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# Long-distance dispersal and recolonization of a fire-destroyed niche by a mite-associated fungus

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## ARTICLE INFO

### Article history:

Received 26 September 2014

Received in revised form

15 December 2014

Accepted 20 December 2014

### Keywords:

Fynbos

Knoxdaviesia

Ophiostomatoid

Recolonize

## ABSTRACT

The Fynbos Biome in the Core Cape Subregion of South Africa is prone to recurrent fires that can clear vast areas of vegetation. Between periods of fire, ophiostomatoid fungi colonize the fruiting structures of serotinous *Protea* species through arthropod-mediated dispersal. Using microsatellite markers, this study considered the process whereby a *Protea*-associated ophiostomatoid fungus, *Knoxdaviesia proteae*, recolonizes a burnt area. The genetic diversity, composition and structure of fungal populations from young *P. repens* plants in a recently burnt area were compared to populations from the adjacent, unburnt *Protea* population. The only difference between *K. proteae* populations from the two areas was found in the number of private alleles, which was significantly higher in the unburnt population. The population structure, although weak, indicated that most *K. proteae* individuals from recently burnt areas originated from the unburnt population. However, individuals from unsampled source populations were also detected. This, together with the lack of isolation-by-distance across the landscape, suggested that long-distance dispersal is important for *K. proteae* to recolonize burnt areas. Similarly, the high level of gene flow and low differentiation observed between two distantly separated *K. proteae* populations also supported the existence of long-distance dispersal. The genetic cohesiveness of populations over long distances and the genetic diversity within populations could be attributed to frequent multiple fungal migration events mediated primarily by arthropods but, potentially, also by birds.

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Abbreviations; CCR, Core Cape Subregion; IBD, isolation-by-distance; MSN, Minimum Spanning Network

<http://dx.doi.org/10.1016/j.funbio.2014.12.010>

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## Introduction

The Fynbos Biome of the Core Cape Subregion (CCR) in South Africa is an unique vegetation type dominated by woody shrubs growing in nutrient-poor soils (Cowling & Richardson 1995). The area is characterized by a Mediterranean-type climate with short, wet winters and long, dry summers (Cowling 1992; Day et al. 1979). Recurrent summer-fires (approximately every 10–15 y) often clear vast areas (more than 4000 ha) of nearly all the above-ground fynbos biomass (Day et al. 1979; Kruger et al. 2000; Southey 2009) and most plants either re-sprout or recruit from seeds stored in the soil (Keeley 1995; Wilgen et al. 1992). In the case of serotinous *Protea* species, mature plants are killed by fire, but their survival is governed by the seeds released from above-ground seed-storage structures (infructescences) that form after flowers mature (Rebello 1995). New *Protea* recruits take ca. four years to reach maturity and flower for the first time (Le Maitre & Midgley 1992).

Between fires, *Protea* infructescences are colonized by numerous organisms such as insects (Coetzee & Giliomee 1985; Roets et al. 2006b), mites (Theron et al. 2012) and fungi (Lee et al. 2003, 2005), including ophiostomatoid fungi (Roets et al. 2005, 2013). *Protea*-associated ophiostomatoid fungi represent a polyphyletic assemblage (Wingfield et al. 1999) that is characterized by occupation of infructescences and arthropod-mediated dispersal (Roets et al. 2013). The long ostiolar necks of the perithcia and the production of sticky spores make these fungi ideally suited for dispersal by arthropods rather than air currents (Cassar & Blackwell 1996). Interestingly, these apparently native ophiostomatoid fungi are not associated with disease symptoms and they have no known adverse effects on growth or reproduction on their hosts (Marais 1996; Roets et al. 2013).

*Knoxdaviesia proteae* was the first *Protea*-associated ophiostomatoid fungus to be discovered (Wingfield et al. 1988). It was isolated from the infructescences of the common sugarbush, *Protea repens*, an indigenous fynbos species that is the only known host of this fungus (Roets et al. 2009b). Since the discovery of *K. proteae*, 11 additional ophiostomatoid fungi associated with serotinous *Protea* species have been identified, bringing the current total of species known in this niche to 12 (De Beer et al. 2013). This extraordinary *Protea*-ophiostomatoid fungus association is not restricted to the CCR, but has also been noted in other areas of South Africa and in Zambia (Crous et al. 2012; Marais & Wingfield 2001; Roets et al. 2010, 2013).

Like many organisms capable of colonizing *Protea* infructescences, the *Protea*-associated ophiostomatoid fungi seem to be specialists of this niche and have not been recorded from any other habitat, including other parts of *Protea* plants, soil and leaf and twig litter (Lee et al. 2005; Marincowitz et al. 2008). Therefore, recolonization of post-fire, newly formed infructescences by these organisms can take place only via dispersal from unburnt areas. This form of dispersal is more easily achieved for winged-groups like insects, than for the mites and ophiostomatoid fungi. However, many of the mites from this niche are phoretic on beetles that pollinate *Protea* species (Roets et al. 2009a). Using these *Protea*-specialist beetles as vectors, mites could easily recolonize infructescences,

presumably over long distances. Some of these phoretic mites also have mutualistic associations with the *Protea*-associated ophiostomatoid fungi and a few even have specialized spore carrying structures for the fungi on which they feed (Roets et al. 2007). While mites appear to be primarily responsible for spore capture and dispersal on a single *P. repens* plant, beetles carry mites to facilitate long-distance dispersal (Aylward et al. 2014b; Roets et al. 2009a).

Previous research has shown that gene flow facilitated by mites and beetles is sufficient to maintain a panmictic *K. proteae* population in a *P. repens* stand covering approximately three square kilometres (Aylward et al. 2014b). Gene flow between fungi in these plants thus exceeds genetic drift, preventing the fungal population from becoming structured based on individual *Protea* plants. The role of beetles in facilitating between-plant dispersal of mites and the ophiostomatoid fungi they carry, therefore, seems to dominate *K. proteae* movement within a *P. repens* stand.

