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Spatial aggregation in *Fusarium pseudograminearum* populations from the Australian grain belt

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Previous studies have evaluated the overall structure of populations of *Fusarium pseudograminearum* (teleomorph, *Gibberella coronicola*), causal agent of cereal crown rot, but there is no information available on spatial relationships of genetic variation in field populations. Three 1-m-row sections in crown-rot-affected wheat fields in the Australian grain belt were intensively sampled to estimate population genetic parameters and the spatial aggregation, or clustering, of disease aggregates and genotypes. Estimates of population genetic parameters based on amplified fragment length polymorphisms (AFLPs) indicated that the genetic diversity in isolates from the 1-m-row populations described a significant portion of the diversity recorded for corresponding field and regional populations. In point pattern analysis, there was physical clustering and aggregation of *F. pseudograminearum* isolates from two of the three sites. Analysis of the spatial distribution of clonal haplotypes (DICE similarity ≥ 97%) indicated significant aggregation of clones in all three 1-m-row populations. Based on matrix comparison tests, both mating types and genetic distances had significant spatial aggregation for at least two of the three 1-m-row populations. This is consistent with the presence of non-random spatial genetic structure due to clonal aggregation. High levels of genetic diversity and spatial structuring of disease and genotypes in at least two of the three 1-m-row populations is consistent with the hypothesis that stubble is a primary inoculum source in no-tillage farming systems, resulting in aggregated patterns of disease and allowing for haplotypes to be maintained in the field over a number of annual cropping cycles.

Keywords: crown rot of cereals, *Gibberella coronicola*, matrix comparison tests, spatial autocorrelation, *Triticum aestivum*

Introduction

Disease is rarely randomly distributed in a population (Hungerford, 1991) and most pathogen populations have a significant spatial structure (Epperson *et al*., 1999). Integrating spatial and epidemiological with genetical studies in plant pathosystems can extend traditional analysis boundaries (Shah *et al*., 2001; Milgroom & Peever, 2003) and actively contribute to disease tracking, management and control (Milgroom *et al*., 1991; Chung *et al*., 1999; Cortesi *et al*., 2004). Uniform, broad-acre cropping systems are ideal for studying spatial dynamics (Real & McElhany, 1996) as the heterogeneity of the pathogen population, in contrast to the homogeneity of the cropping system, can elucidate various aspects of disease epidemiology (Perry, 1995), including local transmission (Pethybridge *et al*., 2004; Schmale *et al*., 2005), sources and patterns of inoculum (Milgroom & Peever, 2003; Cortesi *et al*., 2004; Wilhelm & Jones, 2005) and inferences of reproductive strategies (Chung & Epperson, 2000). Spatial structure, however, is often overlooked in relatively coarse field samplings of plant pathogen populations (Milgroom & Lipari, 1995). Reports of both physical and genetic spatial structure in plant pathosystems typically document distribution of the airborne dispersal spores of primarily outcrossing species on above ground plant parts (Milgroom *et al*., 1991; Milgroom & Lipari, 1995; Linde *et al*., 2002; Zeller *et al*., 2003; Cortesi *et al*., 2004; Schmale *et al*., 2005).

Pathogens in perennial cropping systems, such as *Cryphonectria parasitica*, have aggregated patterns of disease that are stable over time (Milgroom *et al*., 1991).

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Cortesi *et al.* (2000) described grapevine symptoms caused by *Fomitiporia punctata* that were not consistent with clonal spread, and were thought to be the result of colonization by airborne basidiospores. This pattern contrasts with that of known pathogens of annual crops, such as *Stagnospora nodorum* (teleomorph, *Phaeosphaeria nodorum*), whose spatial dynamic patterns are a function of ephemeral pathogen cycles (Shah *et al.*, 2001), and are influenced primarily by inoculum source and distribution. Previous studies of the fusarium head blight pathogen *Fusarium graminearum* (teleomorph *Gibberella zeae*) have implicated sexual ascospores as the primary dispersive spore. Distribution of these spores may result in aggregation and gradients of infection from a concentrated inoculum source (Schmale *et al.*, 2005). In contrast, Munkvold *et al.* (1993) reported random spatial patterns in eutypa dieback of grapevines and attributed them to primary infection from an external ascospore source.

Fusarium pseudograminearum (teleomorph *Gibberella coronicola*) is the primary causal agent of crown rot of wheat and barley, a major limitation to winter cereal production throughout the Australian grain belt. Studies on this pathogen have focused primarily on documenting population structure and formulating control strategies (using both agronomic and resistance breeding methods), with little information available on its epidemiology or spatial dynamics. *Fusarium pseudograminearum* populations collected throughout the Australian grain belt have high levels of genetic diversity (Akinsanmi *et al.*, 2006; Bentley *et al.*, 2008) and genetic differentiation has been shown between regional populations both in Australia (Bentley *et al.*, 2008) and New Zealand (Monds *et al.*, 2005; Bentley *et al.*, 2006). Primary inoculum of *F. pseudograminearum* can come from hyphae surviving in crop residues in no-tillage systems (Summerell *et al.*, 1990), intermediate grass hosts growing in the fallow field or adjacent to cropped areas (Burgess *et al.*, 2001), planting of contaminated seed, and/or movement of immigrant ascospores (Akinsanmi *et al.*, 2006). The distribution of primary inoculum is a fundamental determinant of the site of fungal penetration (Summerell *et al.*, 1990).

