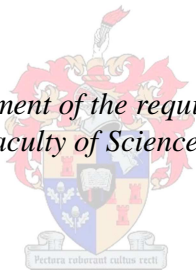


**Diversity and dispersal of the ophiostomatoid  
fungus, *Knoxdaviesia proteae*, within *Protea repens*  
infructescences**

by  
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*Thesis presented in fulfilment of the requirements for the degree of  
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## Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own original work, that I am the authorship owner thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

.....

J. Aylward

10 December 2013

.....

Date

## Abstract

Two genera of ophiostomatoid fungi occur in the seed-bearing structures of serotinous *Protea* species in the Cape Floristic Region. These fungi are dispersed by arthropods, including mites and beetles that visit the *Protea* host plants. Although the vectors of *Protea*-associated ophiostomatoid fungi are known, their dispersal patterns remain unknown – especially the manner in which recently burnt fynbos vegetation is recolonized. Additionally, their reproduction strategy has not previously been investigated. The focus of this study was, therefore, to determine the extent of within- and between-plant dispersal of *Protea*-associated ophiostomatoid fungi at the population level and to investigate their reproductive strategy. One *Protea*-associated ophiostomatoid fungus, *Knoxdaviesia proteae*, is found exclusively in the fruiting structures of *P. repens* and was the focus of this study. In order to interrogate natural populations of this fungus, 12 polymorphic microsatellite markers specific to *K. proteae* were developed with an ISSR-PCR enrichment strategy and pyrosequencing. These markers were amplified in two distantly separated populations of *K. proteae*. The genetic and genotypic diversities of both populations were exceptionally high and neither showed significant population differentiation. The lack of population structure in both populations implies that *K. proteae* individuals within a *P. repens* stand are in panmixia. As one of the sampling sites had burnt recently, the process whereby young fynbos is recolonized could be investigated. Compared to the adjacent, unburnt area, *K. proteae* individuals in the burnt area of this population had significantly less private alleles, suggestive of a young population that had experienced a genetic bottleneck. *Knoxdaviesia proteae* individuals that did not originate from the adjacent unburnt area were encountered within the burnt site and, additionally, isolation-by-distance could not be detected. The parsimony-based haplotype networks and the tests for linkage disequilibrium indicated that recombination is taking place within as well as between the two distantly separated populations. The observed panmixia in *P. repens* stands, widespread recolonization and the high genetic similarity and number of migrants between the two populations emphasizes long-distance dispersal and therefore the role of beetles in the movement of *K. proteae*. This cohesive genetic structure and connection across large distances is likely a result of multiple migration events facilitated by beetles carrying numerous phoretic mites.

## Opsomming

Twee genera ophiostomatoid swamme kom in die saad-draende strukture van bloeiende *Protea* spesies in the Kaapse Floristiese Streek voor. Hierdie *Protea*-verwante ophiostomatoid swamme word gekenmerk deur hul assosiasie met geleedpotige vektore – spesifiek die myt en kewer besoekers van die *Protea* gasheer plante. Alhoewel die geleedpotige vektore van *Protea*-verwante ophiostomatoid swamme bekend is, is die wyse waarop hierdie swamme versprei onbekend; veral die manier waarop onlangse gebrande fynbos geherkoloniseer word. Verder is die voortplantings-strategie van hierdie swamme nog nie voorheen ondersoek nie. Die fokus van hierdie studie was dus om die omvang van binne- en tussen-plant verspreiding van *Protea*-verwante ophiostomatoid swamme te bepaal op die populasie vlak en om hul voorplantings-strategie te ondersoek. Een *Protea*-verwante ophiostomatoid swam, *Knoxdaviesia proteae*, word uitsluitlik in die vrugdraende strukture van *P. repens* aangetref en was die fokus van hierdie studie. Om natuurlike populasies van hierdie swam te ondersoek is 12 mikrosatelliet-merkers spesifiek vir *K. proteae* ontwerp deur ‘n ISSR-PCR strategie en “pyro”-basisvolgorde bepaling te gebruik. Hierdie merkers is geamplifiseer in twee *K. proteae* populasies wat ver van mekaar geskei is. Die genetiese en genotipiese diversiteit van beide populasies was uitsonderlik hoog en nie een het beduidende populasie-differensiasie getoon nie. Die gebrek aan populasie struktuur in beide populasies veronderstel dat *K. proteae* individue binne ‘n *P. repens* stand in panmiksia is. Aangesien een van die steekproef terreine onlangs gebrand het, kon die herkolonisasie proses van jong fynbos ondersoek word. In vergelyking met die aangrensende, ongebrande area, het *K. proteae* individue in die gebrande area beduidend minder private allele gehad. Dit dui op ‘n jong populasie wat ‘n genetiese bottelnek beleef het. *Knoxdaviesia proteae* individue wat nie van die aangrensende, ongebrande area afkomstig is nie is ook binne die gebrande terrein aangetref. Verder is afsondering-deur-afstand nie aangetref nie. Die parsimonie-gebaseerde haplotiepe-netwerke en die toetse vir koppeling-onewewigtigheid het aangedui dat rekombinasie binne sowel as tussen die twee populasies plaasvind. Die panmiksia wat waargeneem is in *P. repens* populasies, wydverspreide herkolonisasie en die hoë genetiese ooreenkoms en hoeveelheid immigrante tussen die twee populasies beklemtoon lang afstand verspreiding en dus die rol van kewers in die beweging van *K. proteae*. Hierdie samehangende genetiese struktuur en die verband oor groot afstande is waarskynlik ‘n gevolg van verskeie migrasies gefasiliteer deur kewers wat talle foretiese myte dra.

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# INTRODUCTION

## 1. Strategies of fungal spore dispersal

The variety contained within the fungal kingdom is enormous, but one characteristic that unifies filamentous fungi is dispersal via spores. Fungi employ an array of different mechanisms and adaptations to achieve spore dispersal to sought-after locations. Before being dispersed, spores have to be released from the structures on which they are borne – a process that may be either active or passive (Dobbs, 1942; Ingold, 1953).

Active spore dischargers directly expend energy to “shoot” their spores to a suitable substrate or other dispersal agent, e.g. a river or air currents. Numerous ascomycete fungi (Fischer *et al.*, 2004; Trail *et al.*, 2005) make use of forcible spore ejection, which is achieved mainly via turgor pressure accumulating in the ascus. The term “passive” discharge is misleading, because these dischargers also expend energy, although indirectly. Some grow spores on long stalks to be accessible to wind, while others invest energy in slime production so that spores are suited for water- or arthropod-mediated dispersal (Money *et al.*, 2009). The principal means of dispersal after discharge is either anemophilous (via air) or hydrophilous (via water) (Dobbs, 1942), but some fungi also employ vector-mediated spore dispersal. In the case of passive dischargers, spore release is often facilitated by physical disturbances such as air flow or raindrops (Carlile *et al.*, 2001).

The spore itself testifies to its mode of dispersal – dry spores tend to be adapted for anemophily and slimy or sticky spores for either hydrophily or vector-mediated dispersal (Ingold, 1953). Spore dispersal through air currents, water, human movement and animals is essential for fungal propagation, as they could travel only a few meters in the absence of these forces (Burnett, 2003; Dowding, 1969). The limitation of these general spore dispersal mechanisms is the low probability of an individual spore reaching a substrate suitable for germination.

### 1.1. Arthropod-mediated dispersal

A special dispersal mechanism, used by some fungi to overcome the limitations of general dispersal, is arthropod exploitation. The fungus may, for example, make use of a pollinating

insect specific to its host as a vector. In this way, it is assured of continuous exposure to a suitable host (Ingold, 1953; Jennersten, 1988). Fungi adapted for arthropod dispersal often produce slimy spores that attach easily to the exoskeleton or hairs of the vector. This has been described as a “paint-brush” method of coating the vectors with spores (Abbott, 2002). It is ideal for fungi to exploit beetles or other arthropods that pollinate their host plant, since the specificity of the pollinators will ensure that the spores reach a suitable niche. Many beetles have mycangia – special sacs – in which they carry a viable inoculum of their symbiotic partner, indicating that the relationship is mutually beneficial (Batra, 1963). Similarly, some mites have primitive spore-carrying structures called sporothecae (Fischer *et al.*, 2004; Lombardero *et al.*, 2003; Moser, 1985).

The best-documented relationships between fungi and arthropods are those that involve insects such as beetles, ants and termites. The basidio-, asco- and zygomycota have members that employ insects to vector their spores to a suitable substrate. Three agriculturally significant groups of insects have members that feed on their symbiotic fungi: attine ants, some termites and ambrosia beetles (Martin, 1979). Although these symbioses are often associated with agricultural and economic pests (Fowler *et al.*, 1989; Robinson & Fowler, 1982; Weber, 1966), many termite- and ant-fungal symbioses are not damaging, but rather essential decomposers and indicators of environmental health (Schultz & Brady, 2008). Ambrosia and bark beetles are also renowned for spreading fungal-associated diseases and have long been known for their relationship with *Ophiostoma* species (Bakshi, 1950; Buchnan, 1940; Dowding, 1969). During the 1900’s, the elm tree populations in Europe and, later, America were destroyed by *O. ulmi* (Buisman) Nannf. (Brasier, 1988) or the more virulent *O. novo-ulmi* Brasier (Brasier, 1991), both carried by bark beetles.

## **2. Protea-associated ophiostomatoid fungi**

Ophiostomatoid fungi are known from the Northern Hemisphere where many are associated with diseases of conifer trees (Brasier, 1988; Brasier, 1991). The ophiostomatoid fungi comprise the orders Ophiostomatales and Microascales, with the genus *Ophiostoma* residing in the Ophiostomatales, and *Ceratocystis* and *Knoxdaviesia*, (previously *Gondwanamyces*) in the Microascales (De Beer *et al.*, 2013b; Marais *et al.*, 1998; Spatafora & Blackwell, 1994). Although the genera themselves are monophyletic, the ophiostomatoid assemblage as a whole is not (Spatafora & Blackwell, 1994; Wingfield *et al.*, 1999). Additionally, the *Protea*-

associated members of *Knoxdaviesia* and *Ophiostoma* form two polyphyletic lineages, indicating that the association of ophiostomatoid fungi with *Protea* species evolved more than once (Wingfield *et al.*, 1999). Additionally, the *Protea*-associated members within each genus are closely related to one another, leading to the belief that divergence occurred recently (Wingfield *et al.*, 1999).

*Knoxdaviesia* was the first genus of ophiostomatoid fungi found associated with an indigenous South African angiosperm (Wingfield *et al.*, 1988). Two *Knoxdaviesia* species descriptions were followed by the identification of nine *Ophiostoma* and one more recent *Knoxdaviesia* species, all from various serotinous proteas (Table A). These fungi do not cause recognizable disease symptoms on their *Protea* hosts and no adverse effects on growth or reproduction have been reported. The relationship between the fungi and their hosts, therefore, remains ambiguous. It may be mutualistic – the fungi receive a unique niche suitable for their growth, reproduction and dispersal, whereas the proteas most likely receive protection by the exclusion of pathogens (Marais, 1996). This notion is supported by the increased number of saprophytes that are encountered in *Protea* species not inhabited by ophiostomatoid fungi (Lee *et al.*, 2005).

Although ophiostomatoid fungi are the dominant colonizers of the *Protea* seed-bearing structures (Marais & Wingfield, 2001; Roets *et al.*, 2005), many other fungal genera are known from these plants (Marincowitz *et al.*, 2008). Saprophytes (Lee *et al.*, 2003) as well as stem and foliicolous pathogens are common on *Protea* plants and may cause considerable damage (Crous *et al.*, 2011; Swart *et al.*, 1998; Taylor & Crous, 2000). Damaged inflorescences are useless from an economic perspective and result in great losses to the cut-and-dried-flower industry of which *Protea* species comprise a considerable portion.

### **3. Dispersal of ophiostomatoid fungi**

The relationship between bark beetles and ophiostomatoid fungi was realized as far back as 1929 (Dowding, 1969). Most of these fungi are dispersed by beetles that carry their spores in mycangia and inoculate them into beetle galleries (Bridges & Moser, 1983; Crone & Bachelder, 1961; Harrington, 2005; Moller & DeVay, 1968; Moser & Roton, 1971). In addition to the bark and ambrosia beetles, the role of mites in the transport of ophiostomatoid fungi is understood to be of great importance. Mites are phoretic on beetles, and therefore a

dual transportation system is available to the fungi (Levieux *et al.*, 1989; Moser *et al.*, 1997; Roets *et al.*, 2009a).

Ophiostomatoid fungi are characteristically associated with arthropods and Northern Hemisphere ophiostomatoid fungi have a close association with the arthropods that facilitate their transmission. The same association is therefore expected in *Protea*-specific ophiostomatoid fungi. The abundance of ophiostomatoid fungi in proteas reaches its peak during the wet winter months (Roets *et al.*, 2005), but their enclosed niche, *Protea* infructescences, presents a problem for spore dispersal. These brown enclosed structures are formed after every flowering season when serotinous *Protea* species close their involucre bracts around the inflorescences. They house the seeds and remain on the plant throughout its lifetime, opening only to release the seeds once the plant dies (Rebelo, 1995). The unique niche within infructescences is also suitable for fungi, bacteria and numerous arthropods. A variety of visiting beetles and mites are implicated in dispersal of the *Protea*-associated ophiostomatoid fungi (Roets *et al.*, 2007; 2009a; Ryke, 1964; Steenhuisen & Johnson, 2012; Theron *et al.*, 2012) and therefore provide a means of escape for the spores.

A certain Cetoniidae beetle, *Trichostetha fascicularis* L. or the Green protea beetle, occurs only on *Protea* species and carries mites with ophiostomatoid spores (Roets *et al.*, 2009a). Due to their size, mites are not capable of long-distance dispersal and, similar to their Northern Hemisphere counterparts, exploit visiting beetles for this purpose (Roets *et al.*, 2009a). When the infructescences desiccate with age, mites move up the stem towards more suitable conditions in younger infructescences, and in doing so spread the fungi between different infructescences on the same *Protea* tree – resulting in “vertical transmission”. Between different *Protea* trees, “lateral” ophiostomatoid dispersal is facilitated by mites phoretic on beetles (Roets *et al.*, 2009a). The number of arthropods in an infructescence increases with the age of the infructescence (Roets *et al.*, 2006) and, provided sufficient moisture is present, older infructescences should have established fungal and arthropod communities with good dispersal abilities. The number of mites carrying ophiostomatoid fungi that have been isolated from beetles and their abundance within the infructescences, indicate that mites are the primary vectors of these fungi (Roets *et al.*, 2009a).

#### 4. The genus *Knoxdaviesia*

The taxonomy of the ophiostomatoid fungi is continually revised as more knowledge concerning their phylogenetic relationships is generated. The first *Protea*-associated ophiostomatoid fungi were described as *Ceratocystiopsis protea* M.J. Wingf., P.S. Van Wyk & Marasas (Wingfield *et al.*, 1988) and *Ophiostoma capense* M.J. Wingf. & P.S. Van Wyk (Wingfield & Van Wyk, 1993). They were also the first ophiostomatoid fungi identified on indigenous South African flora. The taxonomy of both species was revised by Marais *et al.* (1998), because phylogenetic analyses indicated that these species were different from both *Ceratocystiopsis* and *Ophiostoma* and the new genus *Gondwanamyces* was described to accommodate them as *G. proteae* (M.J. Wingf., P.S. Van Wyk & Marasas) Marais & M.J. Wingf. and *G. capensis* (M.J. Wingf. & P.S. Van Wyk) Marais & M.J. Wingf (Marais *et al.*, 1998). The next *Gondwanamyces* species was described from weevil galleries in Costa Rica (Kolařík & Hulcr, 2009). The same authors also described *Custingophora cecropiae* M. Kolařík that was later transferred to *Gondwanamyces* (Van der Linde *et al.*, 2012). Two *Gondwanamyces* species associated with tree decline were identified on diseased *Euphorbia ingens* E. Meyer: Boissier trees in South Africa (Van der Linde *et al.*, 2012) and a third *Protea*-associated species was discovered in South Africa outside of the Cape Floristic Region (Crous *et al.*, 2012).

Most recently, the “one fungus, one name” system (Wingfield *et al.*, 2012) has implicated *Knoxdaviesia*, the anamorph (asexual) genus, as the correct genus to describe the previous *Gondwanamyces* species. Nine *Knoxdaviesia* species are currently known. From South Africa: *K. proteae*<sup>1</sup>, *K. capensis* and *K. wingfieldii* from *Protea* species and *K. serotectus* and *K. ubusi* from diseased *E. ingens* trees. From Costa Rica: *K. scolytodis* and *K. cecropiae* from weevil galleries in *Cecropia angustifolia* Trécul trees. *Knoxdaviesia suidafrikana* and *K. undulatistipes* known from South Africa and Thailand, respectively, were recently transferred to *Knoxdaviesia* from *Custingophora* (De Beer *et al.*, 2013a; Morgan-Jones & Sinclair, 1980; Pinnoi *et al.*, 2003).

Only three of the *Knoxdaviesia* species are associated with proteas. The most recent discovery was *K. wingfieldii* on *Protea caffra* Meisn. in KwaZulu-Natal (Crous *et al.*, 2012). *Knoxdaviesia proteae* occurs only on *Protea repens* L. (Wingfield *et al.*, 1988), whereas its sister species, *K. capensis*, has been isolated from several *Protea* species, but never from *P.*

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<sup>1</sup> Nomenclatural authorities are given in Table A.

*repens* (Roets *et al.*, 2009b; Wingfield & Van Wyk, 1993). Both grow in *Protea* infructescences as ascomatal masses on the flowers (Wingfield *et al.*, 1988; Wingfield & Van Wyk, 1993).

The primary vectors of *K. proteae* and *K. capensis* have been shown to be mites phoretic on beetles (Roets *et al.*, 2009a; 2011b; Wingfield *et al.*, 1988). The association of the Costa Rica species with weevils and the *Euphorbia* species with insect damage may be indicative of similar modes of transmission in these species. Mites of the genus *Trichouropoda* are the most frequent vectors of *Knoxdaviesia*, but *Ophiostoma* species have also been isolated from these mites (Roets *et al.*, 2011b), indicating that the two genera may display overlap in their vectors.

