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Phylogenetic analyses of *Podaxis* specimens from Southern Africa reveal hidden diversity and new insights into associations with termites

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ABSTRACT

Although frequently found on mounds of the grass-cutting termite genus *Trinervitermes*, virtually nothing is known about the natural history of the fungal genus *Podaxis* (*Agaricaceae*) nor why it associates with termite mounds. More than 40 species of this secotioid genus have been described since Linnaeus characterised the first species in 1771. However, taxonomic confusion arose when most of these species were reduced to synonymy with *Podaxis pistillaris* in 1933. Although a few more species have since been described, the vast majority of specimens worldwide are still treated as *P. pistillaris*. Using 45 fresh and herbarium specimens from Southern Africa, four from North America and one each from Ethiopia, and Kenya, we constructed the first comprehensive phylogeny of the genus. Four of the genotyped specimens were more than 100 y old. With the exception of the type specimen of *Podaxis rugospora*, all herbarium specimens were labelled as *P. pistillaris* or *Podaxis* sp. However, our data shows that the genus contains at least five well-supported clades with significant inter-clade differences in spore length, width and wall thickness, and fruiting body length, supporting that clades likely represent distinct *Podaxis* species. Certain clades consistently associate with termites while others appear entirely free-living.

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Introduction

Little is known about the biology of the secotioid fungal genus *Podaxis* (Family: *Agaricaceae*) or the nature of its apparent relationship with termites. Globally distributed between −40° and +40° latitude (Morse 1933), the genus has a long history of being reported in association with the grass-harvesting termite genera *Trinervitermes* (Family: *Termitidae*, subfamily: *Nasutitermitinae*) in Africa and *Nasutitermes* (Family: *Termitidae*, subfamily: *Nasutitermitinae*) in Australia (Massee 1890; Priest & Lenz 1999). Although it has been reported to benefit from growing in association with termites (Bottomley 1948; Herbert 1953), *Podaxis* has not been reported throughout the geographical range of the termite genera serving as its hosts, nor has it been reported as growing exclusively with termites; the association appears to only occur in dry and sandy savannah habitats where host-termite and *Podaxis* ranges overlap (Massee 1890; Bottomley 1948; Herbert 1953; Dring 1964; Alasoadura 1966; Zoberi 1972; Hilton & Kenneally 1981; De Villiers *et al.* 1989; Priest & Lenz 1999).

Linnaeus (1771) described the first species of the genus currently known as *Podaxis*, naming it *Lycoperdon pistillare*, from India, and later described a second species, *Lycoperdon carcinomale*, from the Western Cape, South Africa (Linnaeus 1781). Bosc (1792) described a third species (*Lycoperdon axatum*) from Senegal that was renamed *Podaxis senegalensis* (Desvaux 1809) and designated as the type species of the new genus *Podaxis*. Many more species were subsequently described. However, in the first monograph of the genus, Massee (1890) recognised only seven of these species and suggested several synonymies. Morse (1933), who focused predominantly on North American (mainly Californian) specimens, reduced the then thirty-two described species to synonymy as a single, polymorphic species: *Podaxis pistillaris* (Morse 1933; Bottomley 1948); including the type species of the genus, *P. senegalensis*, as synonym of *P. pistillaris*. Although criticised by some (Heim 1938, 1977), the classification was widely adopted throughout the remainder of the 20th and into the 21st century (Bottomley 1948; Doidge 1950). However, without considering all the older taxa, some authors during this period described new species that they distinguished from *P. pistillaris*.

Of the total of 44 *Podaxis* spp. described to date, 21 were described from Africa, and six original descriptions were of specimens collected from termite mounds (sometimes referred to as 'ant hills' in older literature). These included *Podaxis carcinomalis* (Linnaeus, 1781; Doidge 1950), *Podaxis africana* (De Villiers *et al.* 1989), and *Podaxis ghattasensis* (Hennings 1898) from Africa, *Podaxis termitophilus* from Madagascar (Jumelle and de La Bathie 1907), and *Podaxis beringamensis* from Australia (Priest & Lenz 1999). *P. pistillaris* has also been reported several times from termite mounds (Bottomley 1948; Doidge 1950; Herbert 1953; Alasoadura 1966; Heim 1977), although these collections may represent other *Podaxis* species.

Trinervitermes are distributed in savannah and grassland ecosystems throughout the paleotropics and their dome-shaped mounds are a common sight in Southern Africa. Closed to the exterior environment and only accessible for the termites through subterranean tunnels, the mounds maintain a relatively stable interior temperature and humidity throughout the day (Fig 1A–B) (Priest & Lenz 1999; Uys 2002; Brossard *et al.* 2007; Field & Duncan 2013). The centralised nature of termite colonies concentrates nutrients within and around the nest. In *Trinervitermes*, a large proportion of this nutrient deposition happens in faeces-lined grass-storage chambers (Sands 1970; Priest & Lenz 1999; Uys 2002; Brossard *et al.* 2007; Field & Duncan 2013). Complemented by the removal of soil from deep under ground, this should lead to increased levels of biodiversity in association with termite mounds compared to the surrounding savannah (Brossard *et al.* 2007; Moe *et al.* 2009; Sileshi *et al.* 2010; Bonachela *et al.* 2015). However, aside from *Podaxis*, no other fungi or plants have, to our knowledge, been reported to grow from the mounds of *Nasutitermitinae* (Lee & Wood 1971).

Podaxis fruiting bodies, occurring on *Trinervitermes* mounds, originate from the grass-storage chambers (Fig 1C–D) where white mycelium is visible on the faeces-lined walls (Sands 1970; Priest & Lenz 1999; B.H.C., pers. obs.). It is unknown whether the termites feed on the fungus but it is also possible that *Podaxis* uses the favourable conditions and concentrated nutrients within the nest to grow as a commensal without affecting the termites, or as a parasite growing inside the nest to the detriment of the colony.

While there have been challenges to the reduction of *Podaxis* to a single species (Morse 1933); these have primarily been based on differences in basidiospore morphology (Heim 1977; McKnight & Stransky 1980; De Villiers *et al.* 1989; Priest & Lenz 1999) and no comprehensive studies have explored the phylogenetic diversity of African *Podaxis* species. We sought to test the reduction of *Podaxis* to a single species through phylogenetic analyses; finding evidence pointing towards multiple *Podaxis* species. We therefore also tested if morphological differences in spores support our phylogenetic inferences. Furthermore, we tested whether fruiting body size among *Podaxis* phylogenetic clades is related to the association with termites, or rather a species-specific trait.

