Lack of Evidence for Recombination or Spatial Structure in *Phoma ligulicola* var. *inoxydabilis* Populations from Australian Pyrethrum Fields

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Abstract

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Ray blight, caused by *Phoma ligulicola* var. *inoxydabilis*, causes substantial annual losses in Australian pyrethrum fields. Fifty-nine *P. ligulicola* var. *inoxydabilis* isolates were randomly selected from fields in three distinct geographical regions in Tasmania, Australia. Genetic diversity was characterized using random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP). Based on genetic similarities of less than 99%, 56 distinct genotypes (putative clones) were observed. Mean haploid gene diversity of clone-corrected populations ranged between 0.05 and 0.31, and 0.11 and 0.32, for the RAPD and AFLP data sets, respectively. Cluster analysis indicated two distinct groups of isolates supported by all bootstrap replicates. The

first cluster contained all but four isolates with representatives from all three populations. The second cluster contained two isolates from the Western and Central populations, respectively, while the remaining isolates were not able to be grouped with any distinct cluster. Analysis of the population structure suggested no evidence for spatial autocorrelation at the smallest distance classes. The presence of linkage disequilibrium was indicated regardless of population scale. Collectively, these findings provided further evidence for the absence or minor role of the teleomorph in the epidemiology of ray blight in Australian pyrethrum fields.

Ray blight, caused by *Phoma ligulicola* var. *inoxydabilis* Boerema (syn. *Stagonosporopsis ligulicola* var. *inoxydabilis* (Boerema) Aveskamp, Gruyter & Verkley; teleomorph *Didymella ligulicola* (K.F. Baker, Dimock & L.H. Davis) Arx) (2) is one of the most important diseases affecting pyrethrum (*Tanacetum cinerariifolium* Sch. Bip.) production in Australia (20,22,26). Pyrethrum production in Australia accounts for over 50% of the world market for pyrethrins, which are insecticidal compounds that are extracted from the achenes of the flower (13,45). The pyrethrins are used for the production of domestic and agricultural insecticidal products. Pyrethrum is native to the Dalmatian Coast of Europe and has been produced in Australia since the early 1990s (13). Since the industry's inception, no further plant material has been introduced into the Australian breeding program.

Ray blight was first reported in Australian pyrethrum fields in 1995, causing minor losses of flowers (31). However, symptoms of this disease are also visible on the leaves, stems, and newly emerged shoots in spring (21,27). Pethybridge et al. (26) described the symptoms of this disease on pyrethrum in detail. In the absence of fungicides, these symptoms develop into substantial losses caused by stem and plant death and have led to premature termination of fields (22,23,28). Therefore, yield loss is the result of spring epidemics substantially reducing the number of healthy stems per area, plant density, and the number of flowers produced on each stem (29).

P. ligulicola is split into two varieties, based on the ability of the isolates to produce metabolite “E” which oxidizes sodium hydroxide and changes colorless to the β red to reddish-brown pigment on malt extract agar (5,6,9,30,41). *P. ligulicola* var. *inoxydabilis* is unable to produce metabolite E, whereas the opposite is the case

for *P. ligulicola* var. *ligulicola* (5,41). Mycelial growth rates and the in vitro production of perithecia and pycnidia have also been used to differentiate the two varieties (9). To date, only *P. ligulicola* var. *inoxydabilis* has been associated with ray blight of pyrethrum in Australia, whereas *P. ligulicola* var. *ligulicola* has been reported causing ray blight of chrysanthemum (41). Substantial differences in host range between the two varieties have also been reported. For example, glasshouse studies with *P. ligulicola* var. *ligulicola* have reported the ability to infect lettuce (*Lactuca sativa*) and a range of Asteraceae species (garden chrysanthemum [*Den-drathema* $\times$ *grandiflorum*], endive [*Cichorium endivia*], and sunflower [*Helianthus annuus*]) (8). Moreover, this variety has also been reported to infect an unnamed species of pyrethrum. By contrast, Australian *P. ligulicola* var. *inoxydabilis* isolates from pyrethrum fields were only able to infect garden chrysanthemum and French marigold (*Tagetes patula*) (25). However, *P. ligulicola* var. *inoxydabilis* has also been reported from the related species, *T. parthenium*, and zinnia (*Zinnia elegans*) (41).

