

Population genetic structure of *Rhizoctonia solani* AG 3-PT from potatoes in South Africa

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Highlights

- The genetic diversity and structure of *Rhizoctonia solani* AG 3-PT was investigated by microsatellite analyses.
- High levels of genetic diversity were revealed within and between populations in South Africa.
- The populations were genetically and geographically differentiated.
- Evidence of mixed reproductive system of clonality and recombination was revealed.

Abstract

Rhizoctonia solani AG 3-PT is an important potato pathogen causing significant yield and quality losses in potato production globally. However, little is known about the levels of genetic diversity and population structure of this pathogen in South Africa. A total of 114 *R. solani* AG 3-PT isolates collected from four geographic regions were analyzed for genetic diversity and structure using eight microsatellite loci. Microsatellite analysis found high intra-population genetic diversity, population differentiation and evidence of recombination. A

total of 78 multilocus genotypes (MLGs) were identified with few MLGs shared among populations. Low levels of clonality (13-39 %) and high levels of population differentiation were observed among populations. Most of the loci were in Hardy-Weinberg equilibrium and all four field populations showed evidence of a mixed reproductive mode of both clonality and recombination. The PCoA clustering method revealed genetically distinct geographic populations of *R. solani* AG 3-PT in South Africa. This study showed that populations of *R. solani* AG 3-PT in South Africa are genetically differentiated and disease management strategies should therefore be applied accordingly. This is the first study of the population genetics of *R. solani* AG 3-PT in potatoes in South Africa and results may help to develop knowledge-based disease management strategies in South Africa and elsewhere.

Keywords:

Rhizoctonia solani, AG 3-PT, genetic diversity, population structure, microsatellite, recombination

1. Introduction

Rhizoctonia solani Kühn is a basidiomycete fungus associated with economically important diseases on potatoes globally (Banville et al. 1989). It is considered a species complex which has been classified into 13 anastomosis groups (AG 1 - AG 13) on the basis of hyphal fusion (Carling 1996). Anastomosis group 3 (AG 3) is predominantly associated with Solanaceae crops and is subgrouped into AG 3-PT on potato, AG 3-TB on tobacco and AG 3-TM on tomato (Bartz et al. 2010).

Globally, AG 3-PT has been considered as the predominant AG most commonly associated with potato diseases (Banville 1989; Tsrer 2010). In addition to *R. solani* AG 3-PT, several other AGs, AG 2-1; AG 4; AG 5; AG 7; and AG 8 have been reported as pathogens of potato albeit at lower frequencies (Anguiz and Martin 1989; Balali et al. 1995; Campion et al. 2003; Das et al. 2015; Truter and Wehner 2004; Woodhall et al. 2007).

R. solani AG 3-PT incites several symptoms on potatoes including black scurf, stem and stolon canker, elephant hide, tuber growth cracks and formation of white collar on the base of the stem (Campion et al. 2003; Muzhinji et al. 2015; Woodhall et al. 2008). Despite

the importance of *R. solani* AG 3-PT in potato production systems in South Africa (Muzhinji et al. 2015; Truter and Wehner 2004), the genetic diversity, genetic structure and reproduction mode are still unclear and have never been investigated in the country. Understanding the population genetic structure of *R. solani* AG 3-PT could help in implementing targeted and knowledge-based *R. solani* management strategies on potatoes.

In South Africa and other potato growing regions globally, seed tuber-borne inoculum plays an important role as a mechanism of long distance dispersal of the pathogen between potato growing areas (Cubeta and Vilgalys 2000; Truter and Wehner 2004; Tsrer 2010). In South Africa, the use of disease-free and fungicide treated seed tubers by potato growers restrict and mitigate the dissemination of the *R. solani* seed tuber-borne inoculum (DAFF 2013).

Several strategies have been used to control field populations of *R. solani* on potatoes including; crop rotation, chemical control and the use of disease resistant cultivars. Reports of successful use of crop rotation have been variable due to wide host range and long-term survival of sclerotia in the soil (Ritchie et al. 2013) and concern over the relative importance of seed tuber-borne inoculum (Woodhall et al. 2013). Chemical control has been used successfully, but the effects of chemicals on the environment and the variability of sensitivity of individual AGs to fungicides have led to a search for alternatives (Campion et al. 2003). The use of resistant cultivars has seldom been successful for *R. solani*, although earlier studies have reported potato germplasm with variable responses to *R. solani* AG 3-PT (Khandaker et al. 2011). However, the use of disease resistant cultivars is thought to be ineffective for pathogenic fungi with high genetic variation and evolutionary potential (McDonald 1997). Therefore, successful breeding strategies depend on the knowledge of the pathogen's genetic diversity and structure.

R. solani does not produce asexual spores but exists as mycelium (hyphal growth form) and sclerotia (asexual resting structures comprised of a compact mass of hyphae) in association with plant debris, soil or seed tubers (Keijer 1996; Ritchie et al. 2013). Isolates of *R. solani* AG 3-PT have been traditionally considered to reproduce through asexual or clonal means. Not surprisingly, the *R. solani* disease cycle is hypothesized to be monocyclic and starts from overwintered asexual inoculum, present as sclerotia and/or mycelium in the soil and/or on potato seed tubers (Cubeta and Vilgalys 1997). Based on these observations, *R. solani* AG 3-PT on potatoes, should, presumably in theory, show low levels of genetic diversity and a simple genetic structure.

