



# Three new *Graphium* species from baobab trees in South Africa and Madagascar

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## Key words

*Adansonia digitata*  
*Adansonia rubrostipa*  
fungal biodiversity  
*Microascales*

**Abstract** Baobabs (*Adansonia* spp.) are iconic trees, known for their immense size, strange forms, sources of food and as the subjects of myths and mysteries. It is thus surprising that little is known regarding the fungi that infect these trees. During a survey to determine which wound infecting fungi occur on baobabs, synnematus structures were observed and *Graphium*-like isolates were obtained. Culture characteristics and micro-morphology, together with DNA sequence comparisons for the SSU rRNA, rRNA-ITS and TEF-1 $\alpha$  gene regions were used to characterise these fungi. These data revealed three novel *Graphium* spp. and these are described as *G. adansoniae*, *G. madagascariense* and *G. fabiforme*.

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## INTRODUCTION

Baobab trees are iconic plants and represent some of the most recognisable trees in the world. The eight species of baobabs reside in the single genus, *Adansonia*. Madagascar is their centre of diversity, with six species endemic to the island. These include *A. grandidieri*, *A. madagascariensis*, *A. perrieri*, *A. rubrostipa*, *A. suarezensis* and *A. za* (Baum 1995, Wickens & Lowe 2008). *Adansonia digitata*, the African baobab, has a wide distribution from as far north as the Sahel to a few degrees south of the Tropic of Capricorn in the south of the continent (Wickens & Lowe 2008). This species has also been introduced into Madagascar and other parts of the world (Maheshwari 1971). The remaining species, *A. gregorii*, occurs in the north-western part of Australia, in the Kimberley ranges (Baum 1995, Wickens & Lowe 2008).

The different species of baobab trees are not only part of the local folklore of the regions where they occur, but each has a multitude of uses (Wickens 1982, Gebauer et al. 2002). Every part of the baobab tree can be used for either food, fodder, medicine, shelter or to produce useful utensils. The fruit of *A. digitata* represents a source of vitamin C (Chadare et al. 2009) and the leaves, seeds and young roots can all be eaten. The bark is used to produce ropes and fish traps and the wood can be used as fodder or as thatching for roofs (Wickens 1982).

Ironically, very little is known regarding the fungal associates of baobabs in the countries where they occur. Research on baobab trees has typically concerned the nutritional value of the fruit, seeds and leaves of the trees (Yazzie et al. 1994, Boukari et al. 2001). Some studies have been conducted on the ecology of baobabs in Africa (Dhillon & Gustad 2004, Venter & Witkowski 2009), and also on the effect of elephant populations on baobab survival and regeneration (Swanepoel & Swanepoel 1986, Barnes et al. 1994, Edkins et al. 2008). Baobab trees can survive and recover from an incredible level

of damage by humans and/or elephants. However, in recent years there is evidence of baobab trees dying of unknown cause (Anonymous 1991, Pearce et al. 1994, Alberts 2005, Patrut et al. 2007, Edkins et al. 2008). This has prompted a study to identify the fungi occurring on wounds of these trees and to establish a base of understanding of the mycological diversity associated with baobabs. During these investigations, fungi producing synnemata were observed on wounded as well as dead baobab trees in South Africa and Madagascar. *Graphium*-like fungi were obtained from spore-drops taken from these structures. The aim of this study is to identify and characterise these fungi based on DNA sequence comparisons and morphology.

## MATERIALS AND METHODS

### Isolates examined

Isolates from South Africa used were obtained from an elephant-damaged *A. digitata* tree (Fig. 1) in the Kruger National Park and from an *A. digitata* tree next to the N1 highway close to Musina (Limpopo Province) that had been damaged by a motor vehicle. The Madagascar isolates were obtained from the wood of a recently felled *A. rubrostipa* tree. All isolates (Table 1) are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), Pretoria, South Africa. Representative strains have also been deposited at the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands. Herbarium specimens of types and para-types were deposited in the herbarium of the National Collection of Fungi, Pretoria, South Africa (PREM). Two isolates each, including the ex-type isolates, of five known species in the *G. penicillioides* complex (Table 1) were retrieved from the CMW collection, and included in the study for comparative purposes.