Studies on the population structure of pathogens are essential for understanding evolutionary mechanisms and estimating the relative risk of a pathogen overcoming control strategies, such as fungicide application and host resistance (18). Little is known about the population genetics of *P. ligulicola*, especially within the highly intensive Australian pyrethrum production system. Ascospores or perithecia have yet to be observed in field epidemics of ray blight (26). Additionally, Pethybridge et al. (21) found evidence for only localized spread of inoculum, presumably as rain-splashed conidia, based on spatiotemporal modeling. However, these observations are indirect measures of the mode of reproduction to determine the relative importance of sexual and asexual reproduction on pathogen evolution. Studies, to date, of the genetic diversity of *P. ligulicola* have been limited. Pethybridge et al. (30) observed minimal diversity within DNA sequences from the internal transcribed spacer (ITS) region of a small collection of Australian isolates, suggesting the presence of one species. However, DNA sequence data, especially from the ITS region, typically only provide good differentiation at the species scale and do not provide sufficient resolution to make inferences on population structure. Hence, more polymorphic markers, such as amplified fragment length polymor-

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phisms (AFLPs), are required (17,44). This study aimed to characterize the degree of genetic diversity and its structure within *P. ligulicola* populations in Australian pyrethrum fields. A further objective was to determine whether any evidence for a role of ascospores in the pathogen's evolution could be obtained based on geographically defined population structures.

Materials and Methods

Isolate collection. During 2002 and 2003, diseased shoots were collected in early spring from individual pyrethrum fields that were arbitrarily selected from the major production regions in Tasmania, Australia. These were defined as the geospatial reference centroid for each region, including Eastern (41°11'19"S, 146°29'26"E), Central (41°11'19"S, 146°29'26"E), and Western (40°56'43"S, 145°40'44"E). All sampled fields were within a 10-km radius of this point. Each field was grown using standard production practices which included irrigation, fertilizer, fungicide, and herbicide applications. Fields contained either one of the two dominant cultivar types. Diseased shoots were randomly collected from areas within each field that did not receive the spring fungicide program (10 m long by 24 m wide) for the management of this disease (20).

Isolations from diseased samples were conducted in accordance with the protocol described by Pethybridge et al. (27). Briefly, small pieces (<2 mm³) were excised from the junctions of healthy and necrotic shoot tissue and surface sterilized by immersion in 1% sodium hypochlorite for 2 min, followed by three rinses in sterile distilled water. Tissue pieces were then placed onto 2% water agar (WA) and incubated at 20°C in the dark for 4 days. Mycelia were then transferred to potato dextrose agar (PDA) plates (Difco Laboratories, Detroit, MI). Isolations from pyrethrum seed were undertaken by surface sterilizing whole seed prior to placement on WA (24). After 21 days of growth on PDA, mycelia were hyphal tipped and subcultured onto PDA, malt extract agar (Acumedia Manufacturers Inc., Baltimore, MD), and oatmeal agar (Difco Laboratories) for identification (5,33,41). For long-term preservation, small colonized blocks (10 mm²) of PDA were placed in sterile distilled water and stored at 4°C.

These isolations resulted in 59 *P. ligulicola* var. *inoxydabilis* isolates, from which 20, 31, and 8 were from the Eastern, Central, and Western regional populations, respectively. These regions were separated by approximately 30 km. The *P. ligulicola* var. *inoxydabilis* type-isolate from pyrethrum was also sourced from the New South Wales Agriculture Herbarium, Australia (DAR 70020). The identity of each isolate was confirmed by polymerase chain reaction (PCR) using specific primers designed from the rDNA ITS region (30). All isolates were typed to variety using the sodium hydroxide test for metabolite E (30,33,41).