However, recent studies on the genetic diversity and population structure of *R. solani*

AG 3-PT have revealed a high level of genetic diversity (Fiers et al. 2011) and a mixed reproductive system that includes recombination and clonality (Ceresini et al. 2002, 2003; Ferrucho et al. 2013). Burdon and Roelfs (1985) emphasized that high levels of genetic and morphological diversity in fungi could only be explained parsimoniously by recombination or sexual reproduction. Goodwin et al. (1992) supported the assertion that populations that undergo recombination are more genetically diverse than those that reproduce asexually. Therefore, some of the plausible explanations for high genetic diversity within *R. solani* AG 3-PT populations reported in earlier studies include recombination, and gene flow (McDonald and Linde 2002).

In *R. solani* AG 3-PT populations, the evidence of genetic diversity has led to the speculation that recombination might be occurring in potato fields through formation of meiotic basidiospores bearing hymenia (Cubeta and Vilgalys 1997; Ogoshi 1987). Basidiospores of *Thanatephorus cucumeris* Frank Donk (teleomorph of *R. solani*) have been observed in potato fields during cool and moist periods, but their role in *R. solani* disease epidemics is poorly understood (Ogoshi 1987). Basidiospores produced by some *Rhizoctonia* AGs have been reported to be primary inoculum sources for *Rhizoctonia* disease epidemics in other plant species, such as web blight on beans (Godoy-Lutz et al. 1996), tobacco (Shew and Melton 1995), rice (Chaijuckam et al. 2010) and tomato (Bartz et al. 2010).

The detection of mating reactions in *R. solani* has been hindered by the presence of multinucleate hyphae and lack of clamp connections, making it impossible to distinguish between homokaryons and heterokaryons *in vitro* (Cubeta and Vilgalys 1997). Some AGs have been proposed to possess a heterothallic mating system while others are assumed to possess a homothallic mating system (Cubeta and Vilgalys 1997). Molecular-based methods have provided powerful approaches to infer the reproductive mode of many fungal pathogens (Zhang et al. 2003). Population genetics studies have challenged the perception of a purely asexual reproductive mode in other AGs and subgroups of *R. solani* especially AG 1 (Bernardes-de-Assis et al. 2009; Ciampi et al. 2008; Linde et al. 2005; Rosewich et al. 1999). Analysis with co-dominant restriction fragment length polymorphism (RFLP) loci in AG 1-1A populations collected in United States (Texas and Louisiana) provided evidence of clonal reproduction and recombination (Rosewich et al. 1999). Similar findings were confirmed in subsequent studies as reported for AG 1-1A in India (Linde et al. 2005); Brazil (Ciampi et al. 2008); Louisiana (Bernardes-de-Assis et al. 2008); China (Bernardes-de-Assis et al. 2009) and Latin America (González-Vera et al. 2010). Using microsatellites, Haratian et al. (2012) showed a mixed model of population structure in AG 4 in Iran that includes both

recombination and clonality. Recent studies that have been conducted on the genetic differentiation and the reproductive mechanism of *R. solani* AG 3-PT populations in North Carolina revealed a mixed reproductive system that includes recombination and clonal propagation (Ceresini et al. 2002, 2003). Ferrucho et al. (2013) demonstrated a similar phenomenon for *R. solani* AG 3-PT in Columbia.

Pathogens with higher diversity and evolutionary potential pose a high risk of overcoming host resistance and developing fungicide resistance (Lin and Gudmestad 2013). Currently, information regarding the relationship between genetic diversity, structure and epidemiology of *R. solani* AG 3-PT on potatoes in South Africa is still unknown. Understanding the biology of *R. solani* AG 3-PT in combination with the epidemiology will facilitate development of effective integrated disease management strategies for *Rhizoctonia* in potato production systems in South Africa. The objective of this study was therefore to determine the genetic diversity within populations of *R. solani* AG 3-PT; to evaluate the population genetic structure of *R. solani* AG 3-PT and to infer the reproductive mode of *R. solani* AG 3-PT populations causing potato diseases.

2. Materials and Methods

2.1 Fungal collection and AG identification

Rhizoctonia solani AG 3-PT isolates were obtained from potato fields located in different geographical regions in 2013. Symptomatic plants were collected from a single naturally infected field in each geographic region, viz. Limpopo (LP), Western Cape (WC), North-West (NW), and KwaZulu-Natal (KZN) (Fig 1, Table 1). The geographic regions sampled have different climates and growing seasons. Field isolates were sampled using a hierarchical method similar to that described by Razavi and Hughes (2004). Isolates from each collection site were considered as a separate population. The selected geographic regions are widely distributed throughout South Africa and span an area of about 1 800 km South-West to North and 750 km North to East. Isolates were recovered from black scurf, stem/stolon canker and elephant hide symptoms.