Methods

Material

Nine fresh *Podaxis* fruiting bodies were sampled from seven termite mounds in South Africa from January–February 2015. Core samples from the centre of the stipe, where the tissue is considered to be sterile, of fresh fruiting bodies were stored in RNAlater® (Ambion, USA) while spores were inoculated onto growth medium (see Isolation and culturing). In addition, we obtained spore samples and took photographs of 38 Southern African *Podaxis* herbarium specimens from The South African National Collection of Fungi (PREM) at the Agricultural Research Council at Roodeplaat, Pretoria, and six specimens from The Natural History Museum of Denmark (SNM), Copenhagen (Table 1). Photographs of the herbarium specimens were taken alongside a scale (Supplementary

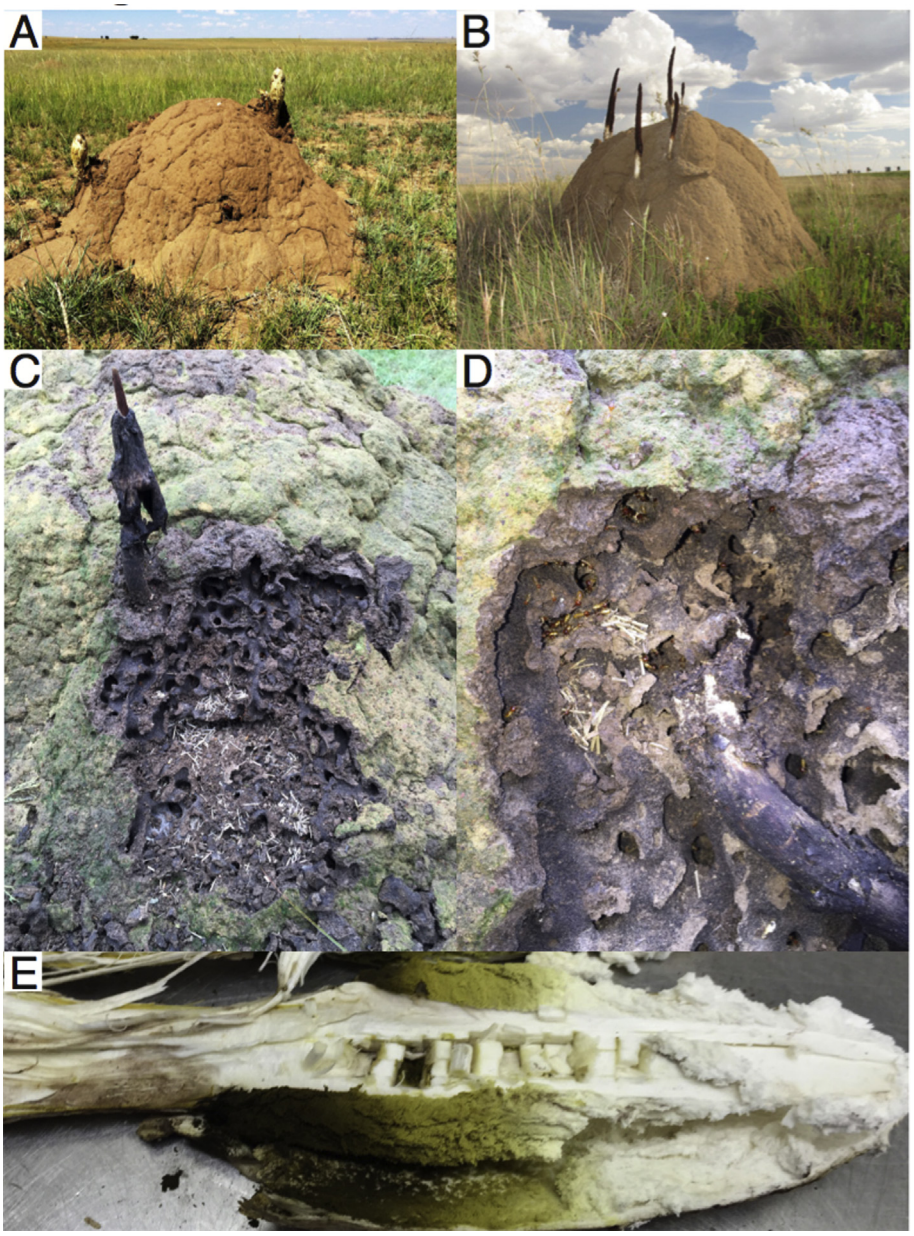

Fig 1 – (A: ZWdB) Immature *Podaxis* fruiting bodies growing on a termite mound in the central Free State province, South Africa. The specimen belongs to Clade D. (B: ZWdB) Mature *Podaxis* fruiting bodies in South Africa in the southern Free State province. (C: BHC) Mature *Podaxis* stalk growing from the grass-storage chamber of an opened *Trinervitermes* mound on the UP experimental farm, Pretoria. (D: ZWdB) Base of *Podaxis* stalk in Fig 1C growing from the grass-storage chamber of *Trinervitermes*. (E: BHC) Maturing fruiting body of *Podaxis* collected in Maropeng, Gauteng province. Immature spores at the tip are white, changing colour to green, and finally dark brown to black when mature. The specimen belongs to Clade D.

material) before their size as the height of the fruiting body measured from the lowest point before the soil to the tip was determined in Photoshop CC 2014 (Adobe Systems, USA).

Isolation and culturing

Our initial attempts to culture *Podaxis* involved placing mature spores and sections from the centre of both mature and immature fruiting bodies onto YMEA (4 g yeast extract, 10 g malt extract, 4 g dextrose, and 20 g agar per litre), Molisch (20 g glucose, 10 g peptone, 0.25 g magnesium sulphate ($MgSO_4$), 0.25 g potassium hydrogen phosphate (K_2HPO_4), and 15 g agar per litre), and Sabouraud (40 g dextrose, 10 g peptone, and 20 g agar per litre) media. Success in these attempts was limited as plates inoculated with material from the centres of the fruiting bodies often did not grow or were contaminated. In an attempt to resolve this problem, we sampled soil and grass from a *Trinervitermes* mound (University of Pretoria

Table 1 – *Podaxis* specimens obtained from South Africa (BHC), The South African National Collection of Fungi (PREM), Pretoria, and The Natural History Museum of Denmark (SNM), Copenhagen, their geographic origins, substrates, whether they were reported with termites and size. All specimens were collected in South Africa unless otherwise specified while 'n/a' in the 'Size (cm)' column indicates the fruiting body was not intact and could not be measured.