## 5. Sexual reproduction strategies in fungi

The reproductive mode of the organism plays an important role in determining how genetic material is inherited. In their *Protea* hosts, both the teleomorph (sexual) and anamorph (asexual) sporulating structures of the *Knoxdaviesia* species are encountered (Wingfield *et al.*, 1988; Wingfield & Van Wyk, 1993), but their reproductive mechanism remains unknown. In *P. repens*, *K. proteae* teleomorphs appear to occur at a greater frequency than the anamorphs (Wingfield *et al.*, 1988; personal observation), but only mitospores are produced in culture. As a result, the mode and relative importance of sexual reproduction in these species still has to be studied in natural populations.

Sexual reproduction in fungi is traditionally divided into two categories: homo- and heterothallism. Heterothallism is the mode of reproduction employed by most fungal species (Taylor *et al.*, 1999a), and requires outcrossing between two individuals for fertilization, whereas homothallic fungi can self-fertilize (Moore & Novak Frazer, 2002). The line separating the two categories is, however, ambiguous and most fungi do not strictly adhere to either. Taylor *et al.* (1999a) describes this phenomenon as a “continuum” from “predominantly homo- to predominantly heterothallic”.

Since thallism governs fungal reproduction, it affects population structure. Homothallic fungi that self-fertilize infrequently will have a similar population structure to heterothallic fungi – one with high genotypic diversity and random allele association (Milgroom, 1996). Haploid organisms that undergo self-fertilization will resemble a clonal population, because the products of meiosis are genetically identical (Fincham & Day, 1963; Milgroom, 1996; Moore



& Novak Frazer, 2002). These fungi will be poor adapters as mutation in the absence of recombination is a slow process and deleterious mutations will be hard to get rid of. However, if conditions are favourable, these fungi can maintain their suitable genotype. Conversely, outcrossing organisms can adapt rapidly, but will not be able to give the “perfect” genotype to all of its progeny. Both the evolution and ecology of an organism is therefore shaped by its mode of reproduction (Nieuwenhuis *et al.*, 2013).

Sexual reproductive mode is regulated by the mating type (MAT) genes. In ascomycete fungi, it is determined by a single locus and two mating types (Kronstad & Staben, 1997; Nelson, 1996). The two MAT idiomorphs – so called because of their sequence disparity – each confer a mating type. Two heterothallic individuals need to have opposite mating types to recombine (Kronstad & Staben, 1997; Nelson, 1996), but homothallic individuals have both idiomorphs residing in the same nucleus, enabling self-fertilization (Turgeon, 1998). Importantly, homothallic fungi have the *ability* to self-fertilize, but are not obligated to do so.

## 6. Genetic measures of dispersal

Molecular techniques are essential for assessing fungal dispersal, as tracking by visual observation is not suitable for micro-organisms (Peay *et al.*, 2008). Traditionally, fungal dispersal has been studied directly by noting the occurrence of disease symptoms. This approach is, however, limited to pathogens that cause clearly recognizable symptoms on their hosts and therefore excludes saprophytic organisms. Furthermore, observations are inaccurate, as the spread can only be tracked as far as symptoms appear and asymptomatic areas will be overlooked. Spore trapping is another direct method that has been used (Lacey, 1996), but analysis of the spores is laborious and identification requires specialized skills. With the advent of molecular markers, several markers were utilized for fingerprinting analyses. These direct techniques, however, have the disadvantage of seeing only a “snapshot” of the overall variation in time (Slatkin, 1985).

Species tend to occur in numerous populations separated geographically. Gene flow or the lack thereof, can be inferred by investigating genetic differentiation or gene flow between the populations – an indirect measure of dispersal (Stenlid & Gustafsson, 2001). Such indirect measures of dispersal use genetic information to infer past dispersal events – therefore dispersal events that have had a genetic impact on the population. In contrast, direct measures (such as spore trapping) focus on current dispersal, but cannot infer whether the subject

reached a suitable substrate or contributed to reproduction (Slatkin, 1987). When investigating the geographical dispersal of an organism we are therefore essentially considering gene flow between different populations.

In the 1960's, protein electrophoresis was the first technique used to investigate molecular variation. Presently, DNA markers are used preferably and many have been developed and used for studying genetic variation. These include restriction fragment length polymorphisms (RFLPs) (Boeger *et al.*, 1993; Moyersoer *et al.*, 2003; Uthicke & Benzie, 2003), amplified fragment length polymorphisms (AFLPs) (Gaudeul *et al.*, 2000), vegetative compatibility groups (VCGs – only in fungi) (Bayman & Cotty, 1991), single nucleotide polymorphisms (SNPs) (Wang *et al.*, 1998) and microsatellites (Barnes *et al.*, 2001).

Thus far, research employing DNA markers to assess gene flow and dispersal has been biased (and understandably so) towards pathogens (McDonald, 1997). Tracking the source and epidemiology of a pathogen is important. The same molecular techniques applied to pathogens can be employed when considering saprophytes. Genetic markers are subject to mutation and therefore closely related isolates should have similar variability within markers. Using this approach, the origin of an isolate can be determined by identifying a source with the same genotype. Examples from pathogenic organisms therefore remain useful for non-pathogens (McDonald & McDermott, 1993).

## **7. Microsatellites – increasingly popular molecular markers**

Microsatellites belong to a class of repeats known as Variable Number of Tandem Repeats (VNTRs). They consist of 1-6 base pair motifs (Chambers & MacAvoy, 2000) that are repeated a variable number of times. The polymorphism of these loci is due to the number of tandem repeats and not the actual sequence (Ellegren, 2004). The hyper-variability of microsatellite loci is due to slippage of the DNA polymerase enzyme and misalignment of the template or nascent strand after re-annealing (Tautz, 1989). The frequency of mutation at these loci is higher than that of other genomic areas, resulting in polymorphism within a population. The frequency is also, however, sufficiently low that a newly evolved allele at such a locus will likely be propagated to the next generation (Charlesworth *et al.*, 1994; Tautz, 1989).

## 7.1. Microsatellite evolution

Similar to other repeat elements (Kidwell & Lisch, 1997; Schaack *et al.*, 2010), the evolution of microsatellites has been described as a “life-cycle” (Amos, 1999; Buschiazzo & Gemmell, 2006). The life-cycle starts with a proto-microsatellite that arises spontaneously from regions with “cryptic simplicity” or is introduced via mobile elements (Nadir *et al.*, 1996; Wilder & Hollocher, 2001). Once the proto-microsatellite gains enough repeats to enable repeat slippage, it has become a microsatellite. The threshold for slippage has been found to be eight base pairs (Shinde *et al.*, 2003) or between four and eight tandem repeats (Rose & Falush, 1998). At this stage, the repeat structure of the microsatellite is perfect and it undergoes unabated repeat slippage. However, due to other mutations that act on the repeat and disrupt its purity, microsatellites do not experience uncontrolled expansion. Point mutations, for example, break a microsatellite into smaller parts, making slippage less effective (Schug *et al.*, 1998). The number of point mutations and slippage events eventually reaches a balance that equalizes the rate of contraction and expansion (Kruglyak *et al.*, 1998; Xu *et al.*, 2000) until the point mutations accumulate to the extent that contraction is favoured (Buschiazzo & Gemmell, 2006; Xu *et al.*, 2000). Slippage becomes less frequent, reducing the mutation rate and therefore the variability of the microsatellite. This ultimately results in “death” when deletions act on the now-stable repeat (Taylor *et al.*, 1999b). The repeat region once again resembles the “cryptic sequence” it started out as and comes full circle by having the potential to become a proto-microsatellite.

In order to use microsatellites for gaining information, an understanding of the mutational processes involved during the life-cycle is required. Ellegren (2000) paradoxically observed that “simple repeats do not evolve simply”. This statement continues to gather support as studies into the mutational mechanisms of microsatellites reveal a highly complex process differing between species, alleles and even loci (Ellegren, 2000; Webster *et al.*, 2002). With a mutation rate of between  $10^{-2}$  and  $10^{-6}$ /generation (Ellegren, 2000; Schlötterer, 2000), microsatellites are among the most rapidly evolving regions in the genome – *Neurospora* microsatellites evolve at approximately 2 500 times the rate of unique ascomycete DNA (Dettman & Taylor, 2004). This complex evolution, coupled with a high mutation rate, is the reason for the variability observed in these regions.

## 7.2. Mutation Models

Numerous theories and models about microsatellite evolution and mutation exist and are continuously changed and improved. These are essential to be able to calculate genetic parameters from microsatellite data. The mutational model affects the inferences that will be made from the data – microsatellite markers are especially sensitive to models because of their high mutation rate (Estoup & Cornuet, 1999). The Stepwise Mutation Model (SMM) and Infinite Alleles Model (IAM) are the two basic models used to determine genetic distance and represent opposite extremes. The SMM assumes that microsatellites change by one repeat unit or “one step” per mutation event (Ohta & Kimura, 1973). Size homoplasies are common in this model, since unrelated alleles may converge to the same state. In contrast, the IAM states that every mutation introduces a new allele so that two alleles can only be identical if they descended from the same parent without mutation (Kimura & Crow, 1964). A microsatellite may change by any number of repeat units per generation, always introducing a novel allele (Estoup & Cornuet, 1999). The key problem with the SMM is its rigidity in allowing only one-step mutations. Nevertheless, it remains useful and applicable for investigating relatedness of individuals and determining population structure (Dettman & Taylor, 2004; Oliveira *et al.*, 2006; Valdes *et al.*, 1993). Although the SMM is sufficient in many cases, it cannot be ignored that changes of more than one repeat unit, although rare, do occur (Di Rienzo *et al.*, 1994; Huang *et al.*, 2002). Di Rienzo *et al.* (1994) developed the Two-Phase Model (TPM) when they realized that allowing a low probability (0.2 to 0.05) of multi-step changes, optimized the fit to their data. Other studies also found that a low level of multi-step changes accounts for data patterns (Renwick *et al.*, 2001). The TPM is therefore appropriate for situations in which the majority of changes are one-step and multi-step changes occur at low probability.

These models are the best known and those used most frequently, however, none of them can simulate the reality of microsatellite evolution, for example, none account for the upper and lower size constraints observed in microsatellite alleles. More complex models have been formulated and many researchers amend the basic ones to suit their individual needs. “All models have some disadvantages when applied to microsatellite data” (Oliveira *et al.*, 2006), it is a matter of identifying the one with the most tolerable disadvantages.

### 7.3. Current Applications

Microsatellites remain popular even though markers such as AFLPs and SNPs have been developed more recently. This is because the advantages associated with microsatellites outweigh most of the disadvantages (Luikart & England, 1999). Microsatellites are co-dominant Mendelian markers that may be highly polymorphic (Tautz, 1989). This, together with their short lengths (< 100 base pairs), make them ideal for historical and biological inferences. Development is the main inconvenience associated with microsatellites. The flanking regions of the microsatellites, although conserved, become more divergent as the phylogenetic distance between two organisms increases (Ellegren, 2004; Primmer & Merilä, 2002). Specific markers therefore have to be developed for the species of interest. Closely related species may have the benefit of transferability – loci identified in one species are amplifiable in another – but this is seldom the case. The process of developing the markers, however, has become much easier in recent years.

Whole genome sequencing and *in silico* methods have reduced the time and effort associated with microsatellite development. Nevertheless, sequence data for many organisms are yet to be generated and traditional methods of isolation therefore remain applicable. Two methods are popular for constructing a microsatellite-enriched library: ISSR-PCR and FIASCO (Zane *et al.*, 2002). In both cases, the motif of the microsatellite to be analyzed has to be “guessed” and probed for (Castoe *et al.*, 2010). ISSR-PCR (Interspersed Simple Sequence Repeat-PCR), analogous to RAMS (Random Amplification of Microsatellite Sequences) (Hantula *et al.*, 1996), relies on amplification reactions with several primers that contain and therefore target microsatellite motifs. The limiting factor of this method is that the isolated microsatellites are often situated on the sequence terminals and additional genome walking steps are required to obtain adequate flanking sequences (Barnes *et al.*, 2001; 2008). FIASCO (Fast Isolation by AFLP of Sequences COntaining repeats) uses the motifs as probes to isolate the DNA fragments containing microsatellite DNA in a pool of restricted genomic DNA. Both methods involve cloning the enriched fragments of DNA and sequencing the inserts. Fortunately, new generation sequencing (NGS) provides a “short-cut” by sequencing the entire enriched library without cloning. This modification to the traditional techniques was first published by Santana *et al.* (2009). Presently, the read lengths of the NGS technologies, specifically the 454 GS-FLX genome sequencer from Roche, has improved to the extent that many researchers simply do shotgun genome sequencing without enrichment to generate sequence

data for identifying microsattellites (Abdelkrim *et al.*, 2009; Rasmussen & Noor, 2009; Yu *et al.*, 2011).

## 8. Problem Statement

Sales and export of *Protea* species form a significant part of the fresh and dried cut-flower industry and is an important contributor to the South African economy (Coetzee & Littlejohn, 2001). Approximately 3 000 hectares of Proteaceae cultivation is undertaken in South Africa (Knoesen & Conradie, 2009), and over 3 000 tons (2011 figures) of fynbos products – of which proteas make up the majority (60%), are exported annually (Kotze, 2012). The economic importance of proteas also extends to the thousands of people that are employed by this industry – approximately 20 000 people owe their monthly income directly to the flora of the Western Cape Province ([www.ars.usda.gov/](http://www.ars.usda.gov/)). The presence of micro-organisms and insects on economically important products is always a concern and merits investigation.

As outlined earlier, much progress concerning the identities, vectors and lifecycle of the *Protea*-associated ophiostomatoid fungi has been made in recent years (Roets *et al.*, 2005; 2007; 2008; 2009a; 2010; 2011a). We wish to extend this knowledge by investigating the population structure of these fungi and inferring how far they are able to spread. The significance of studying the population structure is related to the role the ophiostomatoid fungi play in the lifecycle of their *Protea* hosts. This role remains uncertain and, even more so, their potential effect on harmful fungal competitors. Investigating the gene flow between populations will establish to what extent a local population is independent of this unifying force – in other words, to what extent it will be able to change its genetic make-up (Slatkin, 1985). The *Knoxdaviesia* species known from hosts other than proteas are pathogens, as are their Northern Hemisphere ophiostomatoid counterparts. The presence and function of these fungi within a keystone plant genus in South Africa is therefore a topic of great ecological and economic interest.

Traditional methods of monitoring fungal movement have proven unsuitable and insufficient (Lacey, 1996; Peay *et al.*, 2008; Slatkin, 1985). Access to molecular methodologies will enable the dispersal of the *Protea*-associated ophiostomatoid fungi to be tracked in a reliable manner. Of special interest, is the movement of these fungi across the landscape to recolonize areas destroyed by fire. Massive areas of burnt fynbos vegetation are recolonized, presumably via beetles and mites carrying spores derived from other populations (Roets *et al.*, 2009a).

## 9. Objectives of this study

The four principal objectives of the study were to: 1) develop polymorphic microsatellite loci specific to *Knoxdaviesia proteae*; 2) test the transferability of the microsatellite markers to other *Knoxdaviesia* species; 3) employ the markers to assess the genetic structure and diversity of *K. proteae* in *Protea repens*; and 4) investigate the reproductive strategy of *K. proteae*.

The first two objectives were investigated in Chapter 1 and this manuscript has recently been accepted for publication in *Mycological Progress* (DOI: 10.1007/s11557-013-0951-1). In Chapters 2 and 3, the developed microsatellite markers were used to explore the population genetics of *K. proteae* in two distantly separated *P. repens* populations. Through these population studies, the questions regarding the genetic structure/diversity and reproductive strategy of *K. proteae* could be addressed. The first population genetics study in Chapter 2 considers the population structure of this fungus within a stand of *P. repens* plants, whereas the focus of Chapter 3 is on the fungal recolonization of areas of burnt fynbos.

**Table A: Summary of all the *Knoxdaviesia* and *Protea*-associated ophiostomatoid species known to date**

Species	Origin	Location <sup>A</sup>	References
<i>Knoxdaviesia capensis</i> M.J. Wingf. & P.S. van Wyk	<i>Protea</i> species	Western Cape	Wingfield & Van Wyk 1993; Marais <i>et al.</i> 1998
<i>K. cecropiae</i> (M. Kolařík) Z.W. de Beer & M.J. Wingf.	Weevil galleries in <i>Cecropia angustifolia</i> Trécul	Costa Rica	Kolařík & Hulcr 2009
<i>K. proteae</i> M.J. Wingf., P.S. van Wyk & Marasas	<i>Protea repens</i> L.	Western Cape	Wingfield <i>et al.</i> 1988; Marais <i>et al.</i> 1998
<i>K. scolytodis</i> (M. Kolařík) Z.W. de Beer & M.J. Wingf.	Weevil galleries in <i>C. angustifolia</i> Trécul	Costa Rica	Kolařík & Hulcr 2009
<i>K. serotectus</i> (van der Linde & Jol. Roux) Z.W. de Beer & M.J. Wingf.	<i>Euphorbia ingens</i> (E. Meyer) Boissier	Limpopo	Van der Linde <i>et al.</i> 2012
<i>K. suidafrikana</i> (Morgan-Jones & R.C. Sinclair) Z.W. de Beer & M.J. Wingf.	Decorticated wood	South Africa	Morgan-Jones & Sinclair 1980
<i>K. ubusi</i> (van der Linde & Jol. Roux) Z.W. de Beer & M.J. Wingf.	<i>E. ingens</i>	Limpopo	Van der Linde <i>et al.</i> 2012
<i>K. undulatistipes</i> (Pinnoi) Z.W. de Beer & M.J. Wingf.	<i>Eleiodoxa conferta</i> (Griff.) Burret	Thailand	Pinnoi <i>et al.</i> 2003
<i>K. wingfieldii</i> (Roets & Dreyer) Z.W. de Beer & M.J. Wingf.	<i>P. caffra</i> Meisn.	KwaZulu-Natal	Crous <i>et al.</i> 2012



<i>Ophiostoma africanum</i> Marais & M.J. Wingf.	<i>P. gagedi</i> J.F.Gmel.; <i>P. caffra</i> ; <i>P. dracomontana</i> Beard	KZN; Gauteng	Marais & Wingfield 2001; Roets <i>et al.</i> 2006
<i>O. gemellus</i> Roets, Z.W. de Beer & P.W. Crous	<i>Tarsonemus</i> sp. (on <i>P. caffra</i> )	Gauteng	Roets <i>et al.</i> 2008
<i>O. palmiculatum</i> Roets, Z.W. de Beer & M.J. Wingf.	<i>P. repens</i> (insect tunnels)	Western Cape	Roets <i>et al.</i> 2006
<i>O. phasma</i> Roets, Z.W. de Beer & M.J. Wingf.	<i>P. neriifolia</i> R.Br. <i>P. laurifoli</i> Thunb.	Western Cape	Roets <i>et al.</i> 2006
<i>O. protearum</i> Marais & M. J. Wingf.	<i>P. caffra</i>	Gauteng	Marais & Wingfield 1997
<i>O. protea-sedis</i> Roets, M.J. Wingf. & Z.W. de Beer	<i>P. caffra</i>	Nchila, Zambia	Roets <i>et al.</i> 2010
<i>O. splendens</i> Marais & Wingfield	<i>P. repens</i> ; one isolate from <i>P. neriifolia</i>	Western Cape	Marais & Wingfield 1994; Roets <i>et al.</i> 2005
<i>O. zambiensis</i> Roets, M.J. Wingf. & Z.W. de Beer	<i>P. caffra</i>	Nchila, Zambia	Roets <i>et al.</i> 2010
<i>Sporothrix variecibatus</i> Roets, Z.W. de Beer & P.W. Crous	<i>Trichouropda</i> sp. (on <i>P.</i> <i>repens</i> ); <i>P. longifolia</i> Andrews	Western Cape	Roets <i>et al.</i> 2008

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<sup>A</sup> Provinces are in South Africa, unless otherwise stated.