DNA extraction. For DNA extraction, isolates were recovered from storage and grown on malt extract agar for 10 to 14 days at 20°C in the dark. Mycelial plugs were then taken from the growing margins of cultures and used to inoculate 2% malt extract broth (Oxoid Australia Pty. Ltd., Thebarton, South Australia, Australia). Broth cultures were incubated for 7 days at room temperature on a flat-bed orbital shaker (Ratek Instruments Pty. Ltd., Boronia, Victoria, Australia), set at 50 revolutions/min. Mycelia were removed from the broth cultures and dried on sterile paper towel. DNA was extracted from 100 mg of dried mycelia using QIAGEN DNeasy Plant Mini Kits, following the instructions of the manufacturer (Qiagen, Inc., Valencia, CA).

Genetic analysis. Random amplified polymorphic DNA. Random amplified polymorphic DNA (RAPD) was conducted in accordance with the protocol of Williams et al. (43). RAPD analyses were undertaken using Amersham's Ready-To-Go RAPD Analysis Kit (Amersham Biosciences, now GE Healthcare Biosciences Pty. Ltd., Rydalmere, New South Wales, Australia) with six different primers (Table 1). RAPD reactions were carried out in a total volume of 25 µl consisting of one Ready-To-Go RAPD Bead, 5 µl of primer solution, and target DNA and made to volume with sterile distilled water. RAPD fragments were generated by annealing at 95°C for 5 min, followed by 45 cycles of 95°C for 1

min, 36°C for 1 min, and 72°C for 2 min. Prior to analysis, the quantity of target DNA for each primer was optimized by conducting initial amplifications using serially diluted *Escherichia coli* DNA supplied with the kit (Table 1). Following amplification, fragments were separated by gel electrophoresis in 1× Tris-acetate-EDTA agarose gels stained with ethidium bromide for 15 min. All reactions were conducted in duplicate to identify reproducible bands. Fragments were visualized by UV illumination and sized by comparison with a 100-bp ladder, and those between 100 and 1,000 bp were scored as present = 1 or absent = 0, presumed to be of equal length and identical. For each primer, nonreproducible fragments between the repeated tests were disregarded.

AFLP. AFLP (42) analysis was conducted using the protocol of Radisek et al. (34). Selective amplification was conducted using six primer pairs, consisting of the primers *MspI*+AT, *MspI*+TA, *MspI*+GC, *MspI*+GT, *MspI*+AG, and *MspI*+TC individually paired with *EcoRI*+AC, which was labeled with the WELLRED fluorescent dye, D4 (Sigma-Aldrich, St. Louis). AFLP fragments were capillary-separated and their size determined using a CEQ 8000 Genetic Analysis System with a GenomeLab 600-bp internal standard (Beckman Coulter Inc., Brea, CA). Each reaction was conducted twice. The reproducible amplification fragments between 50 and 600 bp in size were scored as 1 (present) or 0 (absent) for each primer pair.

Loci within both the AFLP and RAPD data sets that contained alleles with frequencies less than 5% were excluded from further analysis. Jaccard's similarity between isolates within each data set was then calculated using FAMD v1.23b (36). Isolates with greater than 99% homology in both the RAPD and AFLP data sets were considered putative clones. Data sets were clone corrected to remove multiple copies of putative clones, with a single putative clone remaining for each regional population.

Data analysis. Haploid gene diversity was calculated from both the RAPD and AFLP data sets, for each population and all isolates combined in GENALEX v.6.2 (19). Estimates were made on data sets both pre- and post-clone correction (h and h_{adj} , respectively).

For a combined data set including clone-corrected data, Jaccard's similarity was calculated using FAMD. These similarities were then used to construct distance matrices for the AFLP and RAPD data. Correlation between AFLP and RAPD distances was tested using a Mantel test (37) in GENALEX, with significance tested using 9,999 random permutations. Clustering of isolates was assessed by the unweighted pair-group method using arithmetic averages (UPGMA) in FAMD. Clustering support was assessed by 1,000 bootstrap replicates. The dendrogram was visualized using TreeGraph v2 (39).

Analysis of molecular variance. Total genetic variance within each data set was partitioned into covariant components, accounting for differences within and between the regional populations using analysis of molecular variance (AMOVA). The haploid analogue, Φ_{PT} , of Wright's fixative index, F_{ST} , was calculated to measure allelic differences between regional populations. All AMOVA-related calculations were conducted in GENALEX (19).

