Evidence for Sexual Recombination in *Didymella tanacetii* Populations, and Their Evolution Over Spring Production in Australian Pyrethrum Fields

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ABSTRACT

Tan spot, caused by *Didymella tanacetii*, is one of the most important foliar diseases affecting pyrethrum in Tasmania, Australia. Population dynamics, including mating-type ratios and genetic diversity of *D. tanacetii*, was characterized within four geographically separated fields in both late winter and spring 2012. A set of 10 microsatellite markers was developed and used to genotype 774 *D. tanacetii* isolates. Isolates were genotypically diverse, with 123 multilocus genotypes (MLG) identified across the four fields. Fifty-eight MLG contained single isolates and P_{sex} analysis estimated that, within many of the recurrent MLG, there were multiple clonal lineages derived from recombination. Isolates of both mating types were at a 1:1 ratio following clone correction in each field at each sampling period, which was suggestive of

sexual recombination. No evidence of genetic divergence of isolates of each mating type was identified, indicating admixture within the population. Linkage equilibrium in two of the four field populations sampled in late winter could not be discounted following clone correction. Evaluation of temporal changes in gene and genotypic diversity identified that they were both similar for the two sampling periods despite an increased *D. tanacetii* isolation frequency in spring. Genetic differentiation was similar in populations sampled between the two sampling periods within fields or between fields. These results indicated that sexual reproduction may have contributed to tan spot epidemics within Australian pyrethrum fields and has contributed to a genetically diverse *D. tanacetii* population.

Pyrethrum (*Tanacetum cinerariifolium* (Trevir) Sch.Bip) is a perennial species of the family Asteraceae grown commercially for the extraction of pyrethrins, which have insecticidal properties and are concentrated within oil glands on the seed (Khater 2012; Soderlund 1995). Australia currently leads global pyrethrum production from an area of approximately 3,000 ha centered in Tasmania and Victoria. Following sowing from seed in austral spring (September to November), plants undergo an establishment phase of 6 to 8 months until the following austral winter (June to August) provides conditions for vernalization and bud initiation (Brown and Menary 1994). Daisy-like flowers are produced annually in late austral spring to summer (November to December) every year thereafter. Ideally, fields produce two to four annual harvests, during which stems are cut and placed in windrows, followed by seed harvest and pyrethrin extraction (Morris et al. 2006). However, declining plant health due to plant diseases often leads to premature termination of fields prior to optimal return to growers (Hay et al. 2015).

A complex of foliar diseases affect pyrethrum in Tasmania (Hay et al. 2015; Pethybridge et al. 2008a). Within this complex, tan spot, caused by the ascomycete *Didymella tanacetii* (syn. *Microsphaeropsis tanacetii*; Pearce et al. 2016a; Pethybridge et al. 2008b), has

been present since at least 2001 (Pethybridge et al. 2003). Initial reports recorded the pathogen at only a low frequency (<10.5%) from diseased leaves (Pethybridge et al. 2003). However, since 2009, the prevalence and incidence of *D. tanacetii* has increased markedly in autumn and winter (Hay et al. 2015). By 2012, *D. tanacetii* was found in ≥96% of fields sampled over the period winter to summer, with mean isolation frequencies of 40.8 to 55.5%. Additionally, 25% of fields sampled in 2012 are reported to have had isolation frequencies >73%. As such, surveys during 2012 and 2013 indicated that *D. tanacetii* is now the dominant foliar pathogen of pyrethrum during winter and spring in Australian crops (Hay et al. 2015).

Secondary spread of *D. tanacetii* is postulated to result from multiple infection cycles following dispersal of pycnidiospores annually. *D. tanacetii* has been putatively identified as heterothallic (Pearce et al. 2016b). However, the gene predictions and functionality of the mating-type genes in *D. tanacetii* are not confirmed and the sexual stage has not been observed or produced in vitro. Thus, sexual reproduction is proposed to occur infrequently when specific environmental and temporal requirements are met. Infested primary seed has also been identified as a major primary source of inoculum into new fields (Pethybridge et al. 2006; Scott et al. 2017), while overwintering on plant stubble carried over from the previous year's harvest and alternative hosts (including many common weed species) may also contribute to inoculum perpetuation between seasons and spread between fields. Annual increases in *D. tanacetii* isolation frequencies have also been proposed to be associated with higher rainfall in winter providing conducive conditions for inoculum build-up through more infection cycles. Increased virulence of *D. tanacetii* isolates and introduction of more susceptible cultivars may also account for the increased prevalence and incidence of tan spot (Hay et al. 2015).

Control of tan spot relies mainly on prophylactic fungicide application and limiting the spread of inoculum from seed or exogenous sources (Scott et al. 2017). The use of fungicides for

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disease control is highly efficacious but may have deleterious effects on populations in selecting for fungicide resistance (van den Bosch et al. 2011). For example, reduced sensitivities of *D. tanacetii* to boscalid were identified within 4 years of its introduction to pyrethrum production (Hay et al. 2015). Furthermore, Hay et al. (2015) reported an increase in the proportion of *D. tanacetii* isolates with boscalid insensitivity between 2009 and 2012.

Analyses of population genetic diversity provides useful information on the epidemiology and evolutionary history of diseases such as tan spot. Multiple factors, including the size of the founder population, modes of reproduction, development and persistence of fungicide-resistant individuals, and seasonal changes have significant effects on the genetic structure of many fungal species (Kretschmer and Hahn 2008; Linde et al. 2010; Shrestha et al. 2017). The use of neutral genetic markers such as microsatellite (simple sequence repeat [SSR]) markers allows the potential of populations to respond by evolutionary adaptation to be assessed (Kirk and Freeland 2011). Knowledge of the genetic structure of *D. tanacetii* populations is limited, and genetic diversity studies have not been undertaken. Knowledge of the distribution of the mating types, together with the amount of genotypic variation observed within a specific fungal population, can provide evidence for whether sexual reproduction is likely to occur (Blixt et al. 2008; Linde and Selmes 2012; Stergiopoulos et al. 2007).

This study aimed to test the hypothesis that the Australian *D. tanacetii* population consists of clonal lineages derived from asexual reproduction. To achieve this, we characterized the amount and structure of genetic diversity within populations of *D. tanacetii* collected from four pyrethrum fields. Changes in *D. tanacetii* population structure in each field over a 3-month period was also evaluated. These times were selected because they encompass the spring epidemic period, during which *D. tanacetii* is most prevalent (Hay et al. 2015; Pethybridge et al. 2008b).

MATERIALS AND METHODS

Sampling and isolation of *D. tanacetii*. *D. tanacetii* populations were collected from each of two pyrethrum fields in the Sassafras (41°17' S, 146°30' E) (fields 51907 and 63242) and Table Cape (40°56' S, 145°43' E) (fields 70047 and 70046) districts in Tasmania, Australia in 2012. Sassafras and Table Cape fall within the pyrethrum cropping region across northern Tasmania but are separated by approximately 70 km and two city areas (Burnie and Devonport). The two fields within the Sassafras and Table Cape districts were separated by approximately 5.2 and 1.4 km, respectively.

All fields were sown between August and October 2011 with seed harvested from four different pyrethrum seed crops. Field 51907 was sown with seed of variety 307, harvested in 2006, while fields 63242, 70047, and 70046 were sown with seed of variety 309, harvested in 2011 from three independent seed crops. Each field received the standard spring fungicide program in August and September 2012. This program consisted of an application of fludioxonil + cyprodinil (Switch; Syngenta), chlorothalonil (Bravo; Syngenta) + boscalid (Filan; BASF), followed by chlorothalonil + azoxystrobin (Amistar 250SC; Syngenta). Applications were conducted at approximately 2- to 3-week intervals and all fields received the same rate per hectare of each fungicide.

Diseased leaves were intensively sampled from each of the four fields in August (late winter) and November (spring) 2012. Sampling in August was conducted the week prior to the application of the first fungicide in the program. Sampling of diseased material conducted in November was conducted 7 to 9 weeks after the final fungicide application. Several (4 to 10) symptomatic leaves were collected from plants in each field at 0.5-m intervals along two parallel 50-m transects (representing 202 sampling units per field). The spatial locations of transects were randomly selected in each field but were a minimum distance of 20 m from the crop edges

and did not target specific diseased areas. The two transects in each field were separated by approximately 50 m. Transect locations were recorded using GPS to allow precise resampling in November.