Flight mill studies on bark beetles have shown that most beetles achieve at least an hour of uninterrupted flight (Atkins 1961; Forsse & Solbreck 1985) and can reach a speed of 2 m s<sup>-1</sup> in still air (Byers 1996). Mark-recapture studies with the southern pine beetle, *Dendroctonus frontalis* Zimm., showed that a third of the released individuals dispersed more than one kilometre, leading the authors to conclude that 'beetles are capable of dispersing quite far' (Turchin & Thoeny 1993). These results suggest that the dispersal kernel (probability distribution) of beetles is typically a fat-tailed one in which long-distance dispersal events are more common (Klein et al. 2006). The geographic distance over which the *Protea*-beetle vectors are capable of supplying sufficient *K. proteae* migrants to maintain panmixia therefore becomes intriguing.

Recolonization of large areas of burnt fynbos by ophiostomatoid fungi presents unique opportunities to study the dispersal patterns of these *Protea*-associated fungi. Fire essentially creates a clean slate so that the origin of fungal inoculants in young *Protea* hosts may be established. Most likely, fungal inoculants enter young plants via short-to medium-distance dispersal from neighbouring *Protea* plants that escaped the fire. In this case, burnt areas will have ophiostomatoid populations that represent a subset of the ophiostomatoid fungi in neighbouring unburnt areas. Young *Protea* plants closest to the source populations may also receive more inoculants than plants further away, producing patterns of isolation-by-distance (IBD). Beetles may, however, be able to facilitate sufficient between-plant dispersal that such patterns do not appear. Additionally, beetles may have the ability to transport ophiostomatoid fungi from more distant source populations into the recently burnt areas (Roets et al. 2009a), adding greater genetic diversity and potentially novel genetic information to the population.

In this study, we used microsatellite markers specific to *K. proteae* (Aylward et al. 2014a) to compare fungal isolates sampled from a recently burnt fynbos area to isolates from neighbouring unburnt areas. The aim was to establish whether adjacent, mature *K. proteae* populations act as the source of inoculants for new *P. repens* stands and whether infructescences can be colonized over long distances, in the first year of

flowering. In addition, we compared genotypic variation within this population to a *K. proteae* population previously genotyped with the same microsatellite markers (Aylward et al. 2014b) to determine the genetic relatedness between two distantly separated populations. In doing so, the role of long-distance dispersal in maintaining *K. proteae* populations could be verified.

## Materials and methods

### Fungal sampling

Sampling of *P. repens* infructescences was conducted along a three kilometre (km) stretch of land in the Franschoek Mountains, Western Cape Province, South Africa (−33.90442; 19.156683). The vegetation in this area burnt in 2008/2009 and the new *P. repens* plants flowered for the first time when infructescences were collected in January 2013. *Knoxdaviesia proteae* individuals isolated from these first-flowering-season infructescences could therefore, not have originated from older infructescences on the plant but had to be a result of between-plant dispersal. Patches of fynbos containing mature (ca. 15–17 y old) *P. repens* plants allowed for sampling of infructescences that presumably contained source populations of *K. proteae* for the recently burnt areas (Fig 1).

Thirty sampling plots were selected depending on availability (11 in the recently burnt and 19 in the unburnt areas), with those in the recently burnt areas chosen at increasing

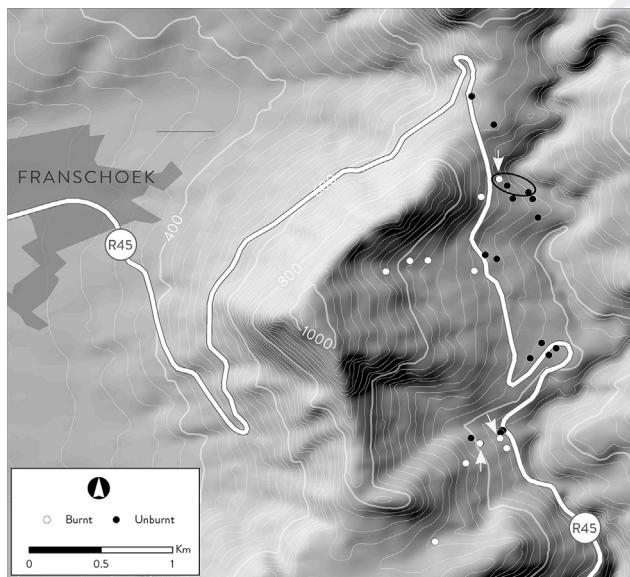
distances from the unburnt areas (Fig 1). A total of 20 ca. 6 month-old infructescences were sampled from various *P. repens* trees in a 10 m radius around the midpoint of each sampling plot. Fungal isolations were performed (Roets et al. 2006a) on Malt Extract Agar (MEA; Merck, Wadeville, South Africa) supplemented with 0.04 g L<sup>−1</sup> Streptomycin Sulfate Salt (Sigma–Aldrich, Steinham, Germany). Individual *Knoxdaviesia* isolates were obtained by sub-culturing hyphal tips from Water Agar (15 g agar L<sup>−1</sup>) onto MEA. DNA extraction and species identity verification via the internal transcribed spacer (ITS) region of the rDNA followed previously described methods (Aylward et al. 2014a). In infructescences where *K. proteae* ascomata were present, at least ten isolations were attempted, but only one *K. proteae* isolate was maintained to prevent re-sampling of the same individual.

### Genetic diversity of the *K. proteae* population and origin of isolates in recently burnt areas

All *K. proteae* individuals were genotyped with 12 microsatellite markers (Aylward et al. 2014a) in three multiplex reactions developed previously (Aylward et al. 2014b). The genetic diversity of the sampled isolates was described by using GENALEX 6.501 (Peakall & Smouse 2006, 2012) to compute the effective number of alleles ( $N_e$ ) (Kimura & Crow 1964), number of private alleles ( $N_p$ ), number of multilocus genotypes and Nei's unbiased estimate of expected heterozygosity ( $H_e$ ) (Nei 1978). The latter is the conventional measure of genetic diversity and describes the probability that two randomly sampled alleles will be different. Using R 3.0.2 (R Core Team 2014), Shapiro–Wilk tests of normality were performed on the data after which Mann–Whitney U tests (W statistic) were used to compare the diversity indices between *K. proteae* populations from the recently burnt and unburnt areas. Genotypic diversity ( $G$ ) and the maximum percentage of this diversity ( $\hat{G}$ ) was calculated according to Stoddart & Taylor (1988) and McDonald (1994), respectively. The evenness index ( $E_s$ ) recommended by Grünwald et al. (2003) was calculated with POPPR, a package implemented in R (Kamvar et al. 2013; R Core Team 2014).