Population structure. Evidence of clustering of genotypes within the populations was assessed using STRUCTURE v.2.3.2 (11,12,32). STRUCTURE uses a Bayesian approach to assign individuals to K clusters based on allele frequencies at each locus. All

Table 1. Primer sequences used for the random amplified polymorphic DNA (RAPD) analysis to quantify population structure of *Phoma ligulicola* var. *inoxydabilis* isolates within Australian pyrethrum fields

Primers	Primer sequences	Target DNA (ng) ^a
1	5'-GGTGC GGGA	10
2	5'-GTTTCGCTCC	10
3	5'-GTAGACCCGT	40
4	5'-AAGAGCCCGT	40
5	5'-AACGCAAC	5
6	5'-CCCCTCAGCA	5

^a Quantity of DNA used in optimized RAPD amplification reactions.

analyses conducted within STRUCTURE used models assuming correlated allele frequencies and admixture. For each data set, 100,000 Monte Carlo-Markov Chain replicates, following 100,000 burn-in replicates, were undertaken for each value of K from 1 to 7, with each analysis undertaken in triplicate. The optimal value of K was selected using the mean estimated maximum value of the natural log of the probability of data ($\ln(P|D)$), and ΔK (10), which uses modal values to estimate the optimal value of K . Estimated ancestry coefficients (Q) for each individual within populations at the optimal value of K were visualized using DISTRUCT v.1.1 (35).

Spatial autocorrelation. The spatial structure of genetic variation within isolates was assessed by spatial autocorrelation analysis (38) in GENALEX. Genetic distance matrices were constructed in FAMD using Jaccard's index for all isolates for each of the AFLP and RAPD data sets, with a complementary geographic distance matrix. These matrices were used to calculate Smouse and Peakall's r (38), which is closely related to Moran's I , over different distance classes. Distance class sizes were varied to maintain even sample sizes within each class. Autocorrelation was considered significant if r was greater than the upper 95% confidence interval calculated from 9,999 random permutations, and the lower 95% confidence error of r , from 9,999 bootstrap replicates, was greater than 0.

Linkage disequilibrium. All clone-corrected data sets were tested for the presence of linkage disequilibrium by calculating the index of association (I_A) (14) and Agapow and Burt's (1) statistic ($\bar{r}_D$), and using the methodology of Burt et al. (7). Both I_A and $\bar{r}_D$ were estimated using MULTILOCUS v1.3 (1), with the significance of index values greater than 0 tested by comparison with the distribution of values obtained from 1,000 randomly permuted data sets. Under the method of Burt et al. (7), the length of the most parsimonious tree generated by maximum parsimony analysis in PAUP* v.4.0b10 (40) was compared with the distribution of lengths from 1,000 data sets randomly permuted in MULTILOCUS. An observed tree length significantly smaller than the generated distribution provided evidence for linkage disequilibrium. The location of missing data points was fixed in the generation of random permutations.

Results

All isolates were confirmed as *P. ligulicola* var. *inoxydabilis* by morphological characteristics and species-specific PCR. In total, 810 marker bands were scored in the AFLP dataset. Of these loci, 78.0% had allele frequencies of less than 5% and were subsequently discarded (Table 2). Seventy markers were scored in the RAPD data set of which 71.4% were discarded for having allele

frequencies below 5%. Overall, 22 RAPD and 176 AFLP loci remained for further analysis. Within the Eastern, Central, and Western regional populations, the percentage of polymorphic loci was 23.5, 20.5, and 46.2% (AFLP data set) and 17.1, 15.7, and 68.6% (RAPD data set), respectively. Over the entire population, 56 putative clones were identified. Following clone-correction, 17, 31, and 8 isolates remained in the Eastern, Central, and Western regional populations, respectively. Representative isolates were lodged with the Department of Employment, Economic Development and Innovation, Plant Pathology herbarium, Queensland, Australia (isolates 2C, 3W, 5E, and 5W = BRIP 54632 to 54635, respectively).