Leaves were surface sterilized by immersion in 10% bleach (0.4% available chlorine) for 1 min, followed by three rinses in sterile, deionized water. From each transect sampling unit, four symptomatic leaves were selected and a small (approximately 0.5 mm²) tissue fragment was excised from the margins of one lesion on each leaf and placed on 2% water agar (Sigma-Aldrich) in Petri plates. Plates were incubated in the dark at 21°C for 5 to 7 days. Resultant mycelial growth was examined at ×63 magnification and putative *D. tanacetii* identified based on colony morphology (Pearce et al. 2016a). A small section of singular hyphae of each putative *D. tanacetii* colony was transferred to potato dextrose agar (PDA; Amyl Media) and incubated at 21°C in the dark for 5 days. Hyphal-tipped cultures were then transferred to a light box under a photoperiod of 14 h of light: and 10 h of darkness using L 36W/860 Lumilux Daylight globes (Osram) at approximately 23°C for 5 days for additional morphological confirmation (Pearce et al. 2016a). For sampling units in which multiple *D. tanacetii* isolates were obtained, a single isolate was arbitrarily selected for further analysis. Isolates were stored at –80°C, as described by Hay et al. (2015).

DNA extraction and quantification. For extraction of genomic DNA, *D. tanacetii* isolates were recovered from –80°C onto PDA and incubated in the dark at 21°C for 7 days. Sterile 50-ml centrifuge tubes containing approximately 20 ml of 2% (wt/vol) malt extract broth (Oxoid Ltd.) were inoculated with mycelium scrapped from the top of the cultures and incubated at approximately 20°C on an orbital shaker (54 rpm) for 12 days. The mycelial mats were blotted with sterile paper towel and ground in liquid nitrogen. DNA was extracted from the ground mycelia using the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol. DNA concentration was quantified using a High Sensitivity Double Stranded DNA QUBIT 2.0 kit (Life Technologies), and diluted to 1 ng/μl for use in polymerase chain reaction (PCR) prior to storage at –20°C.

Mating-type determination. The mating type of each isolate was determined with a *D. tanacetii* MAT-specific multiplex PCR assay using the primers DT-MAT-UniR, DT-MAT1-Spec-F, and DT-MAT2-Spec-F (Pearce et al. 2016b). This species-specific PCR was also utilized to identify any isolates that were initially misclassified as *D. tanacetii*. PCR assays were performed in a C1000 thermocycler (Bio-Rad Laboratories). Amplified products were analyzed by gel electrophoresis in a 1.5% (wt/vol) agarose gel containing GelRed (Biotium Inc.) in 1× Tris-acetate-EDTA buffer. Isolates were differentiated as mating type 1 (MAT1-1) or mating type 2 (MAT1-2) based on the presence of a 1,452- or 1,100-bp amplicon, respectively.

Microsatellite assay development. A nucleotide database provided by high-throughput DNA shotgun 454 sequencing (Rothberg and Leamon 2008) of *D. tanacetii* isolate BRIP 61988 (Pearce et al. 2016b) was utilized for SSR discovery. The open source C++ library QDD v1.3 (Megléc et al. 2010) was used to screen the resultant contigs for SSR loci. From the 162 perfect SSR loci identified, a subset ($n = 50$) was selected which had a minimum of five repeats of a 2- to 4-bp motif. Primer 3 v2.3.3 (Rozen and Skaletsky 2000) was used to design PCR primers using the default parameter settings to flank the SSR loci and amplify markers with a product size <290 bp. For each locus, the primer pair with the lowest paired primer penalty score was selected.

To avoid ascertainment bias (Schlötterer 2004), the selected SSR loci were evaluated on a diverse group of isolates ($n = 35$) collected from Tasmanian pyrethrum fields between 2004 and 2012. Genomic DNA was extracted from the isolates as described previously. Each primer pair or marker was analyzed using real-time PCR followed by high-resolution melt (HRM) analysis to

confirm consistency in amplification and to identify polymorphic loci. Real-time PCR and HRM were undertaken in a Rotor-Gene Q real-time PCR machine (Qiagen). Reaction mixes (10 µl) contained 1× Type-it HRM buffer (Qiagen), 0.7 µM each primer, and 2 ng of genomic DNA. Reaction conditions were an initial denaturation of 5 min at 95°C, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 55 or 57°C (primer dependent) for 30 s, and extension at 72°C for 10 s. Amplified products were subjected to HRM using 0.01°C increments from 65 to 95°C with a 10-s hold at each temperature prior to data acquisition. Samples were run in duplicate. Runs from each marker were analyzed in the Rotor Gene ScreenClust HRM software (v1.10.1.2; Qiagen) (Reja et al. 2010). The raw melt data were analyzed to confirm the amplification of only a single product. An “unsupervised” analysis was used to partition the data into clusters to identify polymorphisms in the amplified region (Reja et al. 2010). For each marker, the SSR loci of representative isolates of each cluster were reamplified and sequenced to identify whether variation was due to differences in the number of motif repeats within the SSR loci. PCR mixtures for sequencing contained 0.05 U of TopTaq polymerase (Qiagen), 1× PCR buffer, 1× CoralLoad, 1.5 mM MgCl₂, 0.7 µM each primer, 200 µM each dNTP (Bioline), and 3 ng of genomic DNA. PCR amplification involved initial denaturation at 94°C for 3 min; followed by 30 cycles of denaturation at 94°C for 15 s, annealing at 55 or 57°C for 15 s, and extension at 72°C for 20 s; and concluded with 72°C for 5 min. Amplified products were visualized and prepared for sequencing using the UltraClean PCR Clean-Up kit (Mo Bio Laboratories Inc.). The amplicons were sequenced in one direction with a single forward or reverse primer to provide coverage of the SSR loci at 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 Inc.). Sequences for each marker were aligned using MAFFT v7.017 (Katoh et al. 2002) in Geneious (v7.1.0; Biomatters Ltd.) to identify polymorphisms within the number of SSR motif repeats.

SSR genotyping. Ten polymorphic SSR markers (Table 1) were used to genotype the *D. tanacetii* population collected in 2012. The forward primer of each SSR marker was labeled with either VIC, FAM, NED, or PET (Invitrogen). PCR assays were performed in 10-µl volumes in 96-well plates. PCR mixtures contained 0.05 U

of TopTaq polymerase, 1× PCR buffer, 1.5 mM MgCl₂, 0.6 µM each locus-specific reverse primer, 0.6 µM each corresponding fluorescent-labeled forward primer, 200 µM each dNTP, and 2 ng of DNA. PCR amplification included denaturation at 94°C for 3 min; 30 to 35 cycles of denaturation at 94°C for 15 s, annealing at 55 or 57°C (Table 1) for 15 s, and extension at 72°C for 20 s; followed by 72°C for 5 min on the final cycle. Within each plate, at least one isolate of each known SSR genotype was included to verify results from capillary electrophoresis. Capillary electrophoresis was conducted at AGRF on a 96-capillary ABI 3730 DNA analyzer using a LIZ 500 size standard (Life Technologies Australia). Allele sizes were determined using Geneious Microsatellite Plugin 1.4 in Geneious. Isolates exhibiting null alleles were repeated and, if necessary, sequenced, as described previously. In addition, the SSR loci of isolates with unidentified or rare alleles were sequenced to confirm the number of motif repeats. To evaluate genotyping error and confirm accuracy of the capillary electrophoresis, 3.2% ($n = 242$) of the examined loci were randomly selected and sequenced. The number of repeats in the sequence data were compared with the number of repeats identified from the fragment length from capillary electrophoresis and used to calculate the genotyping error. SSR alleles are reported as the number of repeats of each motif.

Population structure. The SSR and mating-type data were analyzed as an overall population, populations based on sampling period (August and November; $n = 2$), and individual field populations with two sampling periods (field-sampling period; $n = 8$). In addition, populations consisting of isolates of each mating type ($n = 2$) were also defined. Two datasets were generated for analysis. The first dataset contained all isolates within each population (uncorrected dataset). The second dataset was constructed to minimize bias introduced by the clonality observed in the uncorrected dataset (clone-corrected). Isolates with the same combination of alleles at each locus (multilocus genotype [MLG]) and the same mating type were considered clones. The clone-corrected dataset included one member of each MLG for each mating type in each field population for each sampling period. The clone-corrected dataset was used for analysis of mating-type ratios, analysis of molecular variance (AMOVA), and calculating the index of association (multilocus linkage disequilibrium). Spatial information was used to assess structural patterns of genotypes within the fields for each sampling period.

