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Review

Unseen fungal biodiversity and complex inter-organismal interactions in *Protea* flower heads

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ABSTRACT

A unique microbiome occurs within the flower heads of various *Protea* species endemic to Africa. These include two lineages of ophiostomatoid fungi, *Knoxdaviesia* (Microascales) and *Sporothrix* (Ophiostomatales), that have members occurring exclusively in this environment and that rely on mites as their primary mode of spore dissemination. The mites, in turn, attach to the bodies of *Protea*-pollinating beetles and the beaks and bodies of birds for long-distance movement, establishing a hierarchical dispersal network for the ophiostomatoid fungi. This inter-organismal network is highly successful, achieving fungal dispersal over vast distances. Multiple species of fungi, mites and bacteria have been described from this unique niche over the past four decades. The intricacies of their symbiotic interactions continue to be unravelled. This review covers all current knowledge of the "distinctly African" *Protea*-ophiostomatoid fungus environment and illustrates the depth of a fascinating unseen fungal biodiversity niche.

1. Introduction

Protea is an African genus comprising more than 100 species of iconic flowering plants (Rebelo, 2001). Their diversity is concentrated in the Core Cape Subregion (CCR) at the southern tip of Africa (Valente et al., 2010), although some members occur elsewhere in southern and central Africa (Beard, 1962; Rebelo, 2001). The CCR is characterised by a Mediterranean climate and includes a vegetation type known as fynbos, famous for its rich floral diversity, high levels of endemism and dependence on recurrent summer fires (Cowling et al., 1996; Manning and Goldblatt, 2012). To survive these fires,

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many *Protea* species use serotiny as a recruitment strategy, with seeds maintained above-ground on the plants in woody, old flower heads known as infructescences (Bond and Midgley, 2003).

The infructescences of some *Protea* species provide a unique and stable micro-habitat for a diverse range of organisms (Coetzee and Giliomee, 1985; Lee et al., 2005; Roets et al., 2013). These cone-like structures form after flowering when the colourful *Protea* involucral bracts close around the inflorescence (Fig. 1a). The resulting infructescences may be maintained on the plants for several years until the next fire or severe drought triggers them to open for seed release (Bond, 1985; Fig. 1b). Tightly closed infructescences, found in *Protea* species such as *P. repens* and *P. neriifolia*, offer a warm, moist environment, largely shielded from external fluctuations in weather (Roets et al., 2005, 2006b). A large diversity of microorganisms and arthropods inhabit these structures (Lee et al., 2005; Roets et al., 2006b) and multiple new species have been described as specific inhabitants of this niche (e.g. Ngubane et al., 2018; Theron et al., 2012; Wingfield et al., 1988).

The unique habitat within *Protea* infructescences, which was discovered relatively recently, forms part of the largely unseen, and therefore often overlooked component of biodiversity (Cowan et al., 2013). Unlike most such hidden ecosystems, the *Protea* system has been studied and explored in depth. A previous review of this system (Roets et al., 2013) summarised the fungal and arthropod species known from *Protea* infructescences at that time, and established a foundation from which to unravel their complex ecology and symbiotic interactions. Since then, knowledge of this unique system has continued to expand, including the discovery of new species, studies considering the population biology of some of the fungi, an understanding of vector relationships and dispersal as well as laboratory experiments to better understand the biology of these organisms. Our aim in this review is to assemble and integrate the new knowledge regarding the "distinctly African" (Wingfield and van Wyk, 1993) ophiostomatoid fungi occurring in *Protea* infructescences and their network of inter-organismal associations, which is largely unknown to biologists, including many mycologists.

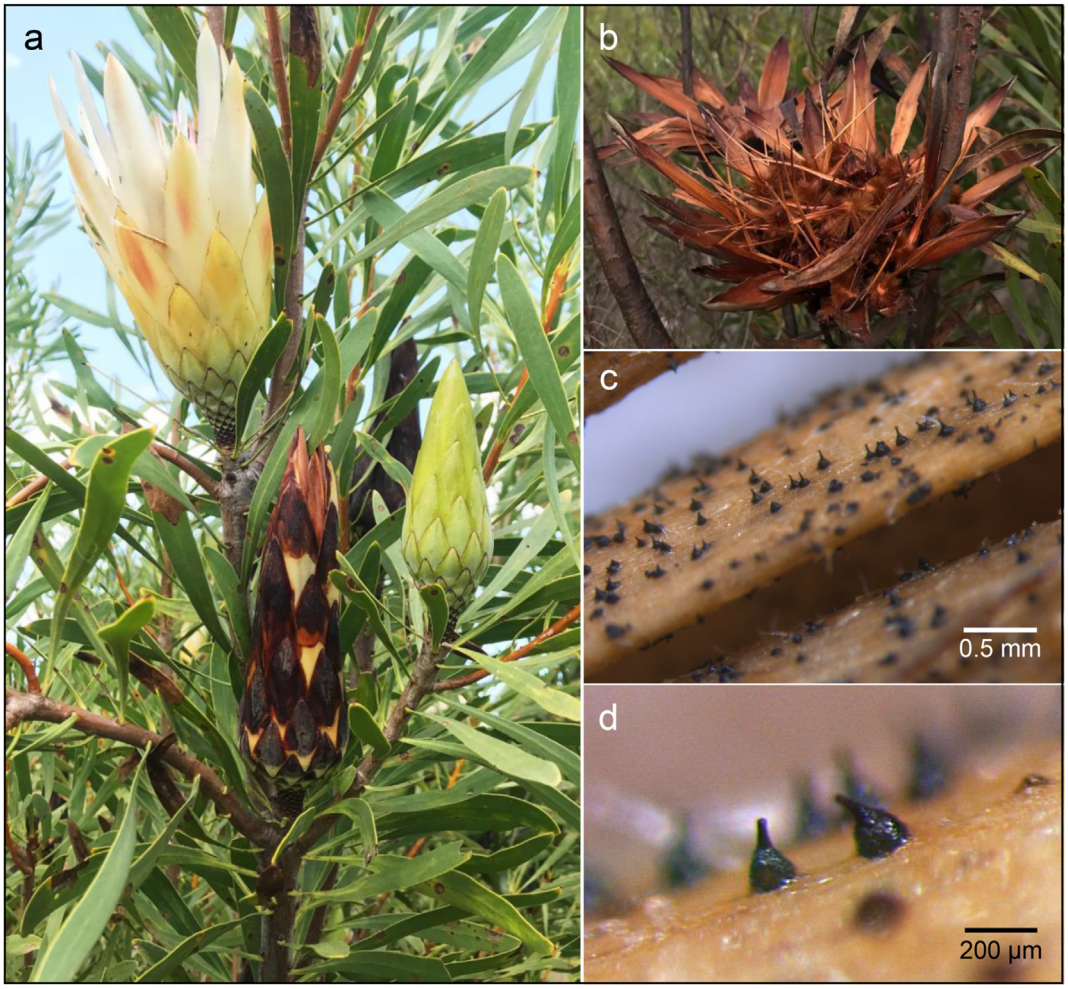

Fig. 1 – *Protea* flowering stages. (a) Bud (right), inflorescence (left) and closed infructescence (middle) of *P. repens*. (b) Infructescence opened to release seeds. (c and d) Ascomata of the ophiostomatoid *Sporothrix phasma* on *P. laurifolia* flowers.

