Bark and ambrosia beetles (*Curculionidae: Scolytinae*), their phoretic mites (*Acari*) and associated *Geosmithia* species (*Ascomycota: Hypocreales*) from *Virgilia* trees in South Africa

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Running Title: *Geosmithia* and its associates from *Virgilia*

ABSTRACT

Bark and ambrosia beetles are ecologically and economically important phloeophagous insects that often have complex symbiotic relationships with fungi and mites. These systems are greatly understudied in Africa. In the present study we identified bark and ambrosia beetles, their phoretic mites and their main fungal associates from native *Virgilia* trees in the Cape Floristic Region (CFR) of South Africa. In addition, we tested the ability of mites to feed on the associated fungi. Four species of scolytine beetles were collected from various *Virgilia* hosts and from across the CFR. All were consistently associated with various *Geosmithia* species, fungi known from phloeophagous beetles in many parts of the world, but not yet reported as *Scolytinae* associates in South Africa. Four beetle species, a single mite species and five *Geosmithia* species were recovered. The beetles, *Hapalogenius fuscipennis*, *Cryphalini* sp. 1 and *Scolytoplatypus fasciatus* were associated with a single species of *Elattoma* phoretic mite that commonly carried spores of *Geosmithia* species. *Liparthrum* sp. 1 did not carry phoretic mites. Similar to European studies, *Geosmithia* associates of beetles from *Virgilia* were constant over extended geographic ranges, and species that share the same host plant individual had similar *Geosmithia* communities. Phoretic mites were unable to feed on their *Geosmithia* associates, but were observed to feed on bark-beetle larvae within tunnels. This study forms the first African-centred base for ongoing global studies on the

associations between arthropods and *Geosmithia* species. It strengthens hypotheses that the association between *Scolytinae* beetles and dry-spored *Geosmithia* species may be more ubiquitous than commonly recognised.

Key words: Insect-fungus interactions, *Hypocreomycetidae*, spore vector, *Fabaceae*, *Scolytinae*

1. Introduction

Bark and ambrosia beetles (*Curculionidae*, *Scolytinae*) are economically and ecologically important pests of trees in urban, forest, plantation and agricultural settings (Avtzis et al. 2012; Harrington 2005; Kirisits 2004; Paine et al. 1997; Six & Wingfield 2011), with about 225 genera and more than 6 000 described species globally (Avtzis et al. 2012; Linnakoski et al. 2012). Some, like the southern pine bark beetle (SPB), *Dendroctonus frontalis* Zimmermann, are capable of killing healthy trees, and causes substantial financial losses (Price et al. 1992). The Redbay ambrosia beetle, *Xyleborus glabratus* Eichhoff, which was introduced to the southeastern USA together with a fungal associate, *Raffaelea lauricola* T.C. Harr., Fraedrich & Aghayeva (Fraedrich et al. 2008; Harrington et al. 2008) is responsible for the extensive wilt and death of Redbay trees (*Persea borbonia* (L.) Spreng.) and other members of the *Lauraceae* (Harrington et al. 2008). However, many scolytine beetles attack only trees that are weakened and/or stressed, or dead (Avtzis et al. 2012; Paine et al. 1997; Raffa et al. 1993; Six & Wingfield 2011). Despite their ecological importance in, for example, initiating nutrient cycling (Christiansen et al. 1987; Stark 1982), they have not attracted much research interest, as they seldom cause economic losses, except for those few that vector detrimental fungi (Lieutier et al. 2009).

Scolytine beetles usually have complex associations with various organisms, including fungi (Linnakoski et al. 2012; Six & Paine 1998; Six & Wingfield 2011; Whitney 1982), bacteria (Bridges 1984), mites (Cardoza et al. 2008; Klepzig et al. 2001; Moser et al. 1995, 2005) and nematodes (Cardoza et al. 2008; Moser et al. 2005). Those associated with ophiostomatoid fungi (e.g., species of *Ceratocystis*, *Ophiostoma* and *Raffaelea*) are of particular interest, as these fungi include important tree pathogens (Klepzig et al. 2001; Moser et al. 1995; Wood 1982). Microbial and scolytine relationships may be incidental or obligatory, mutualistic, commensal or antagonistic (Kolařík et al. 2008; Six 2003; Six & Wingfield 2011). The fungi benefit by being vectored to new plant hosts (Paine et al. 1997; Six 2003; Six & Wingfield

2011), while some beetles and mites feed on their fungal associates (Cardoza et al. 2008; Harrington 2005; Klepzig et al. 2001; Moser et al. 1995; Six 2003; Six & Wingfield 2011). Various other fungi in this system may, in turn, be antagonistic to the beetles (Barras 1970; Harrington & Zambino 1990; Hofstetter et al. 2006; Klepzig et al. 2001; Six & Wingfield 2011). In addition to fungivorous mites, other phoretic taxa can be parasitic, predatory and/or omnivorous (Klepzig et al. 2001).

Most studies on the interactions between scolytine beetles and other organisms have focussed on the ophiostomatoid fungi. However, numerous other fungal taxa may be consistently associated with these beetles. This includes the genus *Geosmithia* Pitt, a mitosporic ascomycete genus belonging to the *Hypocreales* (*Hypocreomycetidae*) (Houbraken et al. 2012; Kolařík et al. 2004, 2005, 2007; Ogawa et al. 1997). It currently contains 31 published species, most of which have not been formally described (Hulcr & Dunn 2011; Kolařík & Jankowiak 2013; Kolařík & Kirkendall 2010; Kolařík et al. 2004, 2005, 2007, 2008). *Geosmithia* has a worldwide distribution (Kolařík et al. 2004, 2005, 2007; Ogawa et al. 1997), but until recently the genus was understudied. However, after it was found to be commonly associated with several scolytine beetle species, there has been a growing body of literature on these fungi (Hulcr & Dunn 2011; Kolařík & Jankowiak 2013; Kolařík & Kirkendall 2010; Kolařík et al. 2004, 2005, 2007, 2008, 2011). Entomochoric adaptations are absent in *Geosmithia* species (Kolařík & Kirkendall 2010; Kolařík et al. 2008). Instead, they produce hydrophobic and dry conidia that are typically air borne (Kolařík et al. 2007, 2008), and some species are sporadically also collected from other substrates such as plant debris, cereals and soil (Kolařík et al. 2004; Pitt & Hocking 2009).