TABLE 1. Microsatellite (simple sequence repeat [SSR]) primer sequences and conditions for amplification and analysis of *Didymella tanacetii* used in this study

SSR marker	Primer sequence (5'–3')	Amplicon length (bp)	Motif	T (°C) ^a	Alleles ^b	PIC ^c	ENA ^d
SSR05	F: ATGCTTGTGGATCTCGACT R: GTGACTGTGCCGCTATCTGA	142–160	TCG	55	7, 10*, 11, 12*, 13*	0.498	1.99
SSR32	F: ATTGCTCGACTGGAAAGCAT R: TGGGCATAACCTCTCGAATC	190–199	TTG	55	5*, 7, 8	0.519	2.08
SSR62	F: CAAGTCTGCTGGTCTTGAC R: CTGCGACTCGTATCCTTGGT	242–257	CAG	55	6*, 7*, 8, 9, 11	0.483	1.93
SRR64	F: CCAGGTGCAGATGTCCCC R: TCGTATGCTACACGAATCGC	109–124	GCA	55	8, 9, 13*	0.324	1.48
SSR53	F: GTCGGCTTGGCTCATGTAGT R: GTCCCCAGCAACACATTGAT	77–86	TGC	55	7, 8, 10	0.551	2.23
SSR54	F: GTGCAATACGGCAACATGAC R: GAATTGCCATGTGGAAAGGT	107–151	TTGC	55	6, 7, 8*, 9*, 10, 12*, 13, 15*, 17*	0.587	2.42
SSR58	F: AGTCGTTCTGATACGTGCC R: ACTTCTCCATGTACCACGCC	252–318	GCT	55	5, 7, 13, 17*, 26*, 27*	0.656	2.91
SSR44	F: GGTGCGACATGACGACTG R: GCGACTGTCAGGCAGTGA	152–158	AGC	57	8, 9	0.102	1.11
SSR31	F: CCAGCAGTACCAGCAGTCAC R: GATCTTGACCTGAACCTGGC	205–208	CAA	55	6, 7	0.219	1.28
SSR56	F: CGTGTGCTGAATAGTCCAA R: CTGCTCAAGGAGAGGAGGG	136–148	CT	55	6, 10*, 11	0.512	2.05
		...	...	...	...	...	...

^a Annealing temperature.

^b Motif repeat numbers observed at SSR locus; as asterisk (*) indicates rare alleles (occurred in less than 5% of the total population).

^c PIC = polymorphic informative content.

^d ENA = effective number of alleles.

SSR marker evaluation. SSR markers were evaluated to determine the presence of sufficient variation for inclusion in the data used for population analysis. First, the Ewens-Watterson neutrality test (Manly 1985) was conducted using 1,000 simulated samples in POPGENE, v. 1.32 (Yeh et al. 1997), to test selective neutrality of each of the microsatellite loci within the overall population. Furthermore, for each locus, the polymorphic informative content (PIC) (Botstein et al. 1980) and the effective number of alleles (ENA) (Kimura and Crow 1964) were calculated for each marker for the overall population and the eight field-sampling period populations in R, v. 3.2.4 (R Core Team 2016). The PIC was defined as $1 - \sum(A_i^2)$ and ENA as $1/\sum(A_i^2)$, where A_i is the frequency of the i^{th} allele of the locus. The interpretation of the PIC by Botstein et al. (1980) was used to evaluate the level of gene variation, whereby a $\text{PIC} > 0.5$, $0.25 < \text{PIC} < 0.5$, and 0.25 to 0.5 indicated the locus was of high, low, and intermediate diversity, respectively. For each SSR locus, gene diversity was examined by calculating the number and frequency of alleles in each population defined by sampling period, field-sampling period, mating type, and overall. The number of private alleles, defined as those found in only one sampling period, field or mating type, were identified. Rare alleles (occurring $\leq 5\%$ of the total population) and their frequency within each sampling period, field, and mating type were also determined.

Gene diversity. Nei's unbiased gene diversity (u_h) was calculated for the uncorrected populations, defined by sampling period, field-sampling period, mating type and overall, by weighting u_h by sample size (Nei 1973) in GenAlEx v6.5 (Peakall and Smouse 2006, 2012). This index is the probability that at a given locus, two randomly drawn alleles from the gene pool are identical. For temporal analysis of gene diversity, homogeneity of gene frequencies for each SSR marker across the two sampling periods was tested within each field through construction of two-way contingency tables and a χ^2 analysis in POPGENE.

Identification of multilocus lineages. To examine whether any of the MLG which differed at only one locus may have occurred due to somatic mutations (Klekowski 2003) and, thus, belong to the same multilocus lineage (MLL) (Arnaud-Haond et al. 2007b), Genetic Distance Spectrums (GDS) were examined in RClone, v1.0.2. (Bailleul et al. 2016). A GDS based on the terms of divergence of SSR motifs (Rozenfeld et al. 2007) was calculated for each pair of unique MLG in the overall population. A second GDS was calculated for each pair of unique MLG in a simulated sexual population size of 774 obtained from the population. The two GDS were superimposed as histograms and manually examined for the occurrence of narrow peaks at the beginning of the distribution followed by a gap preceding the peak at larger distances (Arnaud-Haond et al. 2007a; Meirmans and Van Tienderen 2004). Because this pattern was not observed, no MLL were defined prior to analysis of genotypic diversity.

Genotypic diversity. Multilocus genotypic diversity indices were calculated for the uncorrected populations, defined by sampling period, field-sampling period, mating type, and overall using the R package *poppr* v2.0 (Kamvar et al. 2014, 2015). The number and frequency of each MLG within each population was calculated. To account for differences in samples sizes and allow comparisons between the populations, the number and standard error of expected genotypes ($e\text{MLG} = \text{genotypic richness}$) in each population, using a sample size (n) equal to $0.9 \times N$, where N was the smallest sample size of all populations being compared, was approximated based on rarefaction curves using 1,000 bootstrapped samples (Grünwald et al. 2003). To evaluate the distribution of genotypes within each population, genotypic evenness was calculated using the index E_5 (Ludwig and Reynolds 1988). E_5 converges to 0 when a population consists of a single genotype and is equal to 1 when all genotypes occur at the same frequency (Ludwig and Reynolds 1988). Genotypic diversity was calculated using Simpson's index (D) (Simpson 1949), which estimates the probability that two randomly selected individuals would have

a different MLG, and Stoddart and Taylor's index (G) (Stoddart and Taylor 1988). To compare the genotypic diversity indices (E_5 , D , and G) within populations of different sizes, the indices were estimated following random subsampling of the data using rarefaction in *poppr* with 1,000 jackknife replicates. The number of samples (n) drawn in each replicate was equal to $0.9 \times N$, where N was the smallest sample size of all populations being compared. Furthermore, 95% confidence intervals (CI) of the indices were calculated based on percentiles for the bootstrapped values.

Spatial and temporal structure. To quantify spatial and temporal population structure, AMOVA was conducted to partition the genetic variation within the dataset into three components (among sampling periods, fields within each sampling period, and individuals within fields) (Excoffier et al. 1992) for the uncorrected and clone-corrected datasets in GenAlEx. The distance matrices were constructed using the haploid-SSR distance calculation, which assumed a stepwise mutation model. Significance of differentiation between the sampling period populations was tested using a null distribution of variance (i.e., a random population) computed from 9,999 random permutations of the sample matrices (Excoffier et al. 1992). The variation attributed to genetic differences between fields in each of the sampling periods was also evaluated. Significance of differentiation between each of the field-sampling period populations was conducted as above.

A minimum spanning network (MSN) was constructed to evaluate the relationship between MLG groups and population structure with respect to the field, sampling period, and mating type of isolates. An MSN was constructed on the uncorrected overall populations based on Bruvo's distance (Bruvo et al. 2004). Bruvo's method uses a stepwise mutation model to calculate genetic distance of the individuals. The MSN was implemented in *poppr* and visualized using the R package *igraph* (Csardi and Nepusz 2006) as an unrooted tree network.

To further test for the existence of any underlying structure in *D. tanacetii*, two methods were used and implemented in the R package *adeigenet* (Jombart 2008). First, the best number of supported genetic clusters (k) within the overall population was estimated using k -means clustering of principal components (PC) to identify groups of individuals and the Bayesian information criterion to assess the best supported model (Jombart et al. 2010). Second, discriminant analysis of PC (DAPC) (Jombart 2008; Jombart et al. 2010) was used to create plots representing a single discriminant function (whereby $k = 2$) by plotting the densities of individuals based on predetermined substructures of sampling period and mating type. Twenty PC were retained in the preliminary data transformation step, which contained 99.4% of the total genetic variation for the substructures of sampling period and mating type.

Reproductive mode. MAT ratios. The frequency and ratio of *D. tanacetii* isolates of the two mating types were calculated for each field-sampling period, sampling period, and overall. The χ^2 analysis was used to determine the goodness of fit of a 1:1 ratio of the alternate mating types, and χ^2 analyses were also performed on the clone-corrected dataset to minimize bias introduced by the clonality observed in the uncorrected dataset (Blixt et al. 2008; Linde et al. 2010).

Analysis of random mating. Multilocus linkage disequilibrium was tested using the standardized index of association ($\bar{r}_d$) (Agapow and Burt 2001), implemented in *poppr*, for the field-sampling period populations for both the uncorrected and clone-corrected datasets. A null distribution of values expected for a randomly mating population was estimated from 9,999 permutations. Significance of the obtained $\bar{r}_d$ value was determined from a one-sided P value under a null hypothesis of random association of alleles.