2. A diverse assemblage of *Protea*-associated species

2.1. *The ophiostomatoid fungi*

Novel ophiostomatoid fungi were first detected in *Protea* infructescences in the late 1980s (Wingfield et al., 1988). The sexual structures of these fungi have distinctly long necks bearing ascospores and are the dominant microbial feature in infructescences (Fig. 1c and d; Marais and Wingfield, 2001). Since prominent plant pathogens, such as those in the genera *Ceratocystis* and *Ophiostoma*, also have ophiostomatoid morphologies (Seifert, 1993; Spatafora and Blackwell, 1994), the discovery of these fungi initially raised concern regarding the phytosanitation of the horticulturally important genus *Protea*. Additional surveys of CCR *Protea* plants identified several additional ophiostomatoid fungal species, consistently present in infructescences without any sign of disease (Marais and Wingfield, 1994, 1997, 2001; Wingfield and Van Wyk, 1993). These surveys also provided strong evidence that *Protea* species were the exclusive hosts of these specific ophiostomatoid species (Roets et al., 2013). This was with a single exception in the case of *Sporothrix variecibatus*, a species initially collected from *Eucalyptus* leaf litter and later described from *P. repens* and associated *Trichouropoda* mites (Roets et al., 2008).

Two distinct lineages of ophiostomatoid fungi occur within *Protea* infructescences, suggesting that the *Protea*-ophiostomatoid association evolved more than once (De Beer et al., 2013; Wingfield et al., 1999; Fig. 2). In the CCR, two *Knoxdaviesia* (previously *Gondwanamyces*) species and four *Sporothrix* (previously *Ophiostoma*) species have been identified from diverse *Protea* infructescences. As surveys for these fungi ventured beyond the CCR, an additional *Knoxdaviesia* and seven additional *Sporothrix* species were described, making *Sporothrix* by far the most speciose of the two lineages (Table 1). In contrast to their *Protea* hosts, the diversity of *Protea*-associated *Sporothrix* species is larger outside than within the CCR and phylogenetic analyses suggest that the non-CCR species are likely ancestral (Roets et al., 2009b).

The three *Knoxdaviesia* species form a monophyletic clade (Crous et al., 2012; Roets et al., 2009b), while the diverse *Protea*-associated *Sporothrix* species are polyphyletic (De Beer et al., 2016, 2022). Of the 11 *Sporothrix* species known from the *Protea* habitat, nine reside in three different species complexes that also accommodate species not associated with *Protea* hosts (Table 1; Fig. 2) (De Beer et al., 2016; Ngubane et al., 2018). Close to half of the described *Sporothrix* species form part of the *S. stenoceras* complex (complex C in Fig. 2), but provisional population genetic analyses suggest that two of these species, *S. africana* and *S. protearum*, may be con-specific (Ngubane, 2017). Intriguingly, the *S. stenoceras* and *S. pallida* complexes contain *Protea*-associated species distributed both outside and within the CCR. In addition to separate fungal lineages independently adapting to *Protea* flower heads, the genus *Sporothrix* has, therefore, invaded the *Protea* niche, as well as the CCR, multiple times (Roets et al., 2009b, 2013).

2.2. *The arthropod and bird vectors*

The long ascomatal necks of ophiostomatoid fungi facilitate their dispersal by arthropods (Malloch and Blackwell, 1993),

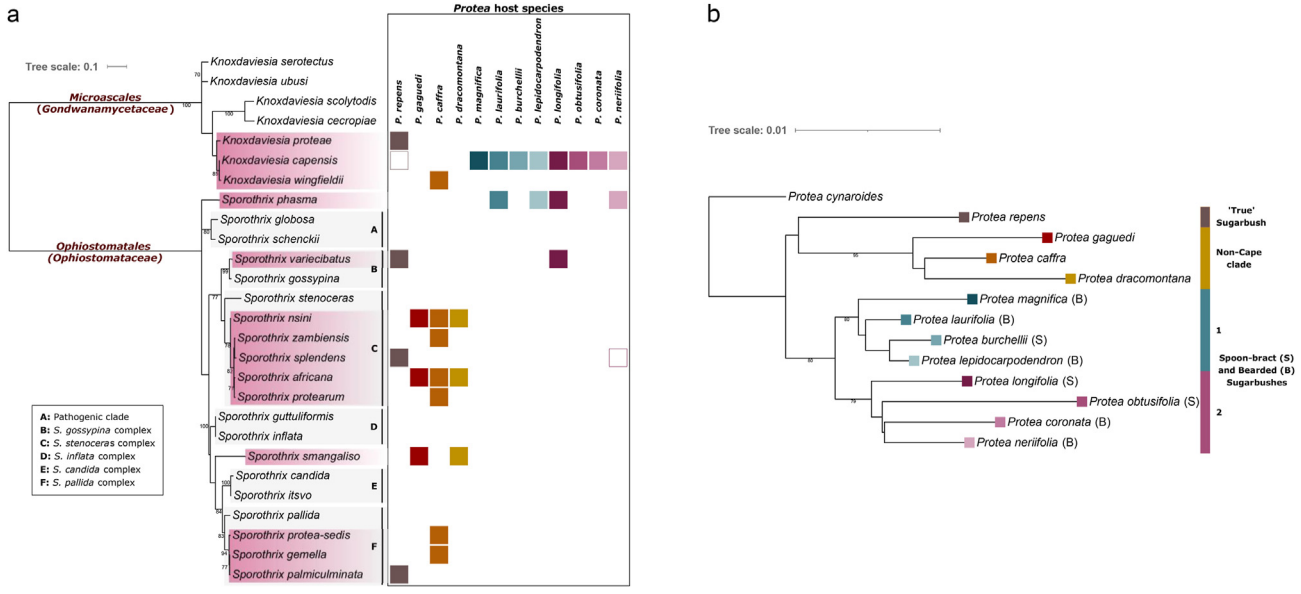

Fig. 2 – Taxonomic relationships of (a) known *Protea*-associated fungal species and (b) the *Protea* hosts known to harbour these fungi. In (a), species found in *Protea* are highlighted in pink. Letters in the *Sporothrix* lineage denote species complexes according to De Beer et al. (2016). Coloured blocks represent *Protea* species from which each fungal species has been confirmed. Open blocks indicate occasional occurrence of a species at low frequency. Only known *Protea* hosts of ophiostomatoid fungi are included in (b). The three non-Cape clade species occur outside of the Core Cape Subregion (CCR), whereas the rest are restricted to the CCR. Phylogenies were inferred based on maximum likelihood using the data of (a) De Beer et al. (2016); van der Linde et al. (2012) and (b) Valente et al. (2010).

because the sticky spore droplets presented at the tips of ascomata attach to the exoskeletons of arthropods as they forage. A combination of an abundance of ophiostomatoid fungi and a high diversity of arthropods in infructescences (Roets et al., 2005, 2006b) made the discovery of mites as the primary spore vectors of these fungi an important, yet not a surprising one (Roets et al., 2007). Five mite species act as primary spore vectors. These are two *Tarsonemus* spp., a *Trichouropoda* sp., *Proctolaelaps vandenbergi* and one initially identified as a species of *Glycyphagus*, but whose identification requires final confirmation (Roets et al., 2007, 2011; Theron-De Bruin et al., 2018). All have been confirmed to carry *Sporothrix* spores, but *Trichouropoda* appears to be the primary vector of *Knoxdaviesia* spores (Roets et al., 2011).