In this study two null hypotheses were tested. First, that small sampling transects adequately capture the genetic variation in field and regional populations of *F. pseudograminearum*, and secondly, that there is neither physical nor genetic aggregation in field populations of this fungus from the Australian grain belt. Specifically, the study aimed to determine the proportion of the total genetic diversity in a field that is present in isolates from intensively sampled 1-m-row section transects. It also determined whether the frequency of successful isolation of *F. pseudograminearum* was spatially dependent on genetic characters such as clonal AFLP haplotypes and mating types, and whether genetic distance could be determined based on point pattern and spatial autocorrelation analyses. Determining spatial and genetic relationships in *F. pseudograminearum* populations allows the (re)consideration of epidemiological questions, especially those related to primary inoculum source and local dispersal, and provides guidance for the formulation of field-based sampling and control strategies.

Materials and methods

Sample collection

Samples of physiologically mature spring bread wheat were collected in November, 2004 from three commercial fields with naturally occurring crown rot. At all three sites, no-tillage rotational cropping systems were employed. Two sites, Boonaldoon (29°28′S/149°26′E) and Moree (29°30′S/149°47′E), were located in northeastern Australia, with the third site, Cambrai (34°38′S/139°18′E) located in south central Australia. The wheat cultivar at Boonaldoon was EGA Wedgetail, at Moree it was Ellison and at Cambrai it was unknown. The cropping histories at Boonaldoon and Moree were predominantly wheat production, with rotation to chickpeas and sorghum, whereas wheat-pasture rotations dominated the cropping at Cambrai. Selected sites had visually estimated incidences of plants infected with *F. pseudograminearum* (as determined by basal stem browning and whitehead formation) of 100% at Boonaldoon, 60% at Moree and 40% at Cambrai.

Within each field, a single row at least 20 m from the edge of the field was arbitrarily selected for sampling. From the row a single section, 1 m in length, was arbitrarily selected for sampling. All plants within the selected section were labelled and collected along the row. Varying numbers of stems were collected from each 1-m-row section depending on seeding rates and varietal differences in tillering: 229 at Boonaldoon, 129 at Moree and 44 at Cambrai. Field populations from outside the selected rows also were collected at two of the three sites – Boonaldoon (70 stems) and Cambrai (100 stems) in the same growing season (November, 2004). These plants were collected in a zigzag pattern beginning 20 m from the edge of the field. Each horizontal line of the zigzag was 5 m in length and five plants were collected at the junction of each horizontal line. All plants were placed in paper bags for return to the laboratory where they were air-dried at room temperature prior to fungal isolations.

The outer leaves and leaf sheaths of each stem were removed and the stems were washed with tap water before surface sterilization with 70% ethanol for 3 min. Nodal stem sections, ~1–2 cm in length, beginning at the crown and then progressing up the plant to each node and concluding at the fifth node, where present, were removed aseptically from each stem and plated on half strength potato dextrose agar (PDA) supplemented with 13 mg L^{-1} dichloronitroaniline, 160 mg L^{-1} streptomycin sulphate and 60 mg L^{-1} neomycin sulphate [modified PDA (MPDA); (Leslie & Summerell, 2006)]. Plant samples from the selected rows and from elsewhere in the field were treated in a similar manner. Plates were incubated at 25°C with alternating light and dark for five days, and red coloured colonies developing on MPDA were subcultured to carnation leaf agar [CLA; (Fisher *et al.*, 1982)] and grown for

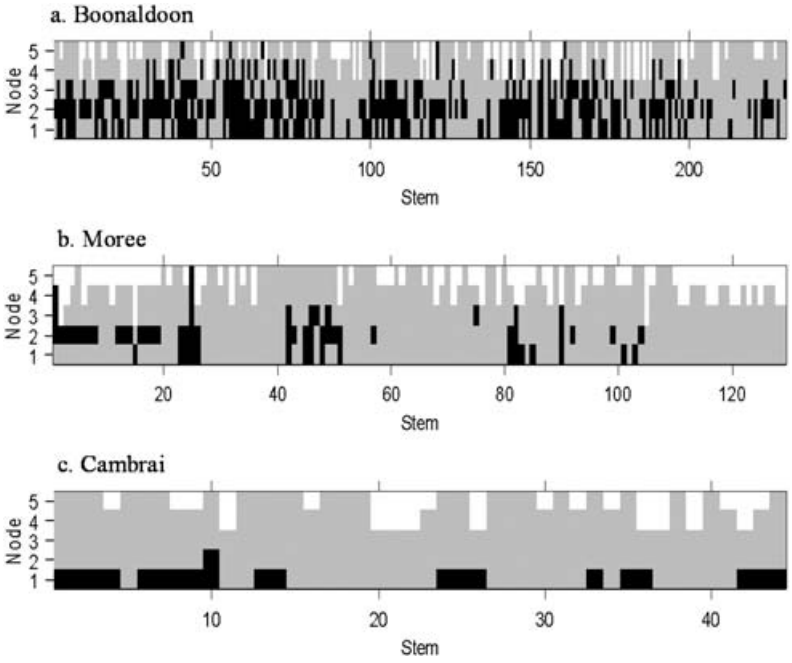

Figure 1 Recovery of *Fusarium pseudograminearum* from each of five possible nodal sections from wheat stems along the 1-m-row population at Boonaldoon (a), Moree (b) and Cambrai (c). Black cells represent the positive recovery of *F. pseudograminearum* from a stem section. Grey cells represent no recovery, and white cells indicate absence of a nodal section from isolation procedures.

five days. Isolates were then transferred to CLA as single germinated conidia (Leslie & Summerell, 2006) and grown for seven to ten days before being identified to species level based on morphological characters (Leslie & Summerell, 2006). For the 1-m-row populations, an individual stem was scored as positive for crown rot if a morphologically identified *F. pseudograminearum* isolate was cultured from any one of the nodes.

