Title: Genetic homogeneity of a recently introduced pathogen of chickpea, *Ascochyta rabiei*, to Australia

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Abstract

The study examined the genetic structure and potential for adaption to host genotype of *Ascochyta rabiei*, a major necrotrophic fungal pathogen of chickpea. For this, *A. rabiei* populations derived from six major chickpea growing regions in Australia were characterized using 20 polymorphic microsatellite markers. The overall gene (*H* = 0.094) and genotypic (*D* = 0.80) diversities among the entire population were low, indicating the establishment of a recent founder population. Since, no significant genetic differentiation was detected among growing regions, subsequent anthropogenic dispersal was proposed, mainly through seed movement. The highest genotypic diversity and allelic richness was detected at Kingsford, South Australia, thought to be the site of industry establishment in the 1970s and hence the centre of introduction. Despite assessing 206 isolates collected in 2010 from host genotypes with differential disease responses, no significant co-occurrence of fungal haplotype with host genotype was detected. Rather a single haplotype that accounted for 70% of the total isolates assessed was detected on all host genotypes assessed and from all regions. Therefore, we propose that up until 2010, host reaction was not a major influence on the Australian *A. rabiei* population structure. Additionally, the detection of a single mating type only, MAT1-2 indicated asexual reproduction, further influencing low haplotype diversity and resulting in a population comprising of multiple clones with relatively few haplotypes compared to populations in other continents.

Keywords  Pathogen invasion, *Ascochyta rabiei*, Microsatellite, Founder populations, genetic diversity
Introduction

Global trading of agricultural products facilitates opportunities for exotic pathogens to be introduced to and invade new geographic areas. An estimated 65 to 85% of exotic plant pathogens are foreign in their current residing locations (Pimentel et al. 2001). An introduced pathogen may result in major ecological and economical consequences (Rossman 2009). For Australia, this has included *Rhynchosporium commune* on barley (Linde et al. 2009), *Phytophthora cinnamomi* on woody plants (Podger et al. 1965) and *Ascochyta rabiei* (Pass.) Labr. (syn *Phoma rabiei* and teleomorph *Didymella rabiei* (Kovachevski)) (Kaiser and Muehlbaur 1988; Navas-Cortés et al. 1998) on chickpea. *A. rabiei* limits both yield and seed quality. First observed on chickpea in South Australia in 1973, it was thought to have arrived via commercially distributed seed (Galloway and MacLeod 2003). Subsequent eradication attempts failed with significant losses reported across southern Australia in 1998, resulting in loss of farmer confidence and near collapse of the industry (Galloway and MacLeod 2003).

*A. rabiei* is a bipolar heterothallic fungus with one mating type locus and two compatible mating forms (MAT1-1 and MAT1-2) (Trapero-Casas and Kaiser 1992; Wilson and Kaiser 1995). The recognition and recombination of two compatible mating type alleles enables sexual reproduction. However, when only one mating type allele is present, the fungus reproduces asexually. The sexual form, *Didymella rabiei* produces pseudothecia early in the growing season and when environmental conditions are conducive; these mature and forcibly discharge ascospores, which are dispersed by wind and can travel several kilometres to infect neighbouring crops (Kaiser et al. 1992). During asexual reproduction, pycnidiospores (conidia) spread mainly through rain-splash over short distances (Shtienberg et al. 2000). Until 2000, only one mating form was detected in Australia (Phan et al. 2002).

Migration of invasive organisms leads to potential selective emergence of adapted isolates in novel geographic regions and on specific host genotypes. Despite the fact that founder populations are generally characterised by low genetic diversity and allelic richness (Templeton et al. 1995), examples of major epidemics subsequent to founder population establishment include the introduction of *Mycosphaerella fijiensis* to banana in Latin America, the Caribbean and Africa from South East Asia (Rivas et al. 2004) and the introduction of *Cryphonectria parasitica* to chestnut from Asia to North America (Milgroom et al. 2008). Evidence suggests that founder events are often followed by genetic expansion within the population through recombination and mutation, producing novel genotypes and quickly increasing the effective population size (Linde et al. 2009). Accordingly, the evolutionary potential of the pathogen may be increased and subsequently adapt to overcome host resistances.
There are two different adaptive patterns that can occur in invasive pathogens; general adaptation to the most common and prevalent host genotype, and maladaptation to different host genotypes, leading to fragmented pathogen populations as a result of genetic drift (Gandon et al. 1996 and 1998). Several factors such as the magnitude of gene flow, population size (Gandon and Michalakis 2000), and the type of host resistance (quantitative vs qualitative) (Gandon 2002) will determine the population structure of the pathogen.