The relationship between the *Protea*-associated ophiostomatoid fungi and some of the mite species is mutualistic, in which the mites provide transport in exchange for nutrients. The *Glycyphagus* and *Trichouropoda* mites feed and reproduce on *Sporothrix* cultures (Roets et al., 2007; Theron-De Bruin et al., 2018) and the *P. vandenbergi* mites do not feed on the fungi, but specialise on *Protea* pollen and nectar (Theron-De Bruin, 2018). The *Tarsonemus* and *Trichouropoda* mites are particularly well-adapted to spore dispersal, having either leg depressions or specialised structures (sporothecae) for carrying *Knoxdaviesia* and *Sporothrix* spores (Roets et al., 2007). The position of fungal spores in transit on the other mite taxa has not yet been investigated.

As the primary vectors of *Protea* ophiostomatoid fungi, the behaviour of the associated mites directly impacts fungal dispersal. Mites seek larger animals for their own dispersal (Roets et al., 2009a) and this phoretic tendency facilitates a multilevel network of symbiotic interactions and fungal dispersal events for the *Protea* ophiostomatoid fungi (Fig. 3). When *Glycyphagus*, *Tarsonemus* and *P. vandenbergi* mites enter inflorescences during the *Protea* flowering season (Winter to Spring), they move towards the nectar wells at the base of the flowers (Theron-De Bruin et al., 2018), depositing *Sporothrix* fungal spores *en route* (Fig. 3a and b). These spores germinate and grow, presumably utilising nectar sugars (Aylward et al., 2017b), and produce conidia.

Large numbers of spore-carrying mites congregate at the tops of *Protea* inflorescences, where they come into contact with pollinators of *Protea* flowers (Fig. 3c and d; Theron-De Bruin et al., 2018). The mites are phoretic on *Genuchus hottentottus* (Protea white grub) beetles and two *Protea*-pollinating *Trichostetha* beetles, *T. capensis* (Brunia beetle) and *T. fascicularis* (Green Protea beetle; Roets et al., 2009a). Additionally, *Glycyphagus* and *P. vandenbergi* mites have been collected in great numbers from the endemic orange-breasted sunbird (*Nectarinia violacea*) and Cape sugarbird (*Promerops cafer*) (Theron-De Bruin et al., 2018). These birds are prominent visitors to *Protea* inflorescences (Collins and Rebelo, 1987) and their discovery as mite and, therefore, fungal vectors, is the first known case of a mite-fungus-bird symbiosis (Theron-De Bruin et al., 2018).

At the end of the *Protea*-flowering season, when the infructescences form, the ophiostomatoid fungi switch to sexual reproduction and exude ascospore droplets at the tips of their long-necked ascomata (Fig. 3e and f; Marais and Wingfield, 1994). At this stage (Summer months) some mites, such as *Glycyphagus* (Theron-De Bruin et al., 2018), feed on the *Sporothrix* fungi and all arthropods that forage in the vicinity are likely to pick up the sticky ascospores. During the next *Protea*-flowering season, short distance dispersal of the *Sporothrix* ascospores takes place by mites that move between flower heads on the same plants (Fig. 3g). For longer-distance dispersal, these mites will attach to larger *Protea*-pollinating arthropods, such as *G. hottentottus* that breeds within *Protea* infructescences, repeating the cycle (Roets et al., 2009a).

It is intriguing that the mite vectors identified in the *Protea*-ophiostomatoid system are related to those known from

Table 1 – *Protea*-associated ophiostomatoid species known to date.

Ophiostomatoid species	Distribution[a]	Species complex[b]	References
Knoxdaviesia capensis	CCR, South Africa	–	Wingfield and Van Wyk (1993)
K. proteae	CCR, South Africa	–	Wingfield et al. (1988)
K. wingfieldii	KZN, South Africa	–	Crous et al. (2012)
Sporothrix palmiculminata	CCR, South Africa (single population in JS Marais Park, Stellenbosch)	*S. pallida* complex (F)	Roets et al. (2006a)
S. phasma	CCR, South Africa	–	Roets et al. (2006a)
S. splendens	CCR, South Africa	*S. stenoceras* complex (C)	Marais and Wingfield (1994)
S. variecibatus	CCR, South Africa	*S. gossypina* complex (B)	Roets et al. (2008)
S. protearum	EC, Gauteng, KZN, South Africa	*S. stenoceras* complex (C)	Marais and Wingfield (1997)
S. gemella	Gauteng, South Africa (single population in W. Sisulu National Botanical Garden)	*S. pallida* complex (F)	Roets et al. (2008)
S. africana	Gauteng, KZN, MP, South Africa	*S. stenoceras* complex (C)	Marais and Wingfield (2001); Roets et al. (2006a)
S. nsini	Gauteng, KZN, MP, South Africa	*S. stenoceras* complex (C)	Ngubane et al. (2018)
S. smangaliso	KZN, South Africa	–	Ngubane et al. (2018)
S. protea-sedis	Nchila, Zambia	*S. pallida* complex (F)	Roets et al. (2010)
S. zambiensis	Nchila, Zambia	*S. stenoceras* complex (C)	Roets et al. (2010)

a Distribution refers to regions from where fungi have specifically been collected, but it is possible that they occur across the distribution ranges of their *Protea* hosts; CCR = Core Cape Subregion, Western Cape Province, KZN = Kwa-Zulu Natal Province, EC = Eastern Cape Province, MP = Mpumalanga.
b According to De Beer et al. (2016), see Fig. 2.

conifer-infesting bark beetle-fungal systems in the Northern Hemisphere (Hofstetter et al., 2013). However, unlike those systems, the fungi in *Protea* infructescences are neither pathogenic nor cause symptoms such as wood sapstain that is common for the fungal associates of conifer-infesting bark beetles (Seifert, 1993; Seifert et al., 2013). For example, most of the research on mite-beetle-insect symbioses stem from the interactions among the blue-stain fungus *Ophiostoma minus*, *Tarsonemus* mites and the southern pine beetle *Dendroctonus frontalis* (Hofstetter and Moser, 2014; Lombardero et al., 2000). In the well-known Dutch Elm Disease system, two mite genera known from *Protea* (*Tarsonemus* and *Proctolaelaps*) are associated with *Scolytus* bark beetles and carry spores of the pathogenic fungus *O. novo-ulmi* (Moser et al., 2010).

3. Population genetics confirms dispersal of endemic ophiostomatoid fungi

3.1. *Highly diverse and mobile fungal populations*

Studies of fungal population structure have been pivotal in understanding the movement of the primary and secondary vectors of the *Protea*-associated ophiostomatoid fungi. Such studies were first conducted on *K. capensis* and *K. proteae*, the first to consider the intraspecific genetic diversity of any native CCR fungus. Microsatellite markers revealed exceptional genetic diversity in *Knoxdaviesia*, with unique genotypes in almost all fungal individuals within a *Protea* stand (Aylward et al., 2014, 2015b). Although different to each other, these genotypes have the same building blocks (i.e. shared alleles), forming a cohesive fungal population in panmixia (Aylward et al., 2014). A population study on *S. splendens* (Aylward et al., 2023) also identified high levels of genetic diversity and closely related populations in the CCR. This confirms that the similar ecologies of these phylogenetically unrelated fungi lead to populations that are similarly structured.