Four hundred and fifty-five morphologically identified isolates of *F. pseudograminearum* from 402 stems from the three 1-m-row populations were accessioned in the Fusarium Research Laboratory collection at The University of Sydney and stored in 15% glycerol (v/v) at –70°C [Site, Region (FRL accession number/s)]: Boonaldoon, northeastern Australia (F18066 to F18440); Moree, northeastern Australia (F18806 to F18868); Cambrai, south central Australia (F18754 to F18773). Of the stems collected outside the 1-m-row populations, 59 morphologically identified isolates of *F. pseudograminearum* from 70 stems at Boonaldoon and 60 isolates from 100 stems at Cambrai were accessioned in the Fusarium Research Laboratory collection at The University of Sydney and stored in 15% glycerol (v/v) at –70°C [Site, Region (FRL accession number/s)]: Boonaldoon, northeastern Australia (F16962 to F16995, F16997 to F17003, F17005 to F17010, F18536 to F18547); Cambrai, south central Australia (F17058 to F17109, F17111 to F17118).

Molecular characterization of *F. pseudograminearum* isolates

Isolates for AFLP analysis were selected from each of the 1-m-row populations to represent the breadth and frequency of *F. pseudograminearum* isolation as determined by the mapped point pattern (Fig. 1). To ensure coverage of the row, all of the isolates selected from Boonaldoon and Moree were isolated from the first node above the crown, whereas all isolates from Cambrai were isolated from the crown region. This difference was due to varying isolation frequencies from different nodes at each site (Fig. 1), which may have resulted from the effects of management practices on the primary zone of infection (Summerell *et al*., 1989). Differing numbers of isolates were used in the AFLP analysis from each row section due to the disparity in isolation frequencies: 60 – Boonaldoon, 36 – Moree and 18 – Cambrai. No more than one isolate from each plated stem was used in the AFLP analyses. From the field populations at Boonaldoon and Cambrai, 29 and 28 isolates, respectively, were randomly selected for use in the AFLP analysis. All isolates were grown on PDA at 25°C in the dark for 5 days. Mycelium was harvested and aseptically transferred to sterile 1·5 mL Eppendorf tubes for DNA extraction by using a FastDNA® Kit (Qbiogene, Inc.) according to the manufacturer's instructions.

The AFLP reactions (Vos *et al*., 1995) were performed as previously described (Leslie & Summerell, 2006). Three primer pairs, *Eco*RI + GG/*Mse*I + CT, *Eco*RI + AA/*Mse*I + AT and *Eco*RI + TG/*Mse*I + TT, were used in the selective amplification step of the AFLP reaction to generate DNA fingerprints of putative *F. pseudograminearum* isolates. All reactions included four positive, and at least two negative controls. AFLP bands between 200 and 800 bp were scored manually as either 1 (presence of a band) or 0 (absence of a band). The DICE coefficient was used to assess the genetic similarity of isolates. Isolates with ≥ 97% similarity were considered to be clones. All genetic analyses were calculated for both the original and

the clone-corrected haplotype data. In addition, loci whose most frequent allele was present at a frequency of > 95% or < 5% were excluded from the final population genetic analysis to avoid any bias that they might contribute. Repeated AFLPs of the positive controls on multiple gels originating from multiple cultures of the same strain indicate little (< 3%) variation in banding patterns in different assays. These standard isolates were used to enable the direct comparison of the results from different AFLP gels and to specify the cut-off for designation of putative clones. The ability to identify clonal genotypes is consistent with limited variation in the AFLP banding patterns due to procedural artifacts.

Mating types were determined by using the degenerate oligonucleotide primers fusALPHAfor, fusALPHArev, fusHMGfor and fusHMGrev developed for asexually reproducing *Fusarium* species (Kerenyi *et al*., 2004) with modifications as previously described (Bentley *et al*., 2008).

Population genetic analyses and gametic disequilibrium

Measures of genetic diversity were calculated for each of the three 1-m-row populations, as well as for the sets of field isolates selected from Boonaldoon and Cambrai. Previously calculated average values for all population genetic parameters from pooled regional populations (northeastern Australia, south central Australia) (Bentley *et al*., 2008) collected in the same growing season also were included in the analyses. In all cases, calculations were made on both the original, and the clone-corrected (≥ 97% DICE similarity) dataset.

Nei's mean gene diversity, *h* (Nei, 1973), and proportion of polymorphic loci were estimated for each population by using POPGENE version 1·31 (University of Alberta, Alberta, Canada). POPGENE was also used to estimate Nei's unbiased measure of genetic identity, *I* (Nei, 1978). For each population, the number of unique haplotypes was calculated as an assessment of genotypic diversity. Arlequin version 3·0 (Excoffier *et al*., 2005) was used for analysis of molecular variation (AMOVA) to determine the distribution of variance components between the 1-m-row, field and regional populations from Boonaldoon and Cambrai based on whole haplotypes. The 1-m-row population from Moree was excluded from this analysis given that no field population data was available.