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## CHAPTER 1

# Development of polymorphic microsatellite markers for the genetic characterization of *Knoxdaviesia proteae* (Ascomycota: Microascales) using ISSR-PCR and pyrosequencing

### Abstract

*Knoxdaviesia proteae* is one of the first native ophiostomatoid fungi discovered in South Africa, where it consistently occurs in the infructescences of the iconic Cape Biome plant, *Protea repens*. Although numerous studies have been undertaken to better understand the ecology of *K. proteae*, many questions remain to be answered, particularly given its unique niche and association with arthropods for dispersal. We describe the development and distribution of microsatellite markers in *K. proteae* through ISSR-PCR enrichment and pyrosequencing. A large proportion of the 31492 sequences obtained from sequencing the enriched genomic DNA were characterized by microsatellites consisting of short tandem repeats and di- and tri-nucleotide motifs. Seventeen percent of these microsatellites contained flanking regions sufficient for primer design. Twenty-three primer pairs were tested, of which 13 amplified and 12 generated polymorphic fragments in *K. proteae*. Half of these could be transferred to the sister species, *K. capensis*. The markers developed here will be used to investigate the reproductive strategy, genetic diversity and dispersal strategies of *K. proteae* and potentially *K. capensis*.

**Keywords:** ISSR-PCR, *Knoxdaviesia*, microsatellites, ophiostomatoid, pyrosequencing

## 1. Introduction

*Knoxdaviesia proteae* M.J. Wingf., P.S. van Wyk & Marasas is a member of a polyphyletic group (Spatafora & Blackwell, 1994) known as the ophiostomatoid fungi (Wingfield *et al.*, 1999) that consistently inhabits the fruiting structures (infructescences) of *Protea* species. All evidence suggests that this biogeographically interesting fungus is confined to the single host species, *Protea repens* L. (Roets *et al.*, 2009b), where it appears as ascomatal masses on the flowers (Wingfield *et al.*, 1988). In addition to *K. proteae*, eight other species belonging to this genus have been described. Of these, *K. capensis* M.J. Wingf. & P.S. van Wyk and *K. wingfieldii* (Roets & Dreyer) Z.W. de Beer & M.J. Wingf. are also known from *Protea* species (Crous *et al.*, 2012; Wingfield & Van Wyk, 1993), while *K. serotectus* (van der Linde & Jol. Roux) Z.W. de Beer & M.J. Wingf. and *K. ubusi* (van der Linde & Jol. Roux) Z.W. de Beer & M.J. Wingf. were isolated from declining *Euphorbia ingens* (E. Meyer) Boissier trees in South Africa (Van der Linde *et al.*, 2012). *Knoxdaviesia scolytodis* (M. Kolařík) Z.W. de Beer & M.J. Wingf. and *K. cecropiae* (M. Kolařík) Z.W. de Beer & M.J. Wingf. originate from weevil galleries on *Cecropia angustifolia* Trécul trees in Costa Rica (Kolařík & Hulcr, 2009; Van der Linde *et al.*, 2012). *Knoxdaviesia suidafrikana* (Morgan-Jones & R.C. Sinclair) Z.W. de Beer & M.J. Wingf. and *K. undulatistipes* (Pinnoi) Z.W. de Beer & M.J. Wingf. were recently transferred to *Knoxdaviesia* from *Custingophora* (De Beer *et al.*, 2013b).

The association of *K. proteae* with *P. repens* represents an intricate symbiosis in which mites act as the primary fungal dispersers between flower heads and beetles act as the vehicles for mite dispersal (Roets *et al.*, 2007a; 2009a; 2011b). Apart from its modes of dispersal, virtually nothing is known regarding the ecology of *K. proteae*. It is presumed to be a saprobe, but early occupation of infructescences suggests that it may also be able to colonize living tissues (Roets *et al.*, 2005; F. Roets, personal observation). This, coupled with its high level of host specificity, suggests a more complex ecological involvement with *P. repens*.

Although a basic understanding of the ophiostomatoid fungi in *Protea* infructescences has emerged in recent years (Roets *et al.*, 2005; 2007a; 2009a; 2011a; 2011b), very little is known regarding the population biology of these fungi, their reproductive strategies and how these contribute to issues such as dispersal and host association. For example, *P. repens* is a re-seeder in which the infructescences open and release the seeds when their water supply is interrupted, such as after fire (Rebello, 1995). Since both plants and fungi die in these often vast fires, it remains a mystery where the ophiostomatoid fungi encountered in new *P. repens*

infructescences come from. The aim of this study was, therefore to develop microsatellite markers as a tool to study the genetic diversity and dispersal biology of *K. proteae*, serving as model for other taxa within this unique ecosystem.

## 2. Materials and Methods

### 2.1. Fungal cultures and identification

Strains of *K. proteae* and other *Knoxdaviesia* species were sourced from the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (Table 1.1). Additional *K. proteae* strains were isolated from *P. repens* infructescences from four different locations in the Western Cape Province, South Africa (Table 1.1). Isolations were made on Malt Extract Agar (MEA; Merck, Wadeville, South Africa) supplemented with 0.04 g/L Streptomycin Sulfate Salt (Sigma-Aldrich, Steinham, Germany) as described by Roets *et al.* (2006a). Individual strains were isolated by sub-culturing a hyphal tip from Water Agar (15 g agar/L) to fresh MEA.

Fungal isolates were grown on MEA overlaid with sterile 3.5 x 3.5 cm<sup>2</sup> cellophane sheets (Product no. Z377597, Sigma-Aldrich, Steinham, Germany). Mycelium was scraped from the cellophane, placed in Eppendorf tubes and shaken vigorously with a vortex mixer in TES buffer (Möller *et al.*, 1992), 70 µg PCR grade Proteinase K (Roche Applied Science, Mannheim, Germany) and glass beads. Subsequent extraction and purification steps followed those of Möller *et al.* (1992). A NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) was used to determine the quality and quantity of the extracted DNA. The species identity of isolates were confirmed by sequencing the ribosomal RNA Internal Transcribed Spacer (ITS) regions and performing BLAST (Basic Local Alignment Search Tool) searches on the NCBI nucleotide data base ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). Amplification and sequencing of the ITS region was done with KAPA Taq ReadyMix (Kapa Biosystems, Inc., Boston, USA) and followed previously described methods and protocols (White *et al.*, 1990).

Table 1.1: *Knoxdaviesia* species and strains studied

<i>Knoxdaviesia</i> species	Strains <sup>A</sup>	Type	Location <sup>C</sup>	Isolated from	Reference
<i>K. capensis</i>	CMW 974	-	Jonkershoek	<i>Protea coronata</i>	Roets <i>et al.</i> 2009b
<i>K. capensis</i>	CMW 997	Holotype	Hermanus	<i>P. longifolia</i>	Wingfield and van Wyk 1993
<i>K. capensis</i>	CMW 11962; CBS 119215	-	Stellenbosch Mountain	<i>P. laurifolia</i>	Roets <i>et al.</i> 2005
<i>K. cecropiae</i>	CMW 22991	Isotype	Finca Morillo, Costa Rica	<i>Cecropia</i> sp.	Kolařík and Hulcr 2009
<i>K. cecropiae</i>	CMW 22993	-		<i>Cecropia</i> sp.	
<i>K. proteae</i>	CMW 738 <sup>B</sup> ; CBS 486.88	Ex-Holotype	Stellenbosch	<i>P. repens</i>	Wingfield <i>et al.</i> 1988
<i>K. proteae</i>	CMW 1043	-	Mossel Bay	<i>P. repens</i>	Roets <i>et al.</i> 2009b
<i>K. proteae</i>	G001 <sup>B</sup>	-	Gouritz	<i>P. repens</i>	
<i>K. proteae</i>	G002 <sup>B</sup>	-	Gouritz	<i>P. repens</i>	
<i>K. proteae</i>	G003 <sup>B</sup>	-	Gouritz	<i>P. repens</i>	
<i>K. proteae</i>	G004 <sup>B</sup>	-	Gouritz	<i>P. repens</i>	
<i>K. proteae</i>	G005 <sup>B</sup>	-	Gouritz	<i>P. repens</i>	
<i>K. proteae</i>	G006 <sup>B</sup>	-	Outeniqua Pass	<i>P. repens</i>	
<i>K. proteae</i>	G007 <sup>B</sup>	-	Outeniqua Pass	<i>P. repens</i>	
<i>K. proteae</i>	G008 <sup>B</sup>	-	Uniondale	<i>P. repens</i>	
<i>K. proteae</i>	G009 <sup>B</sup>	-	Sir Lowry's Pass	<i>P. repens</i>	
<i>K. scolytodis</i>	CMW 22995	-	Finca Morillo, Costa Rica	<i>Cecropia</i> sp.	Kolařík and Hulcr 2009
<i>K. serotexi</i>	CMW 34100	-	Louis Trichardt	<i>Euphorbia ingens</i>	
<i>K. serotexi</i>	CMW 36767	-	Louis Trichardt	<i>E. ingens</i>	Van der Linde <i>et al.</i> 2012
<i>K. serotexi</i>	CMW 36768	Isotype	Louis Trichardt	<i>E. ingens</i>	

<i>K. ubusi</i>	CMW 36769	-	Grahamstown	<i>E. tetragona</i>	Van der Linde <i>et al.</i> 2012
<i>K. ubusi</i>	CMW 36770	Isotype	Grahamstown	<i>E. tetragona</i>	

<sup>A</sup> CMW refers to the Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa; CBS refers to the Centraalbureau voor Schimmelcultures, The Netherlands.

<sup>B</sup> Isolates used for testing microsatellite polymorphism.

<sup>C</sup> Unless stated otherwise, all locations are in South Africa.

## 2.2. Microsatellite enrichment

*Knoxdaviesia proteae* isolates used for microsatellite development included the ex-type strain (CMW 738) collected in the Stellenbosch area and isolate CMW 1043 from the Mossel Bay area (both in the Western Cape Province, South Africa). The ISSR-PCR (Interspersed Simple Sequence Repeat-Polymerase Chain Reaction) technique (Hantula *et al.*, 1996; Zietkiewicz *et al.*, 1994) was used to establish a pool of microsatellite-enriched genomic DNA. ISSR primers ISSR1 - 5'-DDB(CCA)<sub>5</sub>, ISSR2 - 5'-DHB(CGA)<sub>5</sub>, ISSR3 - 5'-YHY(GT)<sub>5</sub>G, ISSR4 - 5'-HVH(GTG)<sub>5</sub>, ISSR5 - 5'-NDB(CA)<sub>7</sub>C, ISSR6 - 5'-NDV(CT)<sub>8</sub>, and ISSR7 - 5'-HBDB(GACA)<sub>4</sub> were used following previously published methods (Santana *et al.*, 2009). These primers were applied in 41 different combinations ranging from one to four primers per reaction and amplified using the Fast Start High Fidelity PCR System (Roche Applied Science, Mannheim, Germany). The 50 µl reactions consisted of 5 µl 10x buffer, 1.8 mM MgCl<sub>2</sub>, 2 µl Dimethyl sulfoxide, 200 µM of each dNTP, 0.1 µM of each primer, approximately 100 ng genomic DNA and 2.5 units of the FastStart High Fidelity Enzyme Blend. Reaction conditions were: 5 minutes at 95°C followed by 40 cycles of 30 seconds at 94°C, 30 seconds at 45°C and 2 minutes at 72°C, with a final extension step for 7 minutes at 72°C.

The 41 PCR reactions were pooled and purified using the Agencourt® AMPure® XP PCR Purification kit (Beckman Coulter, Massachusetts, USA) and eluted in low TE buffer (10 mM Tris-HCl, pH 8.0; 0.1 mM EDTA). A NanoDrop ND-1000 spectrophotometer (Thermo



Fisher Scientific, Wilmington, USA) and Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, California) were used to, respectively, qualify and quantify the microsatellite-enriched DNA in the sample. The final product was sent for pyrosequencing on the 454 Life Sciences/Roche GS-FLX sequencer (Roche Applied Science, Penzberg, Germany) (Margulies *et al.*, 2005) at Inqaba Biotechnological Industries, Pretoria, South Africa.

### **2.3. Microsatellite marker development**

Duplicate sequences were removed from the 454 data using cdhit\_454 (identity threshold = 0.98) (Niu *et al.*, 2010). Microsatellites were identified with MSATFINDER ONLINE 2.0 (Thurston & Field, 2005) using the REGEX search engine and default search parameters to search for perfect repeats. Data were analyzed using Microsoft Office Excel 2010 (Microsoft Corp., Redmond, WA, USA).

Primers were designed with the help of MSATFINDER and PRIMER3PLUS (Untergasser *et al.*, 2007) and sequence analysis was done with BIOEDIT 7.1.3.0 (Hall, 1999). To ensure that the identified tri-nucleotide loci were not located in coding regions, the relevant pyrosequencing reads were subjected to a BLAST search using the least conservative engine, BLASTtx ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). Polymorphism was assessed by amplifying and sequencing all loci in a set of 10 *K. proteae* isolates (Table 1.1). Amplification of these loci in other *Knoxdaviesia* species (Table 1.1) was also tested.

To test random association of loci, the data from the polymorphism tests were used to determine two different measures of assessing pair-wise linkage disequilibrium. Fisher's exact test was calculated with GENPOPOP 4.2 (Rousset, 2008) and  $\bar{r}_d$ , the index of multilocus linkage disequilibrium, was calculated with MULTILOCUS 1.3b (Agapow & Burt, 2001). False discovery rate using GENPOPOP was avoided as recommended by Benjamini & Yekutieli (2001).

## **3. Results and Discussion**

### **3.1. Roche 454 sequence data**

A total of 31492 sequences with an average read length of 281.2 base pairs were generated using pyrosequencing. Of these, 16820 (53%) were unique and 5408 (17%) had sufficient sequence data flanking the microsatellite loci for primer design. Although contigs were not

constructed from our 454 data, the proportion of amplifiable sequences was slightly higher than the 14% noted by Santana *et al.* (2009), who used the same Roche 454 technology. High-throughput sequencing of an enriched pool of microsatellites, therefore, provided an immense improvement on the traditional practice of cloning and Sanger sequencing (Santana *et al.*, 2009). The procedure was not only less laborious and time-consuming, but also afforded the advantage of being able to choose loci of interest.

### 3.2. Identification of microsatellites

Since microsatellites with higher-than-average repeat numbers are more likely to be polymorphic (Dettman & Taylor, 2004; Dutech *et al.*, 2007; Goldstein & Clark, 1995), only those with repeat numbers greater than five were tested. Twenty three primer pairs were initially designed and tested. Of these, 13 loci amplified in *K. proteae* and 12 generated polymorphic fragments (Table 1.2). The other was a compound microsatellite, (ACAG)<sub>6</sub>-<sub>7</sub>(C)<sub>n</sub>, and was discarded as its mutational mechanism may differ significantly from that of perfect repeats. The number of alleles detected ranged from 2 to 9 with a mean of  $4.75 \pm 0.55$  – similar to the average allele number of  $5.4 \pm 0.4$  reported for fungi (Dutech *et al.*, 2007). Neither Fisher's exact test nor the  $\bar{r}_d$  value indicated significant pairwise linkage after performing the linkage disequilibrium tests.

### 3.3. Cross-species transferability

In the three *K. capensis* isolates, six of the 12 microsatellite markers amplified in isolates CMW 997 and CMW 19962 and four in CMW 974. All six markers were polymorphic between *K. proteae* and *K. capensis* and polymorphism could also be detected within *K. capensis* for loci KX4, KX6 and KX11. The cross-species transferability rate was higher than expected based on previous fungal studies. For example, in a survey of fungal studies Dutech *et al.* (2007) reported a transferability rate of approximately 34%. However, this high rate of marker transferability was not surprising, as *K. capensis* is phylogenetically most closely related to *K. proteae*, and these two species group separately from other *Knoxdaviesia* species in phylogenetic trees (De Beer *et al.*, 2013c; Wingfield *et al.*, 1999).

