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ARTICLE

Knoxdaviesia proteae is not the only *Knoxdaviesia*-symbiont of *Protea repens*

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Abstract: Two polyphyletic genera of ophiostomatoid fungi are symbionts of *Proteaceae* in southern Africa. One of these, *Knoxdaviesia*, includes two closely related species, *K. proteae* and *K. capensis*, that have overlapping geographical distributions, but are not known to share *Protea* host species. *Knoxdaviesia capensis* appears to be a generalist that occupies numerous hosts, but has never been found in *P. repens*, the only known host of *K. proteae*. In this study, extensive collections were made from *P. repens* and isolates were identified using DNA sequence comparisons. This led to the surprising discovery of *K. capensis* from *P. repens* for the first time. The fungus was encountered at a low frequency, suggesting that *P. repens* is not its preferred host, which may explain why it has not previously been found on this plant. The basis for the specialisation of *K. proteae* and *K. capensis* on different *Protea* species remains unknown.

Key words:
Gondwanamycetaceae
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INTRODUCTION

Ophiostomatoid fungi are a polyphyletic assemblage (Spatafora & Blackwell 1994, Wingfield *et al.* 1999) that share morphological characters such as flask-shaped ascomata with long necks bearing sticky spore droplets, that make them ideally suited for arthropod-mediated dispersal (Wingfield *et al.* 1993). Species in two genera, *Ophiostoma* and *Knoxdaviesia* (Wingfield *et al.* 1999), occur in the flower heads (infructescences) of serotinous *Protea* species in southern Africa (Fig. 1). They are not associated with disease symptoms on their hosts but could benefit the plant by excluding harmful fungi from the infructescences that must protect viable seeds for long periods of time (Roets *et al.* 2013).

The dispersal biology of *Protea*-associated ophiostomatoid fungi is intriguing. The primary vectors are mites (Roets *et al.* 2011b) that have a mutualistic association with some of the fungi they carry (Roets *et al.* 2007). These mites can self-disperse to other infructescences on a *Protea* tree, but most often they use beetles as long-distance vectors to reach other *Protea* trees (Aylward *et al.* 2014a, Roets *et al.* 2009a). Although the vectors of the *Protea*-associated ophiostomatoid species are the same, the various fungal species display distinct patters of affinity for their host *Protea* species (Roets *et al.* 2005, 2011b). For example, the closely related species *K. capensis* and *K. proteae* have overlapping geographic distributions and similar vectors, yet they have never been encountered together on the same *Protea* host (Wingfield *et al.* 1988, 1999, Wingfield & van Wyk 1993). *Knoxdaviesia proteae* consistently inhabits *P. repens* infructescences and it has not been found in other *Protea* species. In contrast, *K. capensis* occurs in at least eight different *Protea* species including *P. burchelli*, *P. coronata*, *P. laurifola*, *P. lepidocarpodendron*, *P. longifolia*, *P. magnifica*, *P. neriifolia* and *P. obtusifolia*, but has never been found in *P. repens* (Marais & Wingfield 1994, Roets *et al.* 2005, 2011a, Wingfield & van Wyk 1993).

The reason for the difference in host specificity between *K. capensis* and *K. proteae* is unknown. One possibility is that this separation prevents inter-specific competition between these fungi, given that they appear to rely on similar nutritional resources and occupy similar niches. Separation through host-exclusivity could, therefore, have been key to reduce competition and promote speciation (Giraud *et al.* 2008). Inter-species competition could also be avoided through temporal separation (succession) of colonization by ophiostomatoid species (Roets *et al.* 2013), although there is no evidence to support this view. The apparent host separation in the *Knoxdaviesia* species stands in contrast to some *Protea*-associated *Ophiostoma* species, which often co-occur with *K. capensis* or *K. proteae* in a single infructescence (Roets *et al.* 2006, 2013).