Sample id	Collection year	Collection site	Province	Location notes	Size (cm)	GenBank accession number	
						nrLSU	ITS
PREM 1689	1911	Garsfontein, Pretoria	Gauteng	With termites	15.82	KT844822	KT844861
PREM 2119	1912	Skinners Court, Pretoria	Gauteng	With termites	22.73	KT844825	
PREM 5125	1912	Hennops River, Pretoria district	Gauteng		n/a	KT844845	KT844876
PREM 7362	1913	Dundee	KwaZulu-Natal	With termites	n/a	KT844849	
PREM 9789	1916	Sand River Drift, Messina	Limpopo	Open sandy soil	13.9	KT844850	KT844880
PREM 14484	1921	Hammanskraal	Gauteng	On ground	11.48		
PREM 14507	1921	Malcomess Knapdaar	Eastern Cape	With termites (ant heap)	15.4	KT844821	KT844860
PREM 14682	1921	Unknown			21.8		
PREM 18109	1924	Malcomess Knapdaar	Eastern Cape	With termites (ant heap)	13.1	KT844823	
PREM 20585	1925	Near Anenous pass, Namaqualand	Northern Cape	Sandy soil	17.15	KT844824	KT844862
PREM 23672	1929	Pretoria	Gauteng	With termites	10.68	KT844826	
PREM 26602	1932	Johannesburg Road, Pretoria	Gauteng	With termites (ant heap)	12.68		
PREM 27280	1933	Alongside road to Swing Bridge, Pretoria	Gauteng	With termites	10.04	KT844827	KT844863
PREM 28254	1934	Welgevonden, North Brits	Northwest		17.17		KT844864
PREM 28641	1936	Between Windsorton & Klipdam, Barkly West	Northern Cape		12.74	KT844828	KT844865
PREM 28810	1936	Escourt	KwaZulu-Natal	With termites	n/a	KT844829	KT844866
PREM 29955	1938	Escourt	KwaZulu-Natal	With termites (ant heap)	15.38	KT844830	
PREM 30714	1939	Exp. Station, Escourt	KwaZulu-Natal	With termites (ant heap)	12.69		
PREM 36117	Unknown	East London	Eastern Cape		20.55		
PREM 44075	1942	Potchefstroom Rd, Johannesburg	Gauteng	With termites	24.58	KT844836	
PREM 34405	1943	Potchefstroom	Northwest	With termites	11.77	KT844831	KT844877
PREM 41625	1956	Anabib, Kaokoveld	Namibia	Hard stony ground	12.52	KT844832	KT844867
PREM 42236	1962	10 Miles North of Lydenburg	Mpumalanga	With termites	6.34	KT844833	KT844868
PREM 43118	1965	Near the siding Mopane, 53 miles South of Messina	Limpopo		13.81	KT844834	KT844869
PREM 43879	1966	20 Miles West of Thabazimbi, Rustenburg district	Northwest	Gravel road	16.06	KT844835	KT844870
PREM 44240	1968	Winterton	KwaZulu-Natal		24.21	KT844837	
PREM 44293	1969	Half mile S of Kuiseb River, Soutrivier, Namib Desert Park	Namibia	Dune	12.44	KT844838	KT844871
PREM 44294	1969	10 Mile South of Gobabeb, Namib Desert Park	Namibia		13.17	KT844839	KT844872
PREM 44664	1971	Swartkop, Pretoria	Gauteng	With termites	15.8	KT844840	KT844873
PREM 44953	1974	H. Malan Nature Reserve, close to Springbok	Northern Cape	On ground	17.23	KT844841	KT844874
PREM 47477	1984	Groblershoop	Northern Cape	Dune	11.95	KT844842	KT844875
PREM 47484	1984	Okahandja & Gross Barmen	Namibia		21.94	KT844843	
PREM 49048	1988	Erasmusrand, Pretoria	Gauteng	With termites	28.72	KT844844	

BHC – Maropeng 2	2015	Pretoria	Gauteng	With termites	KT844817	
BHC – Maropeng 3	2015	Pretoria	Gauteng	With termites	KT844818	
BHC – Pilanesberg	2015	Pilanesberg National Park (25°25′S 27°17′E)	Northwest	With termites		
BHC – *Podaxis* 1	2015			With termites	KT844820	KT844859
SNM – India: C-F-101402		India				
SNM – California, USA: C-F-101398		California, USA			KT844813	KT844854
SNM – Arizona, USA: C-F-101399		Arizona, USA			KT844811	KT844852
SNM – Mexico: C-F-101400		Mexico			KT844819	KT844858
SNM – Kenya: C-F-101401		Kenya			KT844815	KT844856
SNM – Ethiopia: C-F-92630		Ethiopia			KT844814	KT844855

Experimental Farm, Pretoria; 25°45′S 28°15′E), where we had collected *Podaxis* and used this to make two media: one with soil, grass, and agar (75 g soil and grass and 20 g agar per litre) and one with soil, grass, agar, and yeast (75 g soil and grass, 4 g yeast, and 20 g agar per litre). These media reduced the risk of contamination by opportunistic fungi. The plates of *Trinervitermes*-mound–soil media were inoculated with spores from samples of fresh and herbarium *Podaxis* specimens.

DNA extractions and PCR

Using spores collected from herbarium specimens or sections taken from the centre of the stipe of fresh sporocarps, DNA extractions were performed using a CTAB protocol (Cafaro *et al.* 2011). We chose to use the spores of the herbarium specimens because, due to their adaptations to remain viable in the environment for long periods of time, they seemed to be the most likely part of the fungus to provide high-enough quality DNA. The resulting extracts were assessed spectrophotometrically using NanoDrop ND-1000 (Thermo Scientific, Germany). Based on Schoch *et al.* (2012), we chose to amplify the ITS and nrLSU regions for barcoding our specimens.

The nrLSU gene was amplified using the primers LR0R (5′-ACCCGCTGAACTTAAGC-3′) and LR5 (5′-TCCTGAGGGAAACTTCG-3′) (White *et al.* 1990). PCR reactions were prepared in 25 µl volumes comprising 8.5 µl sterile distilled water, 1 µl of each primer, 2 µl of template, and 12.5 µl of VWR Red Taq DNA Polymerase Master Mix (VWR International, USA). The conditions for PCR were 94 °C for 4 min followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s with a final extension step at 72 °C for 4 min. Target PCR products were visualised by agarose gel electrophoresis and purified using MSB Spin PCRapace (STRATEC Molecular, Germany). The samples were sequenced at Eurofins MWG Operon (Ebersberg, Germany).

For the ITS sequencing, we used either ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) or ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) as forward primers, since it was not possible to amplify samples from all clades using a single primer set, together with ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) as the reverse primer (White *et al.* 1990). PCR reactions were prepared and run with the same conditions as for nrLSU. Target PCR products were visualised with agarose gel electrophoresis, purified using MSB Spin PCRapace (STRATEC Molecular, Germany), and sequenced at Eurofins MWG Operon (Ebersberg, Germany).

