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Short communication

Both mating types of *Phaeosphaeria* (anamorph *Stagonospora*) *nodorum* are present in Western Australia

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Abstract

Phaeosphaeria (anamorph *Stagonospora*) *nodorum* is the most serious fungal pathogen of wheat in the West Australian (WA) wheat belt and is a diallelic heterothallic loculoascomycete. Its population genetics has received considerable attention. A recent study, which sampled isolates from diverse locations worldwide, has indicated that the mating-type idiomorph *MAT1-1* is considerably more frequent than *MAT1-2* in many populations. To investigate this, we developed PCR primers that amplify each idiomorph. In a sample of 23 isolates cultured from ascospores collected in the field, nine amplified DNA with the *MAT1-1* primers and 14 amplified DNA with the *MAT1-2* primers. The virulence of a *MAT1-2* isolate was comparable with *MAT1-1* isolates. Although these sample sizes are small, we suggest that this result is consistent with the presence of equal numbers of both mating types in populations of ascospores in WA.

Phaeosphaeria nodorum (anamorph *Stagonospora*) *nodorum* (syns. *Leptosphaeria nodorum*, *Septoria nodorum*) is a major pathogen of wheat and related cereals, causing serious economic losses in many areas of the world. In Western Australia (WA), yield losses have been recorded in the range of 18–31% (Bhathal et al., 2003; Loughman and Thomas, 1992). The fungus is a heterothallic loculoascomycete with a diallelic mating-type locus (Halama and Lacoste, 1991). The life cycle of the pathogen is believed to involve the release of air-borne ascospores from pseudothecia that develop on decaying stubble. Under moist conditions, the pseudothecia produce ascospores (von Wechmar, 1966; Arseniuk et al., 1998), which establish the disease (Septoria nodorum blotch) on young wheat plants. In the Mediterranean climate of WA, the winter rains, which accompany the initial few weeks of growth of the plant, trigger ascospore release of *P. nodorum*. Ascospores are readily trapped from air currents at this time (Bathgate and Loughman, 2001). On diseased plants, the fungus asexually produces pycnidiospores within brown pycnidia in the dead leaf tissue. Pycnidiospores spread to new crop foliage through rain splash, resulting in polycyclic epidemics. Pseudothecia that form on the decaying stubble probably constitute the main mechanism by which the fungus survives the non-cropping period (Faulkner and Colhoun, 1976).

The presence of both ascospores (which appear to be primarily wind dispersed) and pycnidiospores (mainly splash dispersed) within the annual life cycle creates the opportunity for both large- and small-scale movement of fungal populations. Accordingly, the use of nuclear genetic markers has revealed large amounts of variability within *P. nodorum* populations worldwide. Furthermore each geographical population is highly variable, containing a large proportion of the total genetic variation (McDonald et al., 1994; Keller et al., 1997; Caten and Newton, 2000; Murphy et al., 2000)

If the sexual stage constitutes an important part of the life cycle, the simplest prediction is that both mating-type idiomorphs would be present at equal frequencies in the population. It was therefore both

surprising and intriguing when Halama (2002) reported that 87% of fertile isolates were *MAT1-1*. That study sampled populations collected in many parts of the world and used mating tests with tester strains of known mating type. Of 101 isolates tested, 23 were sterile, 68 were *MAT1-1* and 10 were *MAT1-2*.

To test the mating type frequencies in WA, we collected a sample of isolates firstly from infected lesions. It is therefore likely that these samples originated from splash-dispersed pycnidial inocula. The mating genotype was assayed using degenerate PCR using primers designed by Bennett et al. (2003). Of nine strains tested, all were *MAT1-1*.

This seemed to confirm the skewed mating-type frequencies reported by Halama (2002). However the sample sizes in both studies were small (101 and 9), and were derived from pycnidial lesions. As *P. nodorum* alternates between stages producing limited numbers of ascospores and very large numbers of pycnidia, sampling asexual spores is subject to errors created by local clonal expansion (Keller et al., 1997). Adequate sampling of pycnidia should involve very large numbers of isolates collected quasi-simultaneously over a very large area and then corrected for the presence of clones. Much smaller samples of ascospore-derived isolates can give a better picture of the population structure.

Bathgate and Loughman (2001) collected ascospores in an airtrap, cultured the spores and stored mixed pycnidia. For this study, a total of 25 isolates was revived and tested for mating type. PCR represents a much quicker and less-skill-demanding method of determining mating type than the laborious traditional mating assays (Halama and Lacoste, 1992; Turgeon, 1998). Bennett et al. (2003) defined a set of primers that amplify mating-type idiomorphs of many ascomycetes. They used these primers to determine the sequences of the mating-type genes of *P. nodorum* (GenBank AY212018 and AY212019). Dai et al. (2001) independently determined the sequences of internal regions of the genes (GenBank AY072933 and AY072934). As degenerate PCR is occasionally problematic, we designed specific primers from these sequences. The *MAT1-1* region was amplified using the primers Mat1-1F (5′ agcctggctcaaagctatac 3′) and Mat1-1R (5′ cctttgtcatcagggtccac 3′) yielding a product of 118 bp. Similarly, a 197 bp MAT1-2 product was amplified with primers Mat1-2F (5′ tcgagcaacctgatctcac 3′) and Mat1-2R (5′ ttgtagccagggtaaagcc 3′).