Fungal isolations were made by first washing the infected plant material showing visible signs of *R. solani* symptoms under running tap water. Small pieces of infected tissue were excised from the affected area and plated on 1.5% water agar amended with 50 mg/l of streptomycin sulphate (Sigma-Aldrich). The plates were incubated at 25°C for 48 h. Hyphal

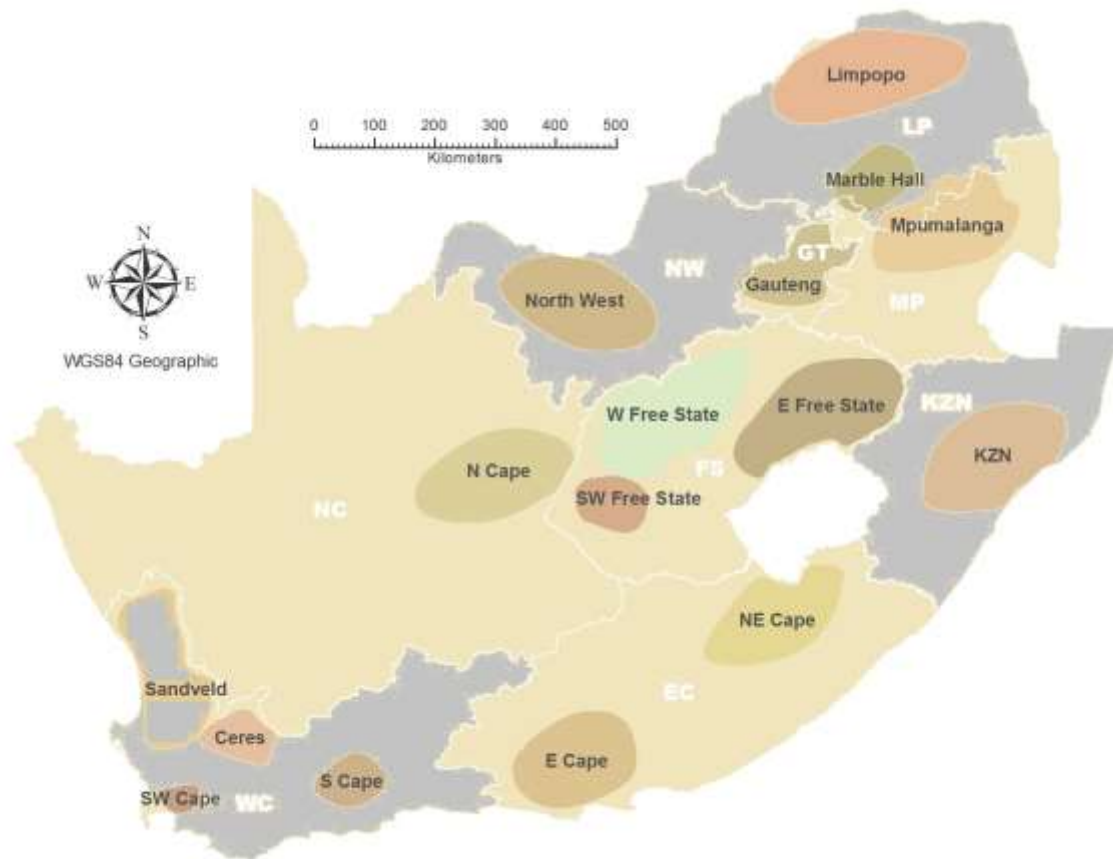


Fig 1 Map showing all the potato growing regions of South Africa. Grey shaded area shows the geographic regions where *R. solani* AG 3-PT isolates used in this study were obtained. (L. van Zyl, TerraGIS)

Table 1 Populations of *R. solani* AG 3-PT

Geographical region	Population code ^a	Linear Distance (km) ^b	Sample	
			Size	Sampling year
Limpopo	LP	0	24	2013
KwaZulu-Natal	KZN	739	31	2013
Western Cape	WC	1487	28	2013
North-Western	NW	687	31	2013

^aPopulation codes to be used hereafter

^bDistance between growing regions were calculated relative to Limpopo

tips of *R. solani* cultures were transferred to 90 mm diameter plates containing Potato Dextrose Agar (PDA, Biolab) and allowed to grow for 7 days. The anastomosis group of each

isolate was confirmed by sequencing of the ribosomal DNA internal transcribed spacer (ITS) region as previously described by Muzhinji et al. (2014, 2015).

2.2 DNA extraction and microsatellite genotyping

Total genomic DNA was extracted from mycelia of all isolates of *R. solani* AG 3-PT by using ZR soil microbe DNA kitTM (Zymo Research Corporation, Irvine CA, USA) according to the manufacturer's protocol recommendations. The concentration and quality of extracted DNA was determined by NanoDrop UV spectrophotometry (NanoDrop Technologies). Eight codominant microsatellites (Table s1) designed by Ferrucho et al. (2009) were used for genotyping isolates. Forward primers labelled with FAM, VIC, NED and PET dye sets were obtained from Applied Biosystems, UK and non-labelled primers were obtained from Integrated DNA Technologies (Iowa, USA). The PCR was performed separately for each locus in a 20 µl volume containing a final concentration of 15 ng of DNA template, 2 µl of 10x PCR buffer, 1.5 mM MgCl₂ 0.4 mM dNTP, primers at 0.3 µM each and 1 U *Taq* Polymerase. Reactions were carried out in a BioRAD thermocycler according to the following programme: 96 °C for 5 min followed by 35 cycles of 96 °C for 30 s, annealing temperature (Table s1) for 30 s and 72 °C for 1 min, and a final extension step at 72 °C for 7 min. Initially, six isolates representing each population were used to optimise the PCR and capillary electrophoresis. Before capillary electrophoresis, 3 µl of PCR products were mixed with loading buffer, separated on 1.5% agarose gel and viewed under UV light to verify the approximate sizes of the amplicons. The amplicons were also evaluated on capillary electrophoresis to determine the appropriate dilution ratio for subsequent analysis. Finally, PCR amplicons were mixed, diluted and capillary electrophoresed on an ABI 3730x1 Genetic Analyser (Applied Biosystems, Forest City, California) at the Bioinformatics and Computational Biology Centre, University of Pretoria, by loading 1 µl of the diluted PCR amplicons, 9 µl formamide, and 0.1 µl GeneScan-LIZ 500 internal size standard (Applied Biosystems). All 8 loci were analysed in one multiplex panel. Allele sizes in base pairs were called using GeneMapper v. 4.1 (Applied Biosystems). Samples that produced ambiguous or negative results on a first attempt were repeated. Reproducibility of the molecular markers was tested by contrasting sizes of each allele at every locus for 8 isolates randomly selected from the three populations (KZN: Rh3a, Rh4a, Rh5a; LP: Rh101, Rh103, Rh108; NW: Rh9801, Rh12).