In order to assess population differentiation, isolates from recently burnt and unburnt plots, respectively, were grouped together. The diversity within ( $\Delta_S/\Delta_T$ ) and between ( $\Delta_{ST}$ ) sub-populations, the relative population differentiation ( $D$ ) and Jost's haploid estimate of  $D$  ( $D_{est(hap)}$ ) (Jost 2008) were determined with SMOGD 1.2.5 (Crawford 2010).  $D_{est}$  is calculated from population estimates that incorporate sample size and ploidy, where a diploid genome is assumed. The estimate was modified to suit haploid data by substituting  $2N$  for  $1N$  in the Nei & Chesser (1983) formulas. Theta, a conventional measure of population differentiation analogous to  $F_{ST}$ , was calculated using MULTILOCUS 1.3b (Agapow & Burt 2001).

The premise that recolonization through short-to medium-distance dispersal may produce an IBD effect was investigated with the ISOLATION BY DISTANCE WEB SERVICE 3.23 (Jensen et al. 2005). A non-parametric Mantel test with 1000 permutations was used to consider whether genetic and logarithmic geographic distances (Rousset 1997) are correlated and the slope on the graph was determined through reduced major axis (RMA) regression analysis. All sampling plots where fungal isolates were obtained were considered as separate



**Fig 1 – Franschoek sampling site.** Dots represent the midpoints of sampling plots in unburnt (black) and recently burnt (white) areas. Downward arrows indicate plots containing the two individuals with identical genotypes. The upward arrow indicates the plot where the nine individuals that cluster separately to the rest of the population were collected. The three unburnt plots that showed weak, but significant isolation-by-distance with the recently burnt plots are circled (the first unburnt plot is hidden behind the recently burnt white plot).



geographic locations and geographic and genetic distances were calculated between them. Geographic distances were calculated from the latitudinal and longitudinal coordinates in GEOGRAPHIC DISTANCE MATRIX GENERATOR 1.2.3 (Ersts 2014). Two genetic distances were used independently – Goldstein's  $\delta\mu^2$  genetic distance for microsatellites (Goldstein et al. 1995), calculated using MSA 4.05 (Dieringer & Schlötterer 2003), and Nei's unbiased standard genetic distance,  $u_D$  (Nei 1978), calculated in POPGENE 1.32 (Yeh et al. 1999). Both distances were included, since they follow different mutation models that represent opposite extremes (Chakraborty & Jin 1992), the Stepwise Mutation Model (SMM) and Infinite Alleles Model (IAM), respectively. The SMM could be inordinately conservative, especially with regards to the di-nucleotide locus KX1, which can often evolve by multi-step changes (Shriver et al. 1993), and the IAM would therefore provide a useful comparison. In contrast, Nei's distance is likely to be too liberal in its estimation, because it does not consider allele size (Nei 1978).

To determine whether fungal dispersal from unburnt to recently burnt areas drives IBD, the test was repeated by comparing each sampling plot in the unburnt area individually with each of the plots in the recently burnt areas (therefore ignoring distance measures between unburnt plots). IBD was further investigated by measuring whether the distance from the nearest unburnt area influenced the number of *K. proteae* isolates obtained from recently burnt sampling plots by calculating Pearson's product-moment correlation in R 3.0.2 (R Core Team 2014).

Population structure was investigated with STRUCTURE 2.3.4 (Falush et al. 2003, 2007; Hubisz et al. 2009; Pritchard et al. 2000). Runs were conducted with an admixture model using correlated allele frequencies, 500 000 burn-in and 750 000 Markov Chain Monte Carlo repetitions. The number of clusters ( $K$ ) in the population was determined based on 10 independent runs for each  $K$  ranging from one to 10. This process was repeated using the two areas (unburnt and recently burnt) as presumed populations of origin (LOCPRIOR model). STRUCTURE HARVESTER (<http://taylor0.biology.ucla.edu/structureHarvester/>) (Earl & von Holdt 2012) was used to determine the optimal number of clusters in the population by computing  $L(K)$  (the mean log-likelihood of  $K$ ) and  $\Delta K$  (Evanno et al. 2005). The optimal alignment of the 10 independent replicates was found with CLUMPP 1.1 (Jakobsson & Rosenberg 2007) and graphical editing of the Q-matrix histogram was performed with DISTRICT 1.1 (Rosenberg 2004).

Relationships between isolates were also investigated by computing a Minimum Spanning Network (MSN) between microsatellite genotypes based on pairwise genetic distances calculated by the molecular variance parsimony technique in ARLEQUIN 3.5.1.3 (Excoffier & Lischer 2010). The MSN, displaying all possible connections, was constructed in HAPSTAR 0.7 (Teacher & Griffiths 2011). The reproductive strategy of the *K. proteae* population in Franschoek was investigated by calculating the linkage disequilibrium index,  $\bar{r}_d$  (Brown et al. 1980), in MULTILOCUS 1.3b (Agapow & Burt 2001), where random recombination ( $\bar{r}_d = 0$ ) is the null hypothesis. To test significance, the observed value of  $\bar{r}_d$  was compared to the values calculated for 1000 random datasets. Previous studies have shown that these 12 microsatellite loci are not in linkage disequilibrium (Aylward et al. 2014a, 2014b).

## Comparisons of genetic diversity and investigation of dispersal between two distantly separated *K. proteae* populations

The Franschoek population data were compared to genotypic data from a previous study on the genetic diversity of a *K. proteae* population in Gouritz, Western Cape Province, South Africa (Aylward et al. 2014b). These two *K. proteae* populations are separated by approximately 240 km and mountainous terrain. Considering all *K. proteae* isolates from both populations, population differentiation and structure was analysed following the methods described above. The respective populations were used as sampling locations in the LOCPRIOR model applied in STRUCTURE. Population recombination was also tested by calculating  $\bar{r}_d$  across all isolates in both populations as explained above. Although the inclusion of *K. proteae* individuals from two different populations would bias the linkage disequilibrium test towards non-random recombination (Taylor et al. 1999), failure to detect non-random recombination would suggest that outcrossing is taking place between the two populations.