DNA was extracted from the isolates. A full loopful of growth (including spores and mycelia) was scraped into 500 μl of DNA extraction buffer (0.5 M NaCl, 10 mM Tris–HCl pH 7.5, 10 mM EDTA, 1% SDS). The solution was then placed at −20 °C and allowed to freeze before being thawed at 37 °C. After repeating the freezing and thawing, the suspension was centrifuged at 12,000*g* for 15 min to remove cellular debris and the supernatant was added to an equal volume of isopropanol. The precipitated DNA was collected by centrifugation at 12,000*g* for 10 min and washed once with 70% ethanol before being air dried and resuspended in 10 μl of sterile water. PCR was then performed using 2 μl of the extracted DNA as template, 1.5 mM $MgCl_2$, 0.4 mM dNTPs, 2 μM of each primer, 2 U of Taq polymerase with the reaction made up to 25 μl with sterile H_2O. The conditions used were 95 °C/2 min, (95 °C/30 s, 55 °C/30 s, 72 °C/1 min) for 40 cycles followed by 72 °C/5 min.

The primer pairs amplified fragments of the predicted sizes (either 118 or 197 bp) in 23 out of 25 isolates tested. In two cases, the PCR failed to produce a band. This result is typical when using such quick and crude methods of preparing DNA. Gel electrophoresis of the 23 successful amplifications showed that 14 were consistent with the size of band expected for *MAT1-2* and 9 were the size expected for *MAT1-1* (data not shown). In all 23 cases, only one of the primer pairs amplified a band, consistent with the reported heterothallism of *P. nodorum.* This lower frequency of *MAT1-1* isolates than *MAT1-2* isolates (9/14), contrasts markedly with the results of Halama (2002) where *MAT1-1* isolates predominated 68/10. Chi-squared analysis indicate that a ratio of 14/9 is not significantly different from equal proportions of each mating type ($\chi^2 = 1.08$, $p = 0.05$).

The predominance of *MAT1-1* isolates in the pycnidial populations, sampled by Halama (2002) and us may have been due to a greater virulence (defined here as the size of the lesion) of *MAT1-1*strains compared to *MAT1-2*. To test this, we compared the virulence of *MAT1-2* isolates with *MAT1-1* isolates. This experiment was partially confounded by our inability to induce sporulation *in vitro* in many of the ascospore-derived cultures (this is common when reviving isolates held in storage for long periods; both mating types were affected). Detached-leaf assays used 2-week-old Amery wheat leaves on 0.15% benzimadazol plates with the ends of the leaves embedded into the agar. The leaves were inoculated with 1 mm^3 blocks of agar from all 25 isolates. The plates were wrapped in parafilm and incubated at 20 °C in a 12 h day/night cycle.

All samples gave lesions in all cases. A quantitative assay was possible with only one *MAT1-2* isolate. The leaves were inoculated with drops (5 μl) of inoculum containing 10^4 spores in 0.02% Tween 20. Spores of this isolate (8799) and a *MAT1-1* control (SN15) were placed on Amery wheat leaves on benzimidazole agar as before. Disease severity was assessed by measuring the size of necrotic tissue 6 days after inoculation The lesion size was measured and found to be 14, 15, 15 and 11 mm for SN15 (*MAT1-1*). For the *MAT1-2* isolate, 8799, the lesion sizes were 15, 14, 12 and 15 mm. *T*-test analysis of these measurements ($p = 0.46$) indicates that the pathogenicity of these two isolates is not significantly different. Hence, by combining the quantitative spore-based assay with the qualitative agar-blocks assay, we tentatively conclude that the virulence of the tested *MAT1-2* isolates is not systematically lower than for *MAT1-1*.

Our studies, therefore, do not explain the prevalence of *MAT1-1* isolates in the pycnidial populations. One possible explanation is that we sampled from within pycnidial clones. The pycnidia used in this study were collected independently from lesions over a time period of several years and at different locations within WA and thus we think this is unlikely, though this has not been excluded. Another possible cause that could be considered is that the two mating types differ in asexual fitness. Whilst this cannot be excluded *in planta*, we have no evidence that fitness differs *in vitro*. Clearly this observation requires further investigation. Bennett et al. (2003) sampled two populations; one had a skewed ratio and one did not in populations of 21 and 31. Overall our results, those of Halama (2002) and Bennett et al. (2003) confirm the view that very large sample sizes are needed to support assertions that genotypic frequencies are skewed (Zhan et al., 2002).

In summary, the suggestion that *S. nodorum* populations exist with skewed mating-type ratios would have had interesting consequences for its population genetics and control strategies. This prompted us to test a limited WA population and we found no evidence of a skewed mating-type ratio. In doing so, we have developed a very rapid and simple method of mating-type testing for this fungus.

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