To test for deviation from panmixis, values for the index of association (I_A) were calculated for all of the 1-m-row and field populations with MultiLocus version 1·2 (Department of Biology, Imperial College, Silwood Park, UK). In order to test for non-random associations between loci (as a result of asexual reproduction), the resulting values were compared to simulated data generated from 1000 randomizations under the assumption of random mating (Agapow & Burt, 2001). Mating type ratios were used to calculate the effective population number for mating types ($N_{e(mt)}$) (Leslie & Klein, 1996) for each 1-m-row population. The lack of female-fertile tester strains for *F. pseudograminearum* prevents tests of female fertility which can also be used to calculate N_e and is a more sensitive indicator of effective population size (Leslie & Klein, 1996).

Point pattern analysis

Three different methods, visual assessment, β-binomial distribution (BBD) and spatial analysis by distance indices (SADIE) were used to determine whether the recovery of *F. pseudograminearum* from individual stems from the 1-m-row populations was spatially random. First, a visual assessment of all the plated stem sections was plotted with R version 2·2·1 for Windows (http://cran.r-project.org) to identify the presence or absence of *F. pseudograminearum* from individual nodes. The vertical and horizontal distances were used to construct the mapped point pattern. Secondly, a physical aggregation analysis was conducted using BBD version 1·3 (Madden & Hughes, 1994), which was used to evaluate the fit of the isolation data to the binomial and β-binomial distributions. BBD was used to estimate the index of aggregation (θ) (an indicator of heterogeneity), and the index of dispersion (D_{isp}) (which tests for variation in dispersal greater than random ($D_{isp} > 1$ suggests aggregation)) (Wilhelm & Jones, 2005) for isolations. All estimates were based on positive or negative recovery in a single dimension from individual stems along the 1-m-row. The $C_{(\alpha)}$ statistic was calculated to test if any overdispersion was characterized specifically by the β-binomial distribution as a further test for aggregation (Madden & Hughes, 1994).

The SADIE process (Perry, 1995; Perry *et al*., 1996) was also used to evaluate the point pattern for the recovery of *F. pseudograminearum* from individual stems in each of the 1-m-row populations using SADIEShell version 1·22 (Conrad, 2001). This analysis is appropriate for characterizing a discrete data set that includes multiple zero counts (Shah *et al*., 2001), as is the case in the current study. The SADIE approach gave indicators of both aggregation [index of aggregation (I_{agg}) and distance to regularity (D_{reg}), with *P*-values based on 5967 random redistributions as the formal test of randomness], and clustering [the mean of clustering indices (v_i) with an expectation of 1, and the mean of gap indices (v_j) with an expectation of –1; (Perry *et al*., 1999)]. Aggregation of isolation in the vertical dimension, i.e. recovery from multiple nodes per stem, was not considered in the point pattern analysis, as any aggregation in a single stem was assumed to result from colonization following a single infection event in the current study.

Aggregation of clonal haplotypes

The aggregation of clonal haplotypes in the 1-m-row populations were examined for spatial autocorrelation by using a matrix comparison test as previously described for vegetative compatibility groups (VCGs) (Milgroom *et al*., 1991). Two matrices, the first containing the distance between isolates in each individual row section (relative to all other stems in the row) and the second containing

Table 1 Comparison of gene diversity, *h* (Nei, 1973), proportion of polymorphic loci, number of unique haplotypes and unbiased genetic identity, *I* (Nei, 1978), estimated from AFLP data on samples of *Fusarium pseudograminearum* affecting wheat from three different spatial scales. Genetic identity indicates the similarity between samples from 1-m-row and field populations collected at random, or between 1-m-row populations and the region (northeastern Australia: Boonaldoon and Moree; south central Australia: Cambrai) as determined by Bentley *et al.* (2008)

	1-m-row population				Field population					Regional population				
Population	n[a]	*h*[b]	PLP[c]	N_h[d]	n	*h*	PLP	N_h	*I*[e]	n	*h*	PLP	N_h	*I*
Boonaldoon	60	0·15	60	33	29	0·18	69	29	0·96	89	0·18	76	76	0·97
Boonaldoon (corrected)*	33	0·15	60		29	0·18	69		0.97	76	0·18	76		0·98
Moree	36	0·21	68	20	nr[f]	nr	nr	nr	nr	89	0·18	76	76	0·96
Moree (corrected)	20	0·21	68		nr	nr	nr		nr	76	0·18	76		0·99
Cambrai	18	0·13	38	10	29	0·10	42	17	0·95	83	0·15	61	53	0·94
Cambrai (corrected)	10	0·16	38		17	0·13	42		0·91	53	0·17	61		0·90

*Individuals sharing ≥ 97% DICE similarity of AFLP haplotypes were considered clonal for clone correction.
[a]Sample size.
[b]Nei's measure of gene diversity, *h* (Nei, 1973).
[c]Percentage polymorphic loci.
[d]Number of unique haplotypes.
[e]Nei's unbiased measures of genetic identity, *I* (Nei, 1978), with the relevant 1-m-row population.
[f]Values not recorded.

clonal haplotypes, defined as isolates sharing ≥ 97% DICE similarity, were compared. Matrix comparisons were made to determine whether the mean distance between individual isolates with clonal haplotypes were not significantly different from the mean distance between clonal isolates assigned to the 1-m-row populations at random. Significance testing was done by using 1000 randomizations under a model assuming a random allocation of clonal haplotypes to positively colonized stem positions within the 1-m-row populations.