Currently, the best available host resistance to \textit{A. rabiei} is partial and governed by multiple quantitative resistance loci (Huettel et al. 2002; Iruela et al. 2006; Tar’an et al. 2007). Quantitative host resistance is thought to be more durable than major gene resistance (Johnson 1984). Although pathogen aggressiveness may evolve slowly (Gould et al. 1991), its gradual evolution and adaptation may lead to an ‘erosion’ of resistance (McDonald and Linde 2002), especially if a monoculture farming system is applied (Gandon 2002). Resistance erosion is caused by selection, increasing frequencies of isolates with high fitness and virulence (Gandon and Michalakis 2000; Andrivon et al. 2007).

Directional selection on plant pathogens generally produces a clonal population, characterised by large numbers of relatively few clonal lineages and an overall low genetic variability (McDonald and Linde 2002). This was previously observed for powdery mildew on barley (Brown and Wolfe 1990) and rice blast, \textit{Magnaporthe grisea} (Babujee and Gnanamanickam 2000). The potential risk from rapid selection for highly virulent clonal lineages within the asexually reproducing Australian \textit{A. rabiei} population is currently unknown. Therefore, to determine the risk of a pathogen, knowledge of the current population diversity and structure is required (McDonald and Linde 2002). This will inform management strategies to hopefully reduce the risk for an epidemic through integrated practices (McDonald et al. 1996; Poczai et al. 2012).

Despite cultivation problems with eroding host resistance, Australian farmers are still encouraged to grow chickpea as it is considered a high return cash crop (FAOSTAT 2013; ABARES 2012). As one of the leading global chickpea producers, Australia has directed 90% of its export market to various legume producing countries such as India, Pakistan, Bangladesh and Sri Lanka (Pulse Australia 2012; AgVantage commodities 2012; Pulse Australia 2013). In addition, chickpea fixes atmospheric nitrogen and is viewed as an important component for rotational cropping with wheat and oilseed, for increased sustainability, and reduced environmental and economic impacts of fertilizer application.

This study aimed to determine the potential risk to disease management from clonal lineages within the Australian \textit{A. rabiei} population through: (i) an extensive study of population structure of isolates from within and across the major chickpea growing regions in Australia
up until 2010, (ii) assessing the mating type distribution and linkage disequilibrium, and (iii) assessing whether host resistance imposes positive (directional) or diversifying selection on the Australian *A. rabiei* population.

### Materials and methods

#### Sample collections

To determine the overall population diversity of *A. rabiei* in Australia, isolates were collected from commercial chickpea crops and National Variety Trials (NVT) sites during 1999 to 2010. A total of 241 isolates from five geographic regions: Victoria (VIC) (37), New South Wales (NSW) (72), Western Australia (WA) (34), Queensland (QLD) (15) and South Australia (SA) (83) were collected. SA comprised two populations, Melton (42) and Kingsford (41) (Online Resource 1).

To investigate local adaptation and selection, 206 isolates were collected in 2010 at each major chickpea growing region in Australia (SA, VIC and NSW) from specific host genotypes with varying degrees of susceptibility to *A. rabiei* (Online Resource 1). At each site, isolates were collected from either a resistant (R) to moderately resistant (MR) host genotype, and from either a susceptible (S) to highly susceptible (HS) host genotype. The selected genotypes were: Genesis090 (R) and Howzat (S) for SA, Genesis090 (R) and Kaniva (S) for VIC, and HatTrick (R) and Flipper (MR) for NSW (DPI 2013; PBA 2009).

For both the overall population diversity and host-selection studies, samples derived from sites that were within a 50 km radius of each other were considered as one population and isolates sampled from sites that were at least 200 km apart were considered as separate according to the dispersion distance of conidiospores (Kaiser et al. 1992). Each commercial chickpea crop was approximated to be a 1 km² quadrat. From within each field, a total of five to ten random samples were taken from each of the five intensive sampling sites (one in each corner and one in the centre). At NVT sites, each row and range number corresponded to a different variety. At these sites, the sampling scheme to determine the overall population diversity comprised of a collection of isolates sought randomly from separate infected plants showing symptoms of ascochyta blight, regardless of the cultivar. For the host-selection study, three to five random samples from each row and range of the chosen varieties were collected from the same field. Each infected leaf sample was collected from a different plant across one row.