Despite being regular scolytine beetle associates, the effects of *Geosmithia* on the beetles still remain vague (Kolařík et al. 2007, 2008) but some probably play a role in beetle nutrition (e.g., Kolařík & Kirkendall 2010). Phytopathogens in this genus include *Geosmithia morbida* M. Kolarík, E. Freeland, C. Utley & Tisserat, which is a serious threat to black walnut trees (*Juglans nigra* L.) as it causes thousand cankers disease, and is dispersed by the walnut twig beetle (*Pityophthorus juglandis* Blackman) (Kolařík et al. 2011). *Geosmithia langdonii* M. Kolarík, Kubátová & Pažoutová and *G. pallida* (G. Sm.) M. Kolarík, Kubátová & Pažoutová, isolated from *Scolytus intricatus* (Ratz.), has the ability to inhibit root formation in *Lepidium sativum* L., probably due to toxin production (Čizkova et al. 2005). *Geosmithia langdonii* was also recently identified as both a bark-beetle associate and an endophyte of coast live oaks in California (McPherson et al. 2013).

Recent reports indicated extensive *Scolytinae* beetle activity on *Virgilia* Pior. (*Fabaceae*) trees endemic to the Cape Floristic Region (CFR) of South Africa. This ornamental and ecologically important tree genus is confined to riparian vegetation, thickets, hillsides and forest margins (Palgrave 1983, 2002; Palmer & Pitman 1972). Nothing is currently known about these beetles and their associated organisms and we, therefore, set out to identify these beetles, their phoretic mites and their associated fungal species. Specific objectives included to: (i) identify bark and ambrosia beetle species that infest *Virgilia* trees from a wide geographical range; (ii) identify mite species phoretic on these beetles; (iii) isolate and identify fungal taxa consistently associated with the *Scolytinae* beetles and their phoretic mites, and (iv) test whether mites that are phoretic on these beetles can feed on the fungi they consistently carry. This study represents one of the first to describe such a system for a natural CFR host tree across its distribution range.

2. Materials and methods

2.1 Scolytine beetle and mite collection

Bark and ambrosia beetles were collected from *Virgilia* populations throughout the CFR between January 2011 and December 2012 (Table 1). Where possible, five declining branches (*ca*. 12 cm diam. and 40 cm in length), colonised by beetles (as indicated by the presence of small bore holes), were collected from random trees per population (one branch per tree), and placed in insect emergence cages (all branches per population combined per cage) constructed from sealed cardboard boxes (49 x 49 x 32.6 cm) fitted with two clear plastic bottles (5.7 cm diameters). Emerging beetles were attracted to light penetrating through these bottles, and were thus easily collected. The total number of beetle individuals per species that emerged from these samples was counted (when below 100), or estimated to the nearest 100 individuals (using average weight) when more individuals emerged. In addition, bark and ambrosia beetles were collected aseptically, directly from galleries, on supplementary collections of bark and branches.

A Leica EZ4 microscope (Leica Microsystems (Schweiz) AG, Taiwan) was used to study the collected beetles and their gallery systems. Emerging beetles were often associated with phoretic mites, and both beetles and their phoretic mites were grouped according to morphotype. Numbers of phoretic mites per individual beetle were determined, and for the

Table 1 – Total number of individuals of four scolytine beetle species (to nearest hundred) collected from different *Virgilia* tree taxa at eight localities throughout the CFR of South Africa.

Site[a]	GPS coordinates	*Virgilia* taxon	*Cryphalini* sp. 1	*Hapalogenius fuscipennis*	*Liparthrum* sp. 1	*Scolytoplatypus fasciatus*
HPNBG, Betty's Bay	S 34' 20.893" E 18' 55.519"	*V. oroboides oroboides*	200	300	800	13
Jonkershoek, Stellenbosch	S 33°58'23.10" E 18°56'11.38"	*V. o. oroboides*			200	
KNBG, Cape Town	S 33°59'11.3" E 18°25'34.4"	*V. o. oroboides*	700	800		
Table Mountain, Cape Town	S 33°57'17.76" E 18°25'29.64"	*V. o. oroboides*	900	1 200	500	
SMNR, Cape Town	S 34°05'27.89" E 18°25'17.55"	*V. o. oroboides*	400	400	700	
George	S 33°54'56.07" E 22°33'11.10"	*V. o. ferruginea*	800	600		1
Knysna	S 34°00'21.44" E 23°07'00.61"	*V. divaricata*	700	900	300	
Storms River	S 33°05'15.54" E 18°25'06.96"	*V. divaricata*	1 000	2 000		

[a]HPNBG – Harold Porter National Botanical Garden; KNBG – Kirstenbosch National Botanical Garden; SMNR – Silver Mine Nature Reserve

two most common bark beetle species, phoretic mite numbers were monitored over a seven week period from placement of branches in emergence cages. Each week, the number of mites per beetle was counted on 20 bark beetle individuals of each of the two species. Normality of the mite numbers data was tested using a Shapiro-Wilk test (Shapiro & Wilk 1965), and subsequently analysed using Kruskal-Wallis ANOVA and Median test procedures in Statistica 10 (Statsoft Corporation, USA). Significant differences are reported when $P \leq 0.05$.

Reference specimens of each beetle species collected were stored in 100% ethanol for later identification. Representative specimens of mites were mounted onto microscope slides (following methods of Theron et al. 2012) for later identification. Reference specimens of all beetle and mite species collected in this study were deposited in the Stellenbosch University Insect Collection (USEC), Stellenbosch, South Africa.

2.2 Fungal isolation

Ten individuals of each beetle and mite species collected per site (where possible) were washed separately in Eppendorf tubes containing 0.1 ml ddH_2O. The suspension was subsequently spread onto malt extract agar (MEA: 20gL-1 malt extract and 20gL-1 agar, Biolab, South Africa) in Petri dishes. An additional 10 individuals per beetle species (per site, where possible) were separately crushed in Eppendorf tubes containing 0.1 ml ddH_2O, where after this solution was spread onto MEA. Plates were sealed with parafilm and incubated at room temperature (20 to 25°C) under normal day/ night conditions until resultant fungi could be purified. Due to the large numbers of colonies of fungi originating from primary extractions, only a single representative of the most common and consistent morphotypes (see below) was chosen at random and purified. To purify the growing fungi, hyphal tips of developing mycelia were transferred to fresh MEA plates under sterile conditions.