Diversity. Mantel comparison of Jaccard distance matrices from the AFLP and RAPD datasets indicated a significant correlation ($R_{xy} = 0.921$, $P < 0.0001$). Based on this result, the AFLP and RAPD data sets were combined and a new Jaccard distance matrix constructed for UPGMA analysis (Fig. 1). Cluster analysis indicated two distinct clusters of isolates sharing at least 75% Jaccard similarity, which was supported by all bootstrap replicates. Cluster 1 contained 52 isolates, with representatives from all three regional populations included. Cluster 2 consisted of two isolates, 5W and 30C, from the Western and Central populations, respectively. Moreover, isolates 7W and 8W (DAR 70020) were not grouped with any distinct cluster.

Mean haploid gene diversity of clone-corrected (h_{adj}) data was 0.11, 0.18, and 0.32 in AFLP data for the Eastern, Central, and Western regional populations, respectively (Table 2). Across all isolates, h_{adj} was 0.19, which did not vary from the gene diversity (h) of the uncorrected data set (Table 2). In the RAPD data set, h and h_{adj} were slightly reduced at 0.12 and 0.13, respectively. Gene diversity of the clone-corrected RAPD data set was 0.05, 0.10, and 0.31 in the Eastern, Central, and Western regional populations, respectively.

Population structure. Analysis of allele frequencies indicated the presence of two groups ($k = 2$; Fig. 2) in each of the AFLP and RAPD data sets. In the RAPD data set, the mean natural log of the probability distribution ($\ln(P|D) = -160.5$), and Δk (70.14) were greatest for $k = 2$. For the AFLP data set, $\ln(P|D)$ were slightly higher at $k = 4$ ($-1,755.5$) than $k = 2$ ($-1,974.3$); however, Δk was higher at $k = 2$ (492.2) than $k = 4$ (40.5).

Assuming $k = 2$ for both data sets, 4 isolates were consistently assigned to group 2, with greater than 70% of their genetic identity coming from that cluster (Fig. 2), while 51 isolates had 75% or greater of their genetic identity from group 1. The remaining isolate (52303) had at least 70% of genetic identity from group 1 in the RAPD data set but only 64% according to the AFLP data set (data

Table 2. Summary of genetic diversity statistics describing Australian *Phoma ligulicola* var. *inoxydabilis* populations based on amplified fragment length polymorphism (AFLP) and random amplified polymorphic DNA (RAPD) markers

Population	N^a	MLG ^b	Polymorphic loci (%)	h (SD) ^c	h_{adj} (SD) ^d	I_A (P) ^e	$\bar{r}_D$ (P) ^f	L (P) ^g
AFLP data set								
Eastern	20	17	23.6	0.104 (0.046)	0.111 (0.044)	8.96 (< 0.001)	0.139 (< 0.001)	95 (< 0.001)
Central	31	31	20.5	0.183 (0.047)	0.183 (0.047)	43.07 (< 0.001)	0.262 (< 0.001)	388 (< 0.001)
Western	8	8	46.2	0.324 (0.024)	0.324 (0.024)	60.16 (< 0.001)	0.359 (< 0.001)	204 (< 0.001)
Overall	59	56	22.0	0.186 (0.061)	0.191 (0.058)	55.79 (< 0.001)	0.328 (< 0.001)	506 (< 0.001)
RAPD data set								
Eastern	20	17	17.1	0.053 (0.079)	0.053 (0.069)	0.83 (0.021)	0.169 (0.021)	6 (0.437)
Central	31	31	15.7	0.100 (0.060)	0.100 (0.062)	9.82 (< 0.001)	0.512 (< 0.001)	23 (0.407)
Western	8	8	68.6	0.313 (0.064)	0.313 (0.064)	6.11 (< 0.001)	0.307 (< 0.001)	28 (< 0.001)
Overall	59	56	28.6	0.122 (0.075)	0.128 (0.076)	0.83 (0.021)	0.169 (0.021)	36 (< 0.001)

^a Total number of individuals.

^b Number of multilocus genotype (MLG) individuals, sharing less than 99% genetic homology.

^c Mean haploid diversity of uncorrected data sets. Values of h vary from 0 (no diversity) to 1 (infinite diversity). Values in parentheses represent the standard deviation of the test statistic.