The host specificities of these *Knoxdaviesia* species are based on numerous randomly made collections of these fungi for taxonomic and biological studies. There has, however, never been a large-scale and systematic survey that would provide confidence in the hypothesis that *K. proteae* is the

Fig. 1. A. Infructescences (brown cones) of *Protea repens*. **B.** Single *Protea repens* flower. **C–D.** Sexual sporing structures of *Knoxdaviesia capensis* (**C**) and *K. proteae* (**D**). Bars C-D = 0.5 mm.

only *Knoxdaviesia* species occurring in *P. repens*. Isolations of *Knoxdaviesia*-like sporing structures were made from infructescences in two natural populations of *P. repens*. These were then used to test the hypothesis that *K. proteae* is the only *Knoxdaviesia* species that colonizes *P. repens* infructescences.

MATERIALS AND METHODS

During November 2012 and January 2013, infructescences were sampled from two *Protea repens* populations in the Western Cape Province of South Africa (Table 1) in order to isolate *K. proteae* individuals as part of a previous study (Aylward *et al*. 2014a, 2015). In the Gouritz population (34.2062°S 21.6812°E), 220 infructescences from the current and 220 from the previous flowering seasons were sampled from 11 different *P. repens* trees (Aylward *et al.* 2014a). The site at Franschhoek (33.9044°S 19.1566°E) had been burnt in 2007, and was sampled just after the new *P. repens* recruits had flowered for the first time. Some *P. repens* trees at this site (*ca* 15-yr-old) had escaped the fire and were included in our surveys. At this site, 20 infructescences were collected from 11 plots (20 m diam) in the burnt area and

Table 1. *Knoxdaviesia capensis* isolates obtained from *Protea repens.*

***Knoxdaviesia capensis* isolate**	**Sampling location**	**GenBank ITS accession**
G024	Gouritz	KP263518
G025	"	KP263519
G027	"	KP263520
G067	"	KP263521
G074	"	KP263522
G075	"	KP263523
G080	"	KP263524
G081	"	KP263525
G084	"	KP263526
G106	"	KP263527
F4.2	Franschhoek	KP263528
F6.3	"	KP263529
F9.4	"	KP263530
F11.6	"	KP263531
F12.3	"	KP263532
F14.1	"	KP263533
F16.1[a]	"	KP263534
F16.2[a]	"	KP263535
F16.7[a]	"	KP263536
F16.8[a]	"	KP263537
F16.9[a]	"	KP263538
F16.10[a]	"	KP263539
F19.10	"	KP263540
F27.2	"	KP263541
F31.2	"	KP263542
R7 (CBS 140644)[b]	"	KT970644

[a] Sampling plot F16 yielded *Knoxdaviesia capensis* isolates, exclusively.

[b] Representative isolate available from the CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands.

19 plots in the unburnt area (Aylward *et al*. 2015). Since the initial aim of the sampling was to collect *K. proteae*, only one *Knoxdaviesia* isolate was maintained per infructescence to prevent repeated isolation of the same individual. Because the sexual morphs of both *K proteae* and *K. capensis* species are indistinguishable under x30 magnification (Fig. 1), fungal isolations were made from randomly selected sporing structures. *Knoxdaviesia* isolation methods and culturing techniques were as given in Aylward *et al*. (2014b). Isolates were identified by sequencing the ITS regions of the rDNA (White *et al.* 1990) as detailed by Aylward *et al*. (2014b).

Statistical analyses were conducted in R v. 3.1.0 (R Core Team 2014). The number of fungal isolates obtained from infructescences at each sampling site (Gouritz or Franschhoek) and for each subdivision (flowering season or burnt/unburnt area) was recorded and tested for normality with Shapiro-Wilk's W test. Subsequently, a Mann-Whitney U test for independent groups and a Pearson's Chi-square test was applied to test for significant differences between the numbers of isolates obtained from each infructescence age class (Gouritz population) and between the burnt and unburnt sampling plots (Franschhoek). These tests were chosen because the Mann-Whitney U test takes into account only the number of positive hits (i.e. the presence of the fungus) whereas the Chi-square test also includes the total number of observations (i.e. number of infructescences sampled) (McKillup 2006).