2.3 Data analysis: Microsatellite information content

For this genetic study, dikaryotic *R. solani* AG 3-PT was treated as a functional diploid organism (Ceresini et al. 2002; Justesen et al. 2003). Data on the number of alleles per locus, identification of private alleles (allele present only in one population), and allelic frequencies per locus and per population were assessed using the program CONVERT v. 1.31 (Glaubitz 2004).

2.4 Genotype diversity

Two types of data sets were generated for the analysis: one using all isolates (non-clone-corrected data) and the other using clone-corrected data set in which isolates with identical multilocus genotypes (i.e. alleles identical at all eight loci) were represented only once in each population (Chen and McDonald 1996). A multilocus genotype (MLG) for each strain was determined using the program GENOTYPE (Meirmans and van Tienderen 2004) and isolates having the same MLG were assumed to be clones. Several parameters, including the number of genotypes per population; site specific genotypes and its evenness (G_o scaled by the maximum number of expected genotypes) were determined using the GENODIVE v 2.0b7 program (Meirmans and van Tienderen 2004). Genotypic diversity ($(n/n-1) (1-p_i^2)$), was computed in the program MULTILOCUS v 1.3 (Agapow and Burt 2001) where p_i is the frequency of the i th genotype and n is the number of individual samples. Genotypic diversity is the probability that two individuals taken at random had distinct genotypes; 1 if every individual was different and 0 if they were clones. To test whether pairs of populations differed in genotype diversity, we used a bootstrapping approach, where individuals were resampled from the populations and the diversity indices compared after every replicate using 1 000 permutations with subsampling to match the size of the smallest population (Grunwald et al. 2003). The clonal fraction, that is, the proportion of fungal isolates originating from asexual reproduction was calculated as $1 - ([\text{number of different genotypes}] / [\text{total number of isolates}])$ (Zhan et al. 2003).

2.5 Analyses of gene diversity

Gene diversity was quantified based on allelic richness and expected heterozygosity. Allelic richness was calculated according to El Mousadik and Petit (1996) as the mean

number of alleles per locus for a standardized minimum sample size of nine using refraction index (Hurlbert 1971) to eliminate the influence of sample size and was implemented in FSTAT v 2.9.3.2 (Goudet 1995). Expected heterozygosity, or Nei's unbiased gene diversity (corrected for sample size), was calculated according to Nei (1978) as $n / (n - 1) \times (1 - \sum_i p_i^2)$ where p is the observed frequency of the i th allele and n is the sample size. To test whether populations differed for allelic richness and Nei's gene diversity, a bootstrapping approach based on 1 000 permutations was performed, also using the program FSTAT 2.9.3.2. Allelic richness and heterozygosity are important measures of population genetic diversity (Petit et al. 1998).

2.6 Genetic variation and population differentiation

Analysis of molecular variance (AMOVA) was used to calculate the overall genetic differentiation of *R. solani* AG 3-PT throughout South Africa using the F - statistics (F_{ST}) (Wright 1965). The relative contributions of within population genetic variation, between population within geographic locations, and between provinces were calculated using 10 000 permutations by a non-parametric approach using the program ARLEQUIN v. 3.5.2 (Excoffier and Lischer 2010). F_{ST} is useful for estimating the genetic uniqueness of individual populations within a group of populations (Gaggiotti and Foll 2010). The null distribution of pairwise F -statistics under the hypothesis of no differentiation between two populations was obtained by computing genotypes between populations. The null hypothesis was rejected when $p \leq 0.05$. A population cluster assignment was determined by Principal Component Analysis (PCoA). The PCoA was performed in GeneAIEx 6.5 (Peakall and Smouse 2012) using pairwise genetic distance between sites. PCoA is a multivariate technique that does not assume random mating or linkage equilibrium (Peakall and Smouse 2012).

2.7 Hardy-Weinberg equilibrium and gametic equilibrium tests

The relative contribution of clonality and sexual recombination to the genetic structure of *R. solani* AG 3-PT field populations was also analysed. The tests of Hardy-Weinberg equilibrium (HWE) and multilocus association were performed to determine the associations within and among loci (Guo and Thompson 1992). Tests for HWE exact test was executed for each locus within each population using the Markov Chain Monte Carlo

(MCMC) approach, with chain length 100 000 and dememorisation 1 000 implemented in ARLEQUIN v 3.5.2. In-breeding coefficient (F_{IS}) was calculated for detection of excess or deficit of heterozygotes, which may be the reason for deviation from HWE (Weir and Cockerman 1984). This was implemented in ARLEQUIN v. 3.5.2 based on 10 100 permutations. Associations among loci were tested using Fisher's exact test (probability test) based on MCMC algorithm with 1 000 batches and 1 000 iteration/batch, implemented in GENEPOP v. 4 (Raymond and Rousset 1995) after Bonferroni correction for large number of multiple comparisons to prevent false rejection of the null hypothesis (Rice 1989).