Fungal isolations were also made directly from bark samples containing fresh beetle galleries. Bark samples were placed in separate moisture chambers (clear plastic bag with moist filter paper) for 7 to 12 days to stimulate sporulation of fungi in the gallery systems. These were stored at room temperature (20 to 25°C) in the dark. Spores from fungal structures that formed within galleries were transferred to MEA plates using a sterile needle and purified. Pure cultures of all isolated fungi were stored at 4°C on MEA until further use.

2.3 Fungal identification

All fungal cultures of the most consistently isolated taxa were grouped according to morphotype based on cultural and micro-morphological characteristics following methods of Kolařík et al. (2004, 2007, 2008). A total of 75 cultures, including representatives of each morphotype, were selected for identification based on DNA sequencing. Representative isolates of all fungal morphotypes identified in this study were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (Table 2).

2.3.1 *DNA extraction, amplification and sequencing*

Fungal mycelia were harvested from actively growing two-week-old cultures using a sterile scalpel. DNA was extracted using the Sigma-Aldrich™ plant PCR kit (Germany) following the manufacturer's instructions. ITS1-f (Gardes & Bruns 1993) and ITS4 (White et al. 1990) primers were used to amplify the nuclear ribosomal internal transcribed spacer regions (ITS1, ITS2) including the 5.8S gene of the rDNA. 20 µL PCR reaction volumes consisted of 5 µL REDExtract-N-Amp PCR ready mix (Sigma-Aldrich™, USA), 10 µL ddH_2O, 0.5 µL (10mM) of each primer and 4 µL extracted fungal DNA. PCR reaction conditions were: initial denaturation at 95°C for 2 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, elongation at 72°C for 1 minute 30 seconds and a final elongation step at 72°C for 8 minutes. All PCR products were visualised by gel electrophoresis on a 1.5 % agarose gel (Promega Corporation, Madison, U.S.A.) stained with 2.5 µL ethidium bromide and visualised under ultraviolet light. All amplified PCR products were cleaned using the Wizard® SV gel and PCR clean-up system (Promega, Madison, Wisconsin, U.S.A.) following the manufacturer's instructions. The purified fragments were sequenced using the respective PCR primers and the Big Dye™ Terminator v3.0 cycle sequencing premix kit (Applied Biosystems, Foster City, CA, U.S.A.), and analysed on an ABI PRISIM™ 3100 Genetic Analyser (Applied Biosystems).

2.3.2 *Phylogenetic analyses*

Fungal sequences generated in this study (Table 2) were compared to published sequences for described *Geosmithia* species and other operational taxonomic units (OTU's) identified in previous studies (Kolařík & Jankowiak 2013; Kolařík & Kirkendall 2010; Kolařík et al. 2004, 2005, 2007, 2008, 2011) available from GenBank (www.ncbi.nlm.nih.gov/genbank). The

Table 2 – Collection details of representative isolates of *Geosmithia* OTU's identified and used for molecular characterisation.

***Geosmithia* OTU**[a]	**Isolate number**[b]	**Host *Virgilia* taxon**	**Site**[c]	**Isolated from**	**Genbank Accession**
Geosmithia flava	40726	*V. oroboides oroboides*	HPNBG	*Hapalogenius fuscipennis*	KJ513210
Geosmithia flava	40727	*V. o. oroboides*	SMNR	*Cryphalini* sp. 1	KJ513209
Geosmithia flava	40728	*V. o. oroboides*	HPNBG	*H. fuscipennis*	KJ513208
Additional collections	n. a.	*V. o. ferruginea, V. divaricata*	Jonkershoek, KNBG, Table Mountain, George, Knysna, Storms River	*Liparthrum* sp. 1, *Elattoma* sp. 1	n. a.
Geosmithia sp. 2	40743	*V. o. oroboides*	HPNBG	*Cryphalini* sp. 1	KJ513232
Geosmithia sp. 2	40744	*V. o. oroboides*	KNBG	*Cryphalini* sp. 1	KJ513233
Geosmithia sp. 2	40745	*V. divaricata*	Storms River	*Cryphalini* sp. 1	KJ513211
Geosmithia sp. 2	40729	*V. divaricata*	Storms River	*Elattoma* sp. 1	KJ513230
Geosmithia sp. 2	40736	*V. o. ferruginea*	George	*Cryphalini* sp. 1	KJ513234
Geosmithia sp. 2	40737	*V. o. oroboides*	HPNBG	*H. fuscipennis*	KJ513229
Geosmithia sp. 2	40730	*V. o. oroboides*	HPNBG	*H. fuscipennis*	KJ513231
Geosmithia sp. 2	40738	*V. o. oroboides*	HPNBG	*Cryphalini* sp. 1	KJ513254
Geosmithia sp. 2	40731	*V. o. oroboides*	HPNBG	*Cryphalini* sp. 1	KJ513253
Additional collections	n. a.	*V. o. ferruginea, V. divaricata*	Jonkershoek, Table Mountain, SMNR, Knysna	*Liparthrum* sp. 1, *Elattoma* sp. 1	n. a.

Geosmithia sp. 8	40739	*V. o. oroboides*	HPNBG	*Cryphalini* sp. 1	KJ513226
Geosmithia sp. 8	40740	*V. o. oroboides*	HPNBG	*Cryphalini* sp. 1	KJ513227
Geosmithia sp. 8	40746	*V. o. oroboides*	HPNBG	*H. fuscipennis*	KJ513258
Additional collections	n. a.	n. a.	n. a.	*Elattoma* sp. 1	n. a.
Geosmithia sp. 10	40733	*V. o. oroboides*	HPNBG	*Elattoma* sp. 1	KJ513217
Geosmithia sp. 10	40734	*V. o. oroboides*	HPNBG	*Liparthrum* sp. 1	KJ513215
Geosmithia sp. 10	40735	*V. o. oroboides*	HPNBG	*Liparthrum* sp. 1	KJ513216
Additional collections	n. a.	*V. o. ferruginea, V. divaricata*	Jonkershoek, KNBG, Table Mountain, SMNR, George, Knysna, Storms River	*Cryphalini* sp. 1 , *H. fuscipennis*	n. a.
Geosmithia sp. A	40732	*V. o. oroboides*	HPNBG	*Scolytoplatypus fasciatus*	KJ533336
Geosmithia sp. A	40741	*V. o. oroboides*	HPNBG	*S. fasciatus*	KJ533337
Geosmithia sp. A	40742	*V. o. oroboides*	HPNBG	*S. fasciatus*	KJ533338
Additional collections	n. a.	*V. o. ferruginea*	George	*Elattoma* sp. 1	n. a.