A Maximum Likelihood (ML) phylogenetic tree was constructed in order to illustrate the difference between the species identified in this study. MAFFT 7 (Katoh & Standley 2013) was used to align the ITS sequences of a subset of the isolated individuals to those of previously characterized species of *Gondwanamycetaceae* obtained from GenBank®. The ML tree was computed in MEGA6 (Tamura *et al.* 2013) under the Tamura-Nei substitution model (Tamura & Nei 1993) and reliability was calculated with 1 000 bootstrap replications.

RESULTS

The intensive sampling effort yielded 224 *Knoxdaviesia* isolates – 103 from the Gouritz and 121 from the Franschhoek population. Surprisingly, the ITS data used to identify the isolates (Aylward *et al.* 2014b) revealed that not all fungal strains collected were *K. proteae*, the only *Knoxdaviesia* species previously known to occur in *P. repens* (Fig. 2). The

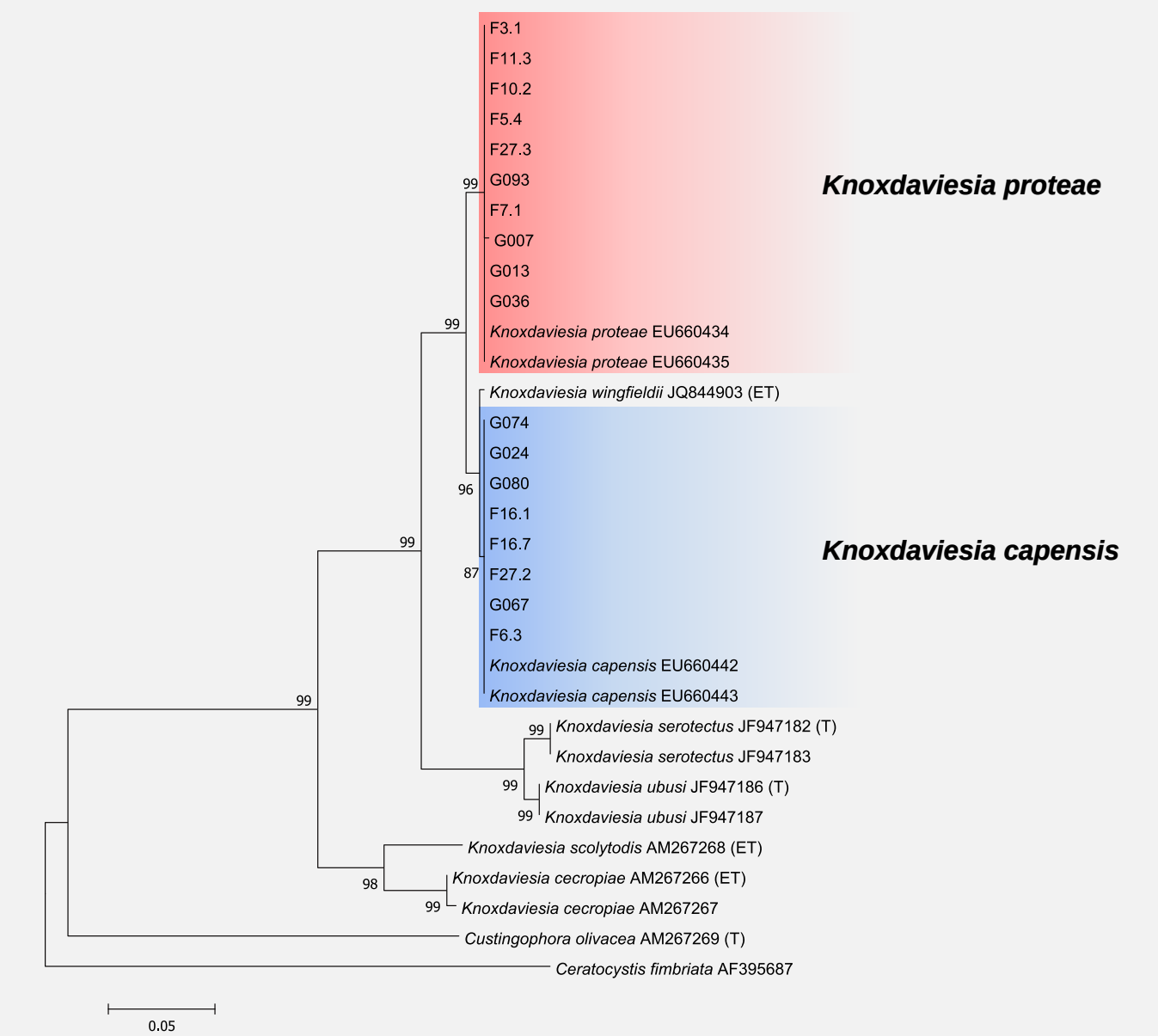

Fig. 2. Maximum Likelihood phylogenetic tree depicting the position of the two *Knoxdaviesia* species sampled from *Protea repens* infructescences. The final dataset consists of 474 characters. *Knoxdaviesia proteae* sequences are from the studies of Aylward *et al*. (2014a, 2015) and *K. capensis* sequences were generated in this study. "T" and "ET" indicate type and ex-type strains, respectively.

closely related *K. capensis* was also encountered, although at a low frequency. Ten *K. capensis* strains were isolated from four of the 11 different *P. repens* plants in the Gouritz population. In Franschhoek, 15 *K. capensis* isolates were found in 10 of the 30 sampling plots, including six from a single plot in which *K. proteae* was not encountered (Table 1). Isolate R7 (CBS 140644) was deposited at the CBS-KNAW Fungal Biodiversity Centre as a representative of *K. capensis* on *P. repens*. The sampling strategy did not enable co-occurrence of the two *Knoxdaviesia* species in one infructescence to be detected.