^d Mean haploid diversity of clone-corrected data sets. Values of h_{adj} vary from 0 (no diversity) to 1 (infinite diversity). Values in parentheses represent the standard deviation of the test statistic.

^e Index of association. Values in parentheses represent probability of test statistic based on 1,000 random permutations.

^f Agapow and Burt's index of multilocus linkage disequilibrium (1). Values in parentheses represent probability of test statistic based on 1,000 random permutations.

^g Burt's measure of linkage disequilibrium, based on the length of the most parsimonious tree. Values in parentheses represent the probability of the test statistic based on 1,000 random permutations.

not shown). Of the four isolates within group 2, three (5W, 7W, and 8W (DAR 70020)) were from the Western population, while 30C was from the Central population.

Spatial autocorrelation. Spatial autocorrelation was not significant in the distance classes 0 to 4, 4 to 5, or 5 to 8 km based on genetic distances calculated from either RAPD or AFLP data (Fig. 3). From the AFLP data set, significant spatial autocorrelation was observed in the 8-to-15-km distance class but this was not supported by the RAPD data set. For the RAPD data set, the 15-to-20-km distance class was significantly autocorrelated but this was not the case for AFLP distances. No significant autocorrelation was observed for distance classes greater than 20 km or less than 8 km for either data set (Fig. 3).

Fig. 2. Relative genetic assignment of *Phoma ligulicola* isolates from three regional populations to two groups ($k = 2$). Assignments based on STRUCTURE analysis, using 100,000 Monte-Carlo Markov chain iterations, with an initial burn-in on 100,000 iterations. Analyses based on clone-corrected data from **A**, amplified fragment length polymorphism and **B**, random amplified polymorphic DNA markers.

Fig. 1. Dendrogram showing Jaccard similarities of *Phoma ligulicola* var. *inoxydabilis* isolates, based on combined amplified fragment length polymorphism and random amplified polymorphic DNA data. Dendrogram created using unweighted pair-group method using arithmetic averages clustering. Clusters with 100% bootstrap support indicated by braces. Values in nodes indicate bootstrap support (%) for clades based on 1,000 replicates. Only bootstrap values greater than 70% are shown. Scale bar indicates Jaccard genetic similarity.

AMOVA. Within the AFLP data set, 97.4% of observed genotypic variance in isolates was attributed to differences within regions (Table 3). Results from analyses of the RAPD data set suggested that 95.3% of variation originated from within the regional populations. The haploid analogue of Wright's Fixation Index (Φ_{PT}) was not significant for either data set (Table 3).

Pairwise comparison of Φ_{PT} between regional populations produced values of 0 (Eastern and Central), 0.031 (Central and Western), and 0.106 (Eastern and Western) for the AFLP data set. In the RAPD data set, values were 0 (Eastern and Central), 0.064 (Central and Western), and 0.156 (Eastern and Western). Pairwise Φ_{PT} values were not significant ($P > 0.05$) for any pair of regional populations in either data set.

Linkage disequilibrium. Both I_A and $\bar{r}_D$ indicated the presence of linkage disequilibrium in all regional populations and across isolates, using both AFLP and RAPD data ($P < 0.05$; Table 2). Using Burt's measure, significant linkage disequilibrium was observed using AFLP data for all isolates and individual regional populations. However, using RAPD data, linkage disequilibrium was only significant across all isolates and within the Western regional population ($P < 0.001$; Table 2).

Discussion

To our knowledge, this is the first quantitative description of the genetic variation in *P. ligulicola* var. *inoxydabilis*. Genotypic variation in this study was high, with 56 putative clones identified from