## Results

### Genetic diversity and recolonization of recently burnt areas

A total of 106 *K. proteae* individuals were isolated from 16/19 of the unburnt and 7/11 of the recently burnt sampling plots (Table S1). Infructescences from the remaining sampling plots were not colonized by *K. proteae* or could not be used for isolation due to insect damage or a high level of contamination by other micro-organisms. During microsatellite amplification, between zero and 4.7 % null alleles were detected in 10 of the loci. However, similar to the previous *K. proteae* population study (Aylward et al. 2014b), loci KX6 and KX9 displayed exceptionally high null allele percentages (29 % and 20 %, respectively) and were excluded from further analyses. The null alleles in the remaining loci were treated as missing data in analyses.

A high genetic diversity was observed in the Franschoek *K. proteae* population (Table 1). The exclusion of two loci did not significantly impact the diversity indices and a plot of the number of loci against diversity (calculated in MULTILOCUS) showed 10 loci to be adequate for describing the diversity (data not shown). Of the 106 isolates, 104 had unique genotypes. Two identical genotypes were encountered in different sampling locations 1.8 km apart; both in recently burnt sampling plots (Fig 1). Stoddart & Taylor's (1988) genotypic diversity ( $G$ ) was 104.04, which corresponds to 98.15 % of the maximum diversity ( $G$ ). The genotypic evenness ( $E_s$ ) was 0.994, identical to the  $E_s$  value of a previous *K. proteae* population (Aylward et al. 2014b).

Comparison of the measures of allele diversity between fungal individuals from the unburnt and recently burnt areas revealed a significant difference ( $W = 96$ ;  $P = 4.7 \times 10^{-4}$ ) in the number of private alleles, with the population from unburnt areas having more private alleles (Fig 2A). Although not significant ( $W = 74$ ;  $P = 0.07$ ), the number of total alleles was also higher in the unburnt area. The expected heterozygosity and

**Table 1 – Genetic diversity of the Franschhoek Mountain *Knoxdaviesia proteae* population across 12 microsatellite loci.**

Locus	N <sub>a</sub> <sup>a</sup>	Null alleles (%)	N <sub>e</sub> <sup>b</sup>	H <sub>E</sub> <sup>c</sup>
KX1	25	0.94	16.53	0.95
KX2	21	2.83	8.88	0.90
KX3	10	0.94	3.29	0.70
KX4	27	0	6.79	0.86
KX5	7	2.83	3.45	0.72
KX6	8	29.25	3.14	0.69
KX7	6	0.94	2.01	0.51
KX8	9	0	4.95	0.81
KX9	16	19.81	4.28	0.78
KX10	11	4.72	4.56	0.79
KX11	12	2.83	4.68	0.79
KX12	8	0	4.64	0.79
Mean ± SEM <sup>d</sup>	13.33 ± 2.09	5.42 ± 2.67	5.60 ± 1.12	0.77 ± 0.03
Excluding KX6 & KX9	13.6 ± 2.45	1.60 ± 0.51	5.98 ± 1.32	0.78 ± 0.04

a N<sub>a</sub> = Number of alleles.

b N<sub>e</sub> = Kimura & Crow's (1964) number of effective alleles; N<sub>e</sub> = 1 / (1 - h).

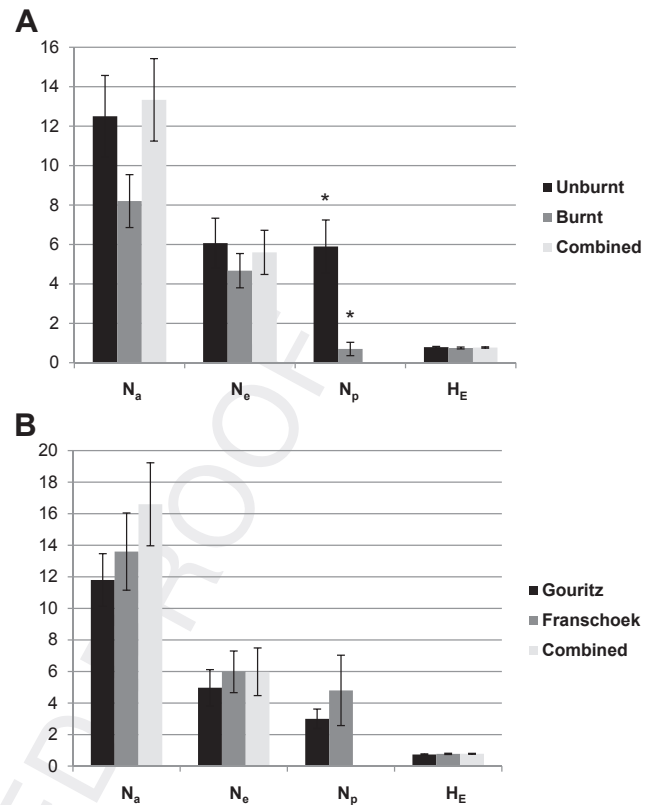
c H<sub>E</sub> = Nei's unbiased expected heterozygosity; H<sub>E</sub> = (n/n - 1) [1 - ∑ p<sub>i</sub><sup>2</sup>].

d SEM = Standard error of the mean.

effective number of alleles were similar between the two groups. Population differentiation statistics could not detect differentiation between *K. proteae* individuals from the unburnt and recently burnt areas (Table 2) and the effective number of sub-populations (Δ<sub>ST</sub>) was one, accounting for 99 % of the total diversity (Δ<sub>S</sub>/Δ<sub>T</sub>).

In the first test for IBD, the Mantel test detected a weak correlation using both δμ<sup>2</sup> (r<sup>2</sup> = 7.02<sup>-3</sup>) and uD (r<sup>2</sup> = 0.03), but only the uD correlation was significantly positive (P = 0.02; Fig 3). Under the IAM, a very weak IBD effect could therefore have been present across this three km stretch of landscape. All but three Mantel tests, conducted between individual unburnt plots and recently burnt plots, indicated that the IBD is not a result of the recolonization of burnt areas. These three tests were conducted under the IAM and suggested weak (r<sup>2</sup> < 0.2), but significant IBD between the burnt plots and unburnt plots 3, 5 and 7 (Table S2). These three plots represent the northernmost unburnt plots from which fungal isolations were successful (Fig 1). The distance of recently burnt from unburnt sampling plots did not significantly influence the number of *K. proteae* isolates obtained in this study (r<sup>2</sup> = 0.26; t = -1.7949; df = 9; P = 0.053; Fig S1). A Pearson's Chi-squared test performed in R 3.0.2 (R Core Team 2014) also showed no difference between the numbers of fungal isolates obtained from infructescences in the unburnt and recently burnt plots (X<sup>2</sup> = 1.42; df = 1; P = 0.23).