The dispersal of ophiostomatoid fungi is primarily medium- to long-distance, confirming the phoretic tendency of mites and the important role of secondary vectors facilitating between-plant dispersal (Aylward et al., 2014, 2015b). This finding for *K. proteae*, which is found on a single *Protea* species, also extended to *K. capensis* that occupies the infructescences of at least nine *Protea* species (Aylward et al., 2015a; Roets et al., 2009b; Fig. 2). Panmixia is maintained between *K. capensis* individuals on its different host species (Aylward et al.,

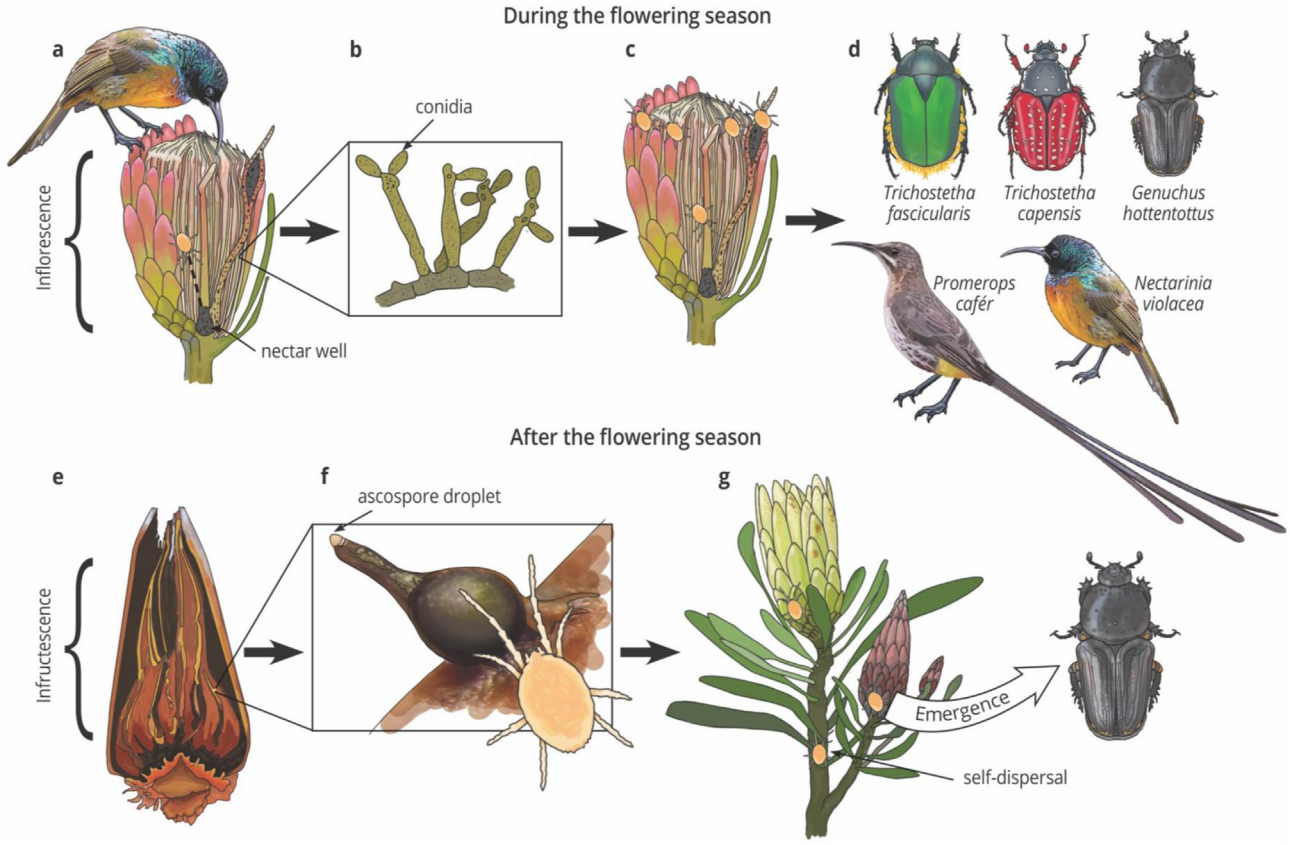

Fig. 3 – Fungal growth and vector movement in *Protea* flower heads, as currently understood. (a) During the flowering season mites are deposited in new inflorescences, inoculating them with fungal spores. (b) Asexual fungal growth and sporulation takes place, and conidia are transferred onto mites. (c) Mites travel to the top of inflorescences and wait for an opportunity to attach to (d) *Protea*-pollinating beetles and birds for dispersal. (e) After flowering, infructescences form and (f) fungi switch to sexual reproduction, exuding ascospores from their long-necked ascomata. Mites grazing on the fungi become coated by ascospores and move them to other infructescences or inflorescences (g) on the same plant as they self-disperse or to other plants if they attach to the adults of *Genuchus hottentotus* emerging from infructescences.

2017a). By implication, its vectors visit different species of *Protea* non-discriminately, congruent with findings that *Trichouropoda* mites move between *Protea* hosts (Roets et al., 2007) and *T. capensis* and *T. fascicularis* beetles occur on both *P. repens* and *P. neriifolia* (Roets et al., 2009a). The fungi move freely between different hosts and the fungal population, therefore, includes all individuals in a *Protea* stand, although the composition of the *Protea* species in the stand may vary.

Gene flow in *Protea*-associated species occurs over vast distances. Both *K. proteae* and *S. splendens* populations in the CCR, separated by >200 km and geographic barriers such as mountains and cities, show only weak genetic structure and have similar genetic compositions (Aylward et al., 2015b, 2023). This could be explained, at least in part, by the widespread geographic range of the *P. repens* hosts that act as 'bridges" for the beetle and bird vectors. Outside of the CCR, dispersal becomes more limited, likely because the abundance of *Protea* populations decreases (Aylward et al., 2023). Despite the isolation by distance effect in *S. splendens*, gene flow still occurs between populations within and outside the CCR. This highlights the prevalence of long-distance dispersal, and therefore the important role that beetles and birds (Theron-De Bruin et al., 2018) play in the dispersal of *Protea*-associated fungi.