### DNA extraction, PCR amplification, and DNA sequencing

DNA extraction was performed using PrepmanUltra (Applied Biosystems, Foster City, CA, USA) following the method described by Linnakoski et al. (2008). A part of the small subunit ribosomal RNA (SSU) gene region was amplified using prim-

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Table 1 Origins, hosts and GenBank accession numbers of the *Graphium* strains used in phylogenetic analyses.

Name	CMW no. <sup>1</sup>	Other no. <sup>2</sup>	Herbarium no. <sup>3</sup>	Host plant / Insect	Origin	Collector or supplier	GenBank accession no. <sup>4</sup>		
							SSU	ITS	TEF-1 $\alpha$
<i>G. adansoniae</i>	30617			<i>Adansonia digitata</i>	South Africa	E. M. Cruywagen		GQ200610	HM630596
	30618 <sup>T</sup>	CBS 124917	PREM 60308	<i>A. digitata</i>	South Africa	E. M. Cruywagen		GQ200611	HM630598
	30619			<i>A. digitata</i>	South Africa	E. M. Cruywagen		GQ200612	
	30620	CBS 124915	PREM 60309	<i>A. digitata</i>	South Africa	E. M. Cruywagen	HM630604	GQ200613	HM630597
	30621			<i>A. digitata</i>	South Africa	E. M. Cruywagen / J. Roux	HM630605	GQ200614	
	30622			<i>A. digitata</i>	South Africa	E. M. Cruywagen / J. Roux			HM630599
	30623			<i>A. digitata</i>	South Africa	E. M. Cruywagen / J. Roux			
30624			<i>A. digitata</i>	South Africa	E. M. Cruywagen / J. Roux				
<i>G. basitruncatum</i>		CBS 320.72		Forest soil	Solomon Islands			GQ200615	
		JCM 8083		Soil	Japan	G. Okada	AB038427		
<i>G. carbonarium</i>	12418	CBS 123611	PREM 60014	<i>Salix babyonica</i> / <i>Pissodes</i> sp.	China	X. Zhou	AB038425		HM630602
	12420 <sup>T</sup>	CBS 123610	PREM 60013	<i>S. babyonica</i> / <i>Pissodes</i> sp.	China	X. Zhou	NA <sup>5</sup>	FJ434980	HM630603
<i>G. fabiforme</i>	30626 <sup>T</sup>	CBS 124921	PREM 60310	<i>A. rubrostipa</i>	Madagascar	J. Roux / M.J. Wingfield	NA <sup>5</sup>	GQ200616	HM630592
	30627	CBS 127181	PREM 60311	<i>A. rubrostipa</i>	Madagascar	J. Roux / M.J. Wingfield	NA <sup>5</sup>	GQ200617	HM630593
<i>G. fimbriisporum</i>	5605 <sup>T</sup>	CBS 870.95		<i>Picea abies</i> / <i>Ips typographus</i>	France	M. Morelet	AY148171	AY148177	HM630590
	5606	CBS 422.94		<i>P. abies</i> / <i>I. typographus</i>	Austria	T. Kirišits	AY148180	AY148183	HM630591
<i>G. lairidis</i>	5601 <sup>T</sup>	CBS 116194		<i>Larix decidua</i> / <i>I. cembrae</i>	Austria	T. Kirišits	AY148182	AY148182	HM630588
	5603	CBS 116196		<i>L. decidua</i> / <i>I. cembrae</i>	Austria	T. Kirišits			HM630589
<i>G. madagascariense</i>	30625	CBS 124918		<i>A. rubrostipa</i>	Madagascar	J. Roux / M.J. Wingfield	HM630606	GQ200618	HM630595
	30628 <sup>T</sup>	CBS 124919	PREM 60312	<i>A. rubrostipa</i>	Madagascar	J. Roux / M.J. Wingfield		GQ200619	HM630594
	30629	CBS 124920		<i>A. rubrostipa</i>	Madagascar	J. Roux / M.J. Wingfield	HM630607	GQ200620	
	5292			<i>Populus nigra</i>	Czech Republic	T. Kirišits		HQ335310	HM630600
5295			<i>P. nigra</i>	Czech Republic	T. Kirišits		HQ335311	HM630601	
<i>G. pseudormiticum</i>	503 <sup>T</sup>		PREM 51539	<i>Pinus</i> sp. / <i>Orthotomicus erosus</i>	South Africa	S. Tribe	AY148186	AY148186	HM630586
	12285	CBS 123608		<i>Tsuga dumosa</i> / <i>Pissodes</i> sp.	China	X. Zhou	HM630608		HM630587
<i>G. putredinis</i>	352			Blackened Agaric	USA	C. T. Rogerson		HQ335312	
<i>Graphium</i> sp. 1		UAMH 10637		<i>Picea</i> sp. / <i>Ips perturbatus</i>	Canada	S. Massoumi Alamouti	GQ266169	DQ268587	
		3YT5P2-G1		<i>Picea</i> sp. / <i>Ips perturbatus</i>	Canada	S. Massoumi Alamouti	GQ266167		
<i>Graphium</i> sp. 2		UAMH 10636		Bark beetle	Canada	J.-J. Kim		DQ268586	
		UAMH 10638		<i>Picea</i> sp. / <i>Ips perturbatus</i>	Canada	S. Massoumi Alamouti	GQ266166	DQ268588	
<i>Graphium</i> sp. 3		3PG4ISW10		<i>Picea</i> sp. / <i>Ips perturbatus</i>	Canada	S. Massoumi Alamouti	GQ266165	GQ266156	
		CBS 470.71		<i>Fagus sylvatica</i>	Germany	H. Butin	AB007681		
<i>Graphium</i> sp. 4		CBS 506.86		<i>Ulmus procera</i>	UK	J. Webber	AB038424		
	5554			<i>Schizolobium parahybum</i>	Ecuador	M.J. Wingfield	AY351894		
<i>Graphium</i> sp. 5	5566			<i>S. parahybum</i>	Ecuador	M.J. Wingfield	AY351895		
		CCF 3566		<i>Cecropia</i> sp. / <i>Scolytodes unipunctatus</i>	Costa Rica	M. Kolařík	AM267260	AM267264	
<i>Graphium</i> sp. 6		CCF 3570		<i>Cecropia</i> sp. / <i>S. unipunctatus</i>	Costa Rica	M. Kolařík		AM267265	
		841EW1 1		<i>Pinus</i> sp.	Korea	J.-J. Kim	GQ266164	GQ266157	
<i>Graphium</i> sp. 7		841EW2 1		<i>Pinus</i> sp.	Korea	J.-J. Kim	GQ266163	GQ266158	