The linkage disequilibrium was assessed using the multilocus index of association (I_A) and the unbiased estimate of multilocus linkage disequilibrium (r_{barD}) executed in Multilocus 1.3b (Agapow and Burt 2001) and were calculated based on 1 000 randomisations and fixing missing data during randomisations. In brief, I_A is a measure of linkage disequilibrium and if I_A is zero it indicates linkage disequilibrium, that is, alleles at different loci are randomly associating in a population and is an indication of clonally reproducing populations. If I_A is significantly different from zero it indicates linkage disequilibrium. The r_{barD} index accounts for differences in number of loci and it ranges from zero, when there is no disequilibrium, to one when there is disequilibrium among tested loci (Agapow and Burt 2001).

3. Results

3.1 Microsatellite information content

Eight microsatellite loci were used to characterise the genetic diversity and structure of four field populations of *R. solani* AG 3-PT in South Africa. A total of 46 alleles were detected from eight microsatellite loci among the 114 *R. solani* AG 3-PT isolates used in this study (Table s2). The total number of alleles ranged from 29 (NW) to 33 (WC). The number of alleles observed at each locus ranged from 2 (TC_AG3_0, TC_AG3_10) to 9 (TC_AG3_18), with an average of 5.8 alleles per locus. A total of 14 private alleles were found in all four field populations (Table 2). The eight microsatellite loci showed high discriminating power among isolates. The percentage of polymorphic loci ranged from 75% to 100% (Table 2).

Table 2 Population genetic parameter of each of the four field populations of *R. solani* AG 3-PT from potatoes in South Africa

Population	% of polymorphic loci	N ^a	Number of genotypes ^b	Site specific genotypes ^c	Genotypic diversity ^d	Clonal fraction ^e	Evenness ^f	Allelic richness ^{g,h}	H _E ⁱ	Private alleles ^j
WC	100	28	18	17(1)	0.94	0.27	0.84	3.51	0.54	7
NW	100	31	20	18 (2)	0.88	0.35	0.89	3.37	0.55	2
KZN	75	31	19	19(0)	0.60	0.39	0.56	3.38	0.48	2
LP	100	24	21	19(2)	0.99	0.13	0.91	3.78	0.52	3
Total		114	78	73 (5)	0.85	0.29	0.8	3.51	0.52	14

^aPopulation sample^bNumber of genotypes per population^cNumber of genotypes shared with other populations are shown in brackets^dGenotypic diversity was calculated using MULTILOCUS v 1.3; diversity was defined as the probability that two individuals taken at random had distinct genotypes, where the value was 1 if every individual was the same (Agapow and Burt 2001)^eClonal fraction was calculated as 1-(number of different MLGs/total number of isolates present in a population) (Zhan et al. 2003)^fAn evenness value = 1.0 indicates that all genotypes have equal frequencies (Grunwald et al. 2003)^gTo test whether pairwise samples differed for Nei's unbiased gene diversity and allelic richness, FSTAT v. 2.9.3.2 was used (Goutet 1995)^hCalculated according to El Mousadik and Peti (1996), with subsampling to match the sample size of the smallest populationⁱNei's unbiased gene diversity (Nei 1978) also known as expected heterozygosity, averaged over all loci, corrected for sample size.^jAlleles occurring only in one population, determined with Convert v 1.31 (Glaubitz 2004)

3.2 Gene and genotypic diversity

Among the 114 *R. solani* AG 3-PT isolates analyzed, 78 MLGs were identified (Table 2). On average, 19.5 MLGs were found per locality and the number of MLGs per clone ranged from two to seven, with two clones represented by seven isolates each and 64 clones represented by five or fewer isolates. MLGs were mainly locality specific indicating that the populations were heterogeneous, and distributed within the same field. In all, two MLGs were shared between LP and NW field populations which are separated by distance of approximately 700 km. No genotypes were shared between the two most distant populations in South Africa, LP and WC.

The average allelic richness per locus was found to be 3.51 with the LP population having the highest value of 3.78 while the NW population had the lowest value of 3.37

(Table 2). The expected heterozygosity (H_E ; Nei's unbiased gene diversity) ranged from 0.48 to 0.55 across populations (Table 2), with the highest H_E in NW and lowest in KZN (0.48). Nevertheless, Nei's gene diversity values were not significantly different ($P \leq 0.05$). The lowest clonal fraction was observed in LP population (0.13) while the highest clonal fraction was found in KZN populations (0.39). Genotypic diversity was very high, ranging from 0.60 to 0.99 indicating a large number of individuals within a population. There were no significant differences between three populations pairs for genotypic diversity except for KZN population which had the lowest value of 0.6 as well as the lowest evenness value of 0.56 (Table 2).

3.3 Distribution of genetic variation and population differentiation

The AMOVA was used to partition total genetic diversity among regions, population within regions, and within populations. The population variation showed that variation among populations was 17.55% of the total variance. The majority of total genetic variation observed in *R. solani* AG 3-PT population, 89.78%, was distributed within a population (Table 3). The overall F_{ST} value was 0.176 indicating high differentiation among the four field populations (Table 3).