Aggregation of mating types and genetic distance

The distribution of mating types in two (Boonaldoon, Moree) of the three 1-m-row populations was examined for the presence of spatial autocorrelation using a method similar to that described above. The distribution of mating types at Cambrai was not considered, as only one *MAT-1* isolate was recovered in the 1-m-row population at this site. Comparisons were made to determine whether the mean distance between individuals with the same mating type was significantly different from the mean distance between isolates of the same mating type assigned to the row sections at random. Significance testing was again done by using 1000 randomizations under a model assuming random allocation of mating types to positively colonized stem positions within the 1-m-row populations.

A matrix correlation test was also used to estimate the correlation between physical and genetic distances between pairs of individuals in each 1-m-row population by using previously described methods (Milgroom & Lipari, 1995). The number of isolates used per 1-m-row population differed from the total number of isolates used in the other analyses, as isolates with > 10% of their AFLP loci scored as missing or ambiguous were removed from the analyses so as to avoid bias in distance calculations due to missing data. Significance testing was again done by using 1000 randomizations as previously described for clonal haplotypes and mating types.

Results

Population genetic analyses

Relatively high levels of gene diversity (Nei, 1973) were recorded for all three 1-m-row populations (Table 1). The highest level of diversity was at Moree, followed by the Boonaldoon and the Cambrai 1-m-row populations. The levels of gene diversity in the Boonaldoon and Cambrai 1-m-row populations were similar to the levels recorded for randomly collected field populations from these two sites and were comparable to previously reported values of gene diversity from pooled regional populations from northeastern and south central Australia (Bentley *et al.*, 2008). However, gene diversity values for the regional populations were higher than the 1-m-row populations at both Boonaldoon and Cambrai. The highest levels of genotypic diversity (as determined by the number of unique haplotypes) were recorded for the northeastern and south central regional populations (Table 1). In the Boonaldoon field population, all 29 isolates had unique haplotypes, whereas lower haplotypic diversity was present in the Cambrai field population. Compared to the regional and field populations, lower levels of genotypic diversity were recorded for the three 1-m-row populations.

Of the 115 AFLP loci scored, the level of polymorphism varied for each of the 1-m-row populations (Table 1), with especially high levels of diversity observed in the Moree (68% polymorphic loci) and the Boonaldoon (60% polymorphic loci) 1-m-row populations. The percentage of polymorphic loci recorded for the 1-m-row populations was always lower than the values recorded for the other field populations and the regional populations.

Table 2 Analysis of molecular variation of regional, field and 1-m-row populations of *Fusarium pseudograminearum* affecting wheat from Boonaldoon and Cambrai

Source of variation	df	Variance component	*P*-value[a]	% of variation
Between regions	1	2·34	< 0·05	41%
1-m-row vs. field	4	0·50	< 0·05	9%
Within populations	305	2·85	< 0·05	50%

[a]*P*-value based on 1000 randomizations.

Table 3 Mating-type ratios, effective population number based on mating type and values for the index of association (I_A) of *Fusarium pseudograminearum* for each of the three 1-m-row wheat populations. Mating-type data are based on both the original and the clone corrected data, whereas linkage disequilibrium values are based only on the original data

	Mating type			Linkage disequilibrium	
Population	*MAT-1*	*MAT-2*	$N_{e(mt)}$[a]	I_A[b]	*P*-value[c]
Boonaldoon	38	22	93	2·7	< 0·01
Boonaldoon (corrected)*	22	11	89		
Moree	3	33	31	9·4	< 0·01
Moree (corrected)	3	17	51		
Cambrai	1	17	20	15	< 0·01
Cambrai (corrected)	1	9	36		

*Individuals sharing ≥ 97% DICE similarity of AFLP haplotypes were considered clonal for clone correction.
[a]$N_{e(mt)}$ = effective population number based on mating types (Leslie & Klein, 1996).
[b]I_A = Index of multilocus association.
[c]*P*-value based on 1000 randomizations.

Estimates of Nei's unbiased measure of genetic identity (Nei, 1978) between individual 1-m-row populations and their respective field and regional populations indicated high levels of genetic identity based on AFLP haplotypes (Table 1). The Boonaldoon and Cambrai 1-m-row populations shared ≥ 91% genetic identity with the randomly collected populations from the same field. In addition, all 1-m-row populations described a large proportion of the genetic diversity in their relevant regional populations (≥ 90% genetic identity in all cases). The AMOVA indicated that approximately 50% of the haplotypic variation recorded for the Boonaldoon and Cambrai populations occurred within populations (Table 2). A further 41% of the variation occurred between populations from northeastern and south central Australia, with the remaining 9% occurring among the 1-m-row population and respective field populations within a region (Table 2).