Table 1.2: Polymorphic microsatellites developed for *Knoxdaviesia proteae*

Locus	Motif	Genbank accession	Primer names	Primer sequences (5'-3')	Fluorescent tag	Size range	Alleles		Transferable
							Gouritz <sup>A</sup>	Total <sup>B</sup>	
KX1	(tc) <sub>16</sub>	KF924611	KX1-1	GAGACATACTGGACTGTACACATTCAT	VIC	107-115	4	9	-
			KX1-2	CGTCCTGTAGTGGCTATCCTG	-				
KX2	(ctt) <sub>16</sub>	KF924612	KX2-1	TGTCGGGTCCTGTGTAAGT	PET	108-144	4	8	-
			KX2-2	CGGATTGTATCAGTTGTCCTCA	-				
KX3	(ctt) <sub>13</sub>	KF924613	KX3-1	CGGGACTCCACTCTCTCAAG	VIC	173-200	1	3	-
			KX3-2	CCGGACTCTAGACGTTGAGG	-				
KX4	(gtc) <sub>12</sub>	KF924614	KX4-1	AAGAAAAGCCAAGGGGAGAG	NED	166-184	2	5	<i>K. capensis</i> <sup>C</sup>
			KX4-2	ACTTACAGGCCAGGACCACA	-				
KX5	(cag) <sub>11</sub>	KF924615	KX5-1	ACGACAGCAGATGCATGAAG	NED	115-127	4	5	-
			KX5-2	GCTGTCTTGCTGCTGAACTG	-				
KX6	(acc) <sub>9</sub>	KF924616	KX6-1	CAAATGGTGCCCTATGACC	6-FAM	189-198	3	4	<i>K. capensis</i> <sup>C</sup>
			KX6-2	ACAAGCCAGAGTTTGGAGGA	-				

KX7	(ctt) <sub>9</sub>	KF924617	KX7-1	AGACGCTTCCATCTCGTTTC	6-FAM	96-108	2	3	<i>K. capensis</i>
			KX7-2	GGAGATGGCGAGAGAAGTTG	-				
KX8	(atgg) <sub>7</sub>	KF924618	KX8-1	GACGACGATCACAGGACGAC	6-FAM	104-116	2	4	<i>K. capensis</i>
			KX8-2	CAAGCCCTCAAAGTTGCTTC	-				
KX9	(agtg) <sub>8</sub>	KF924619	KX9-1	TGAGATTGCGAGTGTGCTTC	VIC	100-132	2	4	-
			KX9-2	GGTGTGCATCAATTGTTCGT	-				
KX10	(attc) <sub>6</sub>	KF924620	KX10-1	CCCCATCAGTTCACTGACATC	PET	183-195	4	5	<i>K. capensis</i>
			KX10-2	GACCACAGTTGGGAAAATCG	-				
KX11	(agtg) <sub>7</sub>	KF924621	KX11-1	AGAGAGCCTTCCCAAAAGGT	6-FAM	185-189	2	2	<i>K. capensis</i> <sup>C</sup>
			KX11-2	GCGAAAGGGAACATAAATCG	-				
KX12	(ctgt) <sub>16</sub>	KF924622	KX12-1	GGGCAGAACCGTTCATATTC	VIC	183-231	4	5	-
			KX12-2	AACCCTGCGTCAGACACC	-				

<sup>A</sup> Alleles from the 5 strains isolated from the Gouritz area.

<sup>B</sup> Alleles from all 10 strains used to test polymorphism.

<sup>C</sup> Polymorphic in *K. capensis*.

### 3.4. Distribution of microsatellites in *Knoxdaviesia proteae* 454 sequence data

Although the microsatellite enrichment strategy employed in this study might have skewed the observed distribution of these motifs in *K. proteae*, our results are consistent with those for fungi. For example, studies considering whole-genome sequences (Karaoglu *et al.*, 2005; Katti *et al.*, 2001; Lim *et al.*, 2004) have shown that fungal microsatellites tend to be short and that mono-, di- and tri-nucleotide motifs predominate. Di- and tri-nucleotides were encountered frequently in our data, while mono-nucleotide motifs comprised only 1.3% of the identified microsatellites (Table 1.3). Such frequencies have also been reported following analysis of an ISSR-PCR enriched library for *Fusarium circinatum* Nirenberg & O'Donnell (Santana *et al.*, 2009), suggesting that the relatively few mono-nucleotide microsatellites observed in this study might be a consequence of the specific enrichment method used.

**Table 1.3: Microsatellite repeat classes in the 454 data<sup>A</sup>**

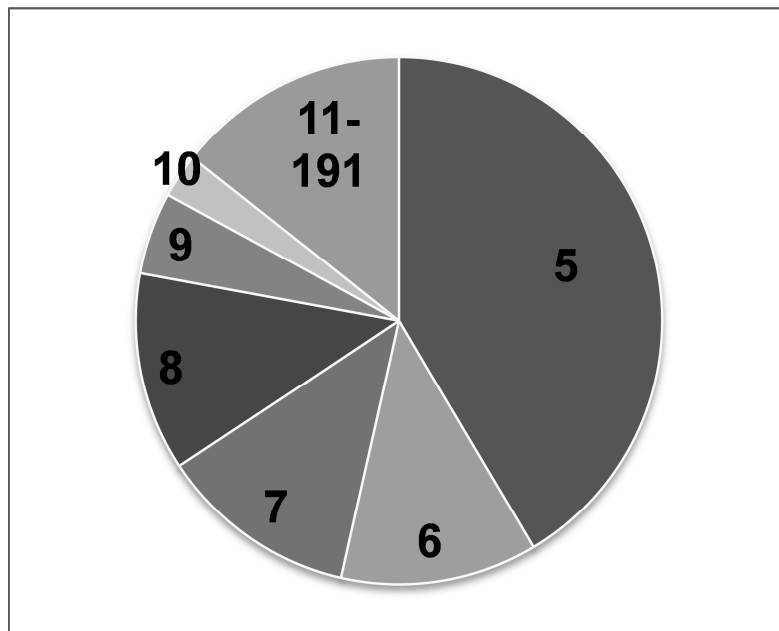
Class <sup>B</sup>	Number of motifs observed	Percentage of total (%)	Most abundant motif	Tandem Repeats	
				Mean ± SEM	Maximum
Mono	4	1.3	a	13.7 ± 0.1	(g) <sub>23</sub>
Di	4	62.4	ac <sup>C</sup>	11.8 ± 0.2	(ac) <sub>191</sub>
Tri	9	34.8	gac <sup>C</sup>	5.4 ± 0.03	(cgt) <sub>78</sub>
Tetra	10	1.4	aacg	6.7 ± 0.4	(cggt) <sub>67</sub>
Penta	6	0.1	aacct	6.0 ± 0.4	(aaccg) <sub>11</sub>
Hexa	4	0.1	aaaccg	5.6 ± 0.2	(agggtt) <sub>7</sub>

<sup>A</sup> Microsatellite distribution in *K. proteae* was described based on the results of the Multipass search implemented in MSatFinder Online version 2.0 (Thurston & Field, 2005). Similar results were generated using the other MSatFinder engines, but Multipass allowed detection of additional microsatellite loci in all classes except the mono-nucleotides. For these analyses, duplicate sequences were excluded at a cdhit\_454 threshold of 0.95.

<sup>B</sup> Motifs in different reading frames (TGA, GAT and ATG), as well as their complementary motifs were treated as belonging to the same class (Jurka & Pethiyagoda, 1995).

<sup>C</sup> Motifs targeted by ISSR primers.

Although tandem repeat numbers of up to 191 were observed, short repeats were most common (Fig. 1.1). More than 40% of microsatellites consisted of 5 tandem repeats and 86.7% had 5-10 repeats. This is roughly the same as reported by Lim *et al.* (2004), who found that 90% of their dataset was comprised of microsatellites with 5-7 repeat units. As expected and observed in previous studies (Karaoglu *et al.*, 2005; Santana *et al.*, 2009), the average tandem repeat number of the microsatellites decreased as the motif complexity increased. This may be due to lower mutation rate and therefore lower polymorphism in long motifs (Chakraborty *et al.*, 1997; Kruglyak *et al.*, 1998), as well as a bias towards contraction mutations in large microsatellite alleles (Xu *et al.*, 2000a).



**Figure 1.1: Distribution of microsatellite tandem repeat units in the 454 data.** Proportion (in %) of microsatellites with different tandem repeat numbers (shown on chart) obtained from the 454 data. Tandem repeat numbers 6, 7 and 8 are in equal proportion.

#### **4. Conclusions**

This study is the first to develop microsatellite markers for a species of *Knoxdaviesia* and also the first to apply microsatellite enrichment coupled with pyrosequencing to ophiostomatoid fungi. The 12 polymorphic markers developed here will be applied to investigate the genetic diversity and dispersal of this arthropod-vectored fungus within the Cape Floristic Region of South Africa. Half of the markers also have the potential to be used to study the closely related *K. capensis*, establishing an opportunity for comparison of these ecologically similar fungi.

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## CHAPTER 2

### **Panmixia defines the genetic diversity of a unique arthropod-dispersed fungus specific to *Protea* flowers**

#### **Abstract**

*Knoxdaviesia proteae*, a fungus specific to the floral structures of the iconic Cape Floral Kingdom plant, *Protea repens*, is dispersed by mites phoretic on beetles that pollinate these flowers. Although the vectors of *K. proteae* have been identified, little is known regarding its patterns of distribution. Seed bearing infructescences of *P. repens* were sampled from current and previous flowering seasons, from which and *K. proteae* individuals were isolated and cultured. The genotypes of *K. proteae* isolates were determined using 12 microsatellite markers specific to this species. Genetic diversity indices showed a high similarity between *K. proteae* isolates from the two different infructescence age classes. The heterozygosity of the population was high ( $0.74 \pm 0.04$ ) and exceptional genotypic diversity was encountered ( $\hat{G} = 97.87\%$ ). Population differentiation was negligible, owing to the numerous migrants between the infructescence age classes ( $N_m = 47.83$ ) and between *P. repens* trees ( $N_m = 2.96$ ). Parsimony analysis revealed interconnected haplotypes, indicative of recombination and homoplasies and the index of linkage disequilibrium confirmed that outcrossing is prevalent in *K. proteae* ( $\bar{r}_d = 0.0067$ ;  $P = 0.132$ ). The high diversity and panmixia in this population is likely a result of regular gene flow and an outcrossing reproductive strategy. The lack of genetic cohesion between individuals from a single *P. repens* tree suggests that *K. proteae* dispersal does not primarily occur over short distances via mites as hypothesized, but rather that long distance dispersal by beetles plays an important part in the biology of these intriguing fungi.

**Keywords:** Dispersal, *Knoxdaviesia*, ophiostomatoid, panmixia

## 1. Introduction

An unique and intriguing assemblage of ophiostomatoid fungi (Wingfield *et al.*, 1993) is specific to the floral parts within the infructescences (seed heads) of serotinous *Protea* L. species. These fungi are encountered primarily in the Cape Floristic Region (CFR) (Marais & Wingfield, 1994; Marais *et al.*, 1998; Roets *et al.*, 2005; 2006; Wingfield *et al.*, 1988; Wingfield & Van Wyk, 1993), but some have also been found where *Protea* species occur beyond this area (Crous *et al.*, 2012; Marais & Wingfield, 2001; Roets *et al.*, 2008; Roets *et al.*, 2010; 2013). In the past, ophiostomatoid fungi have been known as associates of bark beetles and mites that infest trees and while most are saprophytes, some are important pathogens (Bridges & Moser, 1983; Dowding, 1969; Moser & Roton, 1971). The *Protea*-associated ophiostomatoid fungi do not appear to harm their hosts and some have been shown to have a mutualistic relationship with their mycophagous mite vectors (Roets *et al.*, 2007).

*Knoxdaviesia* M.J. Wingf., P.S. van Wyk & Marasas is an ophiostomatoid genus that includes three species occurring in *Protea* infructescences, namely *K. proteae* M.J. Wingf., P.S. van Wyk & Marasas, *K. capensis* M.J. Wingf. & P.S. van Wyk and *K. wingfieldii* (Roets & Dreyer) Z.W. de Beer & M.J. Wingf. The first two species are native to the CFR and have overlapping distributions (Marais & Wingfield, 2001), while *K. wingfieldii* occurs in the KwaZulu-Natal Province (Crous *et al.*, 2012). Whereas *K. capensis* is a generalist that has been found on various *Protea* hosts, *K. proteae* occurs exclusively in the infructescences of *P. repens* L. Despite its apparent lack of host specificity, *K. capensis* has never been encountered in *P. repens* (Marais *et al.*, 1998; Roets *et al.*, 2009b; Wingfield & Van Wyk, 1993).

The dispersal mechanisms of *Knoxdaviesia* species between *Protea* infructescences are not well-understood. In the *Protea*-ophiostomatoid fungus symbiosis, mites appear to be the primary vectors of fungal spores and beetles are believed to act as secondary vectors (Roets *et al.*, 2007; 2009a; 2011). Roets *et al.* (2009a) found that the small mite vectors easily move vertically between infructescences on the same *Protea* plant in search of new and moist environments. These authors also found that the mites are phoretic on beetles associated with *Protea* species and proposed that lateral movement to infructescences of other plants is facilitated by beetles carrying the mites (Roets *et al.*, 2009a). Two arthropod vectors are, therefore, involved in the dispersal of *Protea*-associated ophiostomatoid fungi, probably acting as short- and long-distance dispersal agents, respectively. Because of the sticky spore droplets produced by these fungi and their enclosed niche, dispersal via abiotic agents – such

as air and water – is unlikely. Further, ophiostomatoid fungi have never been recorded from other environmental sources, such as soil.

*Protea* infructescences are formed after every flowering season when serotinous *Protea* species close their involucre bracts around the inflorescences. These brown, cone-shaped structures house the seeds and will be maintained on the plant until severe stress or death triggers seed release (Rebelo, 1995). During their lifetime, infructescences may be colonized by numerous arthropods and microorganisms, including ophiostomatoid fungi (Coetzee & Giliomee, 1985; Marincowitz *et al.*, 2008; Roets *et al.*, 2005; 2006; Theron *et al.*, 2012). New *Protea* infructescences that form after flowering are presumably colonized by ophiostomatoid fungi from older infructescences. The fungal population in these infructescences should therefore represent a subset of the established populations in the older fruiting structures (Roets *et al.*, 2006). If dispersal between infructescences within *Protea* trees were more frequent than between different trees, individual *Protea* trees would be expected to harbour genetically discrete groups of ophiostomatoid fungi. In contrast, if medium- to long-distance dispersal played an important role in the biology of these fungi, fungal migrants from infructescences on other trees would also colonize new infructescences. The dispersal strategy of the fungus would, therefore, be a primary factor in shaping its population structure.

The ecological role that the *Protea*-specific ophiostomatoid fungi play in the biology of these plants is unknown, but they have no harmful effect on *Protea* seeds. Where these fungi are present in an infructescence, they appear to outcompete and exclude other fungi that could also be harmful to long-term seed survival (Lee *et al.*, 2005). If this level of mutualism were to exist, the survival of the ophiostomatoid fungi would be directly linked to that of their *Protea* hosts. In this regard, understanding the relative importance of vertical and lateral dispersal as well as the overall dispersal capacity of the ophiostomatoid fungi would be relevant. The distances over which spores are moved would determine the extent of gene flow, impacting on the diversity and adaptability of these fungi. Even where gene flow is prevalent, the population diversity and structure of these fungi would be largely determined by their sexual reproductive strategy (homo- or heterothallic). Despite extensive research into *Protea*-associated ophiostomatoid fungi, this reproductive aspect has not been studied. Investigation of *Protea*-associated ophiostomatoid dispersal, diversity and reproduction is, therefore, important to further understand the role of these fungi in the unique ecosystem in which they are found.

Gene flow and population structure can be investigated in natural populations using molecular markers (Slatkin, 1985) such as microsatellites. These are hyper-variable DNA loci (Ellegren, 2000; Schlötterer, 2000) that are useful as markers to quantify genetic variability (Chambers & MacAvoy, 2000) and have frequently been applied to study the population genetics of pathogenic ophiostomatoid fungi (Barnes *et al.*, 2005; Engelbrecht *et al.*, 2004; Marin *et al.*, 2009; Tsui *et al.*, 2012; Zhou *et al.*, 2007). Microsatellite-markers specific to *K. proteae* have recently been developed (Chapter 1), providing an opportunity for the population genetics of this fungus to be investigated.

Elucidation of how genetic diversity in *K. proteae* is structured within and/or across populations may reveal the processes responsible for shaping its evolution (Chung *et al.*, 2004; Epperson, 1993). These may include factors such as reproductive strategy, dispersal and ecology. The primary aim of this study was to determine gene flow among *K. proteae* individuals in a *P. repens* population, thus evaluating the extent of lateral and/or vertical migration of *K. proteae* across a *Protea* population. A second aim was to compare the genetic diversity between *K. proteae* individuals in differently aged infructescences to better understand the origin of fungi in newly formed infructescences.

## 2. Materials and Methods

### 2.1. Sampling

Sampling of *P. repens* infructescences was conducted in the Gouritz area, Western Cape Province, South Africa (−34.2062; 21.681217) during September and November 2012. The isolated stand of *P. repens* trees chosen was situated in an area of approximately three square kilometers. It was bordered by roads to the north and west, across from which no other *P. repens* trees were found. Farmland, devoid of *P. repens*, bordered this stand to the east and an irregular distribution of *P. repens* trees was situated to the south. Approximately 20 infructescences from the current (2012) and 20 from the previous flowering season were collected from 11 randomly chosen *P. repens* trees. To prevent repeated isolation of the same individual, only one fungal isolate was maintained per infructescence. Fungal isolations, DNA extraction and species identity verification followed methods described previously (Chapter 1).

## 2.2. Microsatellite amplification

For each *K. proteae* isolate, 12 microsatellite markers (Chapter 1) were amplified in three multiplex reactions with the KAPA2G Fast Multiplex PCR Kit (Kapa Biosystems, Inc., Boston, USA). The 25  $\mu$ l reactions contained 12.5  $\mu$ l KAPA2G, 1 mM additional MgCl<sub>2</sub>, 20 ng DNA and a variable concentration of primers (Table 2.1). PCR conditions were 3 minutes at 95°C followed by 27 cycles of: 15 seconds at 95°C, 30 seconds at 60°C and 1 minute at 72°C. The final extension was 30 min at 72°C. Each PCR plate contained a negative and positive control to indicate contamination and to standardize genotyping, respectively. The amplified products were subjected to a post-PCR clean-up and resolved on a 96-capillary Applied Biosystems 3730xl DNA Analyzer using a GeneScan 500 LIZ size standard (Applied Biosystems). Allele calling was done with GENEMARKER 2.4.0 (Softgenetics LLC, State College, PA, USA).