P_{sex} . The probability of each MLG (P_{gen}) and the likelihood of sampling each MLG at least as many times as observed (P_{sex}) in a panmictic population was estimated using the software MLGSIM2.0 (Ivens et al. 2012; Stenberg et al. 2003). In this

analysis, the null hypothesis was that all representatives of each MLG were produced sexually via recombination. Allele frequencies derived from the entire population and a model which takes into account departures from Hardy-Weinberg equilibrium were used to provide a more conservative P_{sex} estimation (Arnaud-Haond et al. 2007a). Corresponding critical values for testing the significance of the P_{sex} value were estimated by 1,000 random simulations based on the allele frequencies and overall population size. Because rejection of sexual propagation of an MLG under the parameters set by the MLGSIM2.0 analysis does not imply that all representatives of the MLG belong to one clone (Gregorius 2005), the data were also analyzed using a more conservative approach in RClone. In comparison with the MLGSIM2.0 analysis, the RClone analysis aimed to identify how many clones could be maximally expected among the representatives (n) of each MLG within a sample size of 774. Allele frequencies were calculated using a round-robin approach whereby frequencies for a given locus were derived by first clone correcting with the genotypes found at all other loci (Arnaud-Haond et al. 2007a; Parks and Werth 1993). Corresponding critical values for testing the significance of the P_{sex} value were estimated as described. If the P value for testing the significance of the P_{sex} value of a multicopy MLG at $n = 2$ was significant, then all copies of that MLG could be considered to be the product of clonal reproduction. If the P value for testing the significance of the P_{sex} value was significant for some $n > 2$, then the hypothesis that more than $n - 1$ clones exist within n copies of that MLG could be rejected (Gregorius 2005). A P value of 0.05 was used as the cut-off for identifying n .

Relatedness of MAT1-1 and MAT1-2 isolates. To examine relatedness of MAT1-1 and MAT1-2 isolates in the overall population, a distance-based dendrogram was constructed through the unweighted pair-group method with arithmetic mean using Bruvo's distance (Bruvo et al. 2004) in *poppr* for 25 isolates for each of the MAT1-1 and MAT1-2 individuals randomly selected from the total population (Meng et al. 2015). Node support was assessed using 1,000 bootstrap replicates. A further tree was constructed using

a revised clone-corrected dataset, consisting of only one copy of each MLG for each mating type within the overall population, to examine the overall phylogenetic relatedness of MAT1-1 and MAT1-2 isolates.

Identification of clonal spatial patterns in transects. To examine spatial structure within transects in each field, spatial autocorrelation analyses (Smouse and Peakall 1999) were performed in GenAlix to identify clusters of similar MLG within each field. Geographic and genetic distance matrices, summed over all loci under the assumption of independence, were calculated for all pairs of isolates within the two 50-m transects within each field for the two sampling periods. For each transect in each field, multiple analyses were performed using even distance classes of sizes ranging from the minimum to the maximum distance of sampling. A correlation coefficient, r , between linear genetic and geographic distances was calculated for all pairs of isolates within the geographic distance classes and 95% CI of r calculated from 999 bootstrap replicates. Permutations ($n = 999$) were used to generate 95% CI about the null hypothesis of no spatial structure by random shuffling of all individuals among the geographic locations. For each distance class, r was considered statistically significant if it exceeded the 95% CI around the null hypothesis and if the 95% CI around r did not intercept zero. To assess the effect of the different distance classes on spatial autocorrelation, isolates were pooled into successively larger geographic distance classes (e.g., 0 to 1, 0 to 2, up to 0 to the maximum distance between isolates) and the results of the first distance bin of each plotted as a composite graph.

RESULTS

Isolation frequency of *D. tanacetii*. Of the 3,232 symptomatic leaves examined in each sampling period, *D. tanacetii* was isolated from 13% of leaves in August and 26% of leaves in November. *D. tanacetii* was isolated from leaves collected from 44 and 56% of sampling units ($n = 808$) in August and November, respectively. Pure cultures could not be isolated from leaves from all

TABLE 2. Diversity and linkage disequilibrium statistics of *Didymella tanacetii* for each sampling period, field by sampling period and mating-type populations, and overall for the uncorrected and clone-corrected datasets collected in 2012^a

Population ^d	<i>N</i>	Uncorrected ^b											Clone-corrected ^c		
		MLG	eMLG _n ± SE	<i>E</i> ₅	<i>E</i> _{5n} (95% CI)	<i>G</i>	<i>G</i> _n (95% CI)	<i>D</i>	<i>D</i> _n (95% CI)	<i>u</i> _h	<i>r</i> _d	<i>P</i>	MLG _{CC}	<i>r</i> _d	<i>P</i>
Sampling period															
August	325	89	84.3 ± 1.9	0.56	0.57 (0.55–0.60)	27.1	26.9 (25.1–28.8)	0.963	0.963 (0.960–0.965)	0.482	NC	NC	150	NC	NC
November	449	89	73.0 ± 3.1	0.62	0.65 (0.61–0.68)	27.7	26.9 (24.3–29.7)	0.964	0.963 (0.959–0.966)	0.484	NC	NC	153	NC	NC
Field-sampling period															
51907-Aug	112	52	25.0 ± 2.3	0.62	0.78 (0.66–0.88)	21.1	16.3 (11.1–23.4)	0.953	0.937 (0.910–0.957)	0.502	0.09	0.0001	58	0.06	0.0001
51907-Nov	152	55	23.1 ± 2.4	0.53	0.72 (0.58–0.86)	16.7	13.4 (8.40–19.6)	0.940	0.921 (0.881–0.949)	0.482	0.10	0.0001	58	0.03	0.0004
63242-Aug	68	31	21.5 ± 1.9	0.62	0.70 (0.61–0.80)	12.8	11.6 (8.31–15.7)	0.922	0.911 (0.880–0.936)	0.482	0.09	0.0001	32	0.03	0.0102
63242-Nov	64	23	17.3 ± 1.6	0.56	0.63 (0.53–0.75)	8.1	7.8 (5.5–11.1)	0.876	0.867 (0.817–0.910)	0.460	0.15	0.0001	24	0.04	0.0038
70046-Aug	43	23	21.6 ± 0.9	0.82	0.83 (0.80–0.84)	15.3	14.8 (13.2–16.4)	0.935	0.932 (0.924–0.939)	0.471	0.06	0.0001	25	0.02	0.0775
70046-Nov	67	32	23.3 ± 1.8	0.74	0.80 (0.71–0.88)	18.3	15.8 (12.0–20.3)	0.945	0.935 (0.917–0.951)	0.474	0.09	0.0001	32	0.05	0.0001
70047-Aug	102	33	20.6 ± 1.9	0.75	0.82 (0.70–0.90)	17.7	14.3 (10.6–18.8)	0.943	0.929 (0.906–0.947)	0.456	0.11	0.0001	35	0.04	0.0021
70047-Nov	166	35	19.3 ± 2.0	0.70	0.79 (0.67–0.89)	15.9	12.5 (8.7–16.7)	0.937	0.918 (0.885–0.940)	0.474	0.13	0.0001	39	0.04	0.0006
Mating type															
MAT1-1	371	69	65.7 ± 1.7	0.60	0.61 (0.59–0.63)	19.6	19.5 (18.6–20.6)	0.949	0.946 (0.946–0.951)	0.477	NC	NC	69	NC	NC1
MAT1-2	403	69	62.6 ± 2.2	0.58	0.59 (0.57–0.61)	16.9	16.8 (15.7–18.0)	0.940	0.936 (0.936–0.945)	0.476	NC	NC	69	NC	NC
Overall	774	123	NC	0.57	NC	29.8	NC	0.966	NC	0.484	NC	NC	303	NC	NC

^a N = number of individuals; MLG = number of multilocus genotypes (MLG); n = common population sample size, equal to $0.9 \times$ smallest N ($n = 293, 39$, and 334 for sampling period, field-sampling period, and mating-type populations, respectively); eMLG_n ± SE = expected number of MLG ± standard error after rarefaction where the sample size was n (Grünwald et al. 2003); E_5 = genotypic evenness (Ludwig and Reynolds 1988); E_{5n} = genotypic evenness and 95% confidence intervals (CI) (based on quartiles) based on bootstrapping where the number of samples drawn in each replicate was n ; G = Stoddart and Taylor's index of genotypic diversity (Stoddart and Taylor 1988); G_n = Stoddart and Taylor's index of genotypic diversity and 95% CI (based on quartiles) based on bootstrapping where the number of samples drawn in each replicate was n ; D = Simpson's index of genotypic diversity (Simpson 1949); D_n = Simpson's index of genotypic diversity and 95% CI (based on quartiles) based on bootstrapping where the number of samples drawn in each replicate was n ; u_h = Nei's unbiased haploid genetic diversity (Nei 1973); r_d = standardized index of association (Agapow and Burt 2001); P = significance of the observed r_d value from 9,999 permutations; MLG_{CC} = number of MLG reserved in the clone-corrected dataset; and NC = not calculated.

^b Contained all individuals in each population.