The index of multilocus association (I_A) differed significantly from the expectation of panmixis for all 1-m-row populations ($P < 0·01$ in all cases; Table 3). The effective population numbers based on mating types ($N_{e(mt)}$; (Leslie & Klein, 1996) were 20–93% of the count. The severely skewed mating type ratios for Moree and Cambrai limited the effective population numbers for those populations to no more than 51% of the count. For Boonaldoon (where perithecia had previously been observed (Summerell *et al*., 2001)), the skewness in mating type ratios was less, but still significantly different from 1:1, and the effective population numbers were large enough to suggest that the skewness in mating type frequencies was not a significant limitation to random mating.

Point pattern analysis

Visual inspection of the mapped point pattern (Fig. 1) suggested that isolation of *F. pseudograminearum* is aggregated, especially around the stem bases in all three 1-m-row populations. However, statistical analysis of *F. pseudograminearum* recovery along the horizontal row axis at all three sites based on BBD (Table 4) indicates a lack of aggregation as the data are consistent with a binomial distribution, but not with a β-binomial distribution (data not shown), due to the presence/absence counts being recorded in only a single dimension. Although the binomial distribution, which represents randomness, could not be rejected, there was significant support for a deviation from random dispersion in the Boonaldoon and Moree 1-m-row populations based on BBD analysis. SADIE analysis (Table 4) confirms the visual aggregation and clustering at both Boonaldoon and Moree, but not at Cambrai ($P = 0·07$). Measures of clustering (Table 4) indicate clustering significantly greater than the mean in all three cases, further bolstering the case for a non-random distribution, especially at Boonaldoon and Moree.

Aggregation of clonal haplotypes

There was strong evidence for spatial autocorrelation in all 1-m-row populations. The matrix comparison tests for the aggregation of clonal haplotypes indicated a large difference between the observed and expected distances between isolates with the same AFLP haplotype (Table 5). In all cases, the average distance between individuals with the same haplotypes was significantly less than the average distance expected if clonal individuals were assigned to the points of positive *F. pseudograminearum* isolations in the 1-m-row populations at random (*P*-values ranging from < 0·001 to 0·004; Table 5).

Aggregation of mating types and genetic distance

The matrix comparison tests for aggregation of mating type in each of the 1-m-row populations also indicated significant spatial autocorrelation of mating types at both Boonaldoon ($P = 0·026$) and Moree ($P = 0·031$) (Table 6). In both of these 1-m-row populations the average distance between individuals with the same mating type idiomorph was significantly less than the average distance between individuals expected if mating types were assigned at random to the points of positive

Table 4 Spatial patterns of *Fusarium pseudograminearum* recovered from wheat stems in 1-m-row sections at three sites in Australia. Calculations including aggregation index (θ), dispersion index (D) and test for overdispersion ($C_{(\alpha)}$) indicate the appropriateness of the binomial and β-binomial distributions for describing aggregation. Calculations based on Spatial Analysis of Distance IndicEs (SADIE) indicate aggregation and clustering of individual isolates. Results highlighted in bold text indicate statistically significant ($P < 0{\cdot}05$) aggregation or clustering of the point pattern data

		BBD analysis			Aggregation			Clustering	
Population	n[a]	θ[b]	D_{isp} (P)[c]	$C_{(\alpha)}$ (P)[d]	I_{agg}[e]	D_{reg}[f]	P_a[g]	v_i (P)[h]	v_j (P)[i]
Boonaldoon*	229	0·00	**0·33 (0·00)**	–10 (1·00)	**4·0**	**1700**	**0·00**	**4·0 (0·00)**	**–4·1 (0·00)**
Moree	129	0·00	0·82 (0·94)	–2·2 (1·00)	**4·4**	**950**	**0·00**	**4·9 (0·00)**	**–4·6 (0·00)**
Cambrai	44	0·00	0·72 (0·92)	–2·0 (1·00)	1·7	78	0·07	**1·9 (0·04)**	–6 (0·11)

*Boonaldoon data based on average of two 0·5-m-row SADIE runs due to sample size limitation in statistical computations.
[a]Sample size = the number of stems in the 1-m-row population (total number of stems plated in order to potentially isolate *F. pseudograminearum*).
[b]θ = Aggregation index.
[c]D_{isp} (*P*) = Index of dispersion. Values in parentheses indicate the probability that $D_{isp} = 1$.
[d]$C_{(\alpha)}$ (*P*) = value for assessing if overdispersion can be characterized specifically by the beta-binomial distribution. Values in parentheses indicate the probability that $C_{(\alpha)} = 1$.
[e]I_{agg} = Index of aggregation based on SADIE analysis (I_{agg} = value of observed data/mean distance to regularity over 10 000 randomizations).
[f]D_{reg} = observed distance to regularity (based on original data).
[g]P_a = proportion of randomized samples with a distance to regularity ≥ D_{reg}.
[h]v_i (*P*) = the mean of clustering indices in cells with counts > overall mean (expectation = 1) (Perry *et al.*, 1999).
[i]v_j (*P*) = the mean of gap indices in cells with counts < overall mean (expectation = –1) (Perry *et al.*, 1999).

Table 5 Aggregation of clonal haplotypes (isolates sharing ≥ 97% DICE similarity) of *Fusarium pseudograminearum* affecting wheat in each of the three 1-m-row populations

Population	n[a]	N[b]	D_{obs}[c]	D_{exp}[d]	*P*-value[e]
Boonaldoon	60	13	11	83	< 0·001
Moree	36	2	14	45	< 0·001
Cambrai	18	4	8·2	18	0·004

[a]Sample size = the number of isolates used per population.
[b]Number of haplotypes (clones) that occurred in more than one isolate.
[c]D_{obs} = Average observed distance between individuals with the same clonal haplotype.
[d]D_{exp} = Average expected distance between clonal haplotypes assigned to the points of positive *F. pseudograminearum* isolations in the 1-m-row sections at random.
[e]Significance value based on 1000 randomizations.