Without incorporation of the LOCPRIOR model, STRUCTURE could not detect significant population structure. When considering the origin of *K. proteae* isolates (unburnt or recently burnt area), both L(K) and ΔK designated K = 3 as the most likely number of clusters. Inspection of the cluster assignment (Fig 4A) indicated that each cluster contained 106 fungal individuals – i.e. the genotype of each individual was distributed



**Fig 2 – Comparison between the diversity indices of *Knoxdaviesia proteae* from the recently burnt and unburnt areas in Franschhoek (A) and between the Gouritz and Franschhoek populations (B). The mean number of alleles (N<sub>a</sub>), effective alleles (N<sub>e</sub>), private alleles (N<sub>p</sub>) and the unbiased expected heterozygosity (H<sub>E</sub>) is shown. Error bars represent the standard error of the mean. The only statistically significant difference (\*) was observed for N<sub>p</sub> between the burnt and unburnt areas.**

among the three clusters. However, in each case only one of the three clusters contained the majority (>70 %) of an individual's genotype. All of the individuals from the unburnt and most from burnt areas had the majority of their genotype assigned to the green cluster. Eleven individuals from the burnt area were predominantly assigned to either the dark blue or light blue cluster. The MSN (Fig 5) displayed a cohesive assemblage of fungi with numerous loops in the network linking different genotypes. The index of linkage disequilibrium was not significantly different from zero ( $\bar{r}_d = 6.7^{-3}$ ; P = 0.33; Fig 6A), supporting the null hypothesis of random recombination.

#### Dispersal between distantly separated populations

In an earlier *K. proteae* population study, only two identical genotypes were identified in the population (Aylward et al. 2014b). The same phenomenon was found in the current study as 105 unique genotypes were observed out of the 106 sampled isolates. In the combined Gouritz and Franschhoek dataset, shared genotypes were not detected with each

**Table 2 – Population differentiation between the *K. proteae* individuals from unburnt and recently burnt sampling plots in Franschoek and between the two distantly separated populations.**

	Franschoek unburnt vs. burnt	Gouritz vs. Franschoek
$\bar{N}^a$	45.45	98.51
$\Delta_{ST}^b$	$1.01 \pm 0.00$	$1.02 \pm 0.01$
$\Delta_S/\Delta_T^c$	$0.99 \pm 0.00$	$0.98 \pm 0.01$
$D^d$	$0.03 \pm 0.00$	$0.04 \pm 0.01$
$D_{est(hap)}^e$	0	$0.01 \pm 0.01$
$\theta^f$	0.01	0.042**

\*\*P < 0.001 after 1000 randomizations.  
a  $\bar{N}$  = Harmonic mean of the sample sizes.  
b  $\Delta_{ST}$  = Diversity between subpopulations, or the effective number of subpopulations.  
c  $\Delta_S/\Delta_T$  = Proportion of diversity in a subpopulation.  
d  $D$  = Actual (relative) differentiation.  
e  $D_{est(hap)}$  = The haploid estimate of  $D$ ;  
 $D_{est(hap)} = [(H_{T,est(hap)} - H_{S,est(hap)}) / (1 - H_{S,est(hap)})] [n / (n - 1)]$ .  
f  $\theta$  = Conventional measure of relative differentiation;  $\theta = Q - q / 1 - q$ .

individual having at least one private allele in its genotype and the majority of alleles were shared between the populations (Fig 2B). Additionally, the number of effective alleles ( $N_e$ ) calculated for the two populations combined was not significantly different from that of the two separate populations, indicating similar genetic compositions (Fig 2B). The population differentiation measurements  $\Delta_{ST}$  and  $\Delta_S/\Delta_T$  did not provide evidence of genetic isolation (Table 2). However, the non-zero value of Jost's  $D$  and  $D_{est(hap)}$  and the significance of theta suggested some level of differentiation, albeit low, between the Gouritz and Franschoek populations of *K. proteae* (Table 2).

In STRUCTURE, Bayesian inference alone could not detect a difference between the two populations, but incorporation of the LOCPRIOR model revealed  $K = 2$  as most likely (based on both  $L(K)$  and  $\Delta K$ ). Using this method, all *K. proteae* individuals

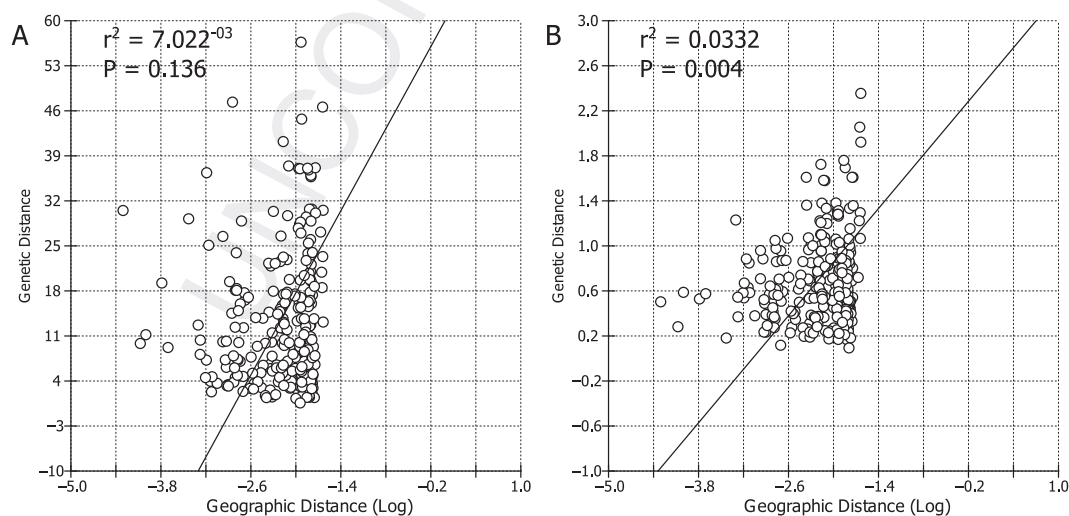
could be assigned to their population of origin (Fig 4B), although all individuals contained elements from both clusters. The inability of STRUCTURE to separate the two populations without prior information of each individual's origin is indicative of weak population structure and is therefore congruent with the measures of differentiation. The constructed MSN (Fig S2) also displayed numerous loops and cohesiveness as observed in the networks for the individual populations (Fig 5; Aylward et al. 2014b). Its failure to cluster the Gouritz and Franschoek populations separately also supports a weak population structure and high similarity between the two populations. The test for random recombination could not reject the null hypothesis ( $\bar{r}_d = -2^{-4}$ ;  $P = 0.66$ ; Fig 6B) and, therefore, indicates general outcrossing between *K. proteae* individuals from the two distantly separated populations.