<sup>1</sup> CMW: Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.<sup>2</sup> CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CCF: Culture Collection of Fungi, Department of Botany, Faculty of Science, Charles University, Prague; JCM: Japan Collection of Microorganisms, Wako, Japan; UAMH: University of Alberta Microfungus Collection & Herbarium, Devonian Botanic Garden, Edmonton, Canada.<sup>3</sup> PREM: National Collection of Fungi, Pretoria, South Africa.<sup>4</sup> Accession numbers of the sequences obtained in this study are shown in bold.<sup>5</sup> No amplification: all attempts to amplify the SSU for these two isolates failed.<sup>T</sup> Ex-type strain.



**Fig. 1** Baobab tree (*A. digitata*) in the northern Kruger National Park damaged by elephants.

ers NS1, NS3, NS4 and NS8 (White et al. 1990). The first and second internal transcribed spacer (ITS 1 and ITS 2) regions of the rRNA gene, including the 5.8S rRNA gene, were amplified with primers ITS1F (Gardes & Bruns 1993) and ITS4 (White et al. 1990), while part of the translation elongation factor 1 $\alpha$  (TEF-1 $\alpha$ ) gene was amplified using primers EF1F and EF2R (Jacobs et al. 2004).

The PCR reaction mixtures for all three gene regions consisted of 2.6 U Expand High Fidelity Taq Polymerase mixture (Boehringer Mannheim, South Africa), 5  $\mu$ L PCR reaction buffer, 3 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP, and 0.2 mM of each primer (total volume: 50  $\mu$ L). For the ITS and TEF-1 $\alpha$  gene regions, PCR conditions were 2 min at 95 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 52 °C and 1 min at 72 °C, and finally one cycle of 8 min at 72 °C. The same reaction mixture as above was used for SSU genes but in this case, PCR conditions were 3 min at 95 °C, then 35 cycles of 30 s at 94 °C, 45 s at 52–56 °C and 2 min at 72 °C, followed by a final cycle of 8 min at 72 °C. PCR products were purified with the High Pure PCR purification kit (Roche, Mannheim, Germany).