Table 3 Analysis of molecular variance (AMOVA) within and among four *R. solani* AG 3-PT field populations in South Africa

Source of Variation	df	Observed partition				P value ^b
		Sum of squares	Variance Components	Percentage of variation (%)	Fixation indices ^a	
Among populations	3	76.69	0.42	17.55	$F_{ST} = 0.176$	0.000*
Among individuals within a population	74	196.55	-0.175	-7.34	$F_{IS} = -0.046$	0.93
Within populations	78	243.5	2.142	89.78	$F_{IT} = 0.102$	0.006
Total	155	516.7	2.86			

^aFixation index, a measure of population differentiation.

^bProbability of obtaining more extreme random variance component and F_{ST} than the observed values by chance alone

* Not significant at $p < 0.05$

Populations from LP and WC were significantly differentiated from other populations, having the highest pairwise F_{ST} of 0.34, $P=0.00$. Pairwise F_{ST} ranged from 0.03 to 0.34 (Table 4). The degree of genetic differentiation F_{ST} , increased with increasing distance and no significant differentiation being detected between populations LP and NW ($F_{ST} = 0.03$, $P = 0.12$). The shortest distance between the two populations was 700 km between NW and LP, while the longest was 1860 km between LP and WC.

Table 4. Measures of population differentiation among four populations of *Rhizoctonia solani* AG 3-PT from South Africa based on F_{ST} values^a

Population	WC	NW	KZN	LP
WC				
NW	0.225			
KZN	0.134	0.212		
LP	0.338	0.033*	0.27	-

^a F_{ST} were computed between pairs of populations with 1023 permutations in ARLEQUIN v 3.5.2

(Excoffier and Lischer 2010)

*Non-significant values ($P \geq 0.05$) based on 1,023 permutations.

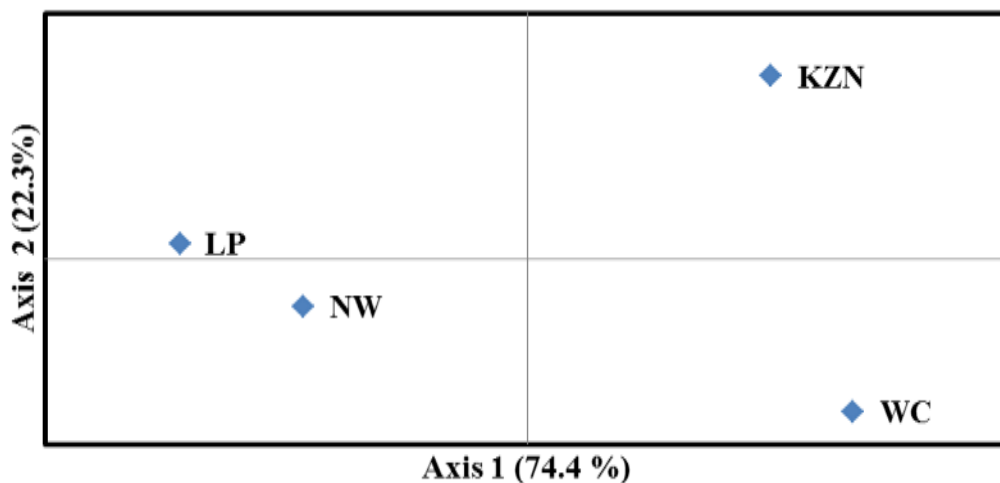


Fig 2 Principal coordinates analysis (PCoA) of four field populations of *R. solani* AG 3-PT in South Africa based on Nei's genetic distance using GenAIEX6. Percentage variation explained by each axis (principal coordinate), is shown in parenthesis

The PCoA analysis indicated that the *R. solani* AG 3-PT populations were genetically differentiated from each other according to geographical origin (Fig 2). The PCoA indicated that each population (WC, LP, NW, and KZN) clustered in different quadrants. The PCoA results corroborated with F_{ST} where LP and NW populations were less differentiated. These findings indicated there may be geographic genetic structure divided into east, south-west and north, north-west and the possibility of isolation-by-distance.

3.4 Hardy-Weinberg equilibrium and multilocus association

The null hypothesis of random mating was tested and results of HWE are shown in Table 5. Populations were in HWE for 37.5% to 75% of the loci. WC and LP population deviated from HWE in four of the eight polymorphic loci analyzed. NW populations deviated from HWE in five of the eight loci tested. KZN was in HWE for six of the eight loci tested. The I_A was significantly differentiated from zero ($p < 0.001$) for three of the four populations. In these populations, the observed I_A values fell outside the distribution for random data sets, which is evidence of non-random association of alleles. In contrast the observed I_A for the WC population fell inside the distribution for random data sets and was in gametic equilibrium (Table 5). The proportion of loci with significant pairwise deviations from equilibrium ranged from 10.2 % to 15.5%. Negative values of F_{IS} were obtained for all the populations (Table 5). Negative values of F_{IS} indicate outbreeding and excess heterozygosity which might be the reason for deviation from HWE. Pudolvkin et al. (1996) hypothesized that excess heterozygosity is due to different allelic frequencies in male and female parents due to a small breeding population caused by binomial sampling error.