**Table 2.1: Primer concentrations in the three multiplex reactions used to genotype *K. proteae***

Reaction 1			Reaction 2			Reaction 3		
Locus	Primers	Concentration (nm)	Locus	Primers	Concentration (nm)	Locus	Primers	Concentration (nm)
KX1	<i>KX1-1</i>	20	KX2	<i>KX2-1</i>	60	KX5	<i>KX5-1</i>	40
	<i>KX1-2</i>	20		<i>KX2-2</i>	40		<i>KX5-2</i>	40
KX3	<i>KX3-1</i>	20	KX4	<i>KX4-1</i>	20	KX6	<i>KX6-1</i>	40
	<i>KX3-2</i>	20		<i>KX4-2</i>	20		<i>KX6-2</i>	40
KX10	<i>KX10-1</i>	20	KX7	<i>KX7-1</i>	40	KX8	<i>KX8-1</i>	40
	<i>KX10-2</i>	40		<i>KX7-2</i>	20		<i>KX8-2</i>	40
KX11	<i>KX11-1</i>	40	KX12	<i>KX12-1</i>	40	KX9	<i>KX9-1</i>	40
	<i>KX11-2</i>	20		<i>KX12-2</i>	40		<i>KX9-2</i>	40

### 2.3. Genetic Diversity

The descriptive diversity indices for fungal isolates occurring in individual *P. repens* trees and the fungal population as a whole were computed using GENALEX 6.501 (Peakall & Smouse, 2006; 2012). Nei's (1978) unbiased estimate of expected heterozygosity ( $H_E$ ) was calculated using the frequency ( $p$ ) of each allele ( $i$ ) and the sample size ( $n$ ) according to the formula  $H_E = (n/n - 1)[1 - \sum p_i^2]$ .

This index gives the probability that two randomly sampled individuals will be different (Nei, 1978; NRC, 1996). Heterozygosity is traditionally used to reflect genetic diversity and infer measures of differentiation, but its non-linearity causes inaccuracies when polymorphism is high (Jost, 2008). Therefore, a linear metric according to Jost (2008) was also employed to describe diversity and calculate differentiation (see below).

To measure genetic diversity, the number of effective alleles ( $N_e$ ) was calculated using  $N_e = 1/1 - h$  (Brown & Weir, 1983; Kimura & Crow, 1964), where  $h$  is the expected heterozygosity ( $1 - \sum p_i^2$ ) (Nei, 1973). Stoddart & Taylor's (1988) genotypic diversity ( $G$ ) was determined according to the formula  $G = 1/\sum [f_x(x/n)^2]$ , where  $f_x$  is the number of distinct genotypes (or haplotypes) occurring  $x$  times and  $n$  is the sample size. This index was used to obtain the maximum percentage of genotypic diversity ( $\hat{G}$ ) with the formula  $\hat{G} = G/N * 100$  (McDonald *et al.*, 1994), where  $N$  is the population size. The distribution of genotypes was investigated by calculating the evenness index (E5) as applied by Grünwald *et al.* (2003), using POPPR, a package implemented in R 3.0.2 (Kamvar *et al.*, 2013; R Development Core Team, 2008).

### 2.4. Population Differentiation

Diversity ratios to describe population differentiation were computed using SMOGD 1.2.5 (Crawford, 2010). However, since SMOGD assumes a diploid organism, the estimated parameters that incorporate sample size and ploidy were calculated independently by substituting  $2N$  for  $1N$  in the Nei & Chesser (1983) formulas. The diversity present between subpopulations ( $\Delta_{ST}$ ) represents the effective number of subpopulations and is the ratio of true diversity ( $\Delta_T$ ; effective number of alleles in the total population) to the within-subpopulation diversity ( $\Delta_S$ ). The inverse ( $\Delta_S/\Delta_T$ ) of this ratio describes the proportion of

diversity that is contained within the average subpopulation. It is a measure of similarity that will decrease as differentiation increases (Jost, 2008).

The haploid estimate ( $D_{est(hap)}$ ) of relative differentiation (Jost's D) was calculated using  $D_{est(hap)} = [(H_{T\_est(hap)} - H_{S\_est(hap)}) / (1 - H_{S\_est(hap)})] [n / (n - 1)]$  (Jost, 2008).  $H_{T\_est(hap)}$  and  $H_{S\_est(hap)}$  are Nei & Chesser's (1983) estimates of total and mean expected subpopulation heterozygosity, respectively, adjusted for haploids and  $n$  is the number of subpopulations. An estimate analogous to the conventional measure of population differentiation,  $F_{ST}$  (Weir & Cockerham, 1984), was calculated with MULTILOCUS 1.3b (Agapow & Burt, 2001) and is given by  $\theta = Q - q / 1 - q$ , where  $Q$  is the probability that two alleles within a population are identical and  $q$  is the probability that two alleles from different populations are identical. Gene flow ( $N_m$ ) was estimated in POPGENE 1.32 (Yeh *et al.*, 1999) from  $G_{ST}$ :  $N_m = 0.5 (1 - G_{ST}) / G_{ST}$  (Slatkin & Barton, 1989), where  $G_{ST}$  is a measure of differentiation relative to the total population (Nei, 1973).

Analysis of molecular variance (AMOVA) was conducted with ARLEQUIN 3.5.1.3 (Excoffier & Lischer, 2010). This test is based on the premise that total molecular variance can be divided into different covariance components within an hierarchical context (within populations, among populations and among groups of populations) (Excoffier *et al.*, 1992). For this purpose an  $F_{ST}$ -like distance matrix and 10 000 permutations to test significance were used.

## 2.5. Population Structure

STRUCTURE 2.3.4 (Falush *et al.*, 2003; Hubisz *et al.*, 2009; Pritchard *et al.*, 2000) was used to determine the number of clusters ( $K$ ) in the population and to assign individuals to these clusters. STRUCTURE implements a Bayesian, model-based approach to cluster individuals based on their allelic frequencies when  $K$  (the number of clusters) is known. Twenty independent runs were conducted for  $K$  values between one and 10, using 500 000 burn-in and 750 000 Markov Chain Monte Carlo repetitions, assuming an admixture model with correlated allele frequencies. Runs were initially conducted without supplying information about the population of origin, after which this information was included with the LOCPRIOR model. The online platform STRUCTURE HARVESTER (<http://taylor0.biology.ucla.edu/structureHarvester/>) (Earl & von Holdt, 2012) was used to

compute  $L(K)$  (the mean log-likelihood of  $K$ ) and  $\Delta K$  (Evanno *et al.*, 2005) to determine the optimal number of clusters.

The relatedness of *K. proteae* individuals was also investigated with the molecular-variance parsimony technique by calculating pairwise distances between the microsatellite haplotypes in ARLEQUIN 3.5.1.3 (Excoffier & Lischer, 2010). A minimum spanning network (MSN), containing all possible connections, was constructed with HAPSTAR 0.7 (Teacher & Griffiths, 2011). The hypothesis of random recombination was tested by investigating multilocus linkage disequilibrium in MULTILOCUS 1.3b (Agapow & Burt, 2001). A modified version of the index of association ( $I_A$ ),  $\bar{r}_d$  (Brown *et al.*, 1980), was calculated and compared to a distribution of  $\bar{r}_d$  for 1 000 simulated random datasets.

### 3. Results

#### 3.1. Genetic Diversity

A total of 92 *K. proteae* isolates were obtained from the sampled *P. repens* infructescences. Inconsistent sample sizes were obtained for the different plants and different aged infructescences, even after a second round of sampling in an attempt to increase numbers. In 10 of the loci, the proportions of null alleles were low (between zero and 4.3%) and were treated as missing data in subsequent analyses. Loci KX6 and KX9 displayed high null allele percentages (36% and 46%, respectively) and were excluded from analyses. Their exclusion, however, did not significantly impact other diversity indices (Table 2.2). Also, a plot of the number of sampled loci against the number of genotypes, calculated with MULTILOCUS (Agapow & Burt, 2001), began to plateau at 9 loci, indicating that the number of loci used was sufficient to capture the diversity of the population (data not shown).

Across the 10 loci, a total of 118 alleles were detected with an average of  $11.80 \pm 1.57$  alleles per locus. Allele frequencies ranged from 0.011 to 0.598 and the expected heterozygosity of the entire population across the 10 loci was  $0.74 \pm 0.04$  (Table 2.2). The genetic diversity or number of effective alleles ( $N_e$ ) was  $4.97 \pm 1.14$ . A t-test for independent samples implemented in STATISTICA 11 (StatSoft Inc., 2012) did not reveal a significant difference between the diversity measures of isolates from new and old infructescences (Fig 2.1). The genetic diversities and genetic composition of the two groups were, therefore, similar. Among the 92 *K. proteae* isolates, 91 different haplotypes were observed – the two identical haplotypes originating from two different old infructescences on the same plant. This yielded

a high maximum percentage of genotypic diversity (97.87%;  $G = 90.04$ ) and a nearly maximal evenness value ( $E_5$ ) of 0.994.

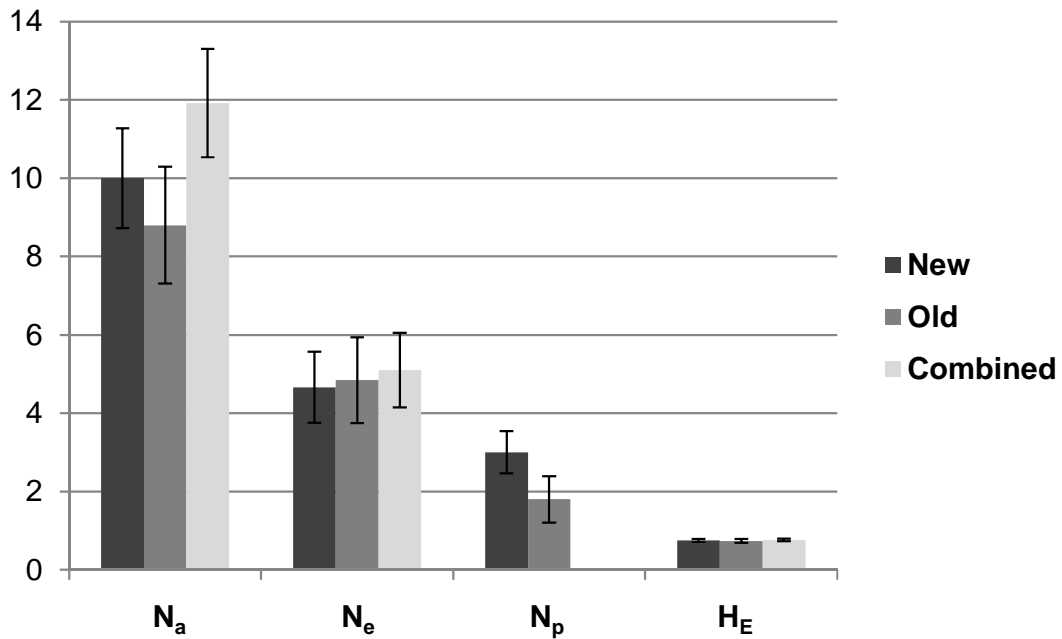
**Table 2.2: Number of alleles and diversity indices for all 12 loci**

Locus	$N_a^A$	Null alleles (%)	$N_e^B$	$H_E^C$
KX1	23	0	14.75	0.942
KX2	14	4.3	5.05	0.811
KX3	11	0	2.83	0.654
KX4	17	0	3.55	0.726
KX5	8	4.3	5.32	0.821
KX6	12	35.9	6.45	0.859
KX7	4	0	2.08	0.525
KX8	8	0	2.49	0.605
KX9	13	45.7	5.08	0.820
KX10	12	1.1	4.96	0.807
KX11	11	1.1	3.92	0.753
KX12	10	1.1	4.74	0.798
Mean $\pm$ SEM	11.92 $\pm$ 1.38	8.00 $\pm$ 4.51	5.10 $\pm$ 0.95	0.76 $\pm$ 0.03
Excluding KX6 & KX9	11.80 $\pm$ 1.57	1.2 $\pm$ 0.55	4.97 $\pm$ 1.14	0.74 $\pm$ 0.04

<sup>A</sup> $N_a$  = Number of alleles

<sup>B</sup> $N_e$  = Kimura & Crow's (1964) number of effective alleles;  $N_e = 1/h$

<sup>C</sup> $H_e$  = Nei's unbiased expected heterozygosity;  $H_E = (n/n - 1)[1 - \sum p_i^2]$



**Figure 2.1: Comparison between the mean genetic diversity indices of *K. proteae* individuals in new and old infructescences across 10 microsatellite loci.** Error bars represent the standard errors of the mean. A t-test for independent samples showed no significant differences between the groups.

### 3.2. Population Differentiation

For calculations of population differentiation (Table 2.3), two different scenarios were considered: 1) Individuals from new and old infructescences, respectively, group together (2 subpopulations); and 2) Individuals from different *P. repens* plants group together (11 subpopulations). The indices calculated for both scenarios describe a situation in which the subpopulations account for all the genetic diversity.  $\Delta_{ST}$  and  $\Delta_S/\Delta_T$  show that the number of effective subpopulations slightly exceeds one and that combining all subpopulations would not greatly increase the observed diversity. This was supported by the low values of  $D$  and the null value of  $D_{\text{est(hap)}}$ , indicating that no differentiation exists. The non-significant values of theta in Scenario 1 and 2 is also congruent with the results of  $D$  and  $D_{\text{est(hap)}}$ .

For AMOVA (Table 2.4), *K. proteae* individuals isolated from each age class of infructescence on every different *P. repens* plant were considered as one population and populations were grouped according to the above scenarios. The AMOVA results supported those obtained using Jost's (2008) indices by showing that more than 96% of molecular variation is contained within the infructescences themselves. Additionally, little (< 5%), but significant ( $P < 0.01$ ) variance was detected between infructescences. Grouping infructescences according to their *P. repens* trees or age class of origin did not, however, account for additional variation.

The number of migrants ( $N_m$ ) in each generation explained the observed lack of population differentiation. This measure was very high ( $N_m = 47.83$ ) between old and new infructescences. Although much lower ( $N_m = 2.96$ ), the value of  $N_m$  between *P. repens* plants is still greater than one, sufficient to prevent differentiation (McDermott & McDonald, 1993).

**Table 2.3: Descriptive measures of population differentiation for the two different subpopulation scenarios. Mean values and the standard error of the mean across the 10 loci are reported<sup>A</sup>**

	Scenario 1	Scenario 2
$\tilde{N}^B$	44.93	6.69
$N_e^C$	4.75 ± 0.02	3.22 ± 0.14
$\Delta_{ST}^D$	1.01 ± 0.00	1.13 ± 0.03
$\Delta_S/\Delta_T^E$	0.99 ± 0.00	0.89 ± 0.02
$D^F$	0.02 ± 0.01	0.13 ± 0.02
$D_{est(hap)}^G$	0	0
$\theta^H$	0	0.01
$G_{ST}^I$	0.01	0.14
$N_m^J$	47.83	2.96

<sup>A</sup> Scenario 1) Individuals from new vs. old infructescences; 2) Individuals from different *P. repens* plants

<sup>B</sup>  $\tilde{N}$  = Harmonic mean of the sample sizes

<sup>C</sup>  $N_e$  = Kimura and Crow's (1964) number of effective alleles;  $N_e = 1/1 - h$

<sup>D</sup>  $\Delta_{ST}$  = Diversity between subpopulations, or the effective number of subpopulations

<sup>E</sup>  $\Delta_S/\Delta_T$  = Proportion of diversity in a subpopulation

<sup>F</sup>  $D$  = Actual (relative) differentiation

<sup>G</sup>  $D_{est(hap)}$  = The haploid estimate of  $D$ ;

$$D_{est(hap)} = [(H_{T\_est(hap)} - H_{S\_est(hap)}) / (1 - H_{S\_est(hap)})] [n / (n - 1)]$$

<sup>H</sup>  $\theta$  = Conventional measure of relative differentiation;  $\theta = Q - q / 1 - q$

<sup>I</sup>  $G_{ST}$  = Gene differentiation relative to the total population (Nei, 1973)

<sup>J</sup>  $N_m$  = estimated gene flow;  $N_m = 0.5 (1 - G_{ST}) / G_{ST}$



**Table 2.4: AMOVA results showing the variance attributable to each hierarchy in the Gouritz *K. proteae* population**

SCENARIO 1					
Variance component	df	Variance	% total	P <sup>A</sup>	Fixation
Among infructescence age classes	1	0	0	0.786	$\theta_{CT} = 0$
Among infructescences within age classes	19	0.135	3.77	<0.01	$\theta_{SC} = 0.035$
Within infructescences	71	3.480	96.74	<0.01	$\theta_{ST} = 0.033$
SCENARIO 2					
Among <i>P. repens</i> trees	10	0	0	0.822	$\theta_{CT} = 0$
Among infructescences within trees	10	0.176	4.88	<0.01	$\theta_{SC} = 0.048$
Within infructescences	71	3.480	96.58	<0.01	$\theta_{ST} = 0.034$