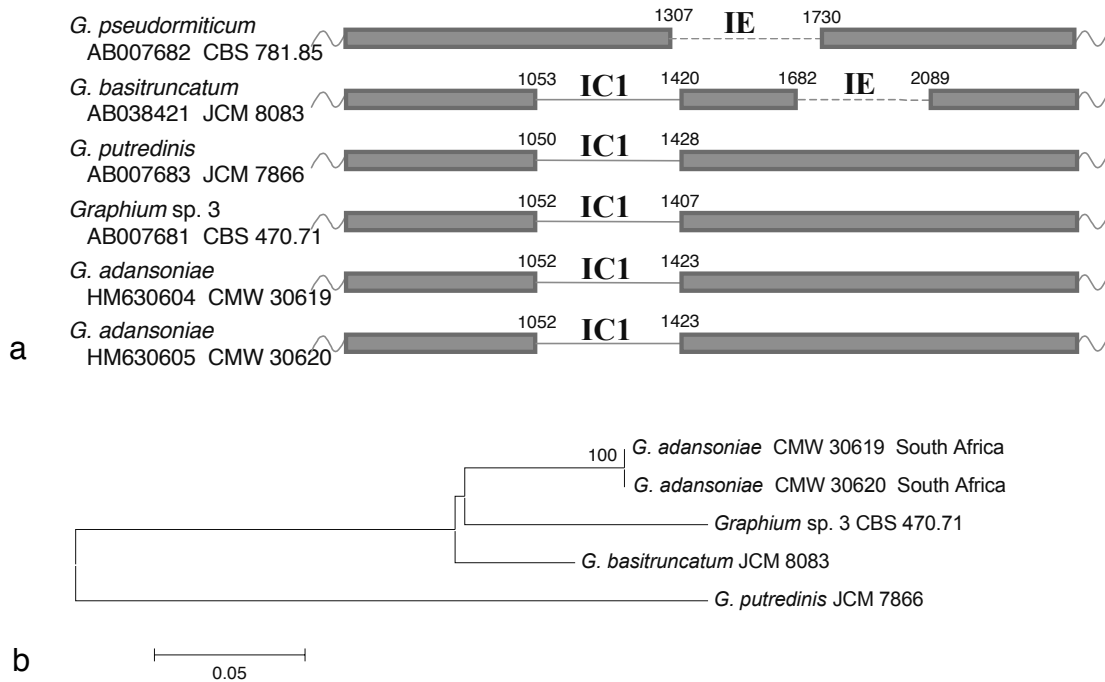
The PCR fragments of each gene region were sequenced using both the forward and reverse primers mentioned above. The ABI Prism® Big Dye™ Terminator v3.0 Ready Reaction Cycle sequencing Kit (Applied Biosystems) was used for the sequenc-

ing PCR. Sequences were determined with an ABI PRISM™ 3100 Genetic Analyzer (Applied Biosystems). DNA sequences of opposite strands were edited and consensus sequences obtained using MEGA v4.0 (Tamura et al. 2007).

#### **Phylogenetic analyses**

Appropriate reference sequences were downloaded from NCBI GenBank (<http://www.ncbi.nlm.nih.gov>) and incorporated into the three separate datasets, which were compiled in MEGA v4.0 (Tamura et al. 2007). Sequences were aligned online with MAFFT 6 (Kato & Toh 2008), using the G-INS-i strategy for the SSU dataset, and the E-INS-i strategy for the ITS and TEF-1 $\alpha$  datasets.

Three different analyses were conducted on each of the three datasets. The introns in the SSU dataset were excluded from the analyses. Maximum likelihood (ML) analyses were conducted in the online version of PhyML v3.0 (Guindon & Gascuel 2003). The best fit substitution models were determined using the Akaike Information Criterion (AIC) in jModelTest v0.1.1 (Posada 2008). The selected model for SSU was HKY+I, for ITS it was GTR+I+G, and for TEF-1 $\alpha$  it was TrN+G. Maximum parsimony (MP) analyses were performed using PAUP v4.0 beta 10 (Swofford 2003) with Tree Bisection-Reconnection (TBR) and 10 trees saved per replicate. Confidence levels for



**Fig. 2** a. Positions of group I introns in SSU gene region of some *Graphium* species. Introns that align with each other are represented with similar lines. Solid lines indicate the rare group IC1 type intron present only in a few *Graphium* and other fungal species, including the one new species from baobabs in South Africa (*G. adansoniae*). Broken lines indicate for the so-called group IE introns (Gibb & Hausner 2003). Numbers indicate the beginning and end positions of the introns in the aligned SSU dataset. — b. Neighbour-joining tree with 1 000 bootstrap replicates obtained from group IC1 intron sequences.