^c Included one member of each MLG for each mating type in each field population for each sampling period.

^d Sampling period = two populations sampled in either August or November; field-sampling period = four fields defined by the period of sampling (Aug = August; Nov = November); Mating type = isolates of MAT1-1 and MAT1-2; and Overall = all isolates.

sampling units at which *D. tanacetii* was identified; however, 774 isolates were acquired for population analysis. A greater number of isolates was acquired from leaves in November ($n = 449$) compared with August ($n = 325$) (Table 2). These results were generally consistent at a field level with *D. tanacetii* isolated from leaves from a greater number of transect sampling units per fields in November for all fields, except field 63242, in which a slightly greater number of isolates were obtained in August ($n = 68$) compared with November ($n = 64$) (Table 2).

SSR marker evaluation. For the 10 SSR markers used to genotype the *D. tanacetii* isolates, the genotyping error was 0%. The Ewens-Watterson test did not support significant deviations in allele distribution from neutral expectation. Within the overall population, all markers were polymorphic, with the number of alleles per locus ranging from 2 to 9 and averaging 4 (Table 1). The PIC averaged 0.45 across all markers and ranged from 0.10 to 0.66 in the overall population, with seven markers with PIC values indicating high diversity (Table 1). The ENA for each marker ranged from 1.11 to 2.91 and averaged 1.95 in the overall population (Table 1). Because the PIC and ENA for marker SSR44 ranged from 0.00 to 0.16 and 1.00 to 1.19, respectively, in each field population, this marker was considered uninformative and, therefore, was removed prior to analysis of gene and genotypic diversity of the populations.

Based on the 9 informative SSR markers, 16 rare alleles were recovered (Table 1). Of these, 6 were identified in only single isolates and, thus, were also private alleles. Of the 10 recurrent rare alleles identified, 9 were recovered from the August and November sampling periods, 8 were recovered from isolates of each of the two

mating types, and 9 were identified in at least two spatially separated fields. One recurrent rare allele was the only private allele recovered multiple times in the dataset and was recovered exclusively ($n = 3$) from field 51907. All other alleles were recovered in both sampling periods, fields, and isolates of the two mating types.

Gene diversity. Nei's unbiased gene diversity, averaged over all markers, was 0.484 for the overall population (Table 2). Small differences in u_h were identified between the August- and November-sampled populations (0.482 and 0.484) and between isolates of each mating type (0.477 and 0.476). In comparison, within the field-sampling period populations, u_h ranged from 0.456 to 0.502 (Table 2). No significant differences in allele frequencies of each marker between the August and November sampling periods were identified in fields 70047 ($P \geq 0.13$) and 51907 ($P \geq 0.06$) (Supplementary Table S1). In field 70046, allele frequencies between sampling periods only differed for marker SSR58 ($P = 0.01$). However, significant differences in allele frequencies between the sampling periods were identified for five of nine markers in field 63242 ($P \leq 0.03$).

Genotypic diversity. The 774 isolates genotyped with the nine SSR markers were assigned to 123 distinct MLG. The number of MLG in each population increased with sample size (Table 2). Of these, 58 (47.15%) were only detected once in the overall population (singleton). A number of MLG detected were unique to each field, the majority of which were singletons (unique MLG/singleton: field 51907 = 40/31, field 63242 = 18/11, field 70046 = 7/6, and field 70047 = 13/10). The most common genotype (MLG24)

Fig. 1. Percentage of *Didymella tanacetii* multilocus genotypes (MLG) containing at least 1% of the total population ($n \geq 8$) for each **A**, sampling period; **B**, mating type; and **C**, pyrethrum field.

was recovered 75 times (9.69% of the overall population) from all fields at both sampling periods (Fig. 1A and C). Only one other MLG (MLG47; $n = 16$) was recovered from all fields at both sampling periods (Fig. 1C). Additionally, when sampling period was ignored, MLG77 ($n = 45$), MLG23 ($n = 43$), MLG13 ($n = 34$), MLG98 ($n = 20$), MLG29 ($n = 13$), and MLG103 ($n = 8$) were recovered from all fields (Fig. 1C). Of the 65 recurrent MLG, 55 were recovered in August and November, including all MLG containing at least eight isolates ($\geq 1\%$ of population) (Fig. 1A). Further comparison of the MLG containing at least eight isolates ($n = 31$) identified that 29 MLG were obtained from at least two fields, regardless of sampling period (Fig. 1C), and 13 MLG contained isolates of the two mating types (Fig. 1B).

Estimated genotypic diversity, scaled to a common sample size of 293, was identical for the populations recovered in August and November ($G_{n=293} = 26.9$, $D_{n=293} = 0.963$) (Table 2). However, the August population had higher average richness ($eMLG_{n=293} = 84.3 \pm 1.9$) than the November population ($eMLG_{n=293} = 73.0 \pm 3.1$). Conversely, average evenness ($E_{n=293}$) was higher in the November (0.65) than the August (0.57) populations (Table 2). Within fields 51907, 63242, and 70047, average values of $E_{n=39}$, $G_{n=39}$, and $D_{n=39}$ were slightly higher for the August sampling period than the November sampling period (Table 2). In field 70046, average $E_{n=39}$ also decreased between August and November; however, average values of $G_{n=39}$ and $D_{n=39}$ were slightly higher in November. For all three indices, observations from the population sampled in November fell within the confidence ranges of the August estimates for each individual field. Within the fields, regardless of the sampling period and based on a sample size of 39, average richness ($eMLG_{n=39}$) ranged from 17.3 to 25.0. Comparison of the genotypic diversity of isolates of the two mating types identified that MAT1-1 isolates had slightly higher values of all the genotypic indices. Based on a common samples size of 334, richness ($eMLG_{n=334}$) was 65.7 ± 1.7 and 62.6 ± 2.2 for the isolates of MAT1-1 and MAT1-2, respectively (Table 2). Genotypic diversity based on average $G_{n=334}$

and $D_{n=334}$ was also higher in MAT1-1 (19.6 and 0.949) than MAT1-2 isolates (16.8 and 0.941). Evenness between the isolates of the two mating types was similar (Table 2).

Spatial and temporal structure. AMOVA indicated that 97.46% of the total genetic variation was attributed to genetic differences between individuals within fields and 0.71% was attributed to among sampling periods (Table 3). Comparative analysis of the clone-corrected dataset resulted in similar distributions of genetic variation for each hierarchical level (Table 3). A negative variance (σ) component was identified among the fields in each sampling period for the clone-corrected dataset, indicating an absence of genetic structure (Table 3). Significant differences were found among the two sampling periods ($\Phi = 0.012$; $P = 0.003$) and the eight field-sampling period populations ($\Phi = 0.017$; $P = 0.0001$) for the uncorrected dataset. Pairwise comparison of the eight field-sampling period populations did not detect significant differences between fields sampled in August ($-0.0013 \geq \Phi \leq 0.015$; $0.057 \geq P \leq 0.359$) (Table 4). Significant pairwise differences were found among all the fields sampled in November, except for between fields 70046 and 70047 ($\Phi = 0.009$; $P = 0.132$) (Table 4). Subsequent evaluation of the variation with each of the sampling periods identified only 0.081 and 3.43% of the total genetic variation attributed to difference between the fields sampled in August and November, respectively. Temporal comparisons within each field identified only significant variation between the August and November populations in field 63242 ($\Phi = 0.061$; $P = 0.006$) (Table 4). Following clone correction, no significant differences were found among the two sampling periods ($\Phi = 0.008$; $P = 0.145$) and the eight field-sampling period populations ($\Phi = 0.009$; $P = 0.157$).

In the MSN of the overall dataset, many of the MLG were only separated by a mutation at a single locus, and no distinct subnetworks were identified (Fig. 2). The dominant MLG (≥ 8 members) were distributed throughout the tree. Examination of the MSN network for which the MLG were partitioned by mating type

TABLE 3. Analysis of molecular variance (AMOVA) for uncorrected and clone-corrected datasets of *Didymella tanacetii*

Population component ^a	df	Sum of squares (SS)	Mean SS	Variance (σ)	Variation (%)
Uncorrected dataset ^b					
Among sampling periods	1	257.16	257.16	0.32	0.71
Among fields within sampling periods	6	730.07	121.68	0.84	1.83
Between individuals within fields	766	34,207.98	44.66	44.66	97.46
Total	773	35,195.21	423.49	45.82	100.00
Clone-corrected dataset ^c					
Among sampling periods	1	207.88	207.88	1.01	1.76
Among fields within sampling periods	6	327.41	54.57	0.00 (−0.06) ^d	0.00
Between individuals within fields	295	16,728.56	56.71	56.71	98.24
Total	302	17,263.85	319.15	57.72	100.00

^a Hierarchical AMOVA, partitioning total genetic variance into the following components: among the two sampling periods (August and November), among the four field populations in each sampling period, and within the eight fields sampled in 2012.

^b The uncorrected dataset contained all individuals in each population.