#### Isolation, culturing and DNA extraction
Isolates were cultured dependant on the visibility of pycnidia. When pycnidia were present, a single pycnidium per lesion was picked with a sterile needle from an infected chickpea leaf, stem or pod and inoculated into 2 mL of sterile distilled water before streaking onto V8 juice growth agar. Samples with no visible pycnidia were surface sterilised and placed on V8 juice growth agar. All plate cultures were incubated for 14 days at 22°C ± 2°C with a 12/12 hour light/dark photoperiod and single spored. *A. rabiei* was isolated from only one lesion per infected plant to minimise the likelihood of sampling clones due to the short distance of conidia dispersal through rain splash.

Single spored isolates were inoculated into 10 mL Czapek Dox broth (Difco, Australia) and incubated for two weeks at 22°C ± 2°C in the dark. Mycelia were then harvested and DNA was extracted with a DNeasy Plant Mini Kit (Qiagen, USA).

**Mating type determination**

The mating type of 604 isolates (Online Resource 1) from 1999 to 2010 was determined with a *MAT*- specific multiplex PCR assay developed by Barve et al. (2003). A combination of three primers amplified the alpha (α) domain of the MAT1-1 and the high mobility group (HMG) box of the MAT1-2 with expected band sizes of 700 and 450 bp, respectively. Multiplex PCR conditions were carried out as described previously in Barve et al. (2003). AR21 (ATCC 76502, MAT1-1) and AR20 (ATCC 76501, MAT1-2) isolates from W.J. Kaiser were used as the mating type testers (T. Peever, Washington State University, pers. comm. 2009).

**Microsatellites**

A total of 20 loci previously identified for *A. rabiei* were used to characterize the Australian *A. rabiei* populations (Geistlinger et al. 2000; Leo et al. 2011) (Table 1). Genotyping was performed using the Multiplex-Ready PCR technique according to the protocol developed by Hayden et al. (2008). PCR products were separated using a 96-capillary ABI 3730 DNA Analyzer at the Australian Genome Research Facility (AGRF, Melbourne). Alleles were analysed using GeneMapper v4.0 software (Applied Biosystems).

To ensure neutrality of all the SSR loci used in this study, W.J. Kaiser mating type testers from the USA and Syria (AR20, AR21, AR628, AR655, and WSFS-03-01) and a population of 84 ascospore progeny isolates resulting from a cross of AR20 and AR21 (T. Peever, Washington State University, pers. comm. 2009) were included.
Data Analysis

Isolates with the same allele sizes at all loci were considered as clones. Clonal fraction was calculated to determine the occurrence and frequencies of clones within a population. The Simpson’s diversity index \((D)\) (Simpson 1949) and its corresponding evenness test \((E_D)\) were used to calculate the genotypic diversity and evenness of each population. These calculations were conducted using GenoDive (Meirmans and Van Tienderen 2004).

For all allele-based analyses, unless otherwise stated, clonal genotypes were removed from populations to minimise bias due to over-representation of alleles in clones. An Ewens-Watterson neutrality test (Manly 1985) was performed on all 21 loci. Loci that were not selectively neutral were excluded from the analyses.

To estimate the genetic diversity of the \(A.\ rabiei\) population in Australia, the number of alleles, number of private alleles and Nei’s gene diversity (Nei 1973) across all loci were calculated in GenAlEx v6.41 (Peakall and Smouse 2006). To account for populations with different sample sizes, rarefaction analyses using HP-Rare (Kalinowski 2005; Department of Ecology, Montana State University, Bozeman, Montana) were performed to determine the allele and private allelic richness of each population. Allelic richness of populations was compared with a pair-wise probability \(t\)-test. Bootstrapping with 999 permutations was conducted to test for population differences in clonal diversity using GenoDive (Meirmans and Van Tienderen 2004).