3.2. *Sexual reproduction and frequent dispersal events drive genetic diversity*

The reproductive genetics of the *Knoxdaviesia* species has been interrogated using their genome sequences, providing an explanation for their exceptional genetic diversity. In this regard, analysis of the mating-type (*MAT*) locus in *K. capensis*

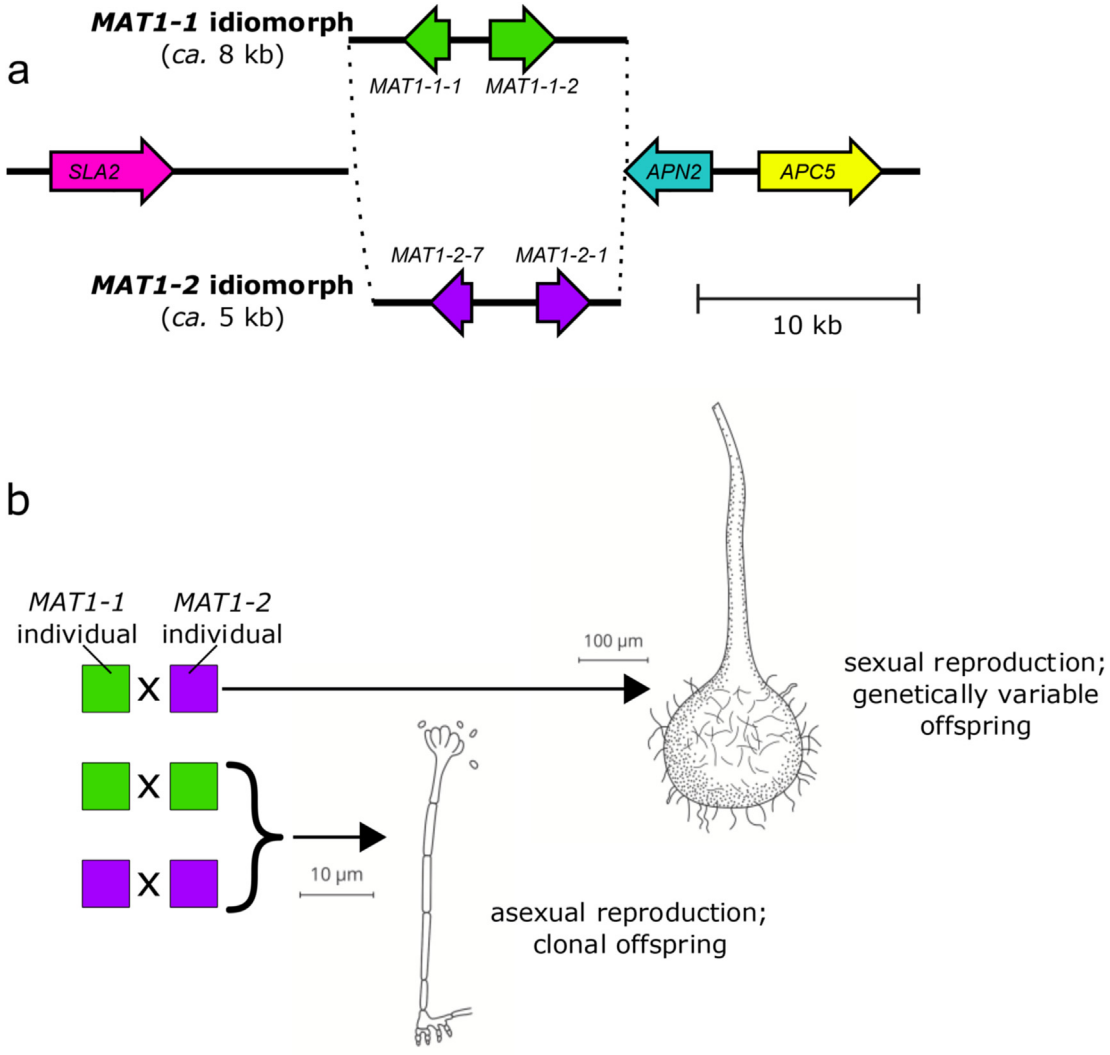

Fig. 4 – The mating-type (*MAT*) locus in the genomes of *Knoxdaviesia capensis* and *K. proteae* indicates that these are obligately outcrossing species. (a) The structure of the *MAT* locus. The single black line represents conserved DNA flanking the two *MAT* "idiomorphs". Coloured arrows represent genes and a scale bar of 10 kilobases (kb) is shown. An individual will encode either the *MAT1-1* or *MAT1-2* idiomorph in its genome, as indicated by the dashed lines, and (b) require a partner encoding the opposite idiomorph to undergo sexual reproduction. Asexual reproduction occurs in the absence of compatible partners.

and *K. proteae* showed that both species are heterothallic (Aylward et al., 2016), with isolates having either the *MAT1-1* or *MAT1-2* idiomorph (Fig. 4), requiring individuals to locate a compatible partner for outcrossing. The sexual structures (ascomata) are the predominant form of these fungi in *Protea* infructescences (Fig. 1; Marais and Wingfield, 1994) and heterothallism implies that each of the ascomata is a source of many ascospores harbouring new genetic combinations (Milgroom, 1996). The mating strategies of *Protea*-associated *Sporothrix* species have not been studied, but the publicly available genome sequences of *S. phasma*, *S. protearum* and *S. variecibatus* show that these species are also heterothallic (Huang et al., 2020). The abundance of the *Sporothrix* sexual states in infructescences and the high genetic diversity observed in *S. splendens* (Aylward et al., 2023) further suggests a situation similar to that of *Knoxdaviesia* where individuals of both mating types are distributed evenly in infructescences and outcrossing generates genetically novel offspring (Aylward et al., 2016).

Due to recombination, the ascospore droplets of *Protea*-associated ophiostomatoid fungi carry spores with multiple genotypes (Aylward et al., 2016). Consequently, dispersal of a single mite potentially facilitates dispersal of multiple genetically unique fungal individuals. In turn, the secondary beetle and bird vectors carry multiple mites (Fig. 5; Roets et al., 2009a; Theron-De Bruin et al., 2018). This hierarchical dispersal system enables frequent fungal migration events and multiple fungal inoculations per event. Combined with obligate outcrossing, it leads to the massive genetic diversity, high rates of gene flow and strong evidence of recombination observed in populations of these fungi (Aylward et al., 2014, 2015b, 2017a).

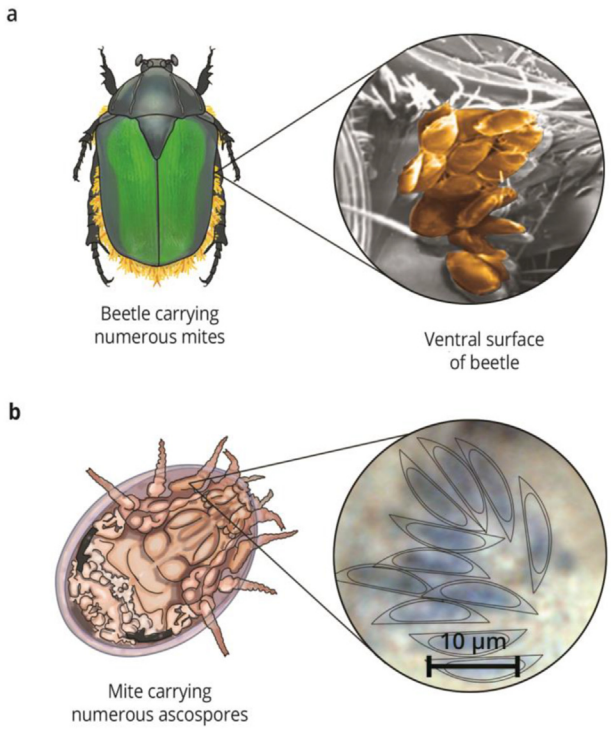

Fig. 5 – The hierarchical dispersal system means that dispersal of a single secondary vector (beetle or bird) causes the dispersal of numerous genetically unique fungi. (a) Beetles (*Trichostetha fascicularis* illustrated) may carry multiple mite individuals (*Tarsonemus* on ventral side of *Genuchus hottentottus* illustrated). (b) In turn, each mite (*Trichouropoda* sp. illustrated) may carry many genetically unique fungal ascospores (*Knoxdaviesia proteae* illustrated), leading to massive fungal dispersal events.