Table 6 Aggregation of mating types and correlation of genetic distances with physical distances between individual isolates from three *Fusarium pseudograminearum* 1-m-row populations from wheat

		Mating type			Genetic distance		
Population	n[a]	D_{obs}[b]	D_{exp}[c]	*P*-value[d]	n_{adj}[e]	r[f]	*P*-value[d]
Boonaldoon	60	79	83	0·026	53	0·61	< 0·001
Moree	36	42	45	0·031	22	0·60	< 0·001
Cambrai	18	18	18	0·329	16	0·30	0·013

[a]Sample size = the number of isolates used per population.
[b]D_{obs} = average observed distance between individuals with the same mating type.
[c]D_{exp} = average expected distance (relative to all other plants along the row) between individuals with the same mating type assigned to the points of positive *F. pseudograminearum* isolations in the 1-m-row section at random.
[d]Significance value based on 1000 randomizations.
[e]Adjusted sample size = value based on elimination of isolates with ≥ 10% AFLP data missing or ambiguous to avoid bias in distance calculations.
[f]r = correlation coefficient of physical and genetic distance between isolates.

F. pseudograminearum isolation in the 1-m-row populations (Table 6). Matrix comparisons for the correlation between genetic and physical distances were significant for all three 1-m-row populations (Table 6), with the correlations between genetic and physical distances significantly greater than if haplotypes were assigned to the 1-m-row populations at random (*P*-values ranging from < 0·001 to 0·013) (Table 6).

Discussion

In this study, spatial aggregation of disease (as measured by successful isolation of the pathogen) and genotype were observed in 1-m-row populations of the soilborne pathogen *F. pseudograminearum* from the Australian grain belt. The genetic diversity amongst the isolates from the 1-m-row populations accounted for a large proportion of the genetic diversity documented at the scale of both field and regional populations. Only 9% of the haplotypic variation observed could be attributed to differences between populations (i.e. 1-m-row vs. field populations) within regions. As their difference was statistically significant, the null hypothesis that small transects can adequately describe population genetic parameters cannot be accepted entirely. The hypothesis that no physical or genetic aggregation or clustering occurs in populations of *F. pseudograminearum* is rejected. These results are interpreted to mean that a stubble-based primary inoculum source in a no-tillage wheat rotation system coupled with

secondary asexual colonization and occasional contributions from sexually produced ascospores are the causes of both the relatively high levels of genetic diversity and the spatial aggregation observed in micro-scale populations of *F. pseudograminearum*. Stubble is likely to maintain the genetic and genotypic diversity that is present, or generated, during the growing season following the planting of contaminated seed, the immigration of ascospores from an external source, or *in situ* sexual recombination. It is thought that these sources can increase genetic and genotypic diversity within fields, and that, even if they occur erratically, that they play an important role in generating diversity that is subsequently preserved in a cropping system where the stubble is retained.

The results indicate that the relatively high levels of genetic diversity in the 1-m-row populations account for a large proportion of the variation present, particularly the variation present in a single field population. Values for Nei's unbiased measure of genetic identity (Nei, 1978) indicate that the row populations are not isolated entities, since they have high genetic affinities with the relevant field and the much more widely dispersed regional populations. Similar findings have been reported previously, in terms of high levels of comparable genotypic diversity, on small geographic scales for the closely related pathogen *F. graminearum* (Zeller *et al*., 2003). However, despite being closely related to *F. pseudograminearum*, the mating systems of the two species are different in that one is homothallic (*F. graminearum*) and the other heterothallic (*F. pseudograminearum*). This difference could be expected to affect levels of genotypic diversity, although, in practice, the genotypic diversity and level of clonality in populations of the two species from the Australian grain belt have been shown to be similar (Akinsanmi *et al*., 2006). The levels of genotypic diversity in *F. pseudograminearum* populations in the current study further support the findings of Akinsanmi *et al*. (2006). In this study, spatial aggregation of clonal haplotypes implies some restrictions on the dispersal of the fungal propagules on a local scale. Such a restriction in dispersal is not consistent with the limited data on genetic differentiation at the regional scale. One possible explanation for this discrepancy could be that equilibrium between migration and drift has not yet been reached and, therefore, the lack of differentiation could be the result of historic, and not current, gene flow.

It is proposed that the presence of relatively high levels of genetic diversity in each of the 1-m-row populations reflects numerous individual primary infection events, presumably from stubble that has been colonized during preceding growing seasons. Previous findings of high levels of genetic diversity in field populations of *F. pseudograminearum* (Akinsanmi *et al*., 2006) are consistent with multiple colonization events occurring over several years or decades in a field's history. Therefore, individual crown rot-affected stems, and the pieces of retained stubble in a field at the end of a growing season due to the lack of tillage, probably represent a multi-year collection of haplotypic diversity. It is proposed that this composite population then initiates primary infection in a subsequent season in which a susceptible winter cereal host is grown. Alternatively, the stubble could be recolonized annually from an external source(s), although this is unlikely, based on the proposed epidemiological model for the disease (Backhouse, 2006).