[a]Following Kolařík & Kirkendall (2010), Kolařík & Jankowiak (2013) and Kolařík et al. (2004, 2005, 2007, 2008).

[b]All isolates collected by Netsai Machingambi and deposited in the University of Pretoria Culture Collection (CMW), Pretoria, South Africa

[c]HPNBG = Harold Porter National Botanic Garden, SMNR = Silver Mine Nature Reserve, KNBG = Kirstenbosch National Botanic Garden

Table 3 – Percentage of individuals of four *Scolytinae* beetle species associated with five *Geosmithia* taxa from *Virgilia* trees throughout the CFR of South Africa.

Site[a]	*Virgilia* taxon	*Scolytinae* taxon	n	*Geosmithia flava*	*Geosmithia* sp. 2	*Geosmithia* sp. 8	*Geosmithia* sp. 10	*Geosmithia* sp. A
HPNBG, Betty's Bay	*V. oroboides oroboides*	*Cryphalini* sp. 1	20	45	20	20	15	
	V. o. oroboides	*Hapalogenius fuscipennis*	20	10	10	70	10	
	V. o. oroboides	*Liparthrum* sp. 1	20	20	15		65	
	V. o. oroboides	*Scolytoplatypus fasciatus*	13					100
Jonkershoek, Stellenbosch	*V. o. oroboides*	*Liparthrum* sp. 1	20	35	55		10	
KNBG, Cape Town	*V. o. oroboides*	*Cryphalini* sp. 1	20	20	65		15	
		H. fuscipennis	20	35	30		35	
Table Mountain, Cape Town	*V. o. oroboides*	*Cryphalini* sp. 1	20	15	50		35	
		H. fuscipennis	20	10			90	
		Liparthrum sp. 1	20	15	85			
SMNR, Cape Town	*V. o. oroboides*	*Cryphalini* sp. 1	20	65	10		25	
		H. fuscipennis	20	10	50		40	
		Liparthrum sp. 1	20	65	10		25	
George	*V. o. ferruginea*	*Cryphalini* sp. 1	20	20	60		20	

		H. fuscipennis	20	50	10	40	
		Scolytoplatypus fasciatus	1				100
Knysna	*V. divaricata*	*Cryphalini* sp. 1	20	15	15	70	
		H. fuscipennis	20		25	75	
		Liparthrum sp. 1	20	10	90		
Storms River	*V. divaricata*	*Cryphalini* sp. 1	20		50	50	
		H. fuscipennis	20	10		90	

[a]HPNBG – Harold Porter National Botanical Garden; KNBG – Kirstenbosch National Botanical Garden; SMNR – Silver Mine Nature Reserve

dataset (available from www.treebase.org, accession number: S15465) was aligned using Clustal W (Thompson et al. 1994) and manually adjusted in BioEdit v7. 0. 5 (Hall 2005). *Acremonium alternatum* Link (GenBank AY566992) was chosen as outgroup taxon following Kolařík & Jankowiak (2013). Phylogenetic analyses were conducted using MrBayes v. 3.0b4 (Ronquist & Huelsenbeck 2003) and PAUP (Phylogenetic Analysis Using Parsimony PAUP*4.0b10) (Swofford 2002). In PAUP, a Maximum Parsimony (MP) analysis was conducted using the heuristic search option with random addition of sequences (1 000 replications), tree bisection-reconnection (TBR) and MULTREES options ON. Bootstrap support values with 1 000 replications were calculated to assess the confidence of resultant nodes in the MP trees with the MULTREES option OFF and 10 random sequence additions in each of 1 000 pseudo-replications. In MrBayes, a Markov Chain Monte Carlo (MCMC) approach was used, using the GTR+I+K model as selected in jModelTest 0.1.1 (Posada 2008) and Akaike information criteria (Akaike 1974). Eight million generations were run, with a sampling frequency of 100 and burn-in trees set at the first 25%. The remaining trees were pooled into a 95% majority consensus tree.

2.4 Mite feeding studies

To test the ability of phoretic mites to feed and reproduce on the fungi they were commonly associated with, 10 mite individuals were placed onto three isolates of each OTU identified in this study. Plates with sterile MEA served as control, and the experiment was replicated three times. Plates (6.4 cm diam.) with fungi that had grown to fully cover the surface of the MEA media were used in these assays to limit growth of potential contaminants. To prevent mites from escaping, plates were sealed with parafilm and placed in 15 L plastic containers that were half-filled with water, thus allowing the plates to float. The lid of the containers was lined with petroleum jelly before closing to prevent entry of contaminating mites and other organisms. After 40 days at 25°C in the dark, the number of live mites in each plate was recorded using a stereo-microscope.

3. Results

3.1 Scolytine beetles

Four species of scolytine beetles were collected from various *Virgilia* taxa (*Virgilia oroboides oroboides, V. o. ferruginea* and *V. divaricata*) at eight sites, including George, Harold Porter National Botanical Gardens (HPNBG), Jonkershoek, Kirstenbosch National Botanical Gardens (KNBG), Knysna, Sivermine Nature Reserve (SMNR), Storms River and Table Mountain (Table 1, Fig 1). The abundance of each beetle species collected varied considerably, with *Cryphalini* sp. 1 and *Hapalogenius fuscipennis* the most abundant overall taxa. *Liparthrum* sp. 1 was also fairly abundant, but very low numbers of *Scolytoplatypus fasciatus* were recorded. *Cryphalini* sp. 1, *H. fuscipennis* and *Liparthrum* sp. 1 constructed their galleries in the cambium/inner bark (Fig 1), while *S. fasciatus* bore straight into the sapwood of its host. *Liparthrum* sp. 1 seemed to prefer smaller branches, but was also commonly found inhibiting only the outer bark layers of larger branches. All species were found to share the same host plant individual with at least one other scolytine beetle at some stage during the course of the study period.