Phylogenetic analyses

Both nrLSU and ITS sequences were subject to BLAST searches with the closest matches included as outgroups in the phylogenetic analyses. These included species of *Macrolepiota*, *Lepiota*, *Leucoagaricus*, and *Leucocoprinus*, which have previously been reported as closely related genera to *Podaxis* (Hopple & Vilgalys 1999; Vellinga *et al.* 2011). The sequences were aligned and concatenated in Geneious 4.8.5 using the MUSCLE algorithm (Edgar 2004). Alignments were inspected and any ambiguities were resolved either by revisiting the original sequence or by resequencing. Bayesian phylogenetic analyses were then performed (Model, HKY; Runs, 2; Burn in rate, 50 %;

Generations, 1 500 000 – concatenated phylogeny/1 250 000 – ITS phylogeny) using TOPALi v2 (Milne *et al.* 2008). We used 0.99 posterior probabilities as the threshold for well-resolved branches. For the ITS phylogeny, we included all ITS sequences of *Podaxis* in GenBank longer than 454 bp, for which all had a herbarium accession number except the specimen from Sinai (GenBank: HE863812). Since no other *Podaxis* specimens than ours had both ITS and nrLSU sequences in GenBank, we were unable to include these in the concatenated analysis.

Spore morphologies

To test whether our phylogenetic inferences of multiple *Podaxis* species were supported morphologically, we selected three specimens from each clade in the ITS phylogeny and measured spore length, spore width, and the thickness of the spore wall for 25 spores per specimen using a Zeiss Axioskop Imager A.2 microscope (Carl Zeiss AG, Germany) with a Nikon DS-Ri2 camera (Nikon Corporation, Japan). We analysed the data using one-way ANOVAs with a paired Tukey test in R (R Development Core Team 2015) and compared the length, width, wall thickness, and the ratio of length to width between specimens assigned to different phylogenetic clades.

Sporocarp size analysis

To test whether fruiting body size differed between phylogenetic clades in association with termites, we used the size data obtained when photographing the herbarium specimens (Material). For samples containing more than one fruiting body (Supplementary material), only the largest fruiting body was analysed. Samples were excluded if the base of the stipe where it emerges from the termite mound or soil surface

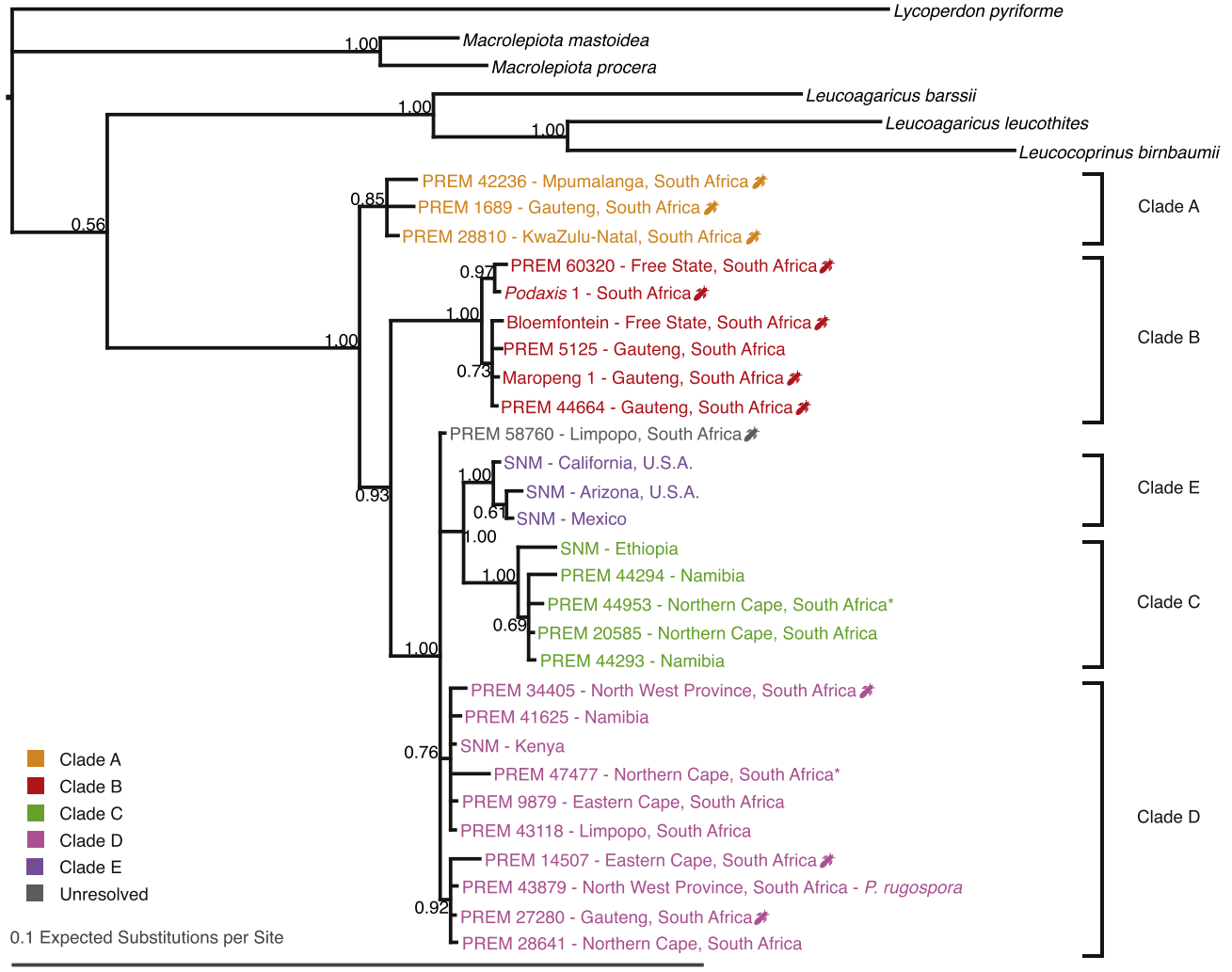

Fig 2 – Phylogenetic placement of *Podaxis* and closely related fungi in a concatenated ITS and nrLSU Bayesian phylogeny with posterior probabilities given at nodes. Herbarium specimens are labelled with their PREM identifiers or SNM followed by their country of origin. Fresh specimens collected during this study are prefixed with BHC followed by their collection location. Colours were designated to specimens based on the clades in this tree. An asterisk indicates that the specimen was successfully cultured while a termite indicates it was reported from termite mounds when added to the collection.

could not be unambiguously determined. We then used a Wilcoxon rank-sum test to test whether *Podaxis* grows taller in association with termites and a one-way ANOVA with a paired Tukey test to see if there is a significant size difference between clades. All statistical analyses were preformed in R (R Development Core Team 2015).

Data deposition

ITS and nrLSU sequences were submitted to GenBank (accession numbers in Table 1) and alignments to treeBASE accession number: http://purl.org/phylo/treebase/phylows/study/TB2:S18314. DNA from the 39 specimens obtained from The South African National Collection of Fungi have been deposited with the herbarium, and *Podaxis* cultures from specimens PREM 44240, PREM 44953, PREM 47477, and PREM 57485 are deposited at The Natural History Museum of Denmark as C-F-101404 (PREM 44240), C-F-101405 (PREM 44953), C-F-101407 (PREM 47477), and C-F-101406 (PREM 57485).