^c The clone-corrected dataset included one member of each multilocus genotype for each mating type in each field population for each sampling period.

^d Negative variance (σ) converted to 0, negative value in parenthesis.

TABLE 4. Pairwise comparison from analysis of molecular variance (AMOVA) for each field-sampling period population of *Didymella tanacetii*^a

Population	51907-Aug	63242-Aug	70046-Aug	70047-Aug	51907-Nov	63242-Nov	70046-Nov	70047-Nov
51907-Aug	...	0.233	0.359	0.358	0.346	0.010	0.009	0.015
63242-Aug	0.004	...	0.193	0.089	0.057	0.006	0.000	0.000
70046-Aug	−0.011	0.010	...	0.358	0.358	0.030	0.099	0.364
70047-Aug	−0.004	0.015	−0.013	...	0.356	0.003	0.018	0.148
51907-Nov	−0.005	0.018	−0.011	−0.006	...	0.004	0.009	0.026
63242-Nov	0.042	0.061	0.040	0.056	0.050	...	0.004	0.000
70046-Nov	0.041	0.094	0.020	0.032	0.036	0.056	...	0.132
70047-Nov	0.021	0.067	0.000	0.006	0.014	0.076	0.009	...

^a Sampling periods = August (Aug) and November (Nov). Pairwise ϕ values from AMOVA are shown below diagonal; pairwise P (based on 9,999 permutations) are shown above diagonal.

of isolates within them found no distinct differentiation between the two mating types (Fig. 2). When the isolates within each of the MLG within the MSN were distinguished by the sampling period or field of collection, no distinct differentiations or patterns were also identified (data not shown).

DAPC analysis on the overall dataset was consistent with the MSN analysis, with no distinct values of k able to be reliably estimated for the dataset based the Bayesian information criterion, indicating lack of structure (Fig. 3A). Additionally, DAPC analysis based on the predetermined substructure of sampling period identified that superimposed density plots of the August and

November sampled populations were almost identical (Fig. 3B). In comparison, DAPC analysis based on the predetermined substructure of mating type identified that superimposed density plots of each mating type population overlapped but differed in density (Fig. 3C).

Reproductive mode. MAT ratios. Both mating types were recovered from all sampling periods and fields. Overall, 47.9% of isolates were identified as MAT1-1 and 52.1% as MAT1-2 (Table 5). Within the field–sampling period populations, the ratio of MAT1-1/MAT1-2 varied from 0.58 to 2.37. The ratio of MAT1-1/MAT1-2 isolates in each field between the two sampling periods was similar,

Fig. 2. Minimum spanning networks, viewed as a tree network, of the relationship between *Didymella tanacetii* multilocus genotypes (MLG) in the overall population. Each node represents an MLG and is characterized by a pie chart to identify the contribution to each MLG of isolates of mating type 1 (MAT1-1) and mating type 2 (MAT1-2). Distances between MLG were estimated using Bruvo's distance (Bruvo et al. 2004). The size of each node is scaled to represent the number of individuals in each MLG. Major MLG (≥8 individuals; ≥1% of total population) are labeled. MLG connected by black lines indicate they differ at a single locus at one marker. Thickness of lines connecting MLG indicates Bruvo's distance. Scale bar indicates Bruvo's distance.

expect in 63242, where it increased from August (1.34) to November (2.37) (Table 5). The ratio of MAT1-1 to MAT1-2 isolates was not significantly different from a 1:1 ratio in the overall population or for the populations collected in August and November ($\chi^2 \leq 1.62$, $df = 1$, $P \geq 0.20$) (Table 5). Within the field populations collected in August, the ratio was not consistent with 1:1 only in field 70047 (0.62, $\chi^2 = 5.65$, $P = 0.02$) (Table 5). However, in November, the ratio significantly differed from 1:1 for fields 70047,

63242, and 70046 ($\chi^2 \geq 4.31$, $df = 1$, $P \leq 0.04$) (Table 5). Following clone correction ($n = 303$), mating-type ratios did not differ from 1:1 within any hierarchical population ($\chi^2 \leq 0.62$, $df = 1$, $P \geq 0.43$) (Table 5).

Analysis of random mating. Estimates of multilocus disequilibrium within each uncorrected field-sampling period population rejected the null hypothesis of random association of alleles ($P \leq 0.004$) (Table 2). In the clone-corrected datasets, the value of the index of association decreased for all fields (Table 2). The null hypothesis was not rejected for the fields 70046 and 63242 ($\bar{r}_d \leq 0.0266$, $P \geq 0.010$) (Table 2), sampled in August. However, a random association of alleles was rejected for the fields 70047 and 51907 sampled in August and in all fields sampled in November ($P \leq 0.004$).

P_{sex} . Based on the estimates and significance of P_{sex} obtained from analysis in MLGSIM2.0, the null hypothesis that all isolates with each recurrent MLG arose independently via recombination was not rejected for 16 of 65 recurrent MLG ($0.052 \leq P \leq 0.829$) (Supplementary Table S2). Sixteen MLG for which all copies of the MLG were considered to be the product of clonal reproduction were identified ($9.23 \times 10^{-05} \leq P \leq 0.045$, at $n = 2$) (Table 6). These included 7 of the 31 MLG with ≥ 8 individuals ($>1\%$ of population). For each of these 16 MLG, the null hypothesis that all isolates arose independently via recombination was also rejected ($0.00 \leq P \leq 0.005$). Of the remaining 24 MLG with ≥ 8 individuals, the number of estimated clonal lineages within each MLG ranged from two to seven (Table 6). MLG24 ($n = 75$) and MLG47 ($n = 16$) were identified in all fields at both sampling periods and were estimated to contain up to three and five clonal lineages, respectively. The highest number of clonal lineages were identified from MLG98 ($n = 20$) and MLG96 ($n = 8$), for which up to six and seven clonal lineages were estimated, respectively (Table 6).

Relatedness of MAT1-1 and MAT1-2 isolates. No evidence for genetic divergence using either a partial (50 isolates) (Fig. 4A) or complete (Fig. 4B) dataset was identified. Low node support (<0.50) in the tree from the partial dataset suggested a lack of genetic structure within the mating-type populations.

Identification of clonal spatial patterns. Analysis of genotypes within the two transects in fields 63242 and 70047 at both sampling periods were not genetically spatially autocorrelated; r was effectively zero for the first bin of each distance class. In transect 2 of field 51907 sampled in August, a significant negative value of r was identified for the 0- to 2-m distance class ($r = -0.112$). This indicated that genetic differentiation of individuals located at distances of 0 to 2 m was higher in this transect than expected. In transect 1 of field 70046 sampled in August, positive and significant

Fig. 3. Discriminant analysis of principal components analyses for the *Didymella tanacetii* population collected in 2012. **A**, Change in Bayesian information criterion within increasing numbers of clusters (k). Individual density plots on the first discriminant function examined using predefined populations ($k = 2$) of **B**, mating type and **C**, sampling period.

TABLE 5. Frequency and ratio of MAT1-1 and MAT1-2 isolates of *Didymella tanacetii* within sampling period and field populations^a

Period, field	Uncorrected dataset ^b				Clone-corrected dataset ^c			
	MAT1-1	MAT1-2	Ratio	χ^2 (P)	MAT1-1	MAT1-2	Ratio	χ^2 (P)
August								
51907	63	49	1.29	1.75 (0.19)	30	28	1.07	0.07 (0.79)
63242	39	29	1.34	1.47 (0.23)	17	15	1.13	0.13 (0.72)
70047	39	63	0.62	5.65 (0.02)	19	16	1.19	0.26 (0.61)
70046	18	25	0.72	1.14 (0.29)	13	12	1.08	0.04 (0.84)
Total	159	166	0.96	0.15 (0.70)	79	71	1.11	0.43 (0.51)
November								
51907	80	72	1.11	0.42 (0.52)	26	32	0.81	0.62 (0.43)
63242	45	19	2.37	10.56 (0.001)	13	11	1.18	0.17 (0.68)
70047	61	105	0.58	11.66 (0.001)	19	20	0.95	0.03 (0.87)
70046	26	41	0.63	4.31 (0.04)	15	17	0.88	0.13 (0.72)
Total	211	238	0.89	1.62 (0.20)	73	80	0.91	0.32 (0.57)
Grand total	371	403	0.92	1.49 (0.22)	152	151	1.01	0.003 (0.95)

^a Ratio = ratio of MAT1-1/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).

^b The uncorrected dataset contained all individuals in each population.

^c The clone-corrected dataset included one member of each multilocus genotype for each mating type in each field-sampling period population.

values of r were only identified for distance classes of 0 to 7 and 0 to 12 m ($0.060 \leq r \leq 0.094$).