both the ML and MP trees were estimated using 1 000 bootstrap replicates. Bayesian inference, based on a Markov Chain Monte Carlo (MCMC) approach, was performed in MrBayes v3.1.2 (Ronquist & Huelsenbeck 2003). Base equilibrium frequencies, instantaneous substitution rates and among-site rate variation values were estimated independently on shared topologies. Two independent Markov chains were initiated from a random starting tree after which runs of 10 M generations (sample frequency of 100) were performed. Burn-in values were determined using Tracer v1.4 (<http://tree.bio.ed.ac.uk/software/tracer/>), and all sampled trees lower than the burn-in values were discarded. The remaining trees were pooled into a 50 % majority rule consensus tree.

### Morphology

Cultures were grown on 2 % malt extract agar (MEA; 20 g malt extract, Biolab, Merck, Midrand, South Africa; 20 g agar, Biolab; 1 000 mL dH<sub>2</sub>O) as well as homemade oatmeal agar (OA; 20 g oats, boiled and filtrated, with 20 g agar added and made up to 1 000 mL with dH<sub>2</sub>O). Fungal structures were mounted in 85 % lactic acid for morphological comparisons. Microscope observations were made using a Zeiss Axioscop compound microscope and photographic images captured using a Zeiss AxioCam MRc digital camera. AxioVision v4 (Carl Zeiss Ltd., Germany) was used for all measurements. Fifty measurements were taken for each taxonomically informative structure of the isolates chosen to represent the new species. Colony colours were defined using the colour charts of Rayner (1970). Nomenclatural novelties and descriptions were deposited in MycoBank [www.MycoBank.org](http://www.MycoBank.org); Crous et al. 2004).

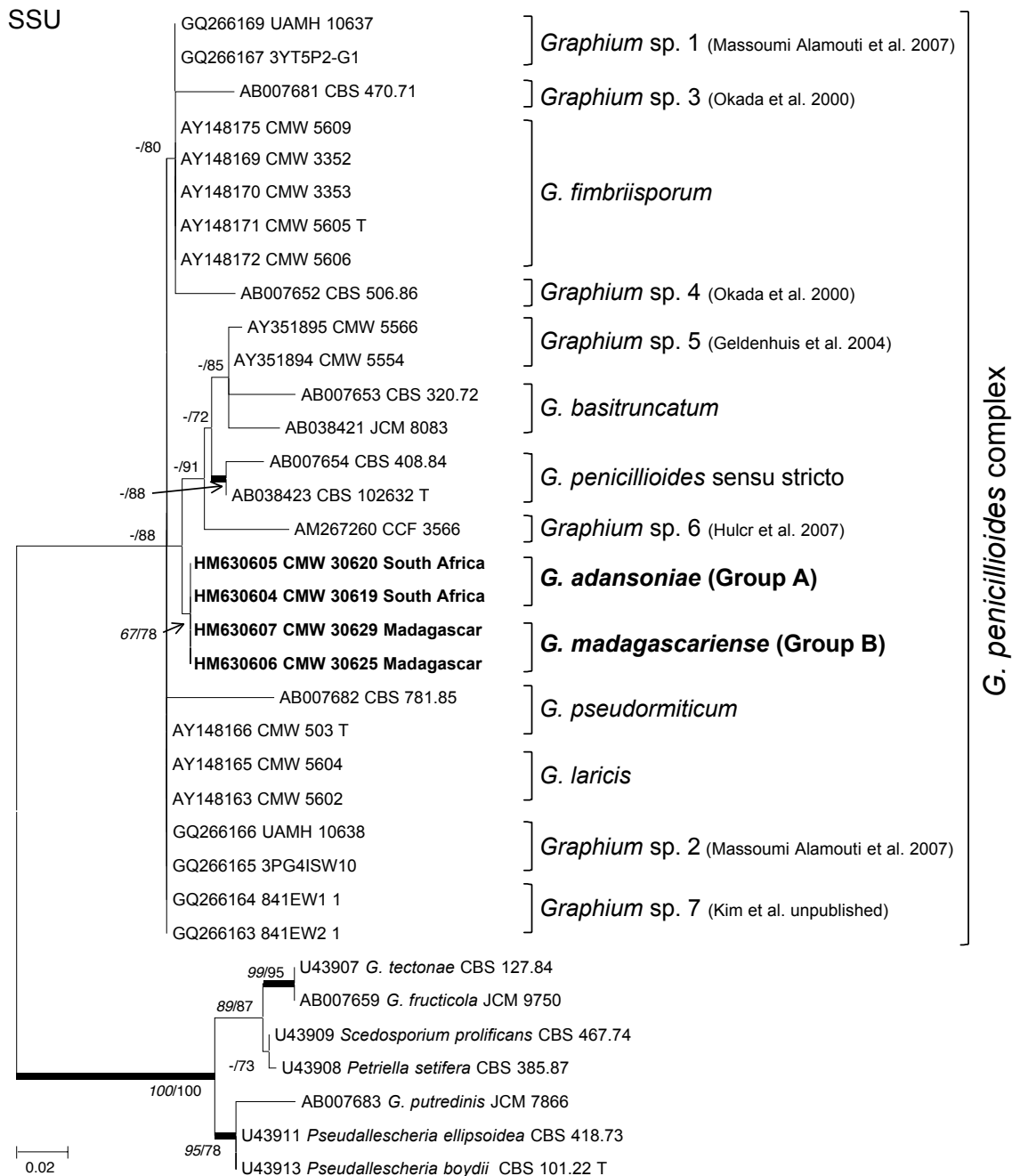
Cultures were grown for 7 d on 2 % MEA to provide inoculum for growth comparisons in culture. A 4 mm cork borer was used to make circular plugs that were placed aseptically at the centres of 2 % MEA plates. Three replicates of each culture were incubated at temperatures 10–35 °C at 5 °C intervals. Measurements of the colony diameters were taken after 7 and 10 d and averages were computed.