Pairwise genetic distances (PhiPT) between individuals were used to construct a principal coordinate analysis (PCA) in GenAlEx v6.41 (Peakall and Smouse 2006) to further define the genetic clusters of individuals within the overall population. Correlations between genetic distance and log (geographical distance) matrices were assessed using a Mantel test (Mantel 1967) in GenAlex v6.41 using 999 randomizations and PhiPT as measures of pairwise genetic distance in the matrix.

To examine the relationships among isolates, minimum spanning trees were constructed with Network 4.6.1.1 (Fluxus Technology Ltd., Clare, Suffold, England) using microsatellite repeat numbers of isolates representing the full data set (Australian collection = 241 isolates) and isolates from specific cultivars in the selection study \((N = 206)\). A median joining network calculation was done with default parameters where the weight of each marker = 10, \(\varepsilon = 0\) and Frequency >1 criterion to simplify the network with a large number of individuals (Bandelt et al 1999). Higher \(\varepsilon\) values were also evaluated, while the haplotype groupings remained the same, the hubs interconnecting different groupings were more complex making the interpretation of results more complicated, hence \(\varepsilon = 0\) was utilised for both haplotype
networks. After calculating the median joining network, the data was further processed with MP calculation to eliminate unnecessary median vectors and links to produce the shortest (MP or Streiner) network trees (Polzin and Daneshmand 2003).

Host genotype selection

Additional to gene, genotypic diversity indexes and the median joining network as calculated above, frequencies of haplotypes within each A. rabiei population derived from specific host genotypes were calculated. Pair-wise and multiple comparisons to detect differences in pathogen populations sampled from different host genotypes were assessed with an analysis of molecular variance (AMOVA) using GenAlEx v6.41 (Peakall and Smouse 2006) using non-clone corrected data.

Linkage disequilibrium

Gametic disequilibrium was calculated with an Index of association (I_A) test (Maynard Smith et al. 1993) on non-clone corrected datasets. The $I_A = 1-V_O/V_E$, where $V_O$ and $V_E$ were the observed and expected variance of $K$ and $K$ was the number of loci for which two individuals differed. Values of $V_O/V_E$ per population were calculated by GenAlEx 6.1 (Peakall and Smouse 2006) using 999 permutations. When the ratio of $V_O/V_E= 1$ and $I_A = 0$, the population was described to undergo sexual recombination or linkage equilibrium. Probability of variance of a randomised data set ($V_r$) to the observed value ($V_O$) at 5% nominal level calculated by the same software was also used to support the linkage disequilibrium results using 999 permutations.

Results

Overall population structure of Australian A. rabiei

Genetic diversity

A total of 241 A. rabiei isolates from six different chickpea growing regions in Australia were analysed with 21 polymorphic microsatellite markers. Twenty loci were selectively neutral when screened against the six USA and Syria (Kaiser) mating type testers and the 84 progeny isolates with an Ewens-Watterson test. The probability segregation ratio of each marker locus was assessed to be approximately 1:1 in the progeny population calculated using 1000 simulated samples. However, locus ArH07D was found to be under directional selection ($P = 0.9350$) and was thus excluded from the analyses. From the 20 microsatellite loci, the number of alleles present in the total Australian population ranged from 2 to 6 per locus, with ArH05T being the most polymorphic. The overall gene diversity for each locus ranged from 0.008 to 0.299. The highest percentage of polymorphic loci was observed in both SA populations Kingsford and Melton (65%) with the least polymorphic loci in NSW (30%)
Gene diversity for the six populations ranged from $H = 0.028$ to 0.174, with an overall diversity of $H = 0.094$ (Table 2). Additionally, allelic richness and private allele richness for each population was low, with an overall population value of 1.55 and 0.22, respectively (Table 2). Allelic richness did not differ significantly among populations ($P > 0.05$), except for between populations with the lowest (NSW) and highest (SA-Kingsford) ($P = 0.04$) allelic richness.

Across all populations, a total of 95 haplotypes were identified from the 241 isolates analysed (Table 2). The highest genotypic diversity ($D = 0.98$) was in Kingsford with 32 unique haplotypes from 41 isolates. Fewer haplotypes were observed in other populations. A two sided bootstrap test of the genotypic diversity showed that SA-Kingsford was significantly different to other populations except for QLD ($P > 0.025$). The genotype evenness of SA-Kingsford was close to 1 ($E_D = 0.76$ (Table 2)), suggesting that haplotypes were distributed evenly within this population. Genotype evenness indices of all other populations were less than 0.5, suggesting uneven frequency distribution of genotypes (Table 2).