Results

Specimens

In sampling at Maropeng, South Africa, we collected one maturing fruiting body, which revealed that the immature spores are white before changing to green and then black as they mature (Fig 1E). We successfully extracted high-enough quantity and quality DNA for PCR and phylogenetic analyses from 42 of the collection of specimens, including four herbarium specimens that were more than 100 y old (Table 1).

Isolations

Using the termite–soil medium, we successfully isolated cultures from four specimens of *Podaxis* from the herbarium samples that had been collected in 1968, 1974, 1984, and 2002. Once purified, these specimens were able to grow on YMEA but still remained highly susceptible to opportunistic contaminants. The identities of these isolates as *Podaxis* and the clades to which they belong (Clades B, C, and D and one unresolved) were confirmed by sequencing of the nrLSU gene and phylogenetic analyses.

Phylogenetic analyses

Phylogenetic analysis of both nrLSU and ITS sequences (Fig 2) revealed five well-resolved taxa (A, B, C, D, and E). All specimens in Clades A and B were reported from termite mounds, with one exception (PREM 5125) in Clade B. Given that PREM 5125 was collected in 1912, any association with termites may have been overlooked or not reported when it was deposited in the herbarium. Clade C did not contain any members reported from termite mounds.

The most basal split within *Podaxis* was between Clade A and all remaining groups. Clade A and the next basal clade of its sister group, Clade B, appear to be more frequently found on termite mounds than the other clades. Some, but not all, specimens from Clade D were identified with termites while no specimens from Clades C and E were reported in association with termites.

Adding available sequence data from GenBank to our data to produce a single-gene ITS phylogeny (Fig 3), four of the clades from Fig 2 (A, B, C, and E) remain well supported, but Clade D is split with strong support (0.98). Based on the ITS phylogeny we identified and named an additional clade 'Clade F' and used it as a group in our analysis of spore morphologies and sporocarp size. Based on the ITS phylogeny, it appears as though Clades C and E exhibit an Old World–New World split, with GenBank specimens from India and Egypt (Fig 3) being more closely related to Clade C than Clade E. Using the ITS phylogeny, we calculated the proportion of each clade that came from the nine South African provinces and Namibia. The one Southern African clade without any reported association with termites (Clade C) was only collected in the western parts of the Northern Cape and Namibia; more specifically, within the Namib Desert.

Spore morphologies

We found that spore length ($F_4 = 28.7$; $p < 0.001$; Fig 4A), spore width ($F_4 = 119$; $p < 0.001$; Fig 4B), and spore wall thickness ($F_4 = 6.83$; $p < 0.001$; Fig 4C) all were significantly different between clades in support of our phylogenetic placements from the ITS phylogeny (Fig 3). The longest and widest spores with the thickest walls were found in the free-living Clade C, while the shortest and narrowest spores with the thinnest walls were found in the termite-associated Clade A (Fig 5). There were also overall ($F_4 = 23.4$; $p < 0.001$) and in some cases pairwise (adjusted p-values for B–A: 0.981; C–A <0.001; D–A <0.001; F–A <0.001; C–B <0.001; D–B <0.001; F–B <0.001; D–C 0.178; F–C 0.011; F–D 0.806) differences in the ratio of spore length to width.

Variation in fruiting body size

There was no significant difference in fruiting body length between specimens reported on termite mounds and those that were not (Fig 6A; $W = 137$; $p = 0.752$). We also tested for differences in length between specimens from the six different clades in our ITS phylogeny (Clades A–F in Fig 3). These results confirmed overall (one-way ANOVA: $F_5 = 2.95$; $p = 0.039$) and pairwise statistically significant differences in fruiting body sizes (Fig 6B) between the different clades, further supporting our classifications.

Discussion

We tested the reduction of *Podaxis* to synonymy as a single species (Morse 1933) by generating a molecular phylogeny of available members of the genus using nrLSU and ITS sequences, analysing spore morphologies and sporocarp size. While several previous studies have suggested the reduction is incorrect (McKnight & Stransky 1980; De Villiers *et al.* 1989; Moreno & Mornand 1997; Priest & Lenz 1999), this is the first comprehensive study to explore the phylogenetic and morphological diversity of primarily Southern African *Podaxis* specimens. Aside from one herbarium specimen (PREM

43879: the type specimen for *Podaxis rugospora*; De Villiers *et al.* 1989), all included specimens were labelled *Podaxis pistillaris* or *Podaxis* sp., although some had previously had their names changed from *Podaxon carcinomalis* to *P. pistillaris*. Despite this, our concatenated phylogeny using the conserved nrLSU gene and the more variable ITS region (Fig 2) showed five well-supported clades (A, B, C, D, and E).

The most basal split in the phylogenies was between Clade A and all other taxa. Clade A and the next most basal clade (B) are the clades most commonly found on termite mounds. Clade D contains the type specimen for *P. rugospora* (PREM 43879), suggesting that all members of this clade belong to that species. The phylogeny with only the more variable ITS region (Fig 3) enabled the inclusion of additional *Podaxis* sequences from GenBank. This phylogeny broadly supported the clades in Fig 2, but there was strong support (98 %) for an additional clade (F) within Clade D. In support of the phylogenetic placement of specimens, we found significant morphological differences between the spores of each clade containing Southern African specimens. The longest and widest spores with the thickest walls were found in Clade C, while the shortest and narrowest spores were found in Clade A. In addition, we found significant differences in spore morphology between specimens from Clades D and E, supporting the classification of these clades as separate lineages. The lack of data from type specimens other than *Podaxis rugospora* prevented the naming of all but one clade, but given the similarly high support for the other clades, it seems unlikely that they all represent *P. pistillaris*. Many of the specimens from GenBank were labelled as *P. pistillaris*, but the naming was typically from their closest matches during BLAST searches against the nr database rather than morphological data (Singh *et al.* 2006). With the level of diversity we found in specimens morphologically identified as *P. pistillaris*, it is therefore questionable whether any of the sequences from GenBank truly represent *P. pistillaris*.