Each beetle species had a distinct gallery system (Fig 1). Parental galleries of *Cryphalini* sp. 1 are short and slightly thicker than those of *H. fuscipennis* and *Liparthrum* sp. 1 (when constructing galleries in smaller branches) and orientated horizontally (against the grain of the vascular tissue). Its larval galleries radiate at right angles from these parental galleries, extending parallel to the grain of the tree (vascular tissue). *Hapalogenius fuscipennis* constructs linear parental galleries that extend parallel to the grain of the host tree. Larval galleries expand at right angles from parental galleries, perpendicular to the vascular tissue. *Liparthrum* sp. 1 makes small parental galleries with larval galleries also diverging at right angles from these. It forms the narrowest larval galleries of the three bark beetle taxa. *Scolytoplatypus fasciatus* bores deep into the wood of host trees. Each parental gallery excavated (n=3) contained a pair of adults.

At HPNBG, *Cryphalini* sp. 1, *H. fuscipennis* and *S. fasciatus* often occupied the same individual trees. At all study sites, except at Jonkershoek, *Cryphalini* sp. 1 and *H. fuscipennis* were often collected from the same individual tree, with their galleries constructed in close proximity to one another and often merging (Fig 1). In HPNBG, Table Mountain, SMNR and Knysna, *Cryphalini* sp. 1, *H. fuscipennis* and *Liparthrum* sp. 1 were often collected from the same individual trees.

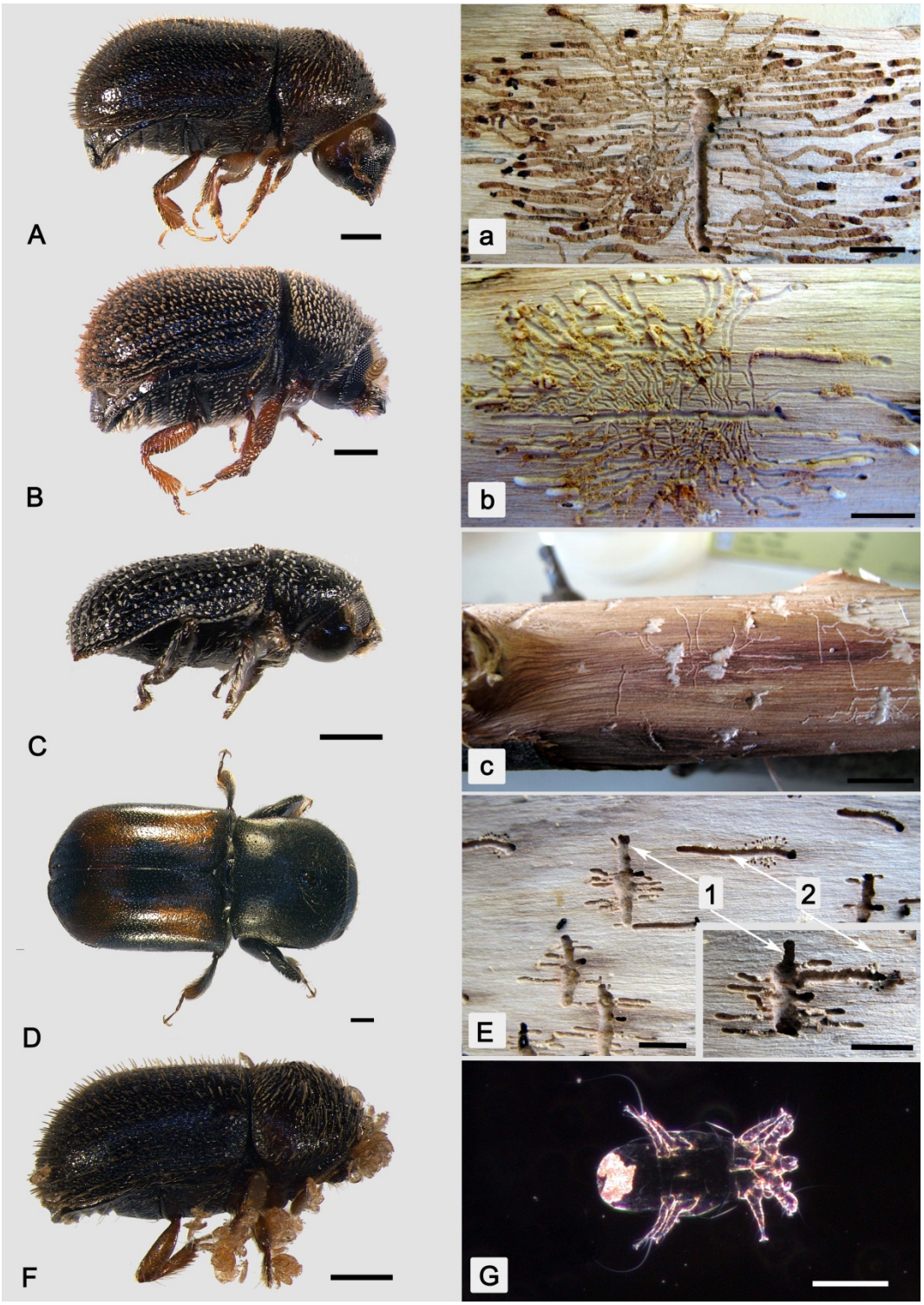

Fig 1 – Scolytinae beetles (in capital letters), their gallery systems (in non-capital letters) and phoretic mites associated with dead and dying *Virgilia* trees in the CFR. (A, a) *Cryphalini* sp. 1; (B, b) *Hapalogenius fuscipennis*; (C, c) *Liparthrum* sp. 1; (D) *Scolytoplatypus fasciatus*; (E) Merging gallery systems of neighbouring *Cryphalini* sp. 1 (1) and *Hapalogenius fuscipennis* (2); (F) *Elattoma* sp. 1 mites phoretic on *Cryphalini* sp. 1; (G) Light micrograph of *Elattoma* sp. 1. Scale bars: A-D and F = 0.25mm, a – c and E = 10 mm, G = 60 μm.