The Shapiro-Wilk's W tests for normality rejected the null hypothesis that the number of *K. capensis* isolates sampled from Gouritz ($W = 0.60$; $p = 1.49^{-6}$) and Franschhoek ($W = 0.46$; $p = 2.39^{-9}$) follows a normal distribution. Additionally, the combined dataset of *K. proteae* and *K. capensis* isolates in each population did not conform to a normal distribution (Gouritz: $W = 0.84$, $p = 3.15^{-5}$; Franschhoek: $W = 0.74$, $p = 5.78^{-9}$). Neither the Mann-Whitney U test for independent groups nor the Pearson's Chi-square test could detect significant differences between the number of *K. capensis* individuals isolated from the burnt and unburnt areas ($U = 93$, $p = 0.56$; $X^2 = 0.73$, $df = 1$, $p = 0.79$). The Pearson's Chi-square test suggested a marginally significant difference between the number of isolates in the current and previous flowering season's infructescences ($X^2 = 3.68$, $df = 1$, $p = 0.05$), but this was not supported by the Mann-Whitney U test ($U = 81.5$, $p = 0.09$). Both tests indicated that the total number of *K. capensis* isolates was significantly lower than the number of *K. proteae* isolates obtained from each population (Gouritz: $U = 455.5$, $p = 2.44^{-7}$, $X^2 = 75.75$, $df = 1$, $p = 2.2^{-16}$; Franschhoek: $U = 732.5$, $p = 1.02^{-5}$, $X^2 = 75.97$, $df = 1$, $p = 0.79$, 2.2^{-16}).

DISCUSSION

Knoxdaviesia capensis has been isolated from numerous serotinous *Protea* species in South Africa (Wingfield & Van Wyk 1993, Roets *et al.* 2005, 2011a). The geographic distributions of the known *Protea* hosts of *K. capensis* often overlap with that of *P. repens*, the host of *K. proteae* (Wingfield *et al.* 1988), yet this study presents the first account of *K. capensis* also occurring in *P. repens.* Given that *K. capensis* is a generalist that occupies numerous *Protea* species (Wingfield & van Wyk 1993, Marais & Wingfield 1994, Roets *et al.* 2005, 2011a), the ability to live in the infructescences of *P. repens* is perhaps not surprising.