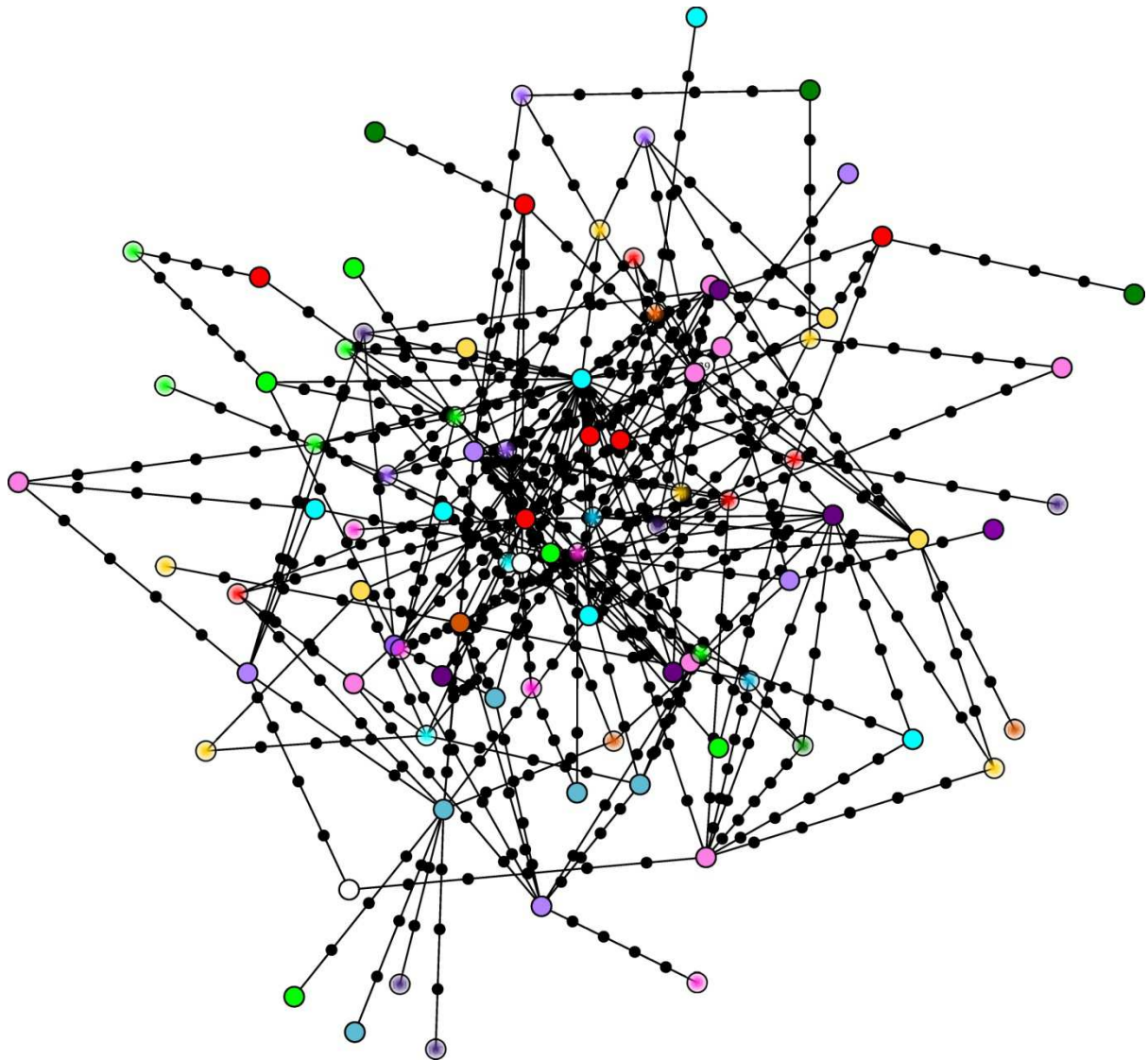
<sup>A</sup> The probability of obtaining a more extreme variance and fixation index by chance

### 3.3. Population Structure

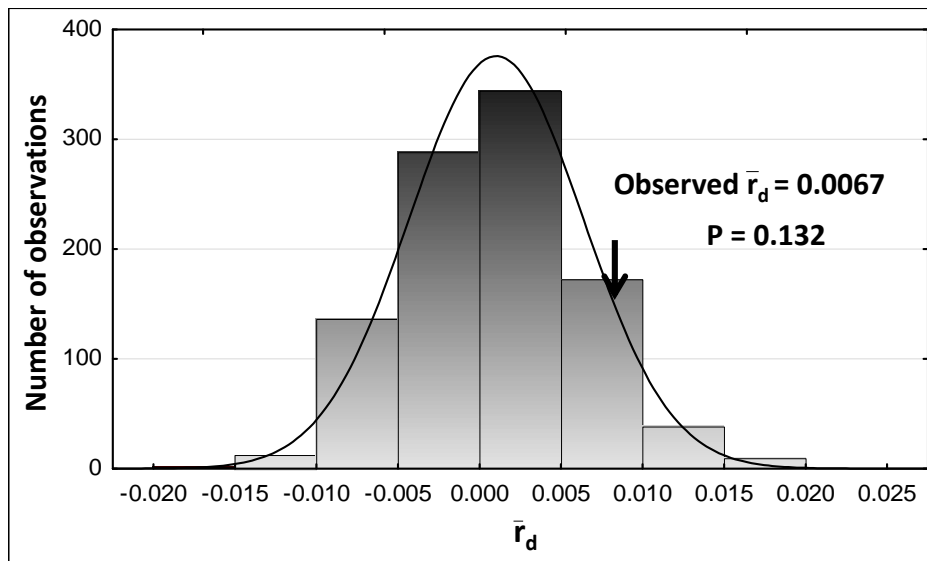
The same scenarios described above were implemented when running STRUCTURE with the LOCPRIOR model. Two different LOCPRIOR runs were therefore conducted, each assuming different populations of origin. The  $\Delta K$  values of the runs both without and including sampling information highlighted  $K$  values between five and nine as the most likely. However, it is important to consider that  $\Delta K$  cannot evaluate  $K = 1$  and the maximum log-likelihood of  $K$ ,  $L(K)$ , was always observed at  $K = 1$ . Evanno *et al.* (2005) also noted that the variance in the mean  $L(K)$  begins to increase after the correct  $K$  value is reached, which was observed in the data for  $K \geq 2$ . Inspection of the clusters highlighted by  $\Delta K$  revealed that they do not provide information on population structure, but rather distribute similar proportions

of the individuals' genetic material to all of the clusters. The formation of such apparently uninformative clusters and  $\Delta K$  values are similar to observations by Waples & Gaggiotti (2006) for a simulated dataset with high gene flow. Mean alpha values for the runs were always greater than one, signifying high levels of admixture between individuals (Falush *et al.*, 2003). A value of  $K = 1$  is therefore the most likely and biologically meaningful when considered together with the population differentiation statistics.

The MSN did not form apparent clusters (Fig. 2.2) and loops were prevalent. Many haplotypes, therefore, had more than one possible ancestor, suggesting the existence of numerous homoplasies and recombination (Posada & Crandall, 2001). This was also evident from the results of the multilocus linkage disequilibrium analysis. The  $\bar{r}_d$  value of *K. proteae* lies within the range of the normal distribution and is not significantly different ( $P > 0.05$ ) from zero (Fig 2.3), indicative of a randomly recombining population. The numerous connections between the different haplotypes in the MSN and the lack of linkage disequilibrium between loci both imply that recombination between individuals is not restricted.



**Figure 2.2: Minimum spanning network displaying the 91 unique haplotypes (nodes) in the *K. proteae* population.** Black circles represent missing haplotypes between samples. Colours specify sampling locations – each colour represents a different *P. repens* plant. Solid fills indicate isolates from new infructescences; gradient fills indicate isolates from old infructescences. The large amounts of loops in the network suggests the presence of recombination and homoplasies in the population.



**Figure 2.3: Histogram depicting the distribution of  $\bar{r}_d$  in *K. proteae* for 1 000 randomizations.** The observed value of  $\bar{r}_d$  and the P-value (shown on the graph) indicate that there is no significant difference from zero, supporting the hypothesis of random recombination.

#### 4. Discussion

While plants and animals in the CFR are well-known and have been intensively studied, there is a relatively sparse knowledge of the microbes in this unique and iconic ecosystem (Crous *et al.*, 2006; Lee *et al.*, 2004; Marincowitz *et al.*, 2008; Slabbert *et al.*, 2010). Ironically, while there is concern that the microbial biodiversity of this and other important ecosystems tends to be overlooked, there is even less knowledge relating to the biology of these microbes. This study represents the first attempt to understand the genetic diversity of any fungus in the CFR. The results have shown intriguing patterns that advance our understanding of an interesting fungus not only in this ecosystem, but also relating to these fungi globally.

Established, native populations are expected to have higher diversities than introduced populations (McDonald, 1997; Nkuekam *et al.*, 2009). The high level of genetic diversity found for *K. proteae*, a native fungus in the CFR, is thus not surprising. The genotypic diversity of *K. proteae*, however, far exceeds that reported in previous studies of ophiostomatoid fungi (Barnes, 2002; Nkuekam *et al.*, 2009; Zhou *et al.*, 2007). This high level of diversity in *K. proteae* appears to be the result of regular gene flow and outcrossing.

Importantly, the similarity in genetic composition and the exceptionally high gene flow between *K. proteae* individuals from old and new infructescences supports the findings of Roets *et al.* (2006; 2009a) that new infections by *K. proteae* found in fresh infructescences originate from the infructescences of previous year that remain on the trees.

Although this study presents support for fungal migration from old to new infructescences (vertical transmission) and *K. proteae* individuals in new infructescences are therefore the offspring of those in old infructescences, this parent-offspring relationship is not restricted to individual trees. The lack of genetic cohesion within individual *P. repens* trees suggests that vertical migration is not the primary method by which gene flow is achieved. *Knoxdaviesia proteae* individuals in old infructescences do not seem to disperse primarily to new infructescences on the same *P. repens* tree, but the observed population structure rather emphasizes medium- to long-distance dispersal. Thus, a *K. proteae* individual in a given infructescence may have the potential to disperse to any other infructescence in the *P. repens* population. The panmixia revealed by the population statistics and graphically represented by the MSN suggests that frequent random dispersal between *P. repens* trees and a recombining reproductive strategy predominates within *K. proteae*. Due to the high mobility of *K. proteae* and the frequent dispersal events, a *K. proteae* population cannot be defined as occurring within a single *P. repens* tree or infructescence age class, but rather as occupying a stand of *P. repens* trees.

At least two avenues are available for short- and long-distance dispersal of ophiostomatoid fungi – mites and beetles. Mites are known to leave previous-year infructescences and self-disperse upwards to new, moist infructescences formed the following year (Roets *et al.*, 2009a). However, as the primary vectors of ophiostomatoid fungi, the predisposition of mites to phoresy greatly affects the fungal population structure. These small arthropods have been found to be phoretic on numerous other organisms, including other arthropods, insects and birds (Krantz & Walter, 2009; Proctor & Owens, 2000). The large numbers of ophiostomatoid-fungus mite vectors that have been found on beetles (Roets *et al.*, 2009a) also show an inclination of mites to utilize larger organisms to facilitate long-distance dispersal. The apparently panmictic structure of *K. proteae* in a stand of *P. repens* trees highlights the importance of long-distance dispersal and, therefore, the role of beetles as ophiostomatoid vectors and mite vehicles. The mite-vectoring beetles have also been implicated as *Protea* pollinators (Coetzee & Giliomee, 1985) and as such, they visit numerous inflorescences

carrying fungus-vectoring mites as well as pollen from plant to plant. This activity may explain the high levels of gene flow observed between different *P. repens* trees.

## 5. Conclusions

This study has shown that *K. proteae* in the CFR is characterized by exceptional genetic and genotypic diversity. The diversity appears to be maintained by high levels of gene flow that prevents population differentiation, thus limiting the effects of genetic drift. This suggests that a panmictic population of *K. proteae* exists within *P. repens* stands in close proximity to each other. Consequently, the role of beetles in the dispersal of *Protea*-associated ophiostomatoid fungi appears to be essential, because they facilitate transport between *Protea* trees and would, therefore, be primarily responsible for the observed panmixia.

Although the extent of vertical and lateral dispersal in *K. proteae* has been addressed in this study, the lack of population structure observed prompts further questions. The geographic range over which panmixia is maintained in *K. proteae* is specifically interesting and will likely be a function of the migration capacity of the long-distance beetle vectors. Furthermore, the ecological role of the ophiostomatoid fungi in this unusual and interesting niche has not yet been elucidated.

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## CHAPTER 3

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

### Abstract

The Fynbos Biome in the Cape Floristic Region is prone to recurrent fires that may clear vast areas of vegetation. Between periods of fire, species of 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 individuals from young *P. repens* plants in a recently burnt area were compared to individuals from the adjacent, unburnt population. The only difference between *K. proteae* isolates from the two areas was 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 and multiple fungal migration events.

**Keywords:** Fynbos, *Konxdaviesia*, ophiostomatoid, recolonize

## 1. Introduction

The Fynbos Biome of the Cape Floristic Region (CFR) 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 to 15 years) often clear vast areas (more than 4 000 hectares) of nearly all the above-ground fynbos biomass (Day *et al.*, 1979; Kruger *et al.*, 2000; Southey, 2009) and many plants resprout or recruit from seeds stored in the soil (Keeley, 1995; Wilgen *et al.*, 1992). In the case of serotinous *Protea* L. 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 (inflorescences) mature (Rebelo, 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.*, 2006), mites (Theron *et al.*, 2012) and fungi (Lee *et al.*, 2003; 2005), including ophiostomatoid fungi (Roets *et al.*, 2005). *Protea*-associated ophiostomatoid fungi represent a polyphyletic assemblage (Wingfield *et al.*, 1999) that is characterized by occupation of infructescences and arthropod-mediated dispersal. The long ostiolar necks of the perithecia and the production of sticky spores make these fungi ideally suited for dispersal by arthropods rather than air currents (Cassar & Blackwell, 1996).

*Knoxdaviesia proteae* M.J. Wingf., P.S. van Wyk & Marasas 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* L., 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). However, this extraordinary *Protea*-ophiostomatoid fungus association is not restricted to the CFR, but has 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. Therefore, recolonization of post-fire, newly formed infructescences can take place only via dispersal from unburnt areas. This form of dispersal is more easily

achieved for winged groups like insects, than for organisms such as mites and ophiostomatoid fungi. However, it was recently shown that 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 within a single *P. repens* plant, beetles carry mites to facilitate long-distance dispersal (Roets *et al.*, 2009a).

Previous research has shown that gene flow facilitated by mites and beetles is sufficient for a *P. repens* stand to harbour a panmictic *K. proteae* population (Chapter 2). 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. Consequently, the geographic distance over which these vectors are capable of supplying sufficient *K. proteae* migrants to maintain panmixia, 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 diversity to the population.

In this study, we used microsatellite markers specific to *K. proteae* (Chapter 1) to compare fungal individuals 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 also compared genotypic variation within this population to a *K. proteae* population previously genotyped with the same microsatellite markers (Chapter 2) 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.

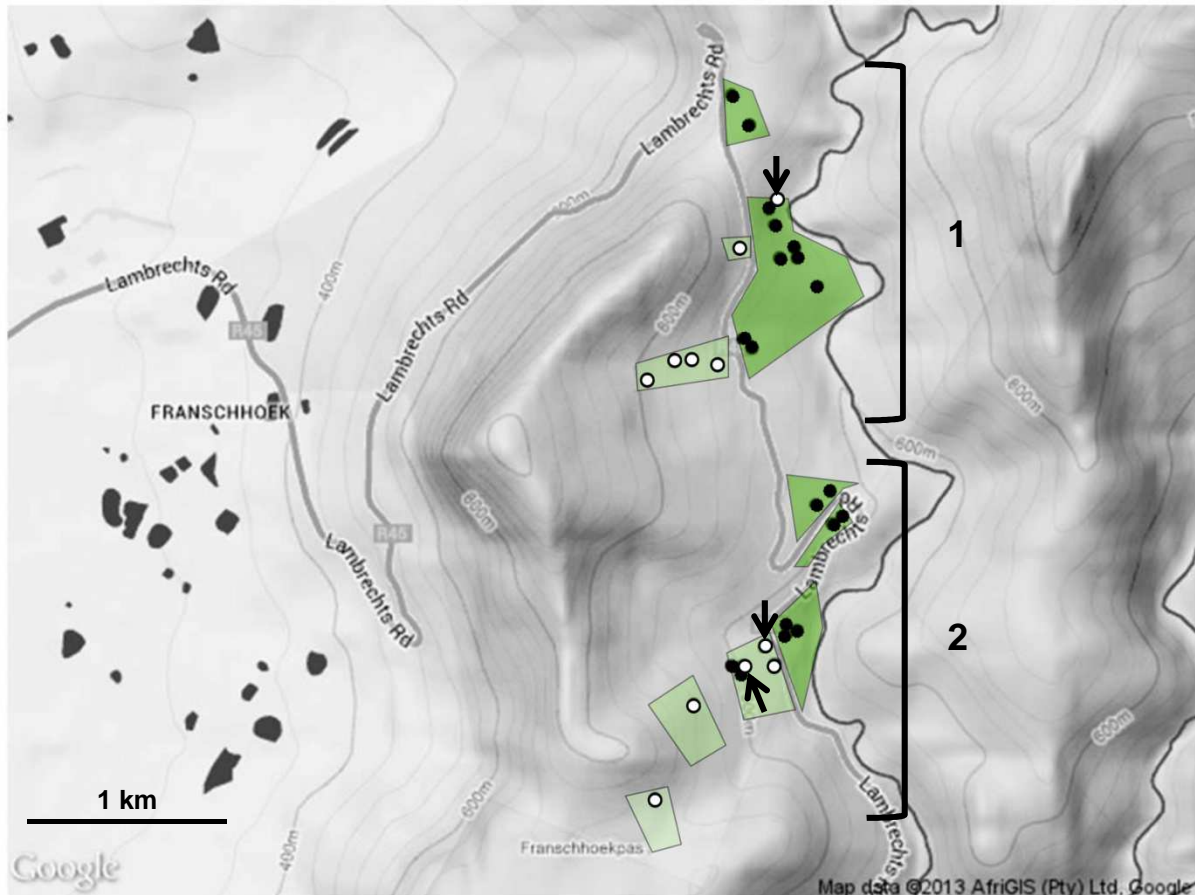
## 2. Materials and Methods

### 2.1. Fungal sampling

Sampling of *P. repens* infructescences was conducted along a three kilometer (km) stretch of land in the Franschoek Mountains, Western Cape Province, South Africa (−33.90442; 19.156683). The fynbos 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. This ensured that the isolated *K. proteae* individuals originated from dispersal during a single flowering season (i.e. between-plant dispersal only). Patches of fynbos containing mature (ca. 15 – 17 years old) *P. repens* plants (Fig. 3.1) allowed for sampling of infructescences that presumably contained source populations of *K. proteae* for the recently burnt areas.

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 3.1). A total of 20 ca. 6 month-old infructescences were sampled from various *P. repens* trees in a 10 metre radius around the midpoints of the sampling plots. Fungal isolations, DNA extraction, and species identity verification followed previously described methods (Chapter 1).