3.2 Phoretic mites

Beetles started to emerge from branches in emergence cages during week four, and carried only a few phoretic mites at that time. *Cryphalini* sp. 1 and *H. fuscipennis* commonly carried a single mite species (*Pygmephoridae*: *Elattoma* Mahunka, Fig 1) at all sites included in this study. The numbers of mites per individual beetle varied between zero and 217. *Liparthrum* sp. 1 never carried phoretic mites. *Scolytoplatypus fasciatus* was very rarely encountered, and phoretic mites were not usually seen on it. However, in one instance (during week six after collection) 217 individuals of the same *Elattoma* species were counted from a single individual. This represented the highest number of phoretic mites on any *Scolytinae* beetle individual collected. During week five and six the numbers of phoretic mites per individual *Cryphalini* sp. 1 and *H. fuscipennis* beetle increased significantly (Fig 2). At week seven numerous mites were still present on emerging beetles. When brood beetles started to emerge after *ca*. five months, a few mite individuals were again present (data not presented). Comparative mite numbers did not vary significantly between *Cryphalini* sp. 1 and *H. fuscipennis* at any given time.

3.3 Fungal identification

Fungal isolates could be grouped into five morphotypes based on colony morphology and micro-morphological characteristics as described by Kolařík et al. (2004, 2007, 2008). Seventy five isolates were selected for identification using DNA sequencing of the ITS gene regions. The aligned ITS data set included 86 sequences and 528 characters of which 382 were constant, 83 were parsimony-informative and 63 variable characters were parsimony-uninformative. Parsimony analyses retrieved a consensus tree with a length of 341 steps. One of the trees resulting from parsimony analyses is presented in figure 3 as the topologies of trees resulting from parsimony analysis and Bayesian inference were similar. Both parsimony analysis and Bayesian inference of the ITS marker placed our fungal isolates into five OTU's (Fig 3) that corresponded to five morphotypes identified using micro-morphological and culture characters. Four of these grouped with previously described OTU's; *Geosmithia* sp. 10, *G. flava* Kolařík, Kubátová & Pažoutová, *Geosmithia* sp. 8 and *Geosmithia* sp. 2 (Fig 3). The fifth OTU grouped in a strongly supported clade, distantly related to previously published

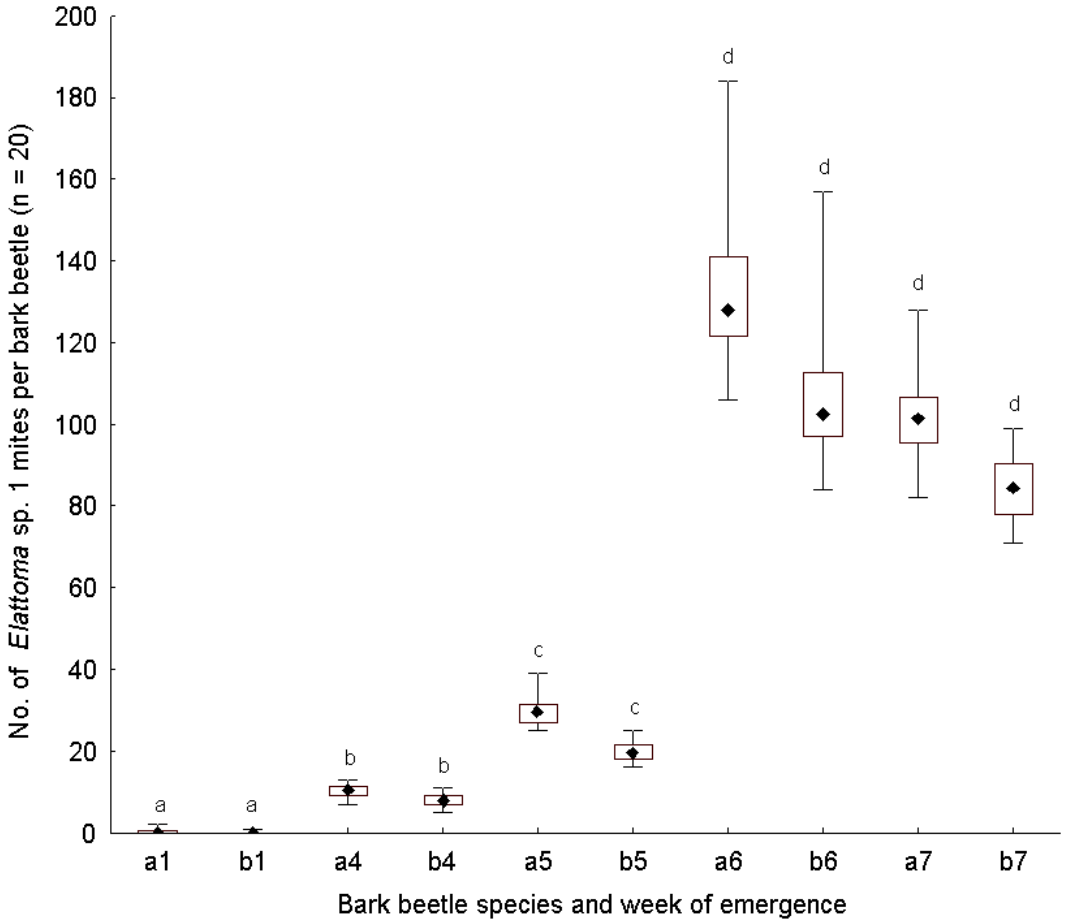

Fig 2 – Median of numbers of individuals of *Elattoma* sp. 1 mites (diamond symbols) phoretic on *Cryphalini* sp. 1 (a) and *H. fuscipennis* (b) as these emerged from *Virgilia* wood over a 7 week period (numbered from 1 to 7 on x-axis label; week 2 and 3 omitted from graph as these were the same as for week 1). Bars indicate 25% to 75% confidence and whiskers depicts data spread. Different letters above bars indicate significant differences in mite numbers encountered per individual beetle (H (df = 9, N = 200) = 92.7595; p = 0.00)..

Fig 3 – One of 1871 most parsimonious trees obtained from parsimony analyses of ITS rDNA sequence data for members of the genus *Geosmithia*. Nodes with support values > 0.70 for Bayesian posterior probability are provided above branches. Taxa in bold indicate isolates that originate from *Virgilia* trees in this study. Taxa labels for other taxa indicate isolate numbers and GenBank accession numbers respectively obtained from Kolařík & Kirkendall (2010), Kolařík & Jankowiak (2013) and Kolařík et al. (2004, 2005, 2007, 2008). Taxon names (species identities and numbered OTU's) followed those proposed in these previous studies.

OTU's, and probably represents an un-described taxon, here referred to as *Geosmithia* sp. A. (Fig 3).