Asexual fruiting structures of the selected isolates were examined with scanning electron microscopy (SEM). Cultures were grown on MEA until sporulation. A colonised block of agar was cut from the agar and immersed in 2.5 % glutaraldehyde in 0.075 M phosphate buffer (pH 7) for 1 h. The specimens were washed three times (10 min each) in 0.075 M phosphate buffer and dehydrated for 10 min in each of 30 %, 50 %, 70 %, 90 % as well as three times in 100 % ethanol. The specimens were critical point dried and sputter coated with gold. Specimens were examined with a JSM-840 SEM (JEOL, Tokyo) at 5 kV and images captured with Orion v6.60.4 (E.L.I. s.p.r.l., Brussels, Belgium).

### RESULTS

#### DNA sequencing, introns in SSU genes and phylogenetic analyses

Amplification of the SSU genes yielded fragments varying between 1 506 and 1 670 bp in size. After alignment with sequences from GenBank, it was found that two isolates of *G. adansoniae* (CMW 30619, CMW 30620) from baobab in South Africa contained introns, 372 bp in size and in a similar position (Fig. 2a) to introns in *Graphium basitruncatum*, *G. putredinis*, and *Graphium* sp. 3. BLAST searches in GenBank revealed that these introns are rare but correspond in length and structure with group I introns as defined by Li & Zhang (2005). The group I introns from the isolates indicated in Fig. 2 were aligned and a neighbour-joining tree was constructed in MEGA v4.0 to determine the similarities between introns from the different isolates (Fig. 2b). The intron of *G. putredinis* was very different from the introns of the four isolates from the *G. penicillioides* complex, which were similar and grouped closer to each other. The introns of the two *G. adansoniae* isolates were identical. A second intron in *G. basitruncatum* and the intron in *G. pseudormiticum* corresponded to group IE introns previously found in another Microascalean species, *Gondwanamyces proteae* (Gibb & Hausner 2003). Three isolates of *G. madagascariense* from Madagascar did not have



**Fig. 3** Unrooted ML tree of the *Graphium penicillioides* complex based on SSU gene sequences. Bootstrap values for MP (*italic*) and ML (roman) above 75 % are given at the nodes. Branches with Bayesian posterior probabilities of more than 0.95 are printed in bold. GenBank accession numbers are shown in bold for isolates newly sequenced in this study.

introns in this position. For two of the Madagascar isolates of *G. fabiforme* (CMW 30626, CMW 30627), it was not possible to amplify the SSU genes despite repeated attempts using different primer combinations. All introns in the SSU dataset were excluded from the phylogenetic analyses of the *G. penicillioides* complex.