Clades A, B, D, and F contain members reported to be associated with termites, while none of the specimens in Clades C and E were reported from termite mounds. This supports previous suggestions that certain *Podaxis* associate with termites, possibly in symbiosis, while others are exclusively free-living (Bottomley 1948; Herbert 1953). The presence of different lifestyles within a single formally recognised species also supports the presence of several cryptic species within *P.*

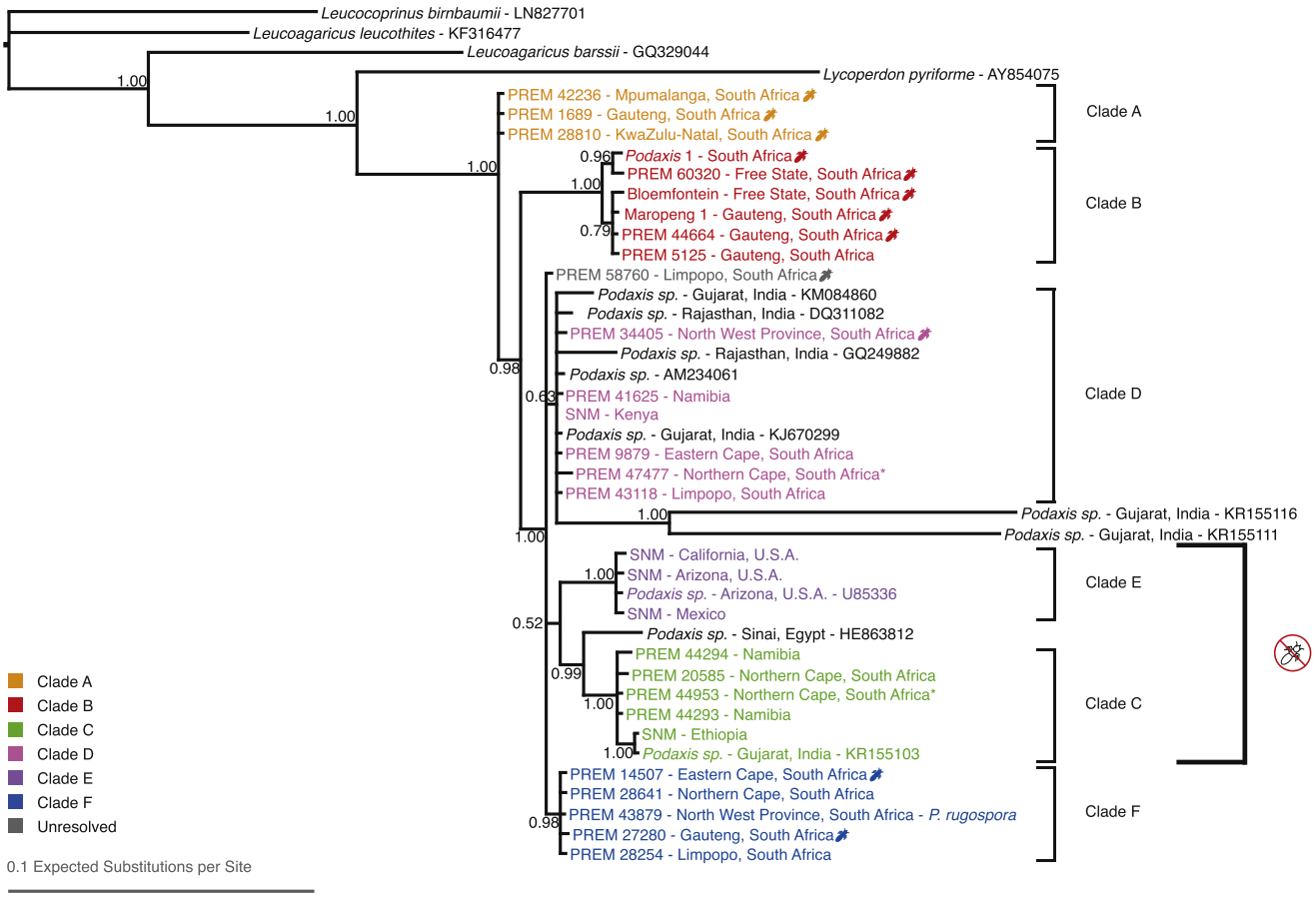

Fig 3 – Phylogenetic placement of 30 *Podaxis* specimens alongside all available GenBank ITS sequences for *Podaxis* and closely related fungi, in an ITS Bayesian phylogeny with posterior probabilities given at the nodes. Herbarium specimens are labelled with their PREM identifiers or SNM followed by their country of origin. Fresh specimens collected during this study are prefixed with BHC followed by their collection location. With the addition of Clade F, the colours show the clade to which a specimen belongs in Fig 2. An asterisk indicates that the specimen was successfully cultured while a termite indicates it was reported from termite mounds when added to the collection.

pistillaris. It has previously been reported that *Podaxis* spp. have only been found within a narrow part of the range of its termite host (Alasoadura 1966; Priest & Lenz 1999). While there are five known species of *Trinervitermes* in Southern Africa (Uys 2002) we were unable to confirm which species the different specimens were from, because host termites were not collected. Consequently, we can at present not determine whether there are host specificities in the termite-*Podaxis* association. Through analysis of fruiting bodies in the herbarium collection, we tested the assertion that *Podaxis* grows taller in association with termites than in isolation (Bottomley 1948; Herbert 1953). While there was no significant overall difference in height between basidiocarps reported in association with termites to those that were not (Fig 6A), termite-associated specimens tended to be larger. Combined with the identification of significant differences in height between clades, this suggests that fruiting body height is likely to vary between *Podaxis* clades and is not directly linked to whether or not a clade is termite associated.

Analysis of the geographical distribution of the Southern African specimens showed that, although all specimens of Clades A, B, D, and F were from semi-arid grassland environments (Massee 1890; Bottomley 1948; Herbert 1953; Dring 1964; Alasoadura 1966; Zoberi 1972; Hilton & Kenneally 1981; De Villiers *et al.* 1989; Priest & Lenz 1999), all Clade C specimens were from the Namib Desert. Clade C is also the only Southern African clade with no specimens reported in association with termites, consistent with the rare presence of *Trinervitermes* in the Namib Desert (Uys 2002). The specimens most closely related to Clade C (Clade E) are also from locations containing desert: Gujarat (India), Sinai (Egypt), Ethiopia, Mexico, California (USA), and two specimens from Arizona (USA). However, as these sequences came from GenBank and other herbaria, we only have precise location data for the specimen from California, which was collected in the Colorado Desert (Table 1). The apparent restriction of Clades C and E to desert areas suggests that some clades of *Podaxis* are adapted to desert living and to not associate with termites. While we do not have specimens for spore morphology measurements from Clade E, the analyses of spores of Clade C showed that they were significantly larger and with thicker walls (Fig 4A–C) than termite-associated clades, suggesting adaptations to particularly harsh conditions. The macromorphology of these specimens was also different from those of other clades, with a much thinner stipe making the cap appear more bulbous (Supplementary material). Similarly shaped specimens of *Podaxis* have been reported in the deserts of Morocco and Iraq (Moreno & Mornand 1997; Muhsin *et al.* 2012) with the Moroccan specimens being classified as *Podaxis saharianus*. Interestingly, specimens from Clades C and E separate into Old World and New World clades, suggesting a biogeographic split in the phylogeny, which should be further explored.