59 isolates. Mean gene diversity was significantly higher in the Western regional population relative to the Eastern and Central populations and all isolates when included as one population, regardless of location. However, no evidence for spatial structure of this variation was detected in this study. Partitioning of genetic variation, indicated between 95 and 97% of the observed genetic variation, was present within regional populations, and pairwise comparisons of Φ_{PT} found no significant differences between regions. Additionally, spatial autocorrelation did not provide any evidence indicating spatial differentiation, with no autocorrelation observed at the smallest distance classes. Spatial autocorrelation was detected between fields 8 to 15 km apart (AFLP) and 15 to 20 km apart (RAPD); however, in the absence of autocorrelation at smaller distances, this is difficult to explain biologically. Allele frequency-based analysis, using Structure, differentiated four isolates (8W [DAR 70020], 5W, 30C, and 7W) from the remainder. UPGMA clustering, based on Jaccard genetic distances, produced a similar result, with the isolates 8W, 5W, 30C, and 7W differentiated from the majority. Three of these isolates were collected from the Western regional population. However, the bias of isolate numbers of group 1 ($n = 52$) to group 2 ($n = 4$) isolates prevents confident inferences of spatial structure from this data. Therefore, overall, the three regional populations could not be differentiated and, thus, are assumed to be part of a single *P. ligulicola* var. *inoxydabilis* population. However, the influence of localized spread of individuals within fields cannot be ascertained with the current sampling strategy, and finer-scale studies should be considered for the future.

Linkage disequilibrium analysis provided no evidence for the presence of the teleomorph, *D. ligulicola*, within Australian pyrethrum fields, which supports previous observations (21,26). Linkage disequilibrium was supported regardless of population regional scale in both the AFLP and RAPD data sets by I_A and $\bar{r}_D$. Similarly, linkage equilibrium was rejected for the AFLP data within all populations by Burt's method based on the length of most parsimonious trees, and two of four populations using RAPD data. This supports findings from spatiotemporal analyses suggesting that short-range spread of inoculum within fields, most likely due to rain-splashed conidia, predominates and discounting the presence of the teleomorph (21,23,26). Finer-scale, hierarchical sampling of isolates from within individual fields would help to further clarify this issue. Interestingly, in chrysanthemum ray blight, perithecia of *D. ligulicola* var. *ligulicola* are frequently observed within diseased tissue and are considered important in long-distance dispersal of inoculum and the flower blight phase of the disease (3,4,15,16).

Thus, although the occurrence of recombination cannot be discounted conclusively, current evidence suggests that it is an infrequent event. Currently, the mating type status of *P. ligulicola* is largely unknown and may differ between varieties. Pethybridge et al. (26) hypothesized that, if *P. ligulicola* var. *inoxydabilis* is heterothallic, only one mating type may be dominant within Australian populations. Conversely, if *P. ligulicola* var. *inoxydabilis* is homothallic, this suggests either unfavorable environmental conditions for perithecial formation or functional inability within the population. The environmental conditions required for perithecial formation for *P. ligulicola* var. *ligulicola* have been characterized

Fig. 3. Spatial correlograms of genetic similarity of *Phoma ligulicola* var. *inoxydabilis* isolates from Australian pyrethrum fields. Analysis conducted on clone-corrected data from **A**, amplified fragment length polymorphism and **B**, random amplified polymorphic DNA markers. Dashed lines indicate 95% confidence intervals from 9,999 random permutations. Error bars indicate 95% confidence intervals from 9,999 bootstrap samples.

Table 3. Summary of analysis of molecular variance of regional populations of *Phoma ligulicola* var. *inoxydabilis* based on amplified fragment length polymorphism (AFLP) and random amplified polymorphic DNA (RAPD) markers

Scale	df ^a	Variance	Var. (%)	Φ_{PT} ^b	Φ_{PT} <i>P</i> ^c
RAPD data set					
Between regions	2	0.078	4.7	...	...
Within regions	53	1.569	95.3	0.047	0.101
AFLP data set					
Between regions	2	0.454	2.6	...	...
Within regions	53	16.854	97.4	0.026	0.160

^a Degrees of freedom.

^b Haploid analogue of Wright's Fixative index, F_{ST} .

^c Probability of the fixative index being significantly greater than 0.

(16), and would be frequently observed in all three pyrethrum regional production districts in Tasmania (27). This suggests that, among other attributes such as host range and aggressiveness, the two varieties of *P. ligulicola* may differ in their mating system. Therefore, whereas current data suggests that ascospores play an infrequent role in population evolution, the mechanism that limits ascospore production by *P. ligulicola* var. *inoxoidabilis* isolates from Australian pyrethrum fields is unknown. Hence, the development of suitable markers to screen populations for mating type idiomorphs is a priority.

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