**Figure 3.1: Franschhoek sampling site.** The approximate distribution of *P. repens* from unburnt (dark green) and recently burnt (light green) areas are highlighted. Dots represent the midpoints of sampling plots in unburnt (black) and recently burnt (white) areas. Based on sampling proximity, the area is divided into two main regions. Downward arrows indicate plots containing the two individuals with identical haplotypes. The upward arrow indicates the plot where the nine individuals that cluster separately were collected.

## 2.2. 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 (Chapter 1) in three multiplex reactions developed previously (Chapter 2). 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 haplotypes 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 identical. T-tests for independent variables implemented in STATISTICA 11 (StatSoft Inc., 2012) were used to compare the diversity indices between *K. proteae* isolates 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 ( $E_5$ ) recommended by Grünwald *et al.* (2003) was calculated with POPPR, a package implemented in R 3.0.2 (Kamvar *et al.*, 2013; R Development Core Team, 2008).

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 ( $\Delta_{ST}$ ) subpopulations, the relative population differentiation ( $D$ ) and Jost's haploid estimate of  $D$  ( $D_{\text{est(hap)}}$ ) (Jost, 2008) were determined with SMOGD 1.2.5 (Crawford, 2010).  $D_{\text{est}}$  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). Gene flow, described by the number of migrants ( $N_m$ ) per generation (Slatkin & Barton, 1989), was estimated from Nei's (1973) measure of total population differentiation,  $G_{ST}$ , in POPGENE 1.32 (Yeh *et al.*, 1999).

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 10 000 permutations was used to consider whether genetic and geographic distances 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 geographic locations and geographic and genetic distances were calculated between them. Geographic distances were

calculated from the latitudinal and longitudinal coordinates in GENALEX 6.501 (Peakall & Smouse, 2012), whereas Goldstein's  $\delta\mu^2$  genetic distance for microsatellites (Goldstein *et al.*, 1995) was calculated using MSA 4.05 (Dieringer & Schlötterer, 2003). Mantel tests were conducted on both the untransformed and log-transformed datasets.

To test 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. Linear regression analysis was performed in STATISTICA 11 (StatSoft Inc., 2012) and 10 000 randomizations of Pearson's  $r$  coefficient were computed with SIMSTAT 2.6.1 (Provalis Research, Montreal, Canada).

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 DISTRUCT 1.1 (Rosenberg, 2004).

Relationships between isolates were also investigated by computing a minimum spanning network (MSN) between microsatellite haplotypes based on 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 1 000 random datasets.

### 2.3. 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 *K. proteae* population study conducted in Gouritz, Western Cape Province, South Africa (Chapter 2). These two *K. proteae* populations are separated by approximately 240 km and mountainous terrain surrounds the Franschoek population. Considering all *K. proteae* isolates from both populations, population differentiation and structure was analyzed 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 will bias the linkage disequilibrium test towards non-random recombination (Taylor *et al.*, 1999), failure to detect non-random recombination will suggest that outcrossing is taking place between the two populations.

## 3. Results

### 3.1. 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. During microsatellite amplification, between zero and 4.7% null alleles were detected in 10 of the loci. However, similar to a previous *K. proteae* population study (Chapter 2), 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 3.1). 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 haplotypes. Two identical haplotypes were encountered in different sampling locations 1.8 km apart; both in recently burnt sampling plots (Fig 3.1). Stoddart & Taylor's (1988) genotypic diversity ( $G$ ) was 104.04 – 98.15% of the maximum diversity ( $\hat{G}$ ). The genotypic evenness ( $E_5$ ) was 0.994, identical to the  $E_5$  value of a previous *K. proteae* population (Chapter 2).

Comparison of the measures of allele diversity between fungal individuals from the unburnt and recently burnt areas revealed a significant difference (t-value = 3.77; df = 18; P = 0.001)

in the number of private alleles, with the population from unburnt areas having more private alleles (Fig 3.2a). Although not significant, the number of total alleles was also higher in the unburnt area. The expected heterozygosity and 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 3.2) and the effective number of subpopulations was one, accounting for 99% of the total diversity. The estimated number of migrants per generation between unburnt and burnt areas was also high ( $N_m = 31.60$ ), explaining the lack of population differentiation.

**Table 3.1: Genetic diversity of the Franschoek Mountain *K. proteae* population across 12 microsatellite loci**

Locus	$N_a^A$	Null alleles (%)	$N_e^B$	$H_E^C$
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 $\pm$ SEM <sup>D</sup>	13.33 $\pm$ 2.09	5.42 $\pm$ 2.67	5.60 $\pm$ 1.12	0.77 $\pm$ 0.03
Excluding KX6 & KX9	13.6 $\pm$ 2.45	1.60 $\pm$ 0.51	5.98 $\pm$ 1.32	0.78 $\pm$ 0.04

<sup>A</sup>  $N_a$  = Number of alleles

<sup>B</sup>  $N_e$  = Kimura & Crow's (1964) number of effective alleles;  $N_e = 1/1 - h$

<sup>C</sup>  $H_e$  = Nei's unbiased expected heterozygosity;  $H_E = (n/n - 1)[1 - \sum p_i^2]$

<sup>D</sup> SEM = Standard error of the mean

**Table 3.2: Population differentiation between the *K. proteae* individuals from unburnt and recently burnt sampling plots in Franschoek and between the two distantly separated populations**

	<b>Franschoek unburnt vs. burnt</b>	<b>Gouritz vs. Franschoek</b>
$\tilde{N}^A$	45.45	98.51
$\Delta_{ST}^B$	1.01 ± 0.00	1.02 ± 0.01
$\Delta_S/\Delta_T^C$	0.99 ± 0.00	0.98 ± 0.01
$D^D$	0.03 ± 0.00	0.04 ± 0.01
$D_{est(hap)}^E$	0	0.01 ± 0.01
$\theta^F$	0.01	0.042**
$G_{ST}^G$	0.02	0.0267
$N_m^H$	31.60	18.2496

<sup>A</sup>  $\tilde{N}$  = Harmonic mean of the sample sizes

<sup>B</sup>  $\Delta_{ST}$  = Diversity between subpopulations, or the effective number of subpopulations

<sup>C</sup>  $\Delta_S/\Delta_T$  = Proportion of diversity in a subpopulation

<sup>D</sup>  $D$  = Actual (relative) differentiation

<sup>E</sup>  $D_{est(hap)}$  = The haploid estimate of  $D$ ;

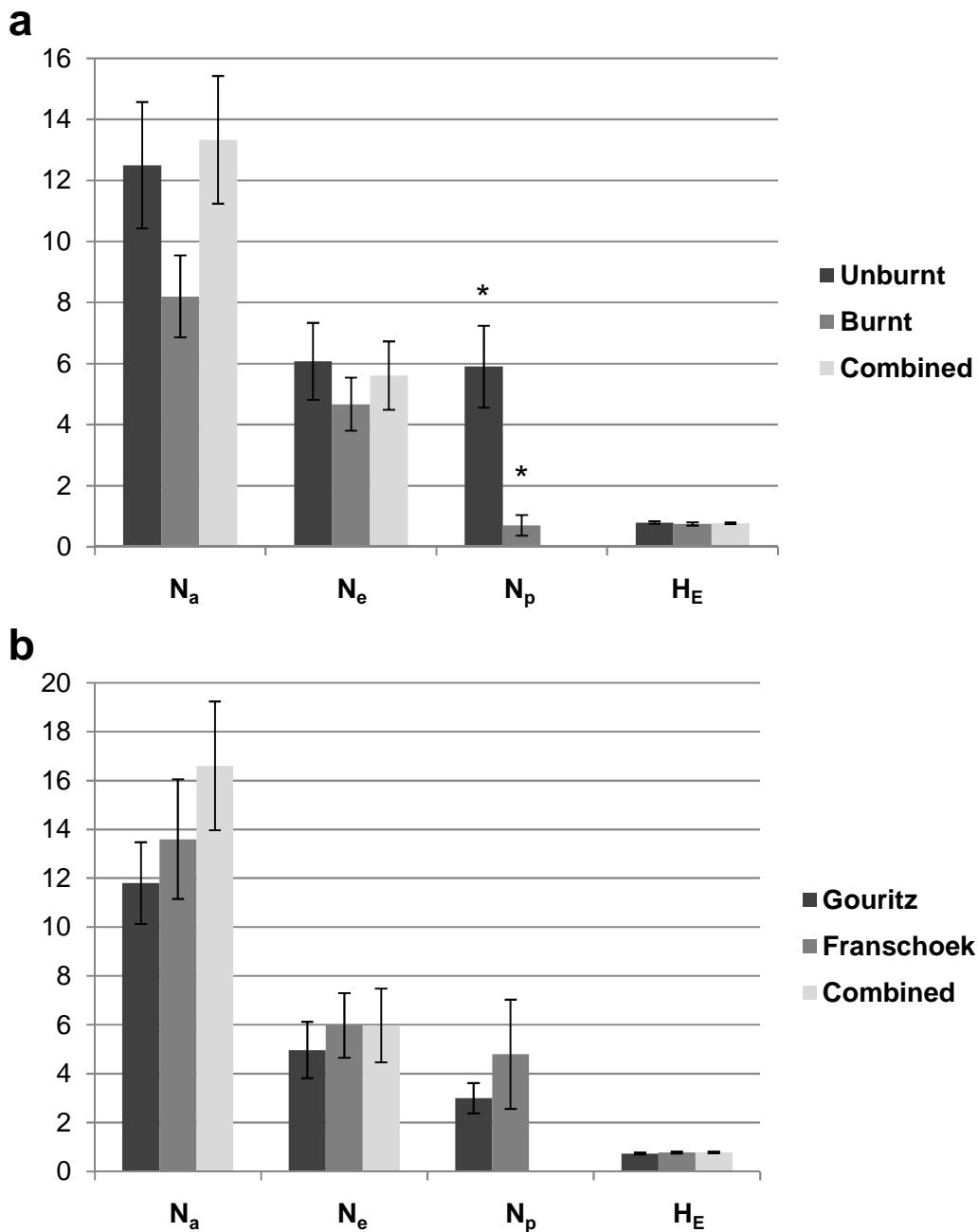
$$D_{est(hap)} = [(H_{T\_est(hap)} - H_{S\_est(hap)}) / (1 - H_{S\_est(hap)})] [n / (n - 1)]$$

<sup>F</sup>  $\theta$  = Conventional measure of relative differentiation;  $\theta = Q - q / 1 - q$

<sup>G</sup>  $G_{ST}$  = Gene differentiation relative to the total population (Nei, 1973)

<sup>H</sup>  $N_m$  = estimated gene flow;  $N_m = 0.5 (1 - G_{ST}) / G_{ST}$

\*\* $P < 0.001$  after 1 000 randomizations

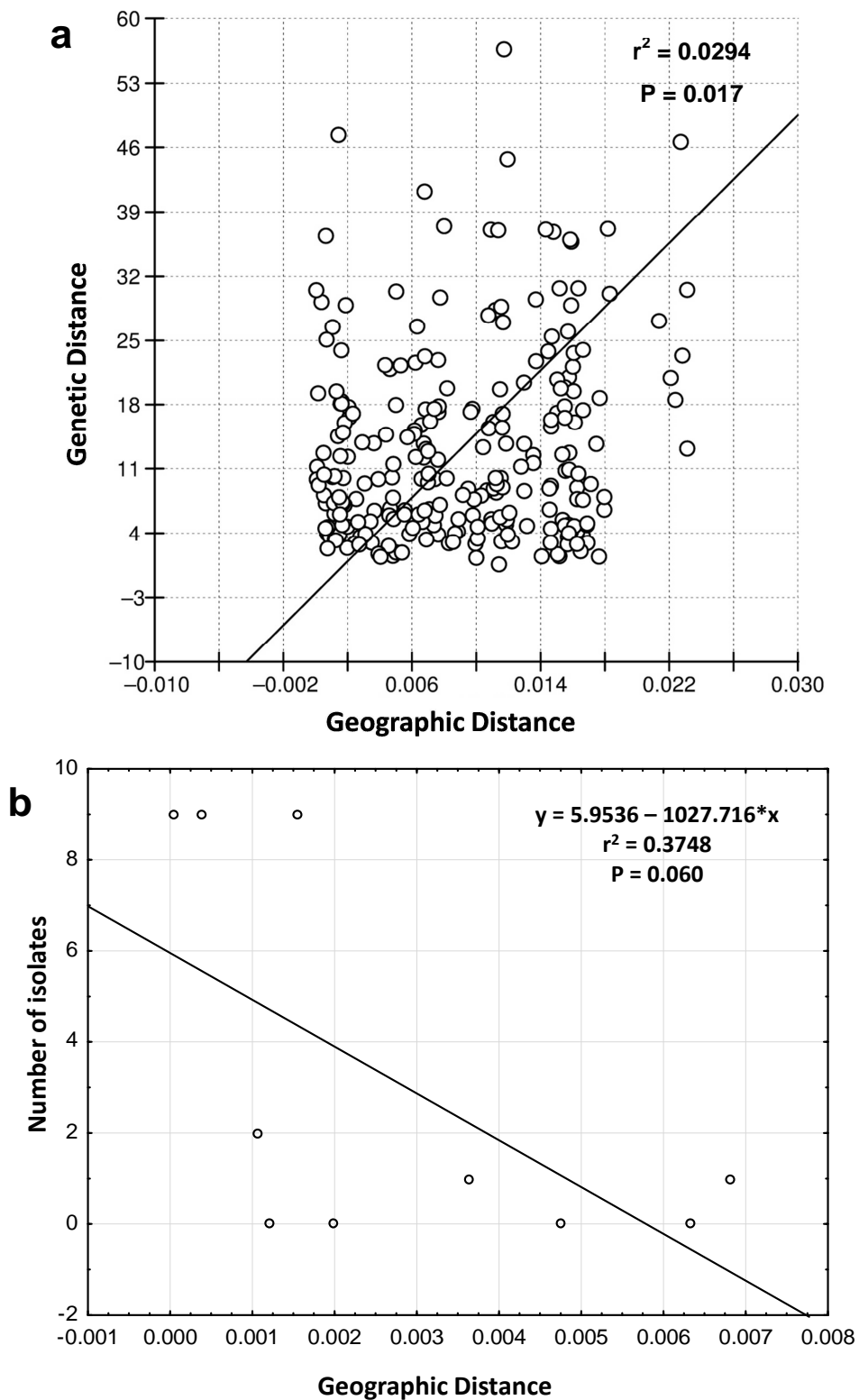


**Figure 3.2: Comparison between the diversity indices of *K. proteae* from the recently burnt and unburnt areas in Franschoek (a) and between the Gouritz and Franschoek populations (b).** The mean number of alleles ( $N_a$ ), effective alleles ( $N_e$ ), private alleles ( $N_p$ ) and the unbiased expected heterozygosity ( $H_E$ ) is shown. Error bars represent the standard error of the mean. The only statistically significant difference (\*) was observed for  $N_p$  between the burnt and unburnt areas.

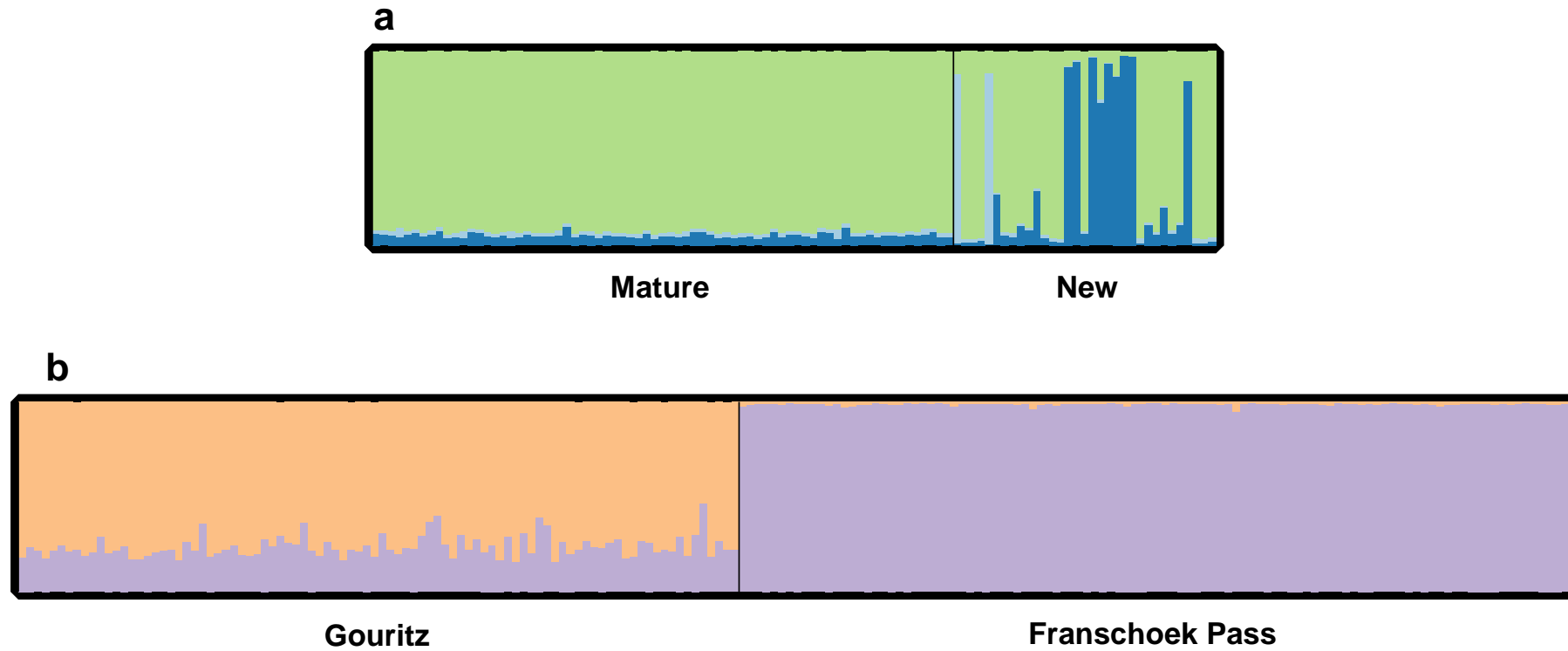
In the first test for IBD, the Mantel test detected a weak, but significantly positive ( $r^2 = 0.0294$ ;  $P = 0.019$ ), correlation between the two distance matrices (Fig 3.3a). Using the logarithm of geographic distance strengthened the correlation slightly ( $r^2 = 0.0164$ ;  $P = 0.044$ ). A weak IBD effect is therefore present across this 3 km stretch of landscape. However, Mantel tests between individual unburnt plots and recently burnt plots indicated that the IBD is not a result of the recolonization of burnt areas. No significant correlations between genetic and geographic distances could be detected between any of the individual unburnt plots and recently burnt plots for the untransformed as well as the log-transformed datasets. Similarly, the distance of recently burnt from unburnt sampling plots did not influence the number of *K. proteae* isolates obtained in this study ( $r^2 = 0.03748$ ;  $P = 0.060$ ; Fig 3.3b). Chi Square tests showed no difference between the numbers of fungal isolates ( $X^2 = 0.083$ ;  $df = 1$ ;  $P > 0.05$ ) obtained from infructescences in the unburnt and recently burnt plots.

