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Knoxdaviesia capensis: dispersal ecology and population genetics of a flower-associated fungus

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ABSTRACT

Protea-associated fungi are dispersed between flower heads by mites, beetles and possibly birds. For the ophiostomatoid fungus, *Knoxdaviesia proteae*, these vectors offer regular dispersal between distant floral hosts. Unlike *K. proteae*, *Knoxdaviesia capensis* occupies multiple *Protea* host species. In this study, we aimed to determine whether the generalist *K. capensis* shares the long-distance dispersal pattern with specialist *K. proteae* and whether it moves freely between different host species. We evaluated the genetic structure of *K. capensis* from five populations of a wide-spread host and between sympatric hosts. Twelve *K. capensis*-specific microsatellite markers were developed and applied to 90 individuals. *K. capensis* showed high genetic diversity and almost maximal genotypic diversity. All populations were poorly differentiated, indicating the presence of long-distance dispersal. No differentiation could be detected between sympatric host populations, suggesting free dispersal between different hosts. This implies that the beetle and bird vectors that pollinate *Protea* species show the same non-specific movement.

1. Introduction

The Greater Cape Floristic region (GCFR; Born et al., 2007) harbors a temperate flora with extreme levels of plant beta- and gamma-diversity and endemism (Linder, 2003). It is characterized by winter rainfall and includes both the Cape Floristic Region (CFR; Manning and Goldblatt, 2012) and the Extra Cape Flora (Snijman, 2013). Within the CFR, the shrubland vegetation known as 'fynbos' is dominant and comprises numerous species of the Proteaceae family (Manning and Goldblatt, 2012). *Protea* is one of the best-known Proteaceae genera and its members are essential to maintaining diversity and organization of numerous other organism communities within the fynbos. The genus carries its flowers in large inflorescences surrounded by involucral bracts that vary in color, depending on the species and its mode of pollination (Carlson and Holsinger, 2010). In the CFR, the main pollinators of the tree-like *Protea* species include various beetles (e.g. *Genuchus* and *Tricostetha* spp.) and birds (e.g. Cape sugarbirds and Orange-breasted sunbirds) (Broekhuysen, 1963; Coetzee and Giliomee, 1985).

Many *Protea* species enclose their seeds in canopy-retained infructescences subsequent to pollination and fertilization, a crucial strategy in fire-prone vegetation like the fynbos (Wright, 1994). Apart from its storage and protective role, infructescences inherently provide micro-niches for a large diversity of organisms such as mites, insects and fungi (Marais and Wingfield, 1994; Roets et al., 2007). Fungal diversity of the fynbos has received little attention in comparison to floral diversity, even though Crous et al. (2006) estimated it contains as many as 200 000 species.

Some species of ophiostomatoid fungi in the genera *Sporothrix* and *Knoxdaviesia* exclusively occur in the infructescences of serotinous *Protea* species (Roets et al., 2013), where they are often the dominant inhabitants (Lee et al., 2005; De Beer et al., 2016). As evidenced by the structure of their ascomata, these fungi are adapted to entomochoric dispersal, mediated primarily by mites

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(Malloch and Blackwell, 1993; Cassar and Blackwell, 1996; Roets et al., 2009a). Mites deposit ascospores of ophiostomatoid fungi in *Protea* flower heads, where they grow vegetatively until the inflorescence matures into an infructescence. In the infructescences, these fungi switch to sexual reproduction, as evidenced by the abundance of ascomata at this stage (Wingfield et al., 1988; Wingfield and Van Wyk, 1993). Recent analyses of the genomes of two *Protea*-associated *Knoxdaviesia* species have revealed that they are heterothallic and that sexual reproduction will, therefore, always create genetic diversity via recombination between two different haploid individuals (Aylward et al., 2016b).

Of the nine described *Protea*-associated members, only *Knoxdaviesia proteae* has received attention in terms of population genetic structure (Aylward et al., 2014b, 2015b). This species is restricted to a single widespread host, *Protea repens*, and displays high genetic diversity due to frequent gene flow and outcrossing. Within a stand of *P. repens* trees, the *K. proteae* population was found to be panmictic (Aylward et al., 2014b). Between distantly separated *P. repens* stands, near-panmixia was observed, likely due to long-distance dispersal (Aylward et al., 2015b). An assortment of *Protea*-visiting insects, such as the *Protea* beetle pollinators *Genuchus hottentottus* and *Trichostetha fascicularis*, are implied as mid- to long-distance vectors of the mites that carry the ophiostomatoid fungi between *Protea* trees (Roets et al., 2007, 2009a). Birds have been suggested as additional dispersal agents of spore-carrying mites in other systems (Proctor and Owens, 2000), leading Aylward et al. (2015b) to speculate that birds may also play a part in facilitating long-distance dispersal of *K. proteae*. Preliminary observations by workers on *Protea* ecology (T. Rebelo), and *Protea*-associated mites (N. Theron) and birds (A. Lee) seem to corroborate this, as *Protea*-associated mites have been observed on Cape sugarbirds (bluehillescape.blogspot.co.za/2012_11_01_archive.html, accessed July 2016).