Table 5 Test for random association of alleles within each locus and between pairs of loci in field populations of *R. solani* AG 3-PT isolates in South Africa

Growing region	Clone corrected	Number of loci under HWE ^a	F_{IS} ^b	p Value	r_{barD} ^c	I_A ^d	p Value	Locus pairs ^e	%
WC	18	4/8	-0.006	0.56	0.17	0.16	<0.01	2/21	9.5
NW	20	3/8	-0.14	0.91	0.05	0.338	0.028	5/28	17.9
KZN	19	6/8	-0.08	0.78	0.017	0.12	0.2	4/28	14.3
LP	21	4/8	-0.153	0.97	0.003	0.02	0.42	3/28	10.7

^aHWE (Hardy-Weinberg Equilibrium) test performed according to an exact test analogous to Fisher's exact test, using a

Markov chain with forecasted length of 10, 1000 (Guo and Thompson 1992)

^bPopulation specific F_{IS} indices and p values calculated based on 10,100 permutations using ARLEQUIN 3.5.2 (Excoffier and Lischer 2010)

^cThe r_{barD} is a modification of IA which accounts for differences in number of loci was computed in MULTILOCUS v1.3b

^dIndex of association (I_A), the random association of alleles among distinct loci pairs was computed in MULTILOCUS 1.3b, significance of I_A was tested by comparing the observed value and the expected value under null hypothesis of random mating with 1,000 randomisations (Agapow and Burt 2001), *indicates significant at $\alpha = 0.05$

^eNumber of pairs of loci with significant disequilibrium according to Fischers's exact test (probability using both a Markov chain with 1,000 batches and 1,000 iteration/batch, implemented in GENEPOP after Benferroni correction for multiple comparisons (Rice 1989)

4. Discussion

Population genetic diversity and structure studies were conducted for *R. solani* AG 3-PT in potato fields located in different geographical locations in South Africa. The null hypothesis of this study was of no population differentiation of *R. solani* AG 3-PT populations between field populations located in different geographic locations as a result of gene flow. An alternative hypothesis was that *R. solani* AG 3-PT field populations are geographically differentiated (isolation-by-distance) and recombining.

4.1 Gene and Genotypic diversity

The expectations from our null hypothesis, for a clonal fungus are low genotypic diversity, high clonal fraction, gametic disequilibrium and markers under HWE (Milgroom 1996). In this study high levels of gene and genotypic diversity were observed in South African *R. solani* AG 3-PT populations. The overall clonal fraction was found to be 0.29 consistent with clonal propagation (asexual). The lowest clonal fraction of 0.13 was found from the LP population and the highest from the KZN population (0.39). Ferrucho et al. (2013) obtained a clonal fraction ranging from 0.06 to 0.33 in *R. solani* AG 3-PT populations from Colombia. The plausible explanation of low clonality would be frequent recombination.

The other likely alternative for a low clonal fraction might be the relatively small population sizes that were used in this study reducing the probability of finding locality specific multilocus genotypes. All the populations exhibited high levels of genotypic diversity (0.6 - 0.99), indicating a large number of different individuals, consistent with sexual reproduction. Sexual reproduction increases genetic variation in populations by generating new alleles through intragenic recombination as well as novel allele combinations (multilocus genotypes) by shuffling existing alleles (Qu et al. 2013).

The observed low to moderate clonal fractions and high genotypic diversity observed in this study are consistent with asexual reproduction and sexual reproduction, respectively. Therefore *R. solani* AG 3-PT populations in South Africa show a mixed reproductive system. The results of a mixed reproduction system corroborate with previous studies on other populations of *R. solani* AG 3-PT (Ceresini et al. 2002; Ferrucho et al. 2013), AG1-IA (Bernardes-de-Assis et al. 2008; Gonzalez-Vera et al. 2010; Linde et al. 2005; Padasht-Dehkaei et al. 2013; Rosewich et al. 1999; Wang et al. 2015), AG 4 (Haratian et al. 2012) and *R. oryzae-sativae* (Chaijuckam et al. 2010).

4.2 Genetic variation and population differentiation

The AMOVA statistic, F_{ST} , was 0.176 ($P < 0.00$), evidence of high genetic differentiation within *R. solani* AG 3-PT populations, with 89.78% of molecular variance explained within the populations and 17.5% among populations. Some studies have led to the generalization that outcrossing species maintain most genetic variability within, rather than among, populations, whereas inbreeding species partition most variability among populations (James et al. 1999).

The results from this study showed that the *R. solani* AG 3-PT populations are geographically differentiated especially for the most distant populations. The south west and eastern populations (WC, KZN) were differentiated from the north western populations (LP, NW). This is supported by moderate to low gene flow between populations (F_{ST}). This is in contrast to the results of Ceresini et al. (2002) who found no population subdivision in *R. solani* AG 3-PT from North Carolina providing evidence of long distance gene flow. Ferrucho et al. (2013) found high levels of population subdivision between the most geographically distance populations in Colombia. High differentiation of populations observed in this study may be because sampled regions are geographically distant from each other and long distance dispersal of the pathogen is less frequent due to restricted geographic

distances. Furthermore, in South Africa potato seed tuber production is decentralized, and is done in each potato growing region and at different times of the year under different climatic conditions (Potatoes South Africa 2015). Centralized production of seed tubers would have facilitated long distance dispersal of seed tuber-borne inoculum of AG 3. Different climatic conditions in the sampled regions could also have contributed to genotypes adapted to that particular climate. In Limpopo, potatoes are produced under irrigation in winter and spring; in the Free State potatoes are produced in summer under dry land production, Western Cape has a Mediterranean climate and potatoes are produced in dry summer (Steyn et al. 2014).