DISCUSSION

The genetic structure of the *D. tanacetii* populations examined was suggestive of high genetic diversity and indicated that inoculum from a sexual morph may be a potentially important contributor to the disease epidemiology (Hay et al. 2015). High isolation frequencies of *D. tanacetii* from fields in August and November corroborated the importance of this pathogen in late winter and spring. However, the incidence of leaves affected by tan spot was higher in the sampling conducted in spring.

In August, genetic diversity of the *D. tanacetii* population was not significantly affected by geographic location. The two main sources of inoculum contributing toward tan spot in these fields in August were hypothesized as infested seed and exogenous sources (Scott et al. 2017). Field 51907 was sown with seed harvested in 2006, while the remaining three fields were sown from seed harvested in 2011. Testing of *D. tanacetii* incidence of seed from these two periods indicated that average seed incidence of *D. tanacetii* in 2006-harvested seed was higher (17.3%) than the average incidence in 2011-harvested seed (5.6%) (Scott et al. 2017). Therefore, it was initially hypothesized that, if seedborne inoculum was significantly contributing to tan spot epidemics, then isolates from field 51907 would be genetically distinct because a greater proportion of inoculum would have been derived from 2006. Although field

51907 contained double the number of isolates with unique MLG and singleton MLG, it was not genetically different from the fields sown with seed harvested in 2011. One explanation for this result is that seedborne inoculum does not significantly contribute to epidemics in late winter. Alternatively, the genetic structure of the pathogen population may not have changed significantly since 2006, despite rapid population expansion and development of fungicide insensitivities (Hay et al. 2015). Despite the fields being established for at least 12 months prior to sampling, common MLG within transects sampled in August with localized aggregation were not identified and isolates sampled within short distances were no more genetically related to each other than expected by random chance. Thus, within each transect, short-range spread of clonal genotypes was minimal. The random distribution of genotypes within each transect was more consistent with either seedborne transmission or random movement of different genotypes through airborne ascospores or medium range dispersal of conidia (Kohn 1995). Scott et al. (2017) hypothesized that fields within 2 km of each other are able to contribute inoculum to each other. The occurrence of many shared MLG further supported the hypothesis of broad-scale movement of genotypes via ascospores or other windblown inoculum. Many fields within the contiguous cropping region in the northwest of Tasmania are separated by only a few kilometers, which would aid broad-scale movement of genotypes. Future analyses may benefit from also selecting populations from outside this main cropping region, including most recently established regions in northeastern Tasmania and on the mainland

TABLE 6. Probabilities of multilocus genotypes (MLG) of *Didymella tanacetii* (P_{gen}) and likelihood of being derived from sexual reproduction (P_{sex}) using MLGSIM2.0 and RClone for multilocus genotypes containing at least 1% of the population

MLG ^c	SSR marker genotype ^b										MLSIM ^c			RClone ^d	Frequency by population ^a							
	05	32	31	62	64	53	54	58	56	<i>P_{gen}</i>	<i>P_{sex}</i>	<i>P</i>	<i>n clones</i>		August				November			
															Total	51907	63242	70047	70046	51907	63242	70047
24	77	8	6	8	8	7	13	5	11	5.72 × 10 ⁻³	0.00	<0.001	3	75	12	11	6	5	27	5	4	5
57	11	7	6	9	8	7	13	5	6	3.05 × 10 ⁻³	0.00	<0.001	4	53	0	0	14	4	2	0	25	8
77	11	8	6	8	9	8	13	13	6	1.90 × 10 ⁻³	2.31 × 10 ⁻¹⁴	<0.001	3	45	14	12	3	3	9	3	0	1
23	7	8	6	8	8	7	13	13	6	6.06 × 10 ⁻³	0.00	<0.001	4	43	8	0	6	1	16	3	8	1
13	7	7	6	9	8	7	13	5	6	1.86 × 10 ⁻³	7.66 × 10 ⁻¹⁵	<0.001	2	34	2	0	6	0	2	6	15	3
75	11	8	6	8	9	8	10	13	11	4.25 × 10 ⁻⁴	0.00	<0.001	2	32	1	0	7	4	0	0	16	4
5	7	7	6	8	8	7	13	13	11	4.28 × 10 ⁻³	1.40 × 10 ⁻¹⁴	<0.001	4	30	6	0	7	1	6	0	7	3
84	11	8	6	8	8	8	10	7	11	7.01 × 10 ⁻⁴	0.00	<0.001	3	28	2	0	6	0	0	0	12	8
4	7	7	6	8	8	7	6	5	11	1.15 × 10 ⁻³	9.77 × 10 ⁻¹⁵	<0.001	3	24	0	5	0	0	0	19	0	0
98	11	8	6	8	8	7	13	5	6	1.02 × 10 ⁻²	1.99 × 10 ⁻⁴	0.003	6	20	0	3	3	0	4	1	7	2
47	11	7	6	8	8	8	7	13	6	6.86 × 10 ⁻⁴	3.76 × 10 ⁻¹⁴	<0.001	4	16	2	1	1	1	1	5	3	2
9	7	7	6	9	8	8	13	5	6	1.43 × 10 ⁻³	1.60 × 10 ⁻¹¹	<0.001	3	14	0	0	6	4	0	0	2	2
64	11	7	7	8	9	10	6	7	11	3.20 × 10 ⁻⁶	0.00	<0.001	1	14	0	0	5	0	1	0	6	2
29	7	8	6	9	8	7	13	5	6	2.42 × 10 ⁻³	9.17 × 10 ⁻⁸	<0.001	2	13	3	1	1	0	6	0	1	1
50	11	7	6	8	8	7	6	5	6	2.06 × 10 ⁻³	1.20 × 10 ⁻⁷	<0.001	4	12	0	0	0	1	2	0	8	1
72	11	8	6	11	9	8	10	7	6	1.61 × 10 ⁻⁵	2.34 × 10 ⁻¹⁴	<0.001	1	12	6	0	0	0	6	0	0	0
110	11	8	7	8	9	8	10	13	11	6.10 × 10 ⁻⁵	4.01 × 10 ⁻¹⁴	<0.001	1	12	1	0	2	2	1	0	6	0
22	7	8	6	8	8	7	13	13	11	5.57 × 10 ⁻³	4.81 × 10 ⁻³	0.034	4	11	0	0	3	1	0	0	5	2
37	11	7	6	11	8	8	10	7	11	4.58 × 10 ⁻⁵	2.22 × 10 ⁻¹⁵	<0.001	2	11	0	1	0	0	0	0	7	3
102	11	8	6	9	8	8	13	13	6	2.99 × 10 ⁻³	2.98 × 10 ⁻⁵	0.001	4	11	4	2	0	0	4	1	0	0
26	7	8	6	9	8	8	13	5	11	1.72 × 10 ⁻³	1.37 × 10 ⁻⁶	<0.001	3	10	2	0	0	2	2	0	2	2
43	11	7	6	8	8	8	6	13	11	1.42 × 10 ⁻³	2.57 × 10 ⁻⁷	<0.001	5	10	0	0	1	4	4	0	0	1
60	11	7	7	11	8	8	6	7	11	7.08 × 10 ⁻⁶	0.00	<0.001	1	10	3	1	1	0	2	1	1	1
65	11	7	7	8	9	8	13	26	6	2.20 × 10 ⁻⁵	0.00	<0.001	1	10	1	1	1	0	6	1	0	0
122	10	5	6	9	8	7	7	13	6	8.99 × 10 ⁻⁷	0.00	<0.001	1	10	1	0	0	0	5	4	0	0
52	11	7	6	8	8	7	13	5	6	7.84 × 10 ⁻³	1.59 × 10 ⁻¹	0.417	5	9	1	0	2	1	3	0	1	1
73	11	8	6	8	9	10	13	7	11	1.11 × 10 ⁻⁴	7.78 × 10 ⁻¹⁴	<0.001	2	8	0	0	1	1	1	0	5	0
96	11	8	6	8	8	7	13	13	6	9.95 × 10 ⁻³	5.06 × 10 ⁻¹	0.791	7	8	0	0	5	0	0	0	2	1
103	11	8	6	9	8	8	7	26	6	3.65 × 10 ⁻⁵	0.00	<0.001	2	8	1	2	2	1	1	0	1	0
107	11	8	6	9	8	7	13	5	11	3.65 × 10 ⁻³	8.45 × 10 ⁻³	0.052	4	8	3	2	0	0	1	1	0	1
114	11	8	7	8	8	8	10	13	10	6.73 × 10 ⁻⁶	0.00	<0.001	1	8	0	4	0	1	1	2	0	0

^a Frequency of each MLG fragmented by sampling period–field and overall (total).

^b Simple sequence repeat (SSR) genotype expressed as motif repeat number.

^c Analysis using MLGSIM2.0 (Ivens et al. 2012); the probability of each multilocus genotype (P_{gen}) and the likelihood of sampling each MLG, at least as many times as observed (P_{sex}), in a panmictic population of sample size $N = 774$. Corresponding critical values for testing the significance of the P_{sex} value (P value) was estimated by 1,000 random simulations. Bolded numbers indicated significant at $P > 0.05$.