4. The competitive infructescence niche

4.1. *Early ophiostomatoid colonisation benefits the* Protea *host*

Early colonisation of *Protea* flower heads by ophiostomatoid fungi appears to be the key to their long-term dominance. Senescing infructescences represent a highly competitive environment that could be colonised opportunistically by many different fungal species. Many of these are common saprotrophic fungi, such as species of *Cladosporium*, *Fusarium* and *Penicillium* (Lee et al., 2005). The *Protea* ophiostomatoid fungi are weak competitors that are easily over-grown when cultivated alongside or after inoculation with commonly occurring saprotrophs (Mukwevho et al., 2021). Their poor competitive ability is also reflected in their very limited secondary metabolite biosynthesis capacity (Aylward et al., 2018). Once established, however, the ophiostomatoid fungi successfully defend the niche that they have colonised, excluding obvious activity of common saprobes (Mukwevho et al., 2021).

Protea nectar sugars likely enable early colonisation of living floral structures by the saprotrophic ophiostomatoid fungi (Aylward et al., 2017b). From the fungal perspective, nectar serves the dual purpose of attracting their secondary pollinator vectors to facilitate early inoculation (Roets et al., 2009a; Theron-De Bruin et al., 2018) and providing a readily available nutrient source. Both *K. capensis* and *K. proteae* are able to grow on fructose, glucose and xylose (Aylward et al., 2017b), the monosaccharides found in *Protea* nectar (Cowling and Mitchell, 1981; Nicolson and Van Wyk, 1998). The *Knoxdaviesia* species also encode glucose oxidase, an enzyme that converts glucose to hydrogen peroxide, leading to the hypothesis that this could be the mechanism by which they capture and become established in the *Protea* niche (Aylward et al., 2018).

Establishment of *Protea*-associated *Knoxdaviesia* and *Sporothrix* must also benefit their hosts (Lee et al., 2005; Mukwevho et al., 2021). Common saprobic fungi are associated with brittle and broken infructescences and dominate only when ophiostomatoid fungi are absent (Mukwevho et al., 2021). Once nectar is depleted and the infructescences develop, simple sugars to sustain fungal growth are trapped as components of the complex structures of plant cell walls (Scheller and Ulvskov, 2010). The *Knoxdaviesia* species encode far less secreted proteins and plant cell wall degrading enzymes (PCWDEs) than other saprotrophs and they have no pectinolytic enzymes (Aylward et al., 2017b). This small repertoire of PCWDs in *K. capensis* and *K. proteae* is sufficient to release monomers from the cellulose and hemicellulose

fraction of cell walls, such as arabinose, glucose, galactose, mannose and xylose, on which they are able to grow (Aylward et al., 2017b). In contrast, commonly occurring saprotrophic fungi, such as *Cladosporium cladosporioides* and *Penicillium* species, are generalist, indiscriminate decomposers. The inability of the *Knoxdaviesia* fungi to degrade pectin seems likely to be the reason why infructescences inhabited by ophiostomatoid species retain the structural integrity necessary to keep these seed-storage structures functional.

4.2. *Generalist and specialist* Protea *fungi*

The ophiostomatoid fungi in *Protea* flower heads (except *S. variecibatus*) are exclusively found in this niche, yet they differ in the number of *Protea* species on which they occur. Some species are specialists, found only on a single *Protea* host species, whereas others are generalists, recurrent on several *Protea* species (Fig. 2a). Particularly notable is the contrast between *K. capensis*, occurring on nine different *Protea* species, and *K. proteae*, exclusive to *P. repens* (Roets et al., 2005; Wingfield et al., 1988). In contrast to the multiple *Protea* hosts of the ophiostomatoid fungi in the CCR, ophiostomatoid fungi are known from three host species outside of this region. Some have been found only in the infructescences of the widely distributed *P. caffra* (Crous et al., 2012; Roets et al., 2010), whereas others occupy all three grassland *Protea* species (Ngubane et al., 2018; Roets et al., 2006a). Host exclusivity for ophiostomatoid fungi occurring outside of the CCR is, however, poorly understood. This is due to their having received a lower level of attention than those in the CCR, but is also likely influenced by the lower diversity and patchy distribution of *Protea* species outside the CCR (Rebelo, 2001).

Reasons for the association of ophiostomatoid fungi with particular *Protea* species and the contrast between the generalist and specialist species of these fungi have been considered in some detail. Adding *Protea* tissues to *in vitro* culture media enhances *Knoxdaviesia* and *Sporothrix* growth, however, different species in these genera grow optimally on tissue of the *Protea* species on which they occur naturally (Roets et al., 2012). The specialist *K. proteae* and *S. splendens* grow best on *P. repens*, whereas other *Protea* species better support growth of the generalists *K. capensis* and *S. phasma* (Roets et al., 2012). Since *P. repens* is phylogenetically distant from the other CCR *Protea* species (Fig. 2b; Valente et al., 2010), their chemistries are likely rather different. This has led to the hypothesis that the specialist ophiostomatoid lineage is the result of a generalist ophiostomatoid ancestor that specialised on *P. repens* to increase its fitness (Aylward et al., 2017b). *Protea repens* is the most widely distributed of all ophiostomatoid-containing *Protea* species in the CCR (Rebelo, 2001).