The spatial analysis of principal coordinates (PCoA) divided the populations into regions. Populations from South Africa clustered into four genetic groups that interestingly corresponded to the geographic localities. WC and KZN had the most differentiated populations. Geographic separation was reported for AG 3-PT (Ferrucho et al. 2013) and AG 1 by (Ciampi et al. 2008). The genetic distance (F_{ST}) between the populations indicate geographical subdivision consistent with limited gene flow among potato growing regions. Geographic distance may have contributed to genetic differentiation of the two closely located populations. The observed highly significant genetic differentiation and limited gene flow contrast with other global reports that confirmed gene flow as shaping the population structure of *R. solani* AG 3-PT (Ceresini et al. 2002; Ferrucho et al. 2013).

4.3 Hardy-Weinberg equilibrium and gametic equilibrium tests

The exact test of HWE revealed a significant proportion of loci that conformed to HWE expectations, consistent with sexual reproduction. The lowest loci under HWE were found in NW population (37%) and a GD of 10.25%. The highest proportion of loci under HWE was found in KZN population (87.5%) and a GD of (15.3%) consistent with sexual production.

The inbreeding coefficients (F_{IS}) were calculated to test if departures from HWE and GD were due to excess heterozygosity. Negative values of F_{IS} indicate excess heterozygosity. No inbreeding was observed in any of the populations in this study indicating random mating of AG 3-PT populations in South Africa. Other AGs, AG 1-1C, AG 2-2, AG 4 and AG 8 have been found to have a bipolar heterothallic (outcrossing) mating system while AG 3 has been speculated to have a homothallic (in-breeding) mating system (Cubeta and Vilgalys 1997). Ceresini et al. (2002, 2007) and Ferrucho et al. (2013) reported evidence of inbreeding in AG 3-PT. This is in contrast to our findings that inferred AG 3 isolates to be

heterothallic (outcrossing) as indicated by negative values of F_{IS} . All of the six populations exhibited non-significant negative values. The negative F_{IS} values, including loci that conformed to HWE showed that an excess of heterozygotes was responsible for departures from HWE. The comparison with the above studies is however problematic due to differences in geographic distance between population sampling sites. High levels of genetic variation are generally expected for isolates sampled from wide geographical locations (James et al. 1999).

Based on the risk model framework proposed by McDonald and Linde (2002), pathogens, with a mixed mode of reproduction (sexual and asexual), have a great potential to adapt to selection pressures and fall into high risk category. *R. solani* AG 3-PT in South Africa would fall into the high-risk category. Therefore, in such given circumstances, disease management strategies that limit gene flow and population size would be the most effective (McDonald and Linde 2002).

In South Africa, farmers use fungicides for the control of *Rhizoctonia* on potatoes and this is likely to continue unabated as no truly resistant cultivars are available. In light of the high genetic diversity and structure of *R. solani* AG 3-PT it is recommended that several isolates representing different pathotypes and genetic backgrounds should be chosen for effective potato germplasm screening for resistance to *Rhizoctonia*.

This study provided initial data on the population diversity and genetic structure of *Rhizoctonia solani* AG 3-PT from potatoes in South Africa. Results from this study showed high genetic and genotypic diversity of *Rhizoctonia solani* AG 3-PT populations in South Africa.

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Supplementary data

Table s1. List of microsatellite used in this study Ferrucho et al. (2009)

Locus	Primer Sequence (5'-3')	Repeat motif	Labelling dye	Annealing T °C	No. of repeats (range)
TC_AG3_0	F: TGAGCGTGTTACGGAGTACA R: GTCTTCACCATCCTCATTGC	(GAC) ₅	PET	55	1-4
TC_AG3_1	F: CGACTTGGGATTTCGTCTAGTTC R: TCTGATCTACGAAGCAGGTCGTC	(TGG) ₁₁	6-FAM	55	1-11
TC_AG3_6	F: GATCTTTAGTCGTAGAGCGATGG R: ATATTGGGGATGTGGAGAGGTG	(TCG) ₈	PET	56	1-10
TC_AG3_7	F: GCGTTCGGTATGTCTATTTGC R: ACGGTTTCGTGATGAGATAGGAG	(GTG) ₉	VIC	56	2-10
TC_AG3_10	F: TTCTTTAGTGGACGCCGTAATC R: GACCAAGGAGGAAGAGATTGAG	(GCT) ₁₄	NED	55	7-9
TC_AG3_16	F: CAACCACCAATCACGGTTTATC R: CCCAGACAACACTCCAACAAC	(CTT) ₁₅	PET	55	1-26
TC_AG3_18	F: ATGTGCGGCTTGGATTGAATAG R: CGTGGCTTTGGTAAATCAGTC	(CTT) ₁₅	PET	56	4-17
TC_AG3_19	F: GGTTACAGAGGGACATCATAGTCG R: ACTCAGATGCACAACAATCGTC	(CAA) ₁₅	6-FAM	55	1-15

^a Range of amplified product sizes obtained in this study

Table s2. List of microsatellite markers and microsatellite content information of *Rhizoctonia solani* AG 3-PT isolates used in this study

Locus	Allelic size range (bp)	Total no. of alleles	Number of alleles per locus and population			
			WC	NW	KZN	LP
TC_AG3_0	151-160	2	2	2	2	2
TC_AG3_1	141-202	8	5	3	6	5
TC_AG3_6	197-215	5	3	5	4	3
TC_AG3_7	212-227	5	3	3	4	4
TC_AG3_10	245-248	2	2	2	2	2
TC_AG3_16	323-356	8	8	5	5	5
TC_AG3_18	321-356	9	6	3	3	4
TC_AG3_19	320-356	6	4	6	4	5
Total number of alleles		46	33	29	30	30
Average		5.8	4.1	3.6	3.8	3.8