## Discussion

### Recolonization of recently burnt areas

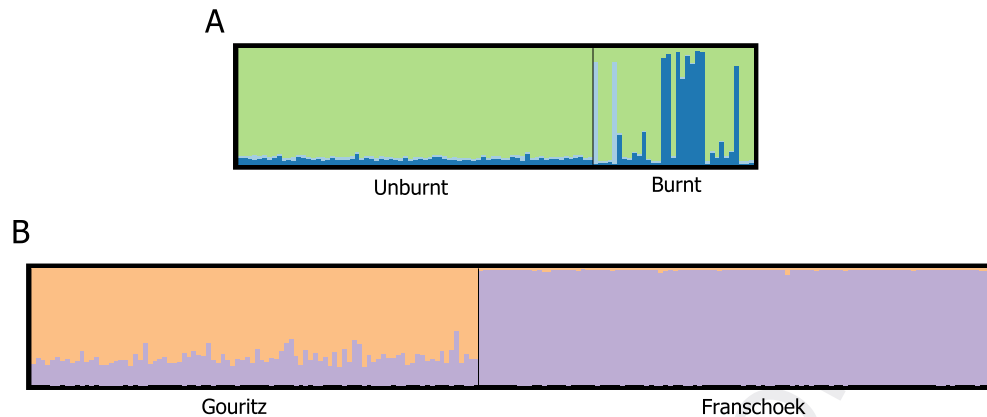
Landscapes that experience prolonged dry conditions, such as Mediterranean, grassland savannah and even some forest areas, are prone to fire damage (Bond & Keeley 2005). It is, therefore, surprising that this study appears to be the first to consider post-fire recolonization of plant-associated fungi. Investigations into the effect of fire on soil microbial biomass in Mediterranean regions (Dumontet et al. 1996) have revealed that fungi are more susceptible to fire and re-establish less easily than bacteria (Bárcenas-Moreno et al. 2011; Guerrero et al. 2005). The niche space availability and elimination of competition provided by fire, however, apparently facilitates rapid re-establishment.

The genetic and genotypic diversity of *K. proteae* described in burnt and unburnt areas was remarkably high. This is consistent with the results of the Gouritz population study (Aylward et al. 2014b) where heterozygosity was  $0.74 \pm 0.04$  and the maximum percentage of genotypic diversity ( $\hat{G}$ ) was



**Fig 3 – Correlations to test isolation-by-distance. The  $\delta\mu^2$  genetic distance (Goldstein et al. 1995) (A) and Nei's unbiased standard genetic distance (Nei 1978) (B) between *Knoxdaviesia proteae* isolates in the different sampling plots versus the logarithmic geographic distance between the midpoints of these plots.**





**Fig 4 – Histograms depicting the inferred vector Q using STRUCTURE 2.3.4 (Falush et al. 2003, 2007; Hubisz et al. 2009; Pritchard et al. 2000).** The proportion of an individual's genotype that originates from each inferred cluster is indicated on the y-axis while each vertical bar represents an individual. The clusters detected are indicated with different colours. **A) In Franschoek, all isolates from unburnt areas and most isolates from recently burnt areas are predominantly assigned to one cluster (green). Two additional clusters (light and dark blue), present only at low levels in the unburnt area, were predominant in eleven individuals in the recently burnt area.** **B) Assignment of all *K. proteae* individuals based on their population of origin (Gouritz or Franschoek).** (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

97.87 %. Contrary to the reduced variation usually observed in recently introduced populations (Dlugosch & Parker 2008), the individual genetic diversities and composition of the burnt and unburnt populations were similar. However, the lack of population differentiation suggests that the recently burnt population is primarily a subset of the adjacent unburnt population. This view is supported by the presence of significantly fewer unique alleles in the burnt populations, which suggests that the fungal populations in the recently burnt sites are primarily derived from the unburnt area and have experienced a genetic bottleneck (Black et al. 1988). The few unique alleles that were detected in the burnt area, however, support the two additional genetic clusters detected by STRUCTURE. Together, they suggest that sources of fungal inoculants other than those from adjacent established populations could play a role in recolonization.