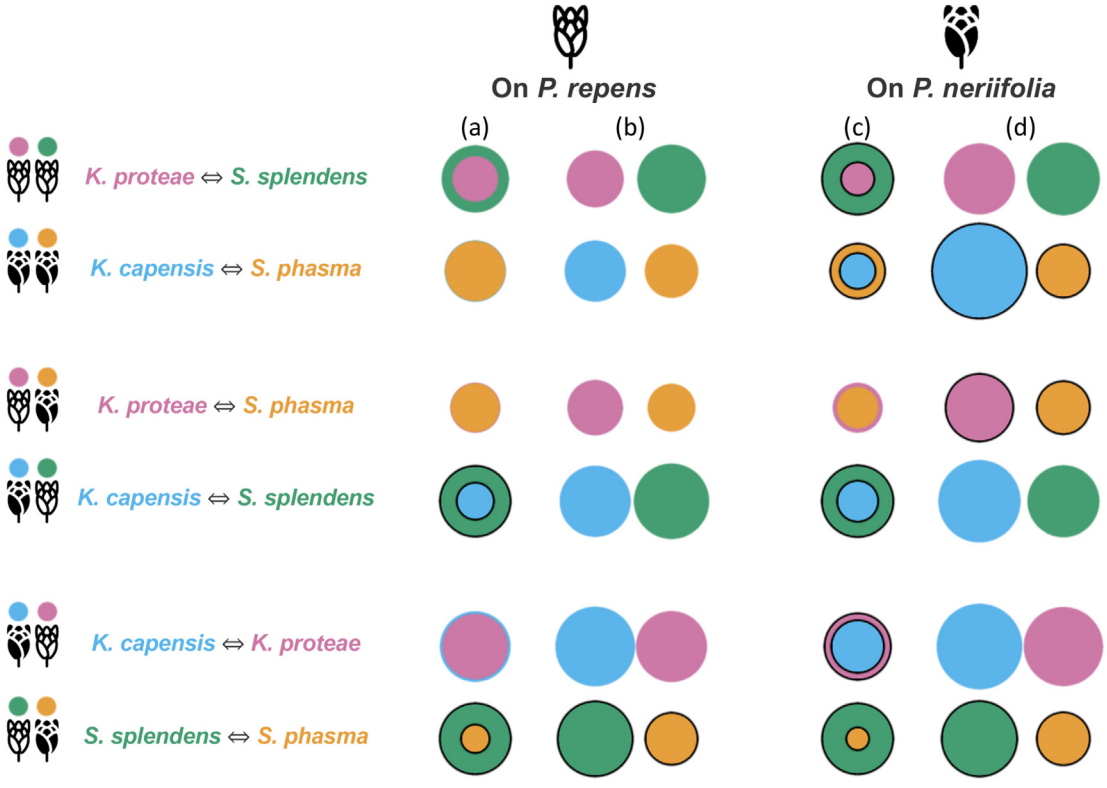

Fig. 6 – Primary resource capture abilities of two *Knoxdaviesia* and two *Sporothrix* species on two different *Protea* hosts, according to Mukwevho et al. (2020). *Protea repens* (white flower) is the usual host of *K. proteae* (pink) and *S. splendens* (green), whereas *P. neriifolia* (black flower) usually hosts *K. capensis* (blue) and *S. phasma* (orange). The average area captured by each species when paired on host-supplemented media *in vitro*, without spatial separation (a and c) and with spatial separation (b and d), is illustrated by coloured circles. Small circles above flower pairings represent 80 mm^2. Circles outlined in black indicate significant differences in the area captured and, therefore, highlight interactions where one species outcompetes the other.

Specialising on this host would, therefore, have had the benefit of retaining host availability.

Other explanations for the host-exclusivity of ophiostomatoid fungi that have been considered are environmental conditions and vector behaviour. Temperature and relative humidity within infructescences do not appear to play a major role and vector behaviour alone is an insufficient explanation for host specialisation (Roets et al., 2012). This is because ophiostomatoid fungal vectors also visit some non-host species, such as *P. nitida*, and the beetle and mite vectors occur on both *P. repens* and *P. neriifolia* that are hosts to specialist and generalist fungi, respectively (Roets et al., 2012). This view is also supported by the fact that the generalist *K. capensis* occurs in *P. repens* at very low levels (Aylward et al., 2015a) and the specialist *S. splendens*, is occasionally found in *P. neriifolia* infructescences (Theron-De Bruin et al., 2018). These reports suggest that the fungi have access to all potential hosts, but that host chemistry and/or fungal competitiveness on specific hosts preclude growth under most circumstances.

Analyses of the *K. capensis* and *K. proteae* genomes have also sought to provide an understanding of the factors underlying host specialisation in these fungi. The genomes of these two species show only 89.5% nucleotide identity and different compositions of repetitive sequences (Aylward et al., 2018). Yet they do not provide a clear picture of one being more metabolically diverse or restricted than the other. In both cases, most predicted secreted enzymes have a carbohydrate-degrading function and species-specific enzymes are rare. In addition, very few differences were found in the carbon usage profile of the two species (Aylward et al., 2017b), despite the fact that these are sufficient to enable the species to be host-specific or not.

4.3. *Competition among ophiostomatoid fungi*

There is considerable competition for niche availability within *Protea* infructescences. This is true not only for the ophiostomatoid and other fungi that occur in this niche, but also amongst species of *Knoxdaviesia* and *Sporothrix*. For example, seven ophiostomatoid species are found on *P. caffra*, five occur on *P. repens*, and *P. longifolia* and *P. neriifolia* each host three species (Fig. 2). At least two species can co-occur within the same infructescence and even sporulate concurrently (Roets et al., 2005, 2013). Culturing ophiostomatoid fungi in pairs, however, always negatively affects the growth of both partners (Mukwevho et al., 2020, 2022).

Competition only partly explains the observed dominance of certain ophiostomatoid species. *Sporothrix splendens* is a particularly strong competitor *in vitro*. This is both with regards to initially colonising media supplemented with *Protea* host tissue and establishment on media already colonised by another ophiostomatoid species (Mukwevho et al., 2020). Only *K. proteae* co-occurs naturally with *S. splendens* and is its strongest competitor for initial colonisation of media containing *P. repens* host material (Fig. 6). Successful concurrent *in vitro* growth of the two generalist species, *K. capensis* and *S. phasma*, relies on physical separation, with direct contact hindering *K. capensis* (Mukwevho et al., 2020). Some interactions, however, do not follow what is known about the natural co-occurrences of these fungi. For example, the *P. repens*-specific species, *K. proteae* and *S. splendens*, can outcompete or capture similar space to *K. capensis* and *S. phasma* when cultivated on *P. neriifolia* host material. In contrast, *K. capensis* competes well with *K. proteae* on *P. repens* material, a host it occupies at low frequency (Aylward et al., 2015a).

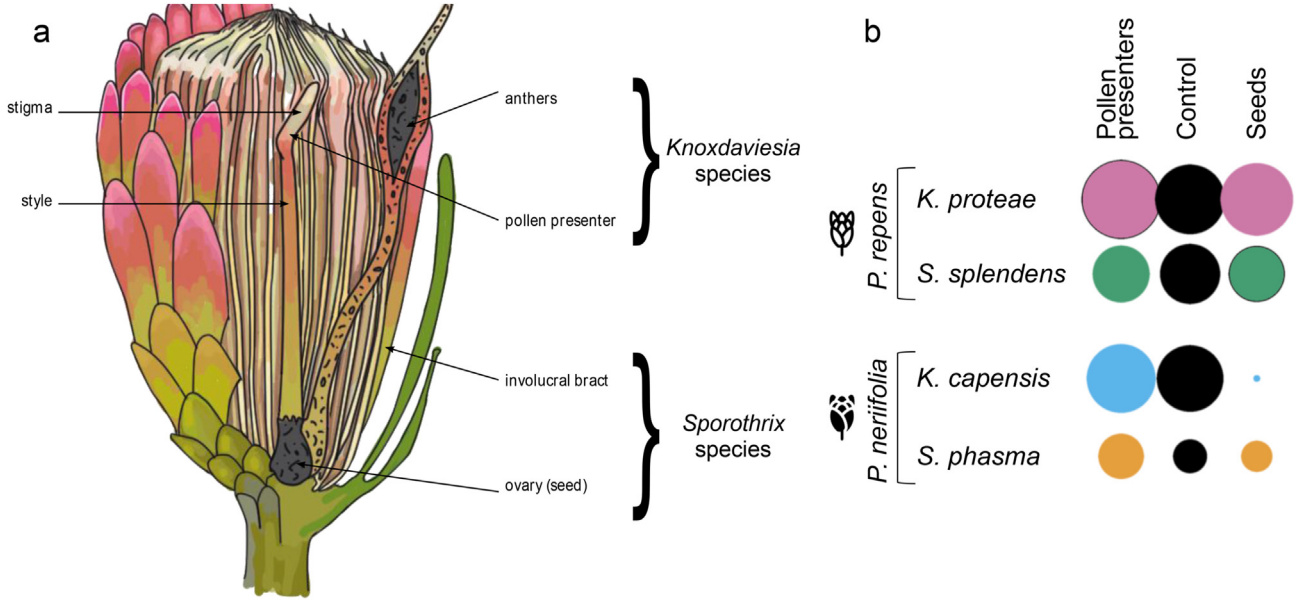

Fig. 7 – Differential growth of *Knoxdaviesia* and *Sporothrix* species on *Protea* pollen presenters and seed. (a) In the field, the ascomata of *Knoxdaviesia* species are typically observed on senescent pollen presenters, while *Sporothrix* species primarily occupy the region at the base of flowers. *Sporothrix* species occasionally, and especially in the absence of *Knoxdaviesia*, grow on pollen presenters. (b) *In vitro* growth of each species on the pollen presenters and seeds of its preferred host. Circle size represents growth area; black outlines indicate growth significantly different from the control (no host tissue). The *S. splendens* control is 1000 mm^2.