A number of haplotypes were common among all six populations. Of the 95 haplotypes identified, 73 haplotypes were found once only, 22 were detected two to seven times in different populations. The two most common haplotypes were detected 89 and 18 times and occurred in all six populations, which accounted for 36.9% and 7.5% of the overall metapopulation, respectively. Median joining haplotype network analyses showed one large node which corresponded to the most common haplotype (89 individuals, ARH01) and another node for the second most common haplotype (18 individuals, ARH046) derived from all collection regions (Figure 1). These two common haplotypes are connected via a single haplotype (ARH074) which had a one step mutational event of a single locus (ME14-1-83) from ARH01 and a two step mutational event of a different locus (ME14-1-42) from ARH046, indicating that the two common haplotypes are closely related. The other single haplotypes found around the two common nodes indicated stepwise evolution from the common haplotypes through mutational events that have occurred at several loci. The maximum number of mutational steps in a locus (ArR12D-L1) between haplotypes is 40, whereas 11 haplotypes separated by long branches indicate separate introductions (Figure 1).

Mating types and gametic disequilibrium

All isolates contained a single amplicon of 450 bp, expected for a MAT1-2 idiomorph. Non-clone corrected data sets from six chickpea regions in Australia were tested for their linkage disequilibrium to further confirm the mating type test and the asexual status of non-identical genotypes. Using the I$_A$ and P ($V$) tests, all populations except for NSW were in significant ($P < 0.05$) linkage disequilibrium (Table 2).
Genetic differentiation among populations

The majority of the total genetic diversity (96%) was detected within populations. Pairwise PhiPT values showed low levels of differentiation among populations, ranging from 0.000 to 0.096. Only four of the pairwise comparisons of PhiPT were significant ($P < 0.05$; Table 3), mostly involving the population from NSW. Likewise, the overall PhiPT value of the total Australian population was low (PhiPT = 0.040 with $P > 0.05$).

PCA did not reveal that isolate clustering corresponded to geographic origin (Figure 2). A few haplotypes, mostly from the NSW population, were outliers and may presumably have contributed to the significant differentiation observed between NSW and other populations. Likewise, the Mantel test also showed no genetic differentiation in relation to geographical distance ($R^2 = 0.284$).

Host genotype selection

Gene and genotypic diversity

Eight of the 20 loci (ME14-1-29, ME14-1-5, ME14-1-37, ME14-1-23, ME14-1-91 ME14-1-41, ME14-1-33, ME14-1-16) were monomorphic among the isolates, independent of host genotype. Up to five alleles per locus were observed from the 12 polymorphic loci (Table 4). The gene diversity per source host genotype was low, ranging from $H = 0.005$ (Flipper, NSW) to 0.038 (HatTrick, NSW) (Table 5). Gene diversities did not differ significantly ($P > 0.05$) among cultivar-derived isolate populations. Across regions and source cultivars, a total of 35 unique haplotypes were identified from 206 isolates ($D = 0.68$), (Table 5). The highest clonality was detected in SA, with isolates from Genesis090 more clonal ($D = 0.43$, clonal fraction = 0.93, $E_D = 0.25$) than isolates from Howzat ($D = 0.32$, clonal fraction = 0.88, $E_D = 0.21$). The least clonal reproduction was observed on the VIC cultivars, Kaniva and Genesis090 (Table 5).

Within regions, sourcing from resistant or susceptible varieties contributed 0 to 3% of the genetic variation observed, with the highest variation observed in SA. None of the allele frequencies in any of the loci tested differed with a chi-square test (data not shown). Similarly, PhiPT values of pairwise comparisons of populations originating from hosts differing in their disease reaction within a region, was low and not significant (Table 5).

Differences between resistant and susceptible cultivars, or regional populations, contributed only 0 to 1% to the genetic variation observed in Australia, with most of the diversity found among isolates. PhiPT pairwise comparisons of populations originating from resistant hosts (Genesis090 and HatTrick) derived from VIC, SA and NSW were very low and non
significant ($P > 0.05$) (Table 65). A similar scenario was found with population comparisons from susceptible hosts (Kaniva and Howzat) from VIC and SA ($\text{PhiPT} = 0.007$ and $P > 0.05$).