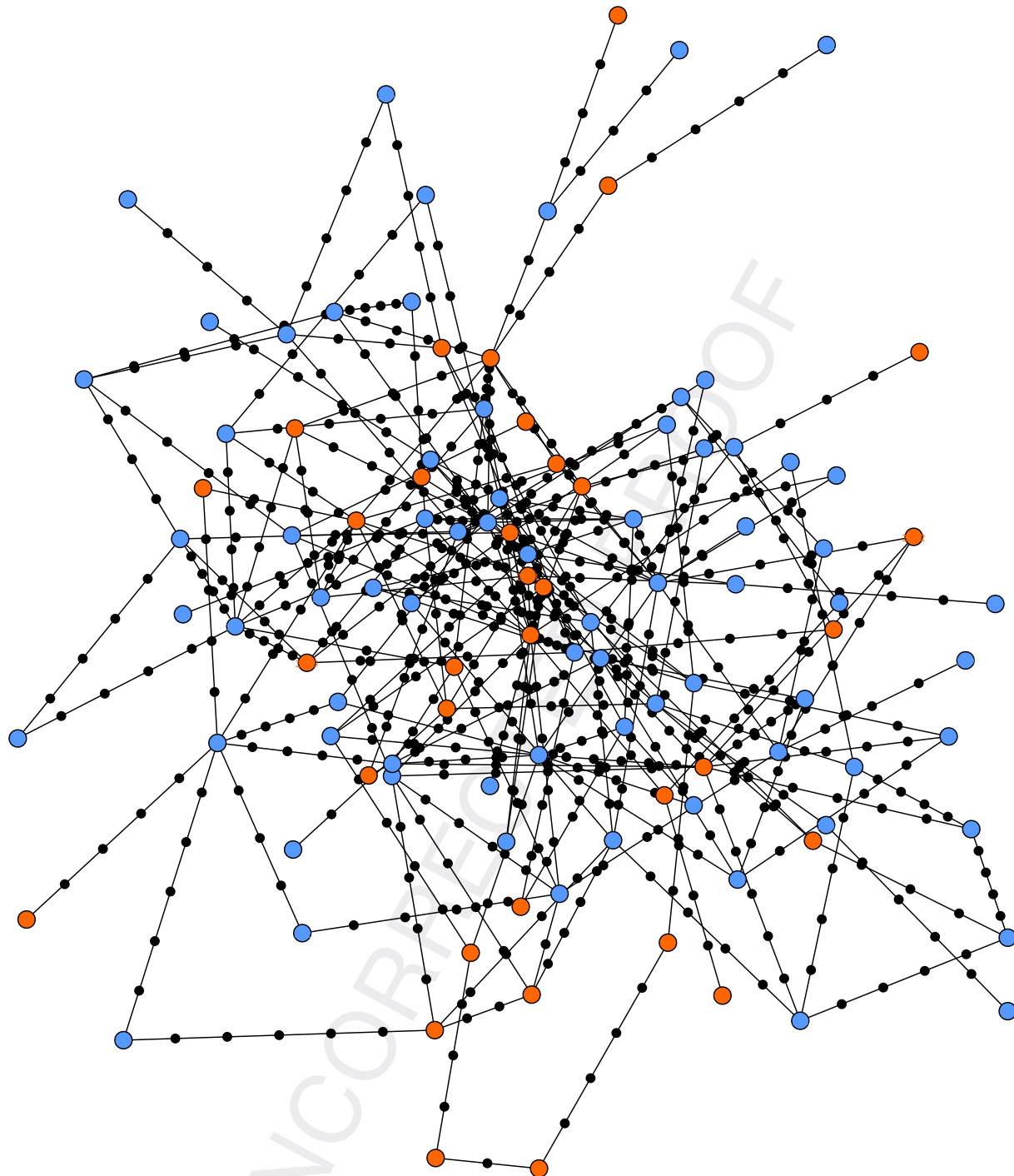
Numerous *K. proteae* migrants within and between the burnt and unburnt areas explain the closely related and weakly structured population. The effects of genetic drift seem to be masked by a high level of gene flow (Slatkin 1987). The high level of genetic variation within the population is probably due to the reproductive strategy of *K. proteae*. Both the asexual and sexual states of *K. proteae* occur in *P. repens* infructescences (Wingfield et al. 1988), but the high genetic and genotypic diversity of the population is explained by sexual outcrossing being the predominant mode of reproduction. Whether *K. proteae* is homothallic (able to self-fertilize) or heterothallic (obligatory outcrossing) has yet to be determined, but would provide further information concerning the population biology of this organism.

Adjacent unburnt areas appear to be the primary source of fungal inoculants for recently burnt regions. Isolation-by-distance (IBD) is a result of limited dispersal across the landscape (Wright 1943) and in three instances such limited dispersal was revealed between the unburnt and recently burnt

areas. These three tests were, however, based on the Infinite Alleles Model (IAM), a model probably less suited to perfect microsatellites than the Stepwise Mutation Model (SMM) described by Shriver et al. (1993) that was also used. The true situation is probably provided by an intermediate between the results emerging from these two models and, since weak IBD was detected in a minority of cases, limited dispersal from the unburnt to the burnt area is unlikely. The lack of (or very weak) IBD pattern observed indicates that *P. repens* plants closer to unburnt areas are not recolonized more readily. In fact, recolonization appears to be a result of widespread dispersal and is therefore primarily long-distance-orientated. This strengthens the premise that long-distance dispersal is the predominant influence in recolonization of burnt areas.

#### Long-distance dispersal between distant *K. proteae* populations

The high level of genetic similarity between the *K. proteae* population in Gouritz and the burnt/unburnt Franschoek population was unexpected because these two sampling areas are separated by 240 km and mountainous geographic barriers. Although the two populations do not appear completely panmictic (randomly interbreeding), their genetic diversities and compositions were alike and population differentiation was low. Also, recombination between fungi in the two populations appears to have occurred frequently, since all *K. proteae* individuals from the combined populations showed random mating and interconnected genotypes based on the Minimum Spanning Network (MSN). Although knowledge about the dispersal of the vectors of *K. proteae* is scarce, it appears that the dispersal of this ophiostomatoid fungus follows a fat-tailed kernel in which long-distance dispersal events are not rare (Klein et al. 2006). In such a case, recolonization would be due to the influence of inoculants from several sources that



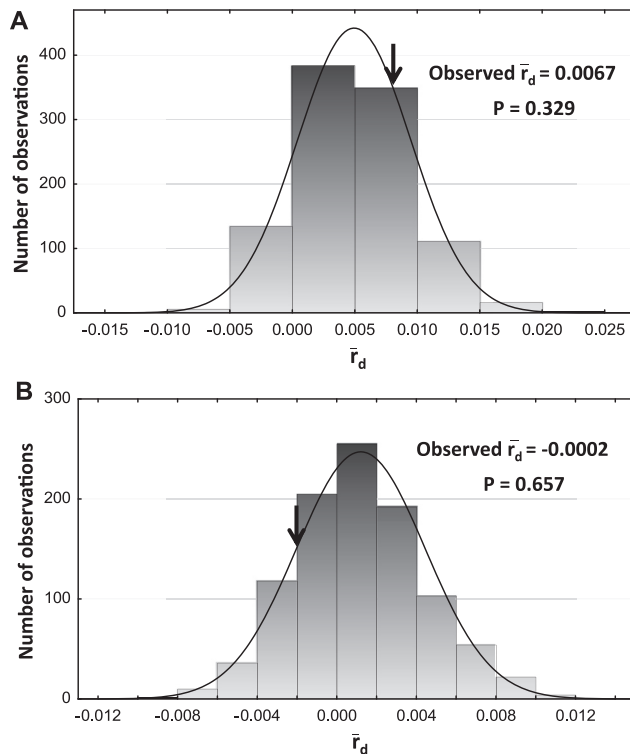
**Fig 5 – Minimum spanning network (MSN) depicting the relationships between 105 unique genotypes encountered in the Franschoek *Knoxdaviesia proteae* population. Black dots represent missing genotypes. Genotypes from the unburnt and recently burnt areas are shaded blue and orange, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)**

maintain genetic diversity during the recolonization process (Szövényi et al. 2012).