The visually observed physical aggregation of *F. pseudograminearum* recovery was supported by point pattern analysis (BBD and SADIE) for the 1-m-row populations from both Boonaldoon and Moree. There were significant differences in the observed distances between clones and those expected when haplotypes were assigned at random to points of positive *F. pseudograminearum* isolation. Spatial autocorrelation analysis for both mating type and genetic distance also indicated that significant aggregation occurred at Boonaldoon and Moree. It is hypothesised that the observed physical and genetic aggregation results from infection initiated from infested stubble retained at a number of points along the 1-m-row.

Hyphae of *F. pseudograminearum* may remain viable in stubble for two to three years (Summerell *et al*., 1989; Burgess *et al*., 1993). Rows are rarely planted in the same configuration season after season, and the cut stubble may be dispersed to some extent by decomposition, environmental factors, e.g. wind and rain, and mechanical disturbances, e.g. sowing and application of chemical weed control. Thus, several physically proximal emerging seedlings could potentially come in contact with a single stubble segment, which is presumably colonized by only one or a few *F. pseudograminearum* individuals. This form of contact would be increased if the infested stubble is no longer standing or if crops are planted at high density, and could explain clonal aggregation due to patchy inoculum distribution. Such a distribution of the primary inoculum source could result in the formation of genetic neighbours and individual disease foci that were observed in the current study. These neighbours should exhibit some evidence of isolation by distance (Milgroom & Lipari, 1995; Milgroom & Peever, 2003) and the gene flow on this micro-scale would be restricted by limited secondary asexual dispersal. This distribution is consistent with the aggregation of mating types and the apparent reliance of the fungus on asexual reproduction in the field (see I_A values; Table 2). Under this hypothesis, emerging seedlings in a single row, or row section, could contact several different haplotypes in different pieces of residue, thus accounting for the relatively high levels of genetic diversity recorded.

Hyphal growth and expansion and the spread of macroconidia via wind and rain-splash also could explain the development of clonal patches in the 1-m-row populations. Presumably though this would occur to a lesser extent, and the conditions for this to occur have not been quantified.

The lack of statistically significant aggregation or clustering of *F. pseudograminearum* isolates at Cambrai could result from the comparably low levels of *F. pseudograminearum* infection in the sparsely planted rows at this site. Under these conditions, the chance of adjacent stems contacting a common stationary inoculum source is

reduced. Alternatively, the absence of aggregation could be due to a lack of statistical power resulting from the smaller sample size at this site. Mating type at Cambrai was dominated by *MAT-2*, with only one *MAT-1* isolate recovered. This imbalance made it impossible to perform matrix comparison tests.

The aggregation of mating types could help determine local gene flow (Sakai & Oden, 1983) due to their role in sexual recombination; however, the role of sexual ascospores in determining the structure of either field (Akinsanmi *et al*., 2006) or row populations is unknown. Ascospores that travel relatively large distances before initiating new infections have been suggested for numerous foliar pathogens, including *F. graminearum* (Zeller *et al*., 2003; Schmale *et al*., 2005). An alternative hypothesis for the results is that multiple haplotypes of *F. pseudograminearum* are introduced into individual fields via ascospores. Such introductions should generate high levels of genotypic diversity in the field populations (Akinsanmi *et al*., 2006; Mishra *et al*., 2006). At least some of this diversity would be retained in the stubble populations as the individual ascospores become established and propagate asexually resulting in clonal aggregation. This could be the case at Boonaldoon, where sexual ascospores from confirmed *G. coronicola* perithecia (Summerell *et al*., 2001) could be the key contributor to the relatively high levels of genetic diversity reported for the field and 1-m-row populations at this site. Effective population numbers based on mating types were relatively low (especially at Moree and Cambrai); however, the I_A values were significantly different from the expectation of panmixis for all three 1-m-row populations. Thus, sexual ascospores probably do not have a direct role in shaping micro-scale spatial arrangements in *F. pseudograminearum* populations at these two sites.

Further work is required to test the stability of spatial patterns over time, which is particularly relevant in the context of no-tillage practices for an annual cropping system. The integration of spatial and temporal analysis would enable evaluation of the effect of crop rotations (both out of, and into winter cereal hosts) in changing the micro-scale dynamics of the pathogen population in no-tillage systems. In the temporal cycle of the foliar pathogen *Erysiphe necator*, there was little evidence for haplotype carry over from one season to the next (over five years), even though the fungus overwintered as mycelium in buds of grapevines (Cortesi *et al*., 2004). These results suggested that other potential primary inoculum sources warranted further investigation (Cortesi *et al*., 2004). Integrated temporal and spatial studies of micro-scale populations of *F. pseudograminearum* under different tillage regimes could be used to verify that stubble is the primary inoculum source and that it directly influences pathogen aggregation (Milgroom & Peever, 2003). Such studies also would enable the quantification of the role of asexual macroconidia and radial hyphal colonization on aggregation (Milgroom & Lipari, 1995; Wilhelm & Jones, 2005). The role, if any, of immigrant ascospores could also be assessed in treatments where stubble was completely removed as a potential inoculum source from the system, e.g. with traditional stubble burning. Thus, the current study provides baseline data against which further studies of the spatial dynamics of *F. pseudograminearum* could be compared.

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