction, has not developed resistance to boscalid (Hay et al. 2015; Vaghefi et al. 2015). Many mating-type studies have used genetic markers to further assess populations for evidence of recombination (Li et al. 2013; Stergiopoulos et al. 2007). Analyzing *D. tanacetii* populations to identify the level and structure of genetic diversity would allow further examination for the occurrence and frequency of a sexual cycle of *D. tanacetii* in pyrethrum fields. This information, combined with results from this study, may facilitate identification of the specific environmental conditions required and allow management strategies to be developed which aim to minimize the inoculum produced from sexual reproduction.

Overall, the detection of both mating-types in equal ratios and under a random spatial distribution within field transect populations indicated the occurrence of a cryptic sexual mode of reproduction. The inability to produce the sexual morph in vitro suggested that specific environmental conditions or temporal requirements are necessary. Undertaking the subsequent analyses outlined will further assist the detection of the sexual mode of reproduction.

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