Although termite mounds contain large concentrations of nutrients compared to the surrounding areas (Brossard *et al.* 2007; Moe, *et al.* 2009; Sileshi *et al.* 2010; Bonachela *et al.* 2015), termites have evolved defences to protect these resources from opportunists that may take advantage of this (Mugerwa 2015). Considering how *Podaxis* breaks through the walls of the mound (Fig 1A; Field & Duncan 2013), reducing mound strength, and exposing the interior to pathogens and predators, it is conceivable that *Trinervitermes* will try to resist the growth of *Podaxis* fruiting bodies. The defences can be physical, such as the nest wall providing an impregnable

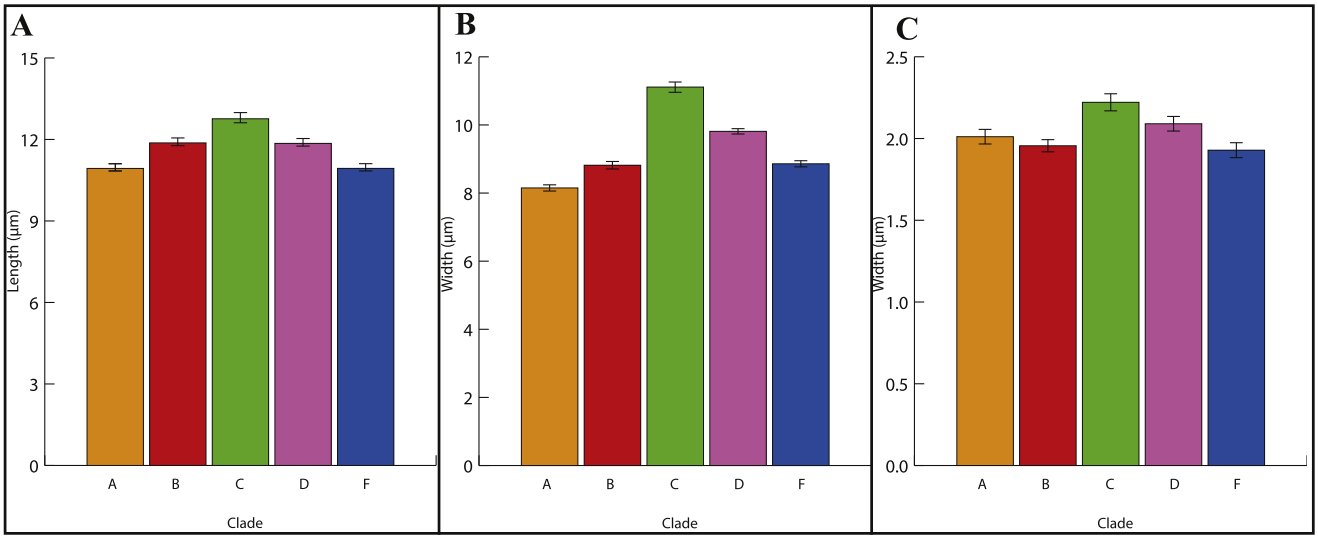

Fig 4 – (A) Mean ± SE spore length from each of the five clades containing Southern African *Podaxis* specimens. Results of the pairwise Tukey test: B–A, $p < 0.001$; C–A, $p < 0.001$; D–A, $p < 0.001$; F–A, $p = 1.00$; C–B, $p < 0.001$; D–B, $p = 1.00$; F–B, $p < 0.001$; D–C, $p < 0.001$; F–C, $p < 0.001$; F–D, $p < 0.001$. (B) Mean ± SE spore width from each of the five clades containing Southern African *Podaxis* specimens. Results of the pairwise Tukey test: B–A, $p < 0.001$; C–A, $p < 0.001$; D–A, $p < 0.001$; F–A, $p < 0.001$; C–B, $p < 0.001$; D–B, $p < 0.001$; F–B, $p = 0.999$; D–C, $p < 0.001$; F–C, $p < 0.001$; F–D, $p < 0.001$. (C) Mean ± SE spore wall thickness from each of the five clades containing Southern African *Podaxis* specimens. Results of the pairwise Tukey test: B–A, $p = 0.908$; C–A, $p = 0.010$; D–A, $p = 0.718$; F–A, $p = 0.698$; C–B, $p < 0.001$; D–B, $p = 0.204$; F–B, $p = 0.993$; D–C, $p = 0.231$; F–C, $p < 0.001$; EFD, $p = 0.010$.

barrier to germination and growth, but this seems to be less the case in *Trinervitermes* than other South African species of termites (B.H.C., pers. obs.), or they can be chemical, with toxins added to the walls of the nest to prevent the growth of vegetation (Lee & Wood 1971; Mugerwa 2015). The latter appears more likely given the diverse array of chemical defences reported in the *Nasutitermitinae* and the lack of plant growth on their mounds compared to other termite subfamilies (Lee & Wood 1971; Šobotnik *et al.* 2010).

It is intriguing to speculate that dissociation from a potential symbiosis with savannah-dwelling termites was selected for in favour of a shift to a free-living lifestyle in extreme