Field observations suggest that interactions among different ophiostomatoid species in *Protea* infructescences could be minimised by niche partitioning (Roets et al., 2013). *Knoxdaviesia* species are typically found on old pollen presenters and *Sporothrix* species on non-viable seeds at the bases of dead flowers (Fig. 7a). *Sporothrix* species, however, occasionally also occur on involucral bracts and pollen presenters (Theron-De Bruin et al., 2018). Differences in substrate specificity, however, do not fully explain this observed niche partitioning (Mukwevho et al., 2022). For example, pollen presenters appear to be a favoured substrate of both *Knoxdaviesia* and *Sporothrix* (Fig. 7b), facilitating either optimal growth (*K. capensis* and *S. phasma*) or similar growth to other substrates (*K. proteae* and *S. splendens*). If co-occurring with *K. capensis*, *S. phasma* can escape competition by growing on seeds, but only pollen presenters can sustain *K. capensis* growth. Both *K. proteae* and *S. splendens* grow well on seeds and pollen presenters, with *S. splendens* being the stronger competitor and also able to grow on other tissue types, such as involucral bracts (Mukwevho et al., 2020, 2022).

Overall, it appears that the host exclusivity of *Protea*-associated ophiostomatoid fungi is the result of interactions among a range of factors. Firstly, the *Protea* species need to have tightly closed infructescences that create a suitable protected micro-environment (Roets et al., 2009b, 2012). The fungi depend on their vectors for dispersal to suitable hosts and likely need to arrive within a particular timeframe to establish successfully. In this regard, weak competitors, such as *K. proteae*, require early colonisation (Mukwevho et al., 2021). It is also evident that the chemistry of the *Protea* host influences both the growth of ophiostomatoid species, as well as their competitive abilities (Mukwevho et al., 2020). Within the infructescence, the chemistry of different floral parts also influences fungal growth, facilitating niche partitioning to minimise competition among the ophiostomatoid fungi (Mukwevho et al., 2022).

5. A possible role for actinomycetes

A question that has arisen is whether actinomycetes might play a role in maintaining an environment conducive to the growth of the ophiostomatoid fungi in *Protea* infructescences. This derives from the fact that these filamentous bacteria are known from other fungus-arthropod systems, such as fungus-growing ants (Dângelo et al., 2016), bark beetles (Hulcr et al., 2011) and the woodwasp, *Sirex noctilio* (Adams et al., 2011). Following this line of thought, Actinomycete bacteria in the genera *Amycolatopsis* and *Streptomyces* have been identified in *P. neriifolia* and *P. repens* flower heads (Human et al., 2016, 2018). Actinomycetes are present in young *Protea* infructescences, but their species richness increases with infructescence age, suggesting that arthropods also play a role in their dispersal (Human et al., 2018), as has been documented for other *Streptomyces* species (Goodfellow and Williams, 1983).

The manner in which actinomycete bacteria influence the inter-organismal interactions within *Protea* flower heads and ophiostomatoid fungal host exclusivity remains to be understood. It has been speculated that the antifungal compounds produced by these bacteria exclude general saprotrophs and, therefore, facilitate niche capture by the ophiostomatoid fungi (Human et al., 2016). However, the ophiostomatoid fungi are sensitive to the antifungal compounds of all three *Streptomyces* groups identified in infructescences (Human et al., 2016), making their co-dominance unlikely. It is possible that there is succession or temporal separation of ophiostomatoid fungi and actinomycetes. The bacteria are unlikely to be present in young flower head stages and the likelihood of encountering them increases with the age of the infructescence (Human et al., 2018). A "seasonal turnover effect" also occurs whereby different *Streptomyces* taxa dominate as the season progresses. In the same way, ophiostomatoid fungi show different levels of dominance in different seasons, being abundant during the wet winter months and almost undetectable in late spring and summer (Roets et al., 2005). The observed succession and seasonal effects of the bacteria and fungi, however, do not merge to form a clear picture of how they affect each other. Further research is necessary to unravel the role of actinomycetes in the complex network of interactions in *Protea* infructescences.

6. Conclusions

The unique habitat created by *Protea* flower heads provides a fascinating example of a diverse ecosystem that has revealed many new species. *Protea* infructescences have contributed substantially to the description of new ophiostomatoid fungi (Marais and Wingfield, 1994; Ngubane et al., 2018), mites (Theron et al., 2012) and bacteria (Human et al., 2016) and to the detection of diverse and complex interactions among them. Biodiversity projections imply that less than 10% of fungi (Blackwell, 2011; Hawksworth et al., 2017) and at best 9% of mite species (Hofstetter et al., 2013) are known. The level of biodiversity thus far detected in the hidden *Protea* infructescence habitat illustrates the fact that the microorganisms known to science are simply the tip of the proverbial iceberg.

The *Protea*-ophiostomatoid fungal symbiosis has been explored relatively deeply, yet several avenues of research deserve further investigation. The identity and distribution of *Protea* species within and outside of the CCR differ markedly (Rebelo, 2001) and the effect of patchy host distribution on fungal dispersal could be considered by studying the population biology of non-CCR ophiostomatoid fungal species. The manner in which the *Protea* niche may have led to fungal specialisation is also poorly understood. *Knoxdaviesia* species appear to encode fewer genes than other saprotrophs (Aylward et al., 2018), but comparisons have not been made with *Protea*-associated *Sporothrix* or non-*Protea*-associated *Knoxdaviesia* species. The influence of actinomycetes and the grazing effect of mites on ophiostomatoid fungi also remains largely unexplored.

The fungi and their arthropod vectors discussed in this review are united by their attraction and specificity to the flower heads of *Protea* plants at the southern tip of Africa. However, much of the fundamental knowledge regarding these organisms stems from studies of plant diseases in which the fungi and arthropods are related to those in the *Protea* system, but are invasive or have some detrimental impact on their hosts (e.g. Hofstetter and Moser, 2014; Lombardero et al., 2000;

Moser et al., 2010). In this regard, understanding the *Protea* system benefits plant health research by providing a benchmark of native species against which disease-causing counterparts can be compared. Ultimately, increasing our understanding of microbial genetic resources, such as that found within *Protea* flower heads, can only be of considerable advantage.

Declaration of competing interest

The authors declare that no competing interests exist.

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