Unlike *K. proteae*, *Knoxdaviesia capensis* colonizes a diversity of *Protea* hosts often present and flowering in sympatry (Marais and Wingfield, 1994; Roets et al., 2009b). Confirmed hosts include *Protea burchellii*, *Protea coronata*, *Protea laurifolia*, *Protea lepidocarpodendron*, *Protea longifolia*, *Protea magnifica*, *Protea neriifolia* and *P. repens* (Wingfield et al., 1993; Marais and Wingfield, 1994; Roets et al., 2005, 2011a; Aylward et al., 2015a). These hosts vary in distribution range, flowering time and flower morphology – all factors that will affect visitation by the *Protea* pollinators that also act as secondary fungal spore vectors. The movement of these vectors between different *Protea* species will, therefore, have a direct impact on the dispersal of *K. capensis* within and between its various hosts.

The extent of gene flow in *K. capensis* will depend on the distance over which its spores are transported as well as whether its vectors show host-specificity (or flower consistency) as *Protea* pollinators. If they tend to move consistently between inflorescences of the same *Protea* species only, *K. capensis* populations on different hosts species will become islands without means of connection. Conversely, pollinators may engage in flights between patches of different species, potentially producing one continuous fungal metapopulation (Levin, 1978). The degree to which the *Protea* pollinators regularly move between different *Protea* species that flower in sympatry is, however, currently unknown. Knowledge about the population genetics of the organisms that travel with these *Protea*-pollinators may shed some light on the possible inter-species visitation of some pollinators.

The aim of this study was to investigate the population structure of *K. capensis* across multiple *Protea* hosts in the CFR. For this purpose, we developed microsatellite markers specific to *K. capensis* according to the protocol of Aylward et al. (2014a). Subsequently, these markers were employed to assess population structure at two levels: (1) within a single host, *P. coronata*, with a widespread, though patchy distribution, and (2) within pairs of sympatric hosts with synchronous flowering times. This strategy enabled us to evaluate both the extent of *K. capensis* dispersal and to test for its movement between different hosts.

2. Material and methods

2.1. Sampling and fungal isolations

Infructescences from different *Protea* species were sampled from randomly selected trees at seven locations in the Western Cape Province, South Africa (Fig. 1), during January and August 2015. *P. neriifolia*, *P. lepidocarpodendron* and *P. longifolia* infructescences were collected from the Kogelberg Biosphere Reserve, an area where multiple *K. capensis* hosts occur in sympatry or near sympatry. A fourth species, *P. coronata*, displays a patchy distribution throughout the Western Cape Province (Rebelo, 2001) and was collected from five sites across its westerly distribution, including a site within the Kogelberg Biosphere Reserve (Fig. 1).

Fungal isolations and DNA extraction followed methods described by Aylward et al. (2014a). A sterile needle was used to isolate ascospores of *K. capensis* from the tip of flask-shaped *Knoxdaviesia* ascomatal necks in infructescences. Ascospores were germinated at room temperature, sub-cultured onto malt extract agar (MEA; Merck, Wadeville, South Africa) and grown at 25 °C. Individual *K. capensis* strains were purified by sub-culturing a single hyphal tip from water agar (15 g L^{-1} agar) onto fresh MEA. To avoid repeated isolation of the same individual, only a single fungal isolate was maintained per infructescence. To verify the species identity of the fungal isolates, the rRNA Internal Transcribed Spacer (ITS) region was amplified in a subset of representative isolates. The 25 μl PCR reaction consisted of 12 μl KAPA Taq ReadyMix (Kapa Biosystems, Inc., Boston, USA), 2.5 μM additional $MgCl_2$, 0.25 μM of the ITS1F (Larena et al., 1999) and ITS4 (White et al., 1990) primers and *ca*. 100 ng template DNA. Cycling conditions were 3 min at 95 °C followed by 40 cycles of 30 s at 94 °C, 1 min at 50 °C and 50 s at 72 °C. The final extension was 7 min at 72 °C. PCR products were sequenced at the Central Analytical Facility (CAF), Stellenbosch University, using ITS1F and the BigDye Cycle sequencing kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions, after which BLAST (Basic Local Alignment Search Tool, Camacho et al., 2009) searches were performed on the NCBI nucleotide data base (www.ncbi.nlm.nih.gov).

2.2. Microsatellite identification and amplification

Microsatellites were extracted from the *K. capensis* genome (GenBank accession: LNGK00000000.1; Aylward et al., 2016a) using the program Msatfinder 2.0.9 (Thurston and Field, 2005). The default search engine and search parameters of this program were used to identify perfect tandem repeat microsatellites. Microsoft Office Excel 2010 (Microsoft Corp., Redmond, WA, USA) was used to filter and analyze the data. Only microsatellites consisting of more than five repeat units were considered, since microsatellites with higher-than-average repeat numbers are more likely to be polymorphic (Dettman and Taylor, 2004; Dutech et al., 2007). The relevant microsatellites were subjected to BLAST searches using BLASTtx (www.ncbi.nlm.nih.gov), the least conservative engine, to ensure that the identified tri- and hex-nucleotide loci were not located in protein-coding regions.

Twenty microsatellite loci were initially selected and primers that flank these loci were designed with Primer3Plus 2.4.0 (Untergasser et al., 2007). Sequence analysis was performed in BioEdit 7.2.5 (Hall, 1999). Polymorphism of the markers was