No evidence of isolate selection by host genotype was found. Howzat and Kaniva shared a common haplotype (ARH01), which accounted for 79% of isolates on susceptible populations. This isolate was also prevalent on Genesis090 and HatTrick where it accounted for 65% of the total population found on the resistant varieties. Susceptible and resistant cultivars shared 12 out of 35 haplotypes in which one of them occurred 131 times (ARH01) (Online Resource 1), accounting for 70% of the total susceptible and resistance populations in Australia. One large central node representing 148 individuals from all resistant and susceptible host genotypes was also seen in the median joining haplotype network (Figure 3).

**Discussion**

Overall gene and genotypic diversity of *A. rabiei* in Australia

Compared to *A. rabiei* genetic diversity detected elsewhere in the world ($H = 0.30$ to $H = 0.78$; Rhaiem et al. 2007; Nourollahi et al. 2010), Australian populations contained a low overall gene ($H = 0.094$) diversity and a high clonal fraction of up to 0.75. This was similar but slightly higher to that detected by Phan et al. (2002) ($H = 0.02$), most likely due to a larger sample size and the utilisation of more informative SSR markers compared to those developed by Geistlinger et al. (2000) (Leo et al. 2011). It is perhaps not surprising to find such low diversity in the Australian population, since the disease became noticeable only in the early 1990s. Coupled with the population’s probable inability to reproduce sexually (only one mating type present) and therefore its inability to create novel genotypes through recombination, the low genetic diversity found in Australia is typical of recent founder populations (Nei 1975).

The number of founder events in Australia is unknown, but could have involved the original introduction of a small number of individuals, which have since become invasive and led to the clusters seen in the median joining haplotype network and PCA. Invasive species form a stochastic loss of genetic diversity as observed in the Australian *A. rabiei* population, with two common haplotypes dominating in all chickpea growing regions (Figure 1). Meanwhile ten unique isolates from QLD, NSW and WA that were explained mostly on principle coordinate 1 (Figure 2), could represent separate multiple introductions to Australia, perhaps from diverse donor populations (Nei et al. 1975; Barrett and Husband 1990). These outliers are also presented on the long branches on the median joining network (Figure 1), suggesting that they are not derived from the most common haplotype and are most likely from separate introductions.

The Australia chickpea industry was established with accessions imported from ICRISAT, India (Brinsmead 1994), Syria (ICARDA) and Turkey (AARI) (Siddique et al. 2004). A
genotypic comparison at the same SSR loci among Australian isolates and those from the regions where the originator accessions were sourced, will aid to determine the likely major routes of invasion. Subsequent widespread dispersal of *A. rabiei* to all the chickpea growing areas in Australia, presumably anthropogenically via seed, may have contributed to the homogenetic pathogen population structure observed.

Anthropogenic dispersal is a major factor in plant disease dispersal and acts to homogenise plant pathogen populations. A lack of isolation by distance (non-significant correlation between genetic and geographic distance), low genetic differentiation among regional populations and little contribution of geographic region to the genetic variation observed, all suggest significant anthropogenic dispersal of *A. rabiei* across Australia. Furthermore, an even distribution and the presence of dominant haplotypes across the country signified introductions of the same haplotypes to all the regions.

Identifying the initial founder population in Australia is important to help determine if genetic changes are caused by local adaptation, genetic drift or other evolutionary forces (Dlugosch and Parker 2008). The original founder population is predicted to have the highest genotypic diversity due to genetic drift further reducing diversity in subsequent founder populations (Hallatscheck and Nelson 2008). Among all the Australian *A. rabiei* populations, the SA-Kingsford population contained the highest genotypic diversity, as well as the most alleles (40 out of 70 total alleles), gene diversity (*H* = 0.174) and allelic richness (*A_r* = 1.83), indicating it as the original founder population and subsequent source of *A. rabiei* diversity in Australia. Indeed, besides the first introduction of desi chickpea, *Tyson at regional trials held in New South Wales and Queensland* (Siddique and Sykes 1997), the first few incursions in Australia through importation and sowing of infected seed in 1973 was near Kingsford (J. Davidson, South Australian Research and Development, pers. comm. 2011) and the first Ascochyta outbreak in Australia occurred in a commercial chickpea crop in SA in 1995 (Kaiser 1997 and Khan et al 1999). Kingsford is also a long running chickpea trial site with a history of a diverse range of host genotypes (J. Davidson, South Australian Research and Development, pers. comm. 2011).