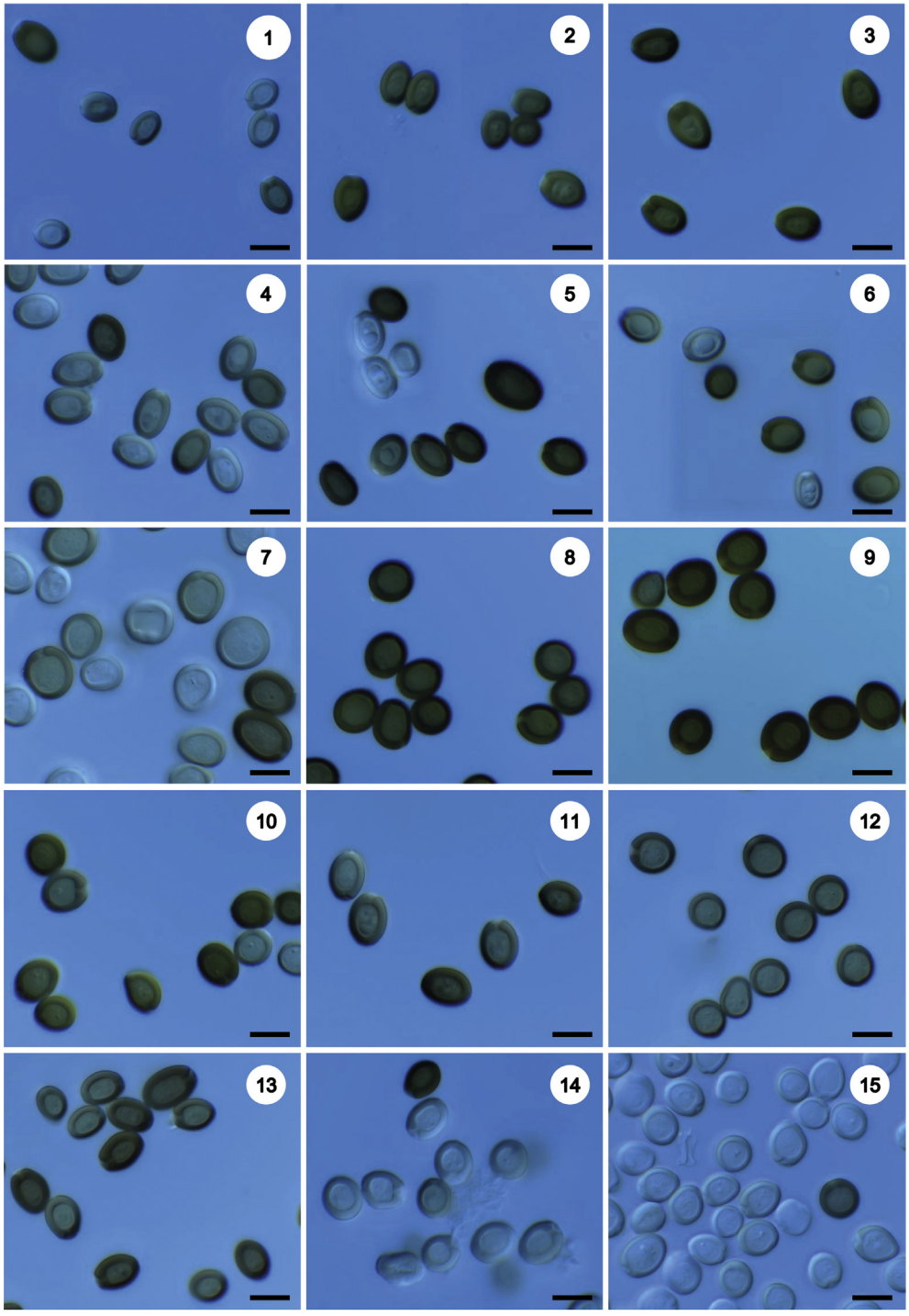

Fig 5 – Basidiospores obtained from Southern African herbarium specimens of *Podaxis*, arranged based on phylogenetic clades from Fig 3. Clade A: (1) PREM 1689, (2) PREM 28810, (3) PREM 42236. Clade B: (4) PREM 5125, (5) PREM 44664, (6) PREM 60320. Clade C: (7) PREM 20585, (8) PREM 44953, (9) PREM 44293. Clade D: (10) PREM 9789, (11) PREM 34405, (12) PREM 41625. Clade F: (13) PREM 14507, (14) PREM 27280, (15) PREM 43879. Scale bar = 10 μm.

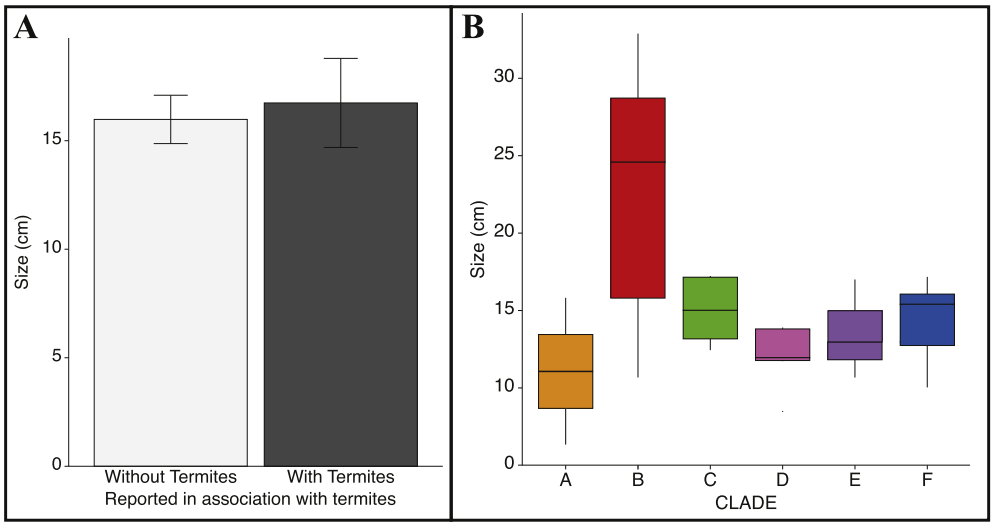

Fig 6 – (A) Mean ± SE fruiting body length of Southern African *Podaxis* specimens reportedly growing free-living (light grey; $n = 12$) *versus* those growing on termite mounds (dark grey; $n = 14$). (B) Boxplot showing median and interquartile range ± 1.5× interquartile range for fruiting body length of *Podaxis* specimens from each of the six distinct phylogenetic clades in Fig 2; Clade A, $n = 2$; Clade B, $n = 5$; Clade C, $n = 5$; Clade D, $n = 5$; Clade E, $n = 3$; and Clade F, $n = 5$. Results of the pairwise Tukey test: B–A, $p = 0.112$; C–A, $p = 0.931$; D–A, $p = 1.00$; F–A, $p = 0.970$; E–A, $p = 0.994$; C–B, $p = 0.210$; D–B, $p = 0.034$; F–B, $p = 0.142$; E–B, $p = 0.183$; D–C, $p = 0.926$; F–C, $p = 1.00$; E–C, $p = 0.998$; F–D, $p = 0.976$; E–D, $p = 0.998$; E–F, $p = 1.00$.

desert environments in more derived *Podaxis* clades. While most clades consist predominantly of specimens reported from termite mounds, it is possible that, through suppression of *Podaxis* from their mounds, *Trinervitermes* provides the selective pressure necessary for certain *Podaxis* to lose this association and switch to a free-living, desert lifestyle. The narrow stipe and bulbous cap of specimens in Clade C and those reported in Morocco and Iraq (Moreno & Mornand 1997; Muhsin *et al.* 2012) suggest that this is associated with adaptations to maximise reproductive success and minimise the cost of growth in a nutrient-poor environment. The large spores seen in Clade C could also be an adaptation to this lifestyle, as the fungus would need more resources to establish in this environment compared to the nutrient-rich termite mound in savannah areas and the thick walls would help to prevent dessication. Alternatively, the thick stipe seen on termite-associated *Podaxis* may represent an adaptation to provide added support when the fruiting body pushes itself through the wall of the mound and the high nutrient concentration in the termite mound allows the fungus to reduce the energy investment in each spore in favour of increased quantities. With the mounds of *Trinervitermes* being sealed to the exterior environment (Uys 2002), this could help to improve the chance that some of the spores find their way into another nest.

Our results show that the genus *Podaxis* is more diverse than previously thought and reveal that several clades appear to consistently interact with termites. While we are still unable to describe the nature of this interaction, our findings show that not all *Podaxis* clades associate with termite mounds. In particular, the desert-living Clades C and E form distinct monophyletic groups in which no specimens have been reported from termites and, for Clade C, with larger spores than the termite-associated clades. Our successful extraction, amplification, and sequencing of DNA from the spores of herbarium specimens over 100 y old also shows the potential that this diverse and accessible resource has for future studies.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funbio.2016.05.011.

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