3.4 Inter-organism associations

Cryphalini sp. 1 and *H. fuscipennis*, the most abundant bark beetle species collected in this study, were found on all host taxa and at all sites except at Jonkershoek (Table 1). Jonkershoek was dominated by *Liparthrum* sp. 1 that was present at most sites and on both *Virgilia* species, but was never recorded from *V. oroboides ferruginea*. *Scolytoplatypus fasciatus* was only recorded at two sites and on *V. oroboides* (both subspecies), but this apparent host range is likely skewed by its low abundance (Table 1).

Geosmithia was the only fungal taxon consistently isolated from all individuals of all four species of *Scolytinae* beetles. It sporulated profusely on artificial media, and was also easily observed in both the maternal and pupal galleries of *Cryphalini* sp. 1, *H. fuscipennis* and *Liparthrum* sp. 1. Due to shortage of material we were unable to isolate directly from the gallery systems of *S. fasciatus*. *Cryphalini* sp. 1 and *H. fuscipennis* were associated with *G. flava*, *Geosmithia* sp. 10, *Geosmithia* sp. 8 and *Geosmithia* sp. 2 (Tables 2 and 3). *Liparthrum* sp. 1 was associated with *G. flava*, *Geosmithia* sp. 10 and *Geosmithia* sp. 2. *Scolytoplatypus fasciatus* was only associated with *Geosmithia* sp. A, and this fungus was never collected from any other *Scolytinae* beetle species (Tables 2 and 3). *Geosmithia* communities were remarkably consistent over the sampled geographical distribution range of *Virgilia*, with *G. flava*, *Geosmithia* sp. 10 and *Geosmithia* sp. 2 recorded from all localities (Tables 2 and 3). In contrast, *Geosmithia* sp. 8 was only recorded from HPNBG, even though it was associated with the two most abundant *Scolytinae* beetle species (*Cryphalini* sp. 1 and *H. fuscipennis*) and the widespread *V. oroboides*. *Geosmithia* sp. A was recorded from both sites where its host beetles were found (Tables 2 and 3).

All individuals of *Elattoma* sp. 1 mites collected from emerging beetles consistently carried *Geosmithia*. The *Geosmithia* taxa isolated from phoretic mites were always the same as those isolated from their associated beetle individuals. Mites were unable to feed or reproduce on any of the *Geosmithia* OTU's, and were all dead at the end of the 40 day period, including those on control plates. These mites were, however, often seen feeding on dead bark beetle larvae within galleries.

4. Discussion

In this study, *Virgilia* trees in the CFR were found in association with three species of bark beetles that are common throughout the region and, less commonly, with one species of ambrosia beetle. All beetles were only found on dead or dying *Virgilia* trees, weakened by storms and/or root pathogens. They were never associated with healthy trees, which suggests that all belong to the secondary group of bark beetles (or "facultative parasitic" beetles) (Raffa et al. 1993).

The beetles *Cryphalini* sp. 1, *Hapalogenius fuscipennis* and *Scolytoplatypus fasciatus* were associated with a single species of phoretic mite (*Elattoma* sp. 1). This mite genus is well known as a bark beetle associate in other parts of the world (e.g., Klepzig et al. 2001; Moser et al. 2005), and is considered to include truly phoretic mites, as they have lost some larval stages normal to non-phoretic taxa (Moser et al. 2005). *Liparthrum* sp. 1 was free of mites, probably because it was so much smaller than the other beetle taxa collected. Similar to what was documented in other systems (Lombardero et al. 2000), there appears to be a synchronization of beetle and mite life histories and emergence times on *Virgilia*. Very few phoretic mites were observed on beetles that still occupied their tunnels. However, after four weeks, when beetles started to emerge from tunnels, the number of phoretic mites significantly increased over time.

The genus *Elattoma* includes members that are fungivorous (Klepzig et al. 2001) and/or parasitoids (Moser et al. 1971), but only the biology of *E. bennetti* has been well studied (Hofstetter & Moser 2014). As the females of this species feed on fungi they become massively swollen with developing larvae inside. The females rupture, releasing phoretic adult mites (Hofstetter & Moser 2014). In the present study, *Elattoma* sp. 1 was unable to feed and reproduce on the various *Geosmithia* species that it was commonly associated with even though it was also the dominant taxon found in beetle galleries. We also did not observe massively swollen females such as were described for *E. bennetti* within beetle galleries. This suggests a commensalistic association between the mites and fungi in this system, as the *Geosmithia* species appear to afford no specific benefit for the mites, while the fungus benefits by being transported to new hosts. Interestingly, *Elattoma* sp. 1 individuals were often observed to feed on dead beetle larvae in larval tunnels. It is unknown if the mites were responsible for killing the larvae, but if not the mites may perform a "cleaning service", ridding galleries of corpses and potentially detrimental microbes.

As with *Elattoma* sp. 1, all beetle species collected in this study were associated with various species of *Geosmithia*. These fungi are well known as associates of phloeophagous beetles in many parts of the world (Belhoucine et al. 2011; Čizkova et al. 2005; Kolařík & Jankowiak 2013; Kolařík & Kirkendall 2010; Kolařík et al. 2004, 2007, 2008, 2011; Six et al. 2009; Tisserat et al. 2009), but has not yet been demonstrated as common associates of *Scolytinae* beetles in South Africa. Interestingly, not a single individual of any *Scolytinae* beetle encountered in this study was free of *Geosmithia*, suggesting a strong association between these two organism groups. This strong association is becoming increasingly apparent globally (Hulcr & Dunn 2011; Kolařík & Jankowiak 2013; Kolařík & Kirkendall 2010; Kolařík et al. 2004, 2005, 2007, 2008), and may include a mutualistic association if it is proven that the fungus plays a role in beetle nutrition. In the case of *Virgilia* species, the beetles only colonise recently dead and dying *Virgilia* trees of poor nutritional quality (Raffa et al. 1993), and ingestion of the fungi may have a direct nutritional advantage to the beetles, as suggested by Kolarik et al. (2008) and Kolařík & Kirkendall (2010).