Since the majority of Australian isolates are clonal and the population does not appear to contain both mating types, it is likely that more recent differences among isolates are due to gradual mutation (Montarry et al. 2006). This was perhaps demonstrated by Elliott et al. (2011), who found clonal isolates with different pathogenicities. Although this theory would require validation since the clonality proposed is based on just 20 independent and selectively neutral loci.

Although the Australian *A. rabiei* population has a low overall genetic diversity, a pair-wise regional comparison of PhiPT and bootstrap values showed that NSW was significantly different from Victoria and two of the South Australian populations (*P* < 0.05). Two plausible reasons for this differentiation are: (i) a separate recent introduction of *A. rabiei* to
NSW which may not have had the chance to spread; ii) adaptive evolution caused by local selective pressure. These may help explain the epidemic recently observed on the newly deployed moderately resistant cultivar, HatTrick in NSW (K. Moore, NSW Department of Primary Industry, pers. comm. 2010). An influx of genetic variation within an invasive population may strengthen adaptive potential (Ellstrand and Schierenbeck 2000; Bossdorf et al. 2005), also directly through the introduction of haplotypes that are well adapted to various environmental traits (Marshall and Brown 1981; Blair and Wolfe 2004). The unique haplotypes detected in northern NSW and QLD, the most recently established chickpea growing regions, may indicate that the population is rapidly evolving within these regions due to selective adaptation to new agro-geographical and host factors (Montarry et al. 2006).

Mating and linkage disequilibrium of the overall Australian isolates

All isolates were MAT1-2, indicating that the population cannot reproduce sexually. This is corroborated by low genotypic diversities and high clonal fractions, indicative of asexual reproduction. Linkage disequilibrium tests on non-clone corrected populations showed that each population except for NSW experienced linkage disequilibrium which is a characteristic of a clonally reproducing population. The exception is the NSW population which is in linkage equilibrium. Apparent linkage equilibrium was most likely achieved by two reasons, the first being because it is a founder population of closely related individuals, as noted by the fact that NSW has the highest clonal fraction value, and the second being an artefact caused by the lack of pattern among loci and population due to an intermediate number of haplotypes in a large sample size rather than an error in the linkage disequilibrium test.

Australia appears to only have one mating type of A. rabiei, likely limiting the pathogen to asexual reproduction, as indicated by the detection of only MAT1-2 in all isolates assessed. However, there is a small probability that MAT1-1 does occur in Australia, albeit at very low frequencies. This is because chickpea seed sourced from overseas locations such as Canada and Syria are infected with both mating types (Amstrong et al. 2001; Atik et al. 2011). Also the teleomorph was reported from chickpea stubble in Western Australia in 2003 (Galloway and MacLeod 2003). In other parts of the world, mating types appear to be present in skewed proportions. For example, in Tunisia and Central Anatolia, more MAT1-1 type isolates were observed than MAT1-2 (Bayraktar et al. 2007; Rhaeim et al. 2007; Rhaeim et al. 2008). In India, Greece, Italy and Morroco, MAT1-2 is dominant (Navas-Cortes et al. 1998; Ali et al. 2010). Considering that many original seed imports to Australia were from India (Brinsmead 1994; Siddique and Sykes 1997), it is possible that more MAT1-2 was introduced, and perhaps MAT1-1 has reduced due to genetic drift. Indeed, mating type analysis of 37 A. rabiei isolates collected from seven different regions in India showed that all are MAT1-2 (Barve et al 2004), rendering the genetic diversity to be less diverse (total of 15 alleles) in comparison to other parts of the world (total of 26 alleles) using one microsatellite locus due to the tendencies of asexual reproduction of the fungus (Barve et al 2004; Santra et al 2001). Since these isolates were analysed with RAPD (Santra et al 2001) and just one microsatellite
locus (Barve et al 2004), its gene diversity of the Indian isolates could not be easily compared to the Australian *A. rabiei* gene diversity. Further research such as utilising more microsatellite loci on the Indian isolates will enable a comparison of its diversity and genetic similarity to the ones collected in Australia.