^d Analysis using RClone (Bailleul et al. 2016) to identify how many clones could be maximally expected among the representatives (n) of each MLG within a sample size of 774. Values represent the largest value of $n - 1$ for which the P value for testing the significance of the P_{sex} value of n was <0.05 .

^e MLG containing at least 1% of the population (≥ 8 isolates).

of Australia in Victoria. The lack of significant population structure between fields, similar allele frequencies for each marker within each field, and low frequency of private alleles suggested that *D. tanacetii* isolates within these populations may have originated from a common genetic pool. It is currently unknown when, or even if, *D. tanacetii* was introduced to Tasmania. Thus, it is unclear whether or not the level of genotypic diversity observed has developed rapidly from a small founder population or multiple introductions, or is the result of evolution over a longer period (Dlugosch and Parker 2008). To further elucidate changes in genetic diversity, sampling of populations may be scheduled just after crop establishment and over successive years.

The hypothesis that a sexual morph of *D. tanacetii* occurs in the field was supported by the high genotypic diversity (including the large number of singleton MLG), high gene diversity, and equal mating-type ratios (Blixt et al. 2008; Meng et al. 2015). Similarly, the lack of population structure suggested that airborne ascospores may be an important source of inoculum and allow spread of individuals (gene flow) among and between fields. For heterothallic fungi with a single MAT gene controlling two idiomorphs, undergoing sexual reproduction requires geographic colocation of individuals, ideally in equal numbers, of each mating type (Billiard et al. 2011; Groenewald et al. 2007). The two mating types of *D. tanacetii* were identified in all fields at both sampling periods, with frequencies of the two mating types at all spatial and temporal population strata consistent with a 1:1 ratio for the clone-corrected datasets. A 1:1 distribution of isolates of each mating type has been observed in a number of fungal species where sexual reproduction

has not been observed but was hypothesized to occur infrequently (Bihon et al. 2014; Groenewald et al. 2008; Linde et al. 2003). Further supporting the hypothesis of a mixed mating system, analysis of P_{sex} and its significance indicated that, within many of the recurrent MLG, there were multiple clonal lineages derived from recombination. However, significant linkage disequilibrium within all populations, with the exception of two of the clone-corrected field populations sampled in August, was identified, indicating that the populations are not in complete panmixia. The significant linkage disequilibrium in the uncorrected populations most likely resulted from the asexual component of the reproduction system. Linkage disequilibrium is expected in populations that do not undergo regular sexual reproduction but may also be attributed to other factors (Brewer et al. 2012; Falush et al. 2003; Hartl and Clark 1989). According to Brown et al. (1980), 30 diploid organisms is defined as the minimum requirement to adequately detect significant linkage. Because *D. tanacetii* is a haploid organism, this would be equivalent to a population of 60 individuals. Therefore, almost all of the clone-corrected field-sampling period populations had an inadequate number of samples to detect significant linkage (Brown et al. 1980) and, as such, the results should be interpreted with caution (Brown et al. 1980). Furthermore, linkage disequilibrium has been observed in a number of pathogen populations with known mixed modes of reproduction, including other *Didymella* spp. (Bogacki et al. 2010; Brewer et al. 2012; Dale et al. 2011; Ozkilinc et al. 2015). It has been suggested that specific factors are required for sexual reproduction in *D. tanacetii* (Pearce et al. 2016b) and, therefore, sexual reproduction

Fig. 4. Phylogenetic relatedness of mating type 1 (MAT1-1) (purple) and mating type 2 (MAT1-2) (orange) isolates from the 2012 *Didymella tanacetii* population. **A**, Unweighted pair group method with arithmetic mean (UPGMA) tree of 25 each of MAT1-1 and MAT1-2 isolates randomly selected from the total population. At the nodes, bootstrap percentages (>50%) from 1,000 replicates are presented. **B**, UPGMA radial tree based on Bruvo's distance of all *D. tanacetii* isolates with mating-type assignment. Scale bars indicate branch lengths.

may only occur infrequently relative to asexual reproduction. Linkage disequilibrium between unlinked loci can only decay by a maximum of 50% with recombination from each sexual generation; therefore, it may take several sexual recombination events for the disequilibrium to be eliminated (Ardlie et al. 2002).

Few studies have examined differences in genotypic indices and population structure between isolates of known mating types. If sexual reproduction was absent within *D. tanacetii* populations, genetic interaction between isolates of each mating type would be limited due to the heterothallism and, thus, genetic drift would differentiate these populations over time (McDonald 1997). The two mating types of *D. tanacetii* have been identified from isolates collected in 2004 (Pearce et al. 2016b), indicating that isolates of each mating type have had at least 8 years to diverge if sexual reproduction was absent. In this study, mating-type ratios consistent with a mixed reproductive mode were identified. Moreover, 80% of the rare alleles and 100% of the nonrare alleles were present within both mating types. Additionally, common MLG were identified that contained isolates of both mating types, which has been interpreted as evidence of sexual recombination (Henk et al. 2012; Short et al. 2014). Similar magnitudes of the genotypic indices for MAT1-1 and MAT1-2 isolates, the lack of aggregated structure of the MSN, and absence of genetic convergence of phylogenetic trees further support the hypothesis of exchange of genetic material through recombination. The conclusion that *D. tanacetii* has the potential to undergo a mixed reproductive system differs from that found for *Stagonosporopsis tanacetii*, which only undergoes only asexual reproduction (Vaghefi et al. 2015a,b). Since its first record (Pethybridge and Wilson 1998), *S. tanacetii* had been the dominant pathogen of pyrethrum crops in Australia (Pethybridge et al. 2003, 2008b). However, in recent years, *D. tanacetii* has displaced *S. tanacetii* during the winter and spring periods (Hay et al. 2015). Thus, the ability to undergo sexual recombination may have provided *D. tanacetii* an opportunity to outcompete *S. tanacetii* in the same ecological niche (Xu 2006).

Because the sexual morph has yet to be detected, the timing of its putative formation is unknown. *D. tanacetii* is known have increased prevalence over the winter and spring period (Hay et al. 2015). A number of closely related species produce ascospore inoculum in winter and spring, including *Ascochyta rabiei* (Chilvers et al. 2007; Rhaïem and Cherif 2014) and *D. pinodes* (Davidson et al. 2013; Schoeny et al. 2007). Thus, it was hypothesized that this was the most likely time period for *D. tanacetii* to reproduce sexually and sampling periods were selected to encompass the period. Overall, genotypic diversity was similar in August and November, and genetic variation between the sampling periods was low. Few new MLG were identified in November, suggesting that clonal reproduction was dominant between the sampling periods. Significant genetic differentiation was found within populations between some of the fields in November according to AMOVA but was not supported by isolation–distance effects. Field 63242 was the only location in which the population significantly differed between the two sampling periods, suggesting a limited impact of genetic drift and purifying selection in the other fields. Allele frequencies and mating-type ratios in field 63242 also shifted significantly over this period. These results were attributed to MLG4, the occurrence of which increased four-fold between August and November, suggesting that this genotype may be linked to favorable traits such as increased virulence, aggressiveness, fecundity, or fungicide resistance (Pariaud et al. 2009; Shrestha et al. 2017). MLG4 was not detected in any of the other fields.

Despite being subjected to the standard commercial fungicide program for the control of foliar pathogens, the number of *D. tanacetii* individuals identified was higher in November than August in all fields except 63242. The increased isolation frequency of *D. tanacetii* in November coincided with decreased genotypic richness but increased genotypic evenness, which may indicate

selection of a specific genotypes. Decreased sensitivity to boscalid within *D. tanacetii* populations has been reported (Hay et al. 2015). The development of fungicide resistance in populations can have differential effects on genetic population structure. Knapova and Gisi (2002) found no association between SSR genotype and sensitivity to phenylamide in *Phytophthora infestans*, while Shrestha et al. (2017) found that quinone outside inhibitor-resistant *Cercospora sojina* isolates clustered in a single group and were represented by a dominant clonal lineage. In the absence of phenotypic data on the sensitivity of each *D. tanacetii* individual within the population to boscalid, the effect on genetic population structure and the effect of the fungicide program and frequency of resistant *D. tanacetii* individuals is difficult to infer. The potential selection pressure applied by fungicides to *D. tanacetii* field populations is the subject of future studies.

The results from this study imply that the rapid expansion in tan spot since 2009 has been aided by a genetically diverse population, which has developed through mutation and infrequent sexual recombination. Windborne dispersal of ascospores has likely facilitated the rapid dispersal of new genotypes over the pyrethrum growing region and provided a potential advantage over competing pathogens such as *S. tanacetii*, which is hypothesized to rely solely on asexual reproduction. Future studies into temporal changes in the genotypic composition of *D. tanacetii* populations over the last decade would be beneficial in identifying the advantageous genotypes that have aided this expansion.

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