The low frequency of *K. capensis* individuals isolated from *P. repens* (9.7 % in Gouritz and 12.4 % in Franschhoek) illustrates the dominance of *K. proteae* in this niche. It also offers an explanation for the previous oversight of *K. capensis* in *P. repens*. This low frequency is also congruent with the suggestion that *P. repens* is not a preferred host of *K. capensis*. *In vitro* host exclusivity experiments conducted by Roets *et al*. (2011a) showed that *K. capensis* produces significantly more aerial hyphae on 1.5 % Water Agar (WA) supplemented with *P. repens* material than on WA alone. However, these authors also found that when supplementing nutrient-rich 1.5 % Malt Extract Agar (MEA), *K. capensis* grew significantly better on its natural host, *P. neriifolia*, than on *P. repens*. Indeed, compared to MEA alone, *P. repens* supplemented media "slightly inhibited" the growth of *K. capensis*. These results suggest that although *K. capensis* is able to utilize *P. repens* as a substrate, it is not the preferred host of this species. However, the low level of occurrence of *K. capensis* in *P. repens* is unlikely to be due to inadequate nutrition, but more likely to be attributable to competition between *K. capensis* and other ophiostomatoid species, specifically the most prevalent species, *K. proteae*. Inter-species competition is known to occur between Northern Hemisphere ophiostomatoid fungi associated with the southern pine beetle, where *Ophiostoma minus* consistently out-competes *Ceratocystiopsis ranaculosus* (Klepzig & Wilkens 1997). Further investigation of the interactions between *Knoxdaviesia* species in *Protea* are, however, necessary to resolve this question.

An alternative explanation for the dominance of *K. proteae* over *K. capensis* in *P. repens* could be the succession of these fungi during initial colonization. The infructescences sampled from the burnt area in the Franschhoek population represent the first flowering season of those plants. Because of the absence of older infructescences, fungi in these new infructescences must have originated from sources outside the population of burnt *P. repens* trees. *Protea neriifolia* trees observed in the vicinity of the burnt area were most likely to be the source of the *K. capensis* colonizers. Where *K. capensis* spores from *P. neriifolia* reach new, uncolonized *P. repens* infructescences, this species is able to grow and sporulate. This is illustrated by our results from the Franschhoek sampling plot that exclusively yielded *K. capensis* (Table 1). However, once *K. proteae* is introduced, it apparently dominates *K. capensis* and reduces the prevalence of that species. However, *K. capensis* individuals were also isolated from mature *P. repens* plants in the unburnt area as well as from new and old infructescences in the Gouritz population. This implies that *K. capensis* can survive in a *P. repens* population even though *K. proteae* is dominant. Statistically, however, this study does not offer support for the premise of succession, since there was no difference in the number of *K. capensis* individuals isolated from infructescences of different ages (Gouritz) or burnt and unburnt areas (Franschhoek). However, the low numbers of *K. capensis* individuals found in this study, preclude us from completely disregarding the possibility that a succession of species could occur.

Roets *et al*. (2009b) hypothesized that the specificity of ophiostomatoid fungi to different *Protea* species may be more dependent on the vectors associated with the fungi than the specificity of the fungus to the *Protea* host. Results of recent studies (Roets *et al.* 2011a), including those of the present investigation, suggest that vectors are not a primary factor underlying specificity. *Knoxdaviesia capensis* is clearly capable of growing in *P. repens* infructescences and has the opportunity of being vectored to this suitable habitat. The apparent difference in prevalence of the two *Knoxdaviesia* species in *P. repens* must, therefore, be determined by other factors, the most plausible being inter-species competition. Future studies should consider the timing of colonization, and the interaction between and the potential effects that these *Knoxdaviesia* species may have on each other's growth.

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