Although current Australian *A. rabiei* is reproducing asexually and generating clonal populations, this does not preclude a significant breakdown in host resistance. Whilst sexually reproducing fungi have the advantage of recombination, which may lead to higher fitness and faster adaptation in changing environments (Browne and Hoopes 1990), asexually reproducing fungi may contain combinations of well-adapted alleles that also lead to high pathogenic fitness and are preferentially inherited to result in high frequencies within a population (Browne and Hoopes 1990; Stukenbrock et al. 2006). Through positive selection, genotypes that have broad tolerance to host resistance and low fitness variance to abiotic factors such as fungicides will generally be rapidly replicated. Either as a result of founder effect and/or positive selection in our study, high frequencies of two dominant clonal haplotypes were found in all growing regions. Since these are obviously adapted over a wide range of environments and hosts, and mutations of these haplotypes may pose the greatest risk to disease management strategy including host resistance in the near future,

Host genotype selection

Progressive deployment of resistant cultivars causes pathogen populations to evolve in response to changes in the host population (McDonald et al. 1996). Environmental heterogeneity plays a significant role in shaping the pattern of species diversity (Rosenzweig 1995). Two types of adaptations are usually observed in heterogeneous environments; generalised adaptation resulting in specialised fungal haplotypes which are optimally adapted to the most prevalent host, and localised adaptation (maladaptation) of fungal haplotypes that are uniquely adapted to a specific host. Both lead to the selection of fungal haplotypes with the highest pathogenic fitness that generally increases in frequency as they have higher reproductive and survival capacities (Mettler and Gregg 1969; Leonard 1977). Due to the cultivation of a wide range of host genotypes, we expected that localised adaptation will occur and there will be a significant difference in the genetic composition among the populations sampled from different host genotypes with differing levels of susceptibility. Instead, we found low genetic diversity, high clonality and no significant difference among all host genotypes, which provided little evidence of selection or adaptation towards any of the host genotypes. Furthermore, no or very low genetic differentiation was found between populations from different host genotypes within the same geographic region, and the majority of genetic variation was distributed among individuals and was not due to differences among source host genotypes. The resistant and susceptible host genotypes within and among regions were found to share 12 out of 35 haplotypes in which one of them was shared 131 times out of 206 isolates tested, indicating it to be one of the fittest common
isolates in the current populations as shown on the median joining network. The most common isolate may be the first to be introduced in Australia and the high frequency of the isolate may demonstrate that it had the most extensive time to spread in the chickpea growing regions. A single large node comprising most of the haplotypes in the median joining network also indicated that the populations are derived from a single haplotype. These indicated no selection for a particular fungal clone on either the resistant or susceptible chickpea genotypes.

Selection is thought to have the least impact on population subdivision and adaptation in comparison to other evolutionary forces such as mating system, mutation and gene flow (McDonald et al. 1996). However, this does not mean that diversifying selection is not operational in the Australian A. rabiei pathosystem. To detect selection, the population tested needs to be in equilibrium (Kassen and Bell 1998). The current A. rabiei populations were in linkage disequilibrium likely due to the asexual reproduction system. Therefore, although there may be a trade-off between fungal haplotypes that are fit and aggressive, selection processes will not be detectable. Populations with clonal lineages are usually highly mutable (Lebreton et al 1998; Montarry et al 2006), and beneficial mutations can aid adaptation of specific fungal haplotypes. However, in some cases, accumulation of mutations in several fungal haplotypes may cause little functional interference in the environment and result in a more generalised pathogen adaptation (Reboud and Bell 1997). In a study by Elliott et al. (2011), pathogenicity on Australian A. rabiei isolates was diverse, even within clonal SSR lineages as identified in this study, further suggesting that selection does not play a major role in these populations.

However, Australia's position of population uniqueness and any advantages for disease management may be on the cusp of change with the recent wide adoption of new and different resistance genes and the ever present potential for further introductions. To identify the risk for directional selection and consequent erosion of specific resistant cultivars through introduction of potential increased pathogenicities, a more thorough spatial and temporal investigation of the population is required (Ahmed et al. 1996; Otto 2000) Therefore, it will be important to monitor for changes in allele or haplotype frequencies of the unique and clonal isolates in subsequent seasons.

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