Without incorporation of the LOCPRIOR model, STRUCTURE could not detect significant population structure. When considering the origin of *K. proteae* isolates (burnt or recently burnt area) both  $L(K)$  and  $\Delta K$  designated  $K = 3$  as the most likely number of clusters. Inspection of the cluster assignment (Fig 3.4a) indicated that all fungal isolates from the unburnt and most from burnt areas were assigned to the same cluster. Two additional clusters were, however, identified in isolates from the recently burnt area. Nevertheless, the MSN (Fig 3.5) displayed a cohesive assemblage of fungi with numerous loops in the network linking different haplotypes. The index of linkage disequilibrium was not significantly different from zero ( $\bar{r}_d = 0.0067$ ;  $P = 0.329$ ; Fig 3.6a), supporting the null hypothesis of random recombination.

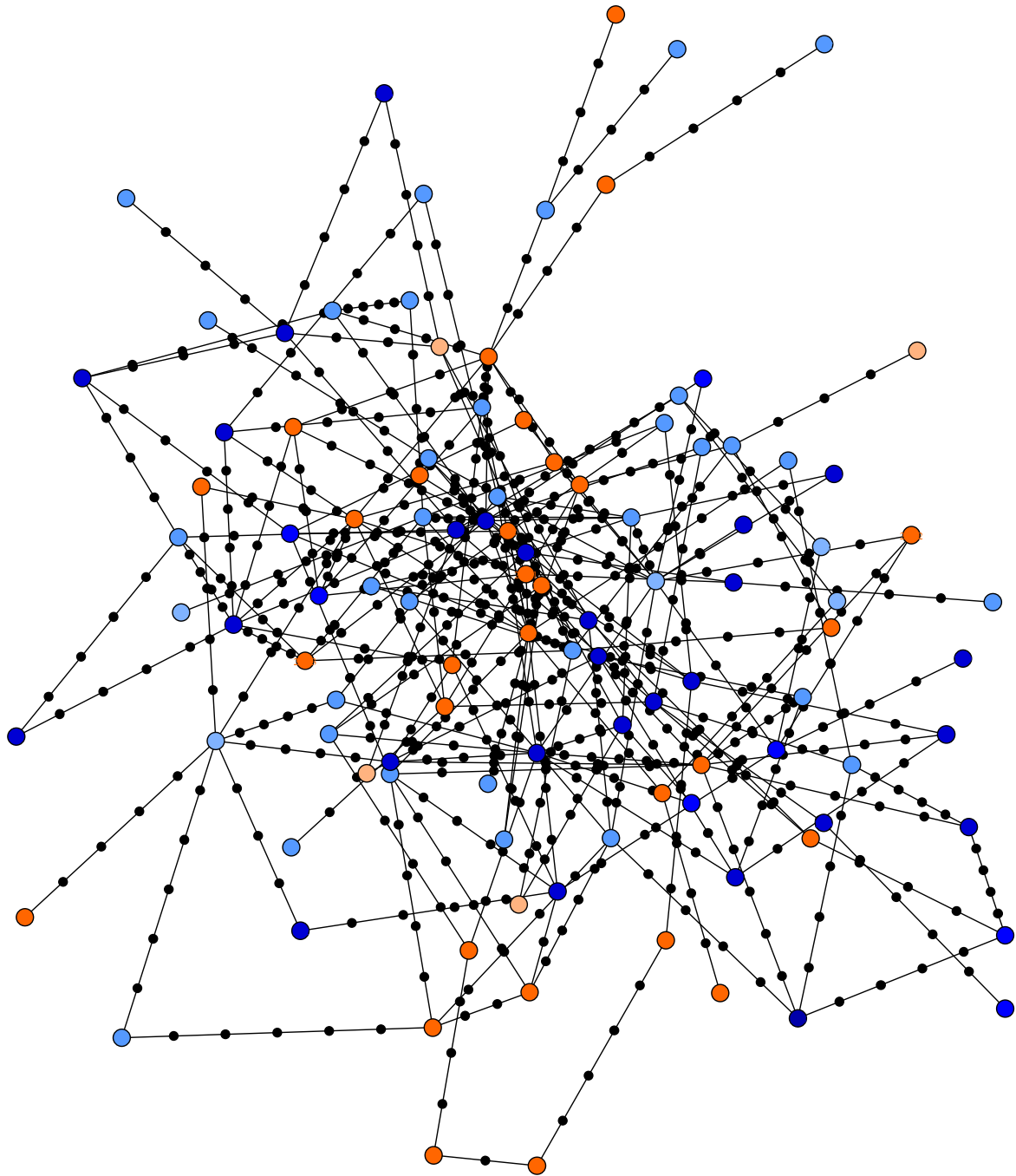




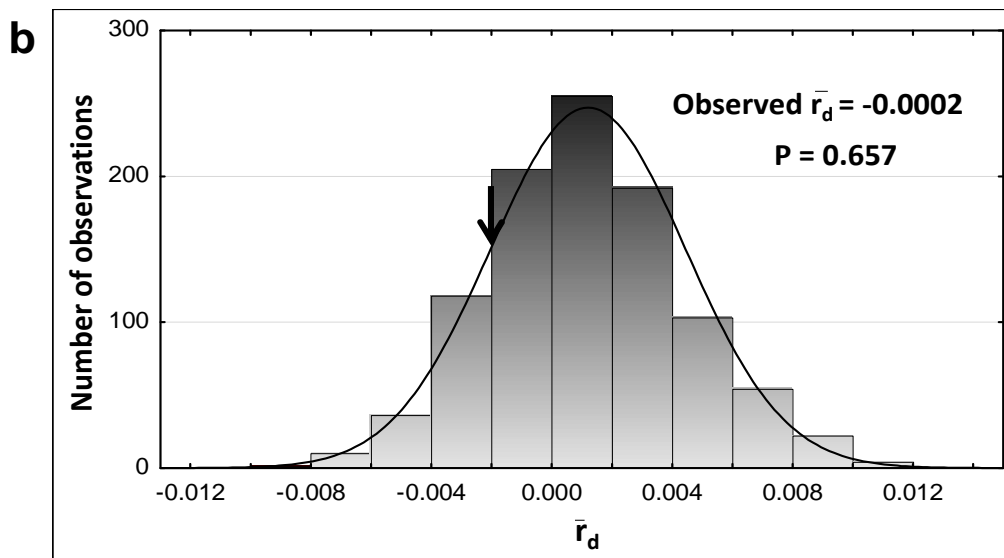
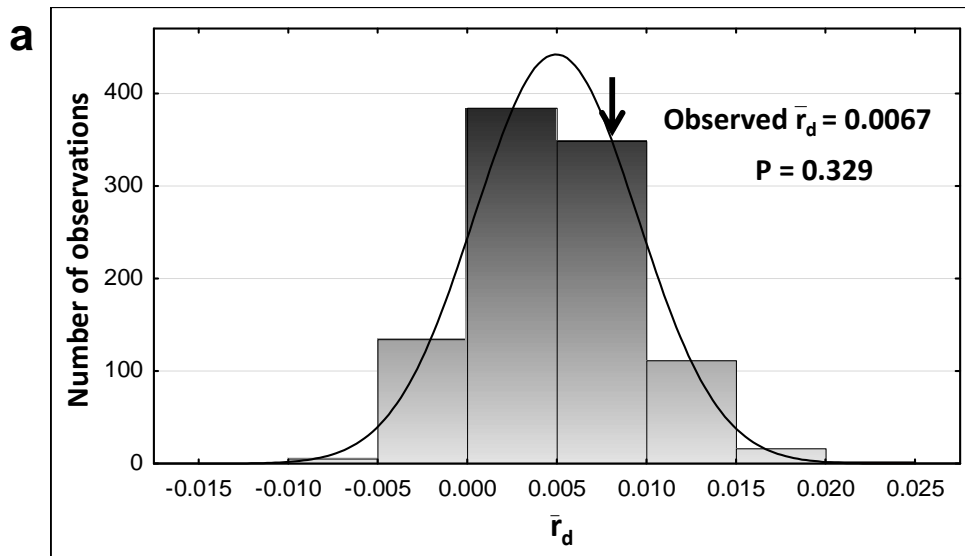
**Figure 3.3: Correlations to test IBD.** a) The  $\delta\mu^2$  genetic distance between *K. proteae* isolates in the different sampling plots versus the geographic distance between the midpoints of these plots in Franschoek and b) the number of *K. proteae* isolates obtained from each sampling plot in the recently burnt areas versus the geographic distance to the nearest unburnt sampling plot.



**Figure 3.4: Histograms depicting the inferred vector  $Q$  (the proportion of an individual's genome that originates from each cluster).** a) In Franschoek, all isolates from unburnt areas and most isolates from recently burnt areas grouped into one cluster (green). Two additional clusters (light and dark blue) not identified in the unburnt areas were observed in the recently burnt area. b) Assignment of all *K. proteae* individuals based on their sampling origin (Gouritz or Franschoek). The two populations are clearly distinguishable.



**Figure 3.5: Minimum spanning network (MSN) depicting the relationships between 105 unique haplotypes encountered in the Franschoek *K. proteae* population. Black circles represent missing haplotypes. Haplotypes from the unburnt and recently burnt areas are shaded blue and orange, respectively. Isolates from region 1 (see Fig 3.1) are shaded lighter than those from region 2.**



**Figure 3.6: The observed values of the index of linkage disequilibrium,  $\bar{r}_d$ , for a) the Franschoek and b) Gouritz and Franschoek *K. proteae* populations combined. Observed values are indicated on a normal distribution of  $\bar{r}_d$  values calculated for 1 000 random datasets. The  $\bar{r}_d$  and P values are shown on the graphs.**

### 3.2. Dispersal between distantly separated populations

In both an earlier *K. proteae* population study (Chapter 2) as well as the present study, only two identical haplotypes were identified in each population. This remained true for the combined dataset as additional shared haplotypes were not detected. Each individual in the two populations had at least one private allele in its haplotype and the majority of alleles were shared between the populations (Fig 3.2b). Additionally, the combined index of genetic diversity ( $N_e$ ) was not significantly different from that of the two separate populations, indicating similar genetic compositions. The population differentiation measurements  $\Delta_{ST}$  and  $\Delta_S/\Delta_T$  did not provide evidence of genetic isolation (Table 3.2). However, the non-zero value of Jost's  $D$  and  $D_{est(hap)}$  and the significance of theta suggested some level, albeit low, of differentiation between the Gouritz and Franschoek populations of *K. proteae* (Table 3.3).

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 are assigned to their population of origin (Fig 3.4b). 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 also displayed numerous loops and cohesiveness as observed in the networks for the individual populations (figure not shown). 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 ( $\bar{r}_d = -0.0002$ ;  $P = 0.657$ ; Fig 3.6b) and, therefore, indicates general outcrossing between *K. proteae* individuals from the two distantly separated populations.

## 4. Discussion

### 4.1. Recolonization of recently burnt areas

The genetic and genotypic diversity of *K. proteae* described in burnt and unburnt areas was remarkably high. This is consistent with the results of a previous study (Chapter 2) where heterozygosity was  $0.74 \pm 0.04$  and the maximum percentage of genotypic diversity ( $\hat{G}$ ) was 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 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 not fully established and have probably experienced a genetic bottleneck (Black *et al.*, 1988).

The numerous *K. proteae* migrants within and between the burnt and unburnt areas resulted in a closely related and weakly structured population. The effects of genetic drift seem to be masked by this high level of gene flow (Slatkin, 1987) and the great genetic variation within the population is therefore explained by the reproductive strategy of *K. proteae*. Both the asexual and sexual states of *K. proteae* occur in *P. repens* infructescences, 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. Since IBD is a result of limited dispersal across the landscape (Wright, 1943), the lack of an IBD pattern in the recently burnt area 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. Two additional population clusters in the recently burnt area, not associated with either the Franschoek or Gouritz (Chapter 2) *K. proteae* isolates, signify that sources of fungal inoculants other than adjacent established populations could play a role in recolonization. This strengthens the premise that long-distance dispersal is the predominant influence in recolonization of burnt areas.

#### **4.2. 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 population that was considered in this was unexpected. This is because these two sampling areas are separated by 240 km and mountainous geographic barriers. Although the two populations do not appear completely panmictic, 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 MSN.

The high level of gene flow observed between these two populations probably demands reconsideration of the vectors known to disperse *K. proteae*. Dispersal via flying insects, especially robust beetles, may allow much 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.

Sunbirds and sugarbirds are common avian visitors of *Protea* species (Collins & Rebelo, 1987) and are also regarded as important pollinators of *P. repens*. 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). It is therefore possible that mites carrying ophiostomatoid fungi are not exclusively phoretic on beetles, but also on birds. Since birds will be able to travel further than beetles, involvement of *Protea*-pollinating birds in the dispersal of ophiostomatoid fungi may explain the widespread gene flow between these two distantly separated populations. The Cape Sugarbird, *Promerops cafer* L., and the Malachite Sunbird, *Nectarinia famosa* L., have both been noted to travel up to 160 km (Fraser *et al.*, 1989; Harrison *et al.*, 1997) within the CFR and they would be good candidates for further investigation of this question.

The question arises as to how the small, primary mite vectors would accomplish such extensive dispersal even through 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) may 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, seem 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 may 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.

## 5. 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, long-distance dispersal of *Protea*-associated ophiostomatoid fungi also introduced inoculants from other areas. Long-distance dispersal remained prevalent even over large distances and across geographic barriers, although fungal panmixia was not perfectly maintained. In addition to beetles, 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 possibly sexual outcrossing.

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. *Protea repens* plants separated by the same distance and geographic barriers as the two *K. proteae* populations may therefore be similarly structured to the fungi. This study may therefore guide future studies on the population genetics of this iconic and economically important (Coetzee & Littlejohn, 2001; Knoesen & Conradie, 2009) CFR plant.



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## GENERAL CONCLUSIONS

This study has shown that the unique assemblage of *Protea*-associated ophiostomatoid fungi can provide insight into the microscopic biodiversity of the Cape Floristic Region (CFR). Microsatellite markers were developed to allow for the study of the population genetics of *Knoxdaviesia proteae*, elucidating questions concerning its dispersal, diversity and reproduction. As such, these markers have proven to be valuable tools to answer both ecological and evolutionary questions. Although only a single species was examined in this study, it can be considered a model taxon for future studies on *Protea*-associated ophiostomatoid fungi in the CFR.

Examining *K. proteae* dispersal at the population level revealed exceptional genetic and genotypic diversity within individuals distributed over several *P. repens* trees in close proximity to each other. Since a panmictic fungal population was maintained, medium- to long-distance dispersal between *P. repens* infructescences on different trees was prevalent. Long-distance dispersal also appeared to be the most important dispersal strategy for *K. proteae* to recolonize recently burnt fynbos areas. Although the neighbouring, unburnt areas acted as the predominant source of ophiostomatoid inoculants, more distant populations also contributed fungal inoculants to the recolonization process – emphasizing the role of long-distance dispersal in the movement of *K. proteae*. The remarkable fungal genetic diversity revealed in this study suggests that even the most intense sampling strategies may not be sufficient to discover the full extent of diversity in *K. proteae*. Furthermore, new diversity may be formed continuously through recombination.

The high level of gene flow that was observed, coupled with a predominantly outcrossing reproduction strategy, appeared to overwhelm the effects of genetic drift within *K. proteae* populations. The result was highly diverse and weakly differentiated populations, even between distantly (*ca.* 240 km) separated areas. Long-distance dispersal facilitated by beetles carrying phoretic mites, therefore, seemed to play a prominent role in the movement of *K. proteae* individuals. The great distances across which fungal gene flow can be facilitated in the CFR, implicates a possible role for larger fauna in the dispersal process. In addition to beetles, birds have previously been implicated as agents of mite phoresy (Krantz & Walter, 2009; Proctor & Owens, 2000) and the avian visitors of *P. repens* may, therefore, also be involved in facilitating long-distance dispersal of *Protea*-associated ophiostomatoid fungi.

As mites are the primary carriers of the spores of ophiostomatoid fungi (Roets *et al.*, 2011), the number of mites that are phoretic on a beetle or bird will determine the amount of fungal migrants and consequently the level of gene flow. The genetic structure of *K. proteae* populations observed in this study may be explained by frequent multiple migrations between *P. repens* infructescences and into burnt areas. Since a large number of mites may be phoretic on a single beetle (Roets *et al.*, 2009; Roets *et al.*, 2011), one dispersal event of a beetle potentially facilitates numerous ophiostomatoid migration events simultaneously.

This population genetics study is the first to consider a fungus within the CFR. The results are intriguing and prompt more questions about the state of microbial biodiversity in the CFR. Since the other *Protea*-associated ophiostomatoid fungi have similar vectors (Roets *et al.*, 2009), their dispersal strategies and consequently their population structures are likely to be similar to that of *K. proteae*. However, since many of the other *Protea*-associated ophiostomatoid fungi occur in more than one *Protea* host (Marais *et al.*, 1998; Marais & Wingfield, 2001; Roets *et al.*, 2006; 2008; Wingfield & Van Wyk, 1993), it would be interesting to see how or if the fungal population structure changes between different hosts. Using the present study as model, *Protea*-associated ophiostomatoid fungi seem to have a remarkable capacity to disperse and recombine, creating metapopulations across the CFR.



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