Long-distance dispersal is a common trait in fungi that disperse via air or water and is well-studied for plant pathogenic fungi (Brown & Hovmöller 2002). Human activity and movement of plant materials is also commonly implicated in the

dispersal of fungi and has resulted in the spread of devastating pathogens between countries and continents (Brown & Hovmöller 2002; Wingfield et al. 2001; 2010). Examples of migration at an inter-continental or even global scale are available for lichen-forming (Buschbom 2007), saprobic (Moncalvo & Buchanan 2008), ectomycorrhizal (Moyersoen et al. 2003)





**Fig 6 – Observed values of the index of linkage disequilibrium,  $\bar{r}_d$ , for (A) the Franschoek and (B) Gouritz and Franschoek *Knoxdaviesia proteae* populations. Observed values are indicated on a normal distribution of  $\bar{r}_d$  values calculated for 1000 randomly generated datasets.**

and marine fungi (Pang et al. 2013). Extreme long-distance dispersal of arthropod-vectored fungi in the absence of human involvement is, however, unlikely. The high level of relatedness between the two populations in this study, therefore, probably demands reconsideration of the vectors known to disperse *K. proteae*. Dispersal via flying insects, especially robust beetles such as *Genuchus hottentotus* (F.), *Trichostetha fascicularis* L. and *T. capensis* L. (Roets et al. 2009a), may allow substantial contact between fungal individuals in a population and facilitate a high number of migration events. It is, however, difficult to attribute the extent of admixture observed between these distantly separated *K. proteae* populations to beetles alone, particularly given the structured populations observed in other ophiostomatoid fungi with beetle vectors (Lee et al. 2007; Morin et al. 2004; Tsui et al. 2012). Additionally, these beetles are unlikely to fly great distances, at least not in large numbers.

Ultimately, the dispersal of *Knoxdaviesia* species is linked to the behaviour of the mites that act as their primary vectors. Due to their small size, autonomous dispersal is not effective and most mites utilize either wind or habitat-specific vectors as dispersal vehicles (Mitchell 1970). Many mites are known to associate with birds for phoresy (Krantz & Walter 2009; Proctor & Owens 2000) and this relationship has been documented between birds and mites associated with *Protea* species (Collins & Rebelo 1987; N. Theron, pers. com.). It is therefore possible that mites carrying ophiostomatoid fungi

are not exclusively phoretic on beetles, but also on birds. Since birds are able to travel further than beetles, involvement of *Protea*-pollinating birds, such as sunbirds and sugarbirds (Collins & Rebelo 1987), in the dispersal of ophiostomatoid fungi could explain the widespread gene flow between these two distantly separated populations. The cape sugarbird, *Promerops cafer*, and the malachite sunbird, *Nectarinia famosa*, have both been noted to fly to 160 km (Fraser et al. 1989; Harrison et al. 1997) within the Core Cape Subregion (CCR) and would be good candidates for further investigation of this hypothesis.

The question arises as to how the small, primary mite vectors of *Knoxdaviesia* species would accomplish such extensive dispersal, even considering phoresy on larger arthropods or birds. It is possible that successive migration events between interspersed *K. proteae* populations or transfer of mites between birds (Proctor & Owens 2000) could lead to gene flow over distances far exceeding the capacity of one pollinator. In fact, multiple introductions of *K. proteae*, both from the unburnt to the burnt area and between the two *K. proteae* populations, seems to explain the patterns of gene flow and genetic diversity observed in this study (Dlugosch & Parker 2008). Considering the dual dispersal system available to the ophiostomatoid fungi, numerous fungal inoculants are expected. A single beetle can carry a large number of mites (>100) (Roets et al. 2009a; 2011) and thereby facilitate many migration events. Individual mites may even carry more than one fungal strain, further increasing the potential number of fungal migrants.

## Conclusions

*Knoxdaviesia proteae* colonizers of *P. repens* infructescences arise primarily from plants in adjacent unburnt areas. Although the *K. proteae* population in the recently burnt area considered in this study is primarily a subset of its neighbouring unburnt population, our data suggest that inoculants were also introduced from other areas by long-distance dispersal. Long-distance dispersal further remained prevalent even over large distances and across geographic barriers, although fungal panmixia was not perfectly maintained. In addition to beetles that are already known to carry mites vectoring *Protea*-associated ophiostomatoid fungi, avian *Protea* pollinators may therefore also play a significant role in the phoresy of ophiostomatoid mite vectors. Ultimately, the lack of population structure appears to be a result of multiple fungal introductions and sexual outcrossing. Future studies should include *K. proteae* populations across the natural range of its host and examine IBD at this larger spatial scale.

Since the agents responsible for long-distance dispersal of fungal spores (i.e., beetles and presumably passerine birds) are also carriers of *Protea* pollen, the gene flow observed for *K. proteae* between *P. repens* populations may also reflect gene flow for the plants themselves. As a result, *P. repens* plants separated by the same distance and geographic barriers as the two *K. proteae* populations may be similarly structured to the fungi. This study could therefore guide future studies on the population genetics of this iconic and economically

important (Coetzee & Littlejohn 2001; Knoesen & Conradie 2009) CCR plant.

## Acknowledgements

The National Research Foundation (NRF) and the Department of Science and Technology (DST)-NRF Centre of Excellence in Tree Health Biotechnology (CTHB) are acknowledged for financial support. We thank the Western Cape Nature Conservation Board for the necessary collection permits and two anonymous reviewers for their comments on this manuscript.

## Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.funbio.2014.12.010>.

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