The genus *Geosmithia* currently contains 31 recorded species with only 11 described to date, most of which are associated with phloeophagous beetles (Hulcr & Dunn 2011; Kolařík & Jankowiak 2013; Kolařík & Kirkendall 2010; Kolařík et al. 2004, 2005, 2007, 2008). In the present study we recorded five distinct OTU's of *Geosmithia* based on morphological, culture and molecular characterisations. Four of these were closely related to previously recorded taxa, and included *Geosmithia* sp. 10, *Geosmithia* sp. 8, *Geosmithia* sp. 2 and *G. flava* (Kolařík & Jankowiak 2013). *Geosmithia* sp. 10, *Geosmithia* sp. 2 and *G. flava* are known from various bark beetles from temperate Europe and the Mediterranean area, and from a wide range of host trees (Kolarik et al. 2007). *Geosmithia* sp. 8 is known from *Scolytus intricatus* in *Quercus* trees in Bulgaria, Slovakia and the Czech Republic (Kolarik *et al*. 2008).

Currently, the identification of *Geosmithia* species based on DNA sequence data relies on sequencing of the ITS 1 and 2, including the 5.8S gene region of the nuclear encoded ribosomal DNA. It is important to note that ITS rDNA data is not very diagnostic of many species of *Geosmithia* (Kolařík & Kirkendall 2010; Kolařík & Jankowiak 2013; Kolařík et al. 2011), and alternative markers should be used in future studies for clear species delimitations in this genus. It is, therefore, likely that *Geosmithia* sp. 10, *Geosmithia* sp. 8 and *Geosmithia* sp. 2 identified in this study represent undescribed taxa that are distinct from these formerly identified species.

Scolytoplatypus fasciatus and its phoretic mites were exclusively associated with *Geosmithia* sp. A, and this fungus was not isolated from any other *Scolytinae* beetle. It seems to be closely related to the *G. pallida* species complex, but can be distinguished from these taxa by its brownish to greyish colour during sporulation. This character is also present in the closely related *Geosmithia* sp. 27 that is associated with *Pityogenes bidentatus* from *Pinaceae* in Poland (Kolařík & Jankowiak 2013). As *S. fasciatus* is an ambrosia beetle, *Geosmithia* sp. A may play a role in its nutrition. Morphological characters suggesting that this fungus may be ambrosial include the formation of dense palisades of hyphae, the production of large, solitary, globular spores, and the presence of a short-lived yeast like phase after conidial germination (Kolařík & Kirkendall 2010). Like *G. rufescens*, *Geosmithia* sp. A, therefore, seems to possess ambrosial states intermediate of the usual adaptations (Kolařík & Kirkendall 2010).

Bark and ambrosia beetles and their associated *Geosmithia* species were not specific towards any particular *Virgilia* taxon. Geographical distance between sites surveyed did not seem to affect the association as the same *Geosmithia* communities were constantly isolated from the same *Scolytinae* beetle species at the near extreme ends of our sampling area (ca. 600 km apart). Our results, therefore, support those of Kolařík et al. (2008, 2013) who found similar *Geosmithia* communities from *Scolytinae* beetles that shared similar host plants (same host genus or family). The maintenance of these constant *Geosmithia* communities over large geographical ranges further suggests strong symbiotic interactions between these taxa. *Geosmithia* sp. 10, *Geosmithia* sp. 2 and *G. flava* were consistently associated with *Cryphalini* sp. 1, *H. fuscipennis* and *Liparthrum* sp. 1. These beetles often co-inhabited the same logs, and we often observed galleries of *Cryphalini* sp. 1 and *H. fuscipennis* to overlap, with the beetles moving around galleries of neighbouring co-existing taxa. This would facilitate fungal contact with other beetle individuals and taxa, rendering it unsurprising that the communities strongly overlap. The strong overlap between the *Geosmithia* communities of these beetles and those of *Liparthrum* sp. 1 is probably the result of construction of galleries in the outer bark of *Virgilia* by the latter, directly above the gallery systems of the former species which are constructed in the phloem. The close proximity of these gallery systems will easily allow the fungus to grow from one gallery system into an adjacent one. The only anomaly for this phenomenon of shared *Geosmithia* communities and associated beetles was in the association between *S. fasciatus* and *Geosmithia* sp. A. *Scolytoplatypus fasciatus* occupies an isolated niche (deep within wood), that probably does not allow it to come into frequent contact with the other *Geosmithia* spp. from other co-occurring beetles.

The frequent isolation of *Geosmithia* sp. 8 from only one site (HPNBG), but on the two most common and widespread beetle taxa collected in this study and their associated *Elattoma* sp. 1 mites, is intriguing. *Virgilia* trees have reportedly been introduced into the HPNBG when this garden was established more than 80 years ago (J. Forrester pers. com.). Since then it has become naturalised in native vegetation surrounding the gardens. Despite this, all *Scolytinae* beetle species and *Geosmithia* OTU's identified in this study from *Virgilia* species throughout its natural range are present at this site. It is unlikely that these beetles were introduced with the host plants as they only invade dead and dying trees, individuals that would not be transplanted normally. It is, therefore, possible that these beetle taxa may also occur on plant species other than *Virgilia* and, following the same logic, the *Geosmithia* taxa isolated from these may also be found on other trees in natural systems. It is possible that *Geosmithia* sp. 8 was initially only associated with a plant taxon particular to this area, but shifted host to *Virgilia* using bark beetles or their associated mites. Because this is a botanical garden setting, a potential host shift from non-native plants cannot be ruled out. Some evidence for polyphagy for the beetles identified in this study includes documented polyphagy in *S. fasciatus* (Schedl 1962) and the identification of *Millettia grandis* (*Fabaceae*) as a host for *H. fuscipennis* in the northern parts of South Africa (Beaver 2010). Both beetle taxa also have very wide distribution ranges that include other African countries (Beaver 2010; Schedl 1962), well past the distribution range of *Virgilia* species.

This study presents the first record of *Geosmithia* species and their association with secondary bark beetles, ambrosia beetles and phoretic mites on *Virgilia* trees in South Africa. We have shown that *Geosmithia* communities are relatively similar for co-occurring scolytine beetles, and different for those with isolated ecological niches. In addition, geographic distance is not a determining factor for *Geosmithia* associates of the beetles. The relationship between *Scolytinae* beetles, mites and fungi on *Virgilia* trees is complex, and may include commensualisms, parasitism and/or mutualisms. The present study will serve as platform for further scolytine beetle-*Geosmithia*-mite association studies in South Africa and globally.

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