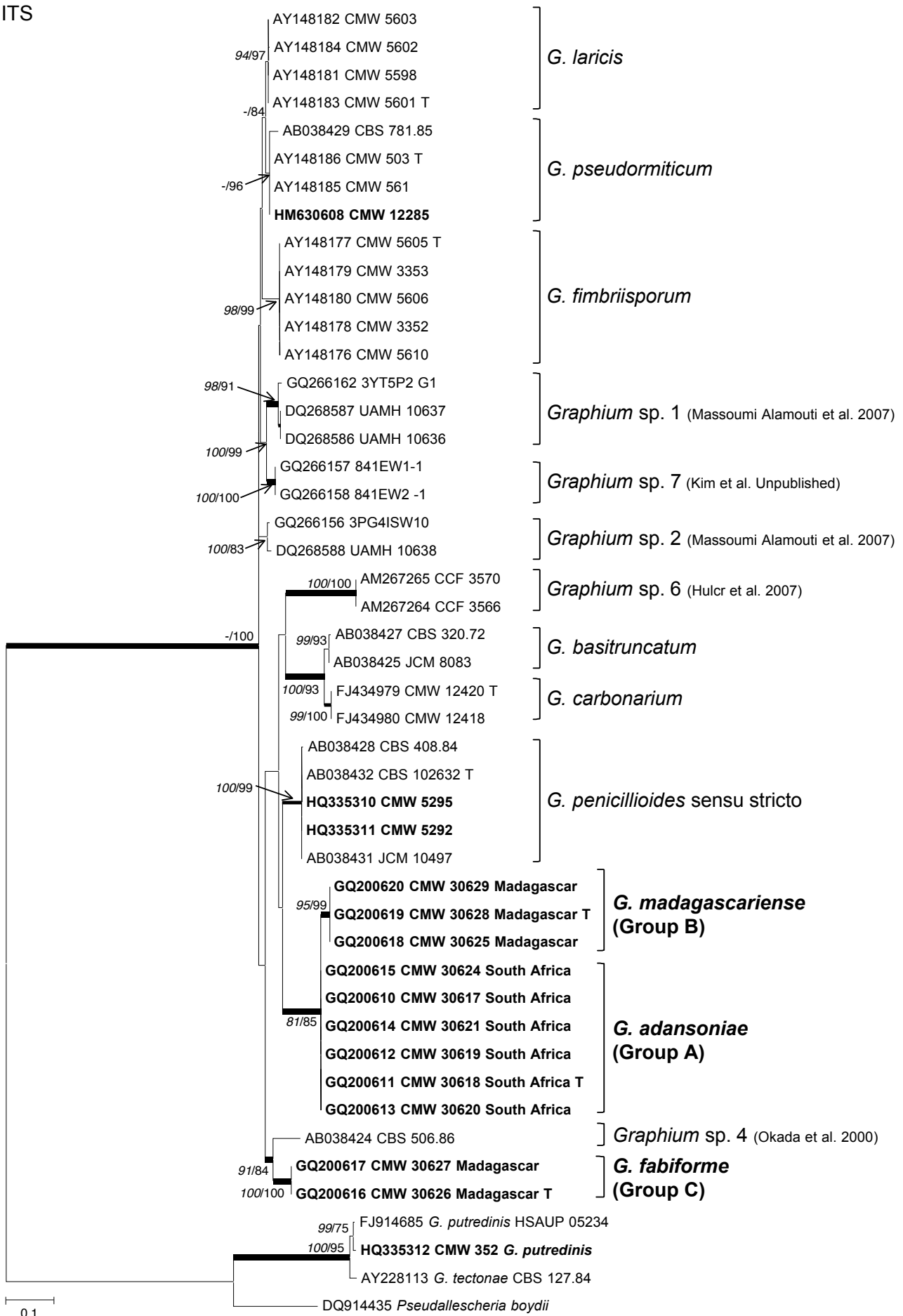
The aligned SSU dataset without introns contained a total of 1 668 characters of which 1 517 were constant and 70 characters were parsimony informative. The ITS dataset consisted of 556 characters with 256 constant and 252 parsimony informative. There were 614 characters in the TEF-1 $\alpha$  dataset including 357 constant and 255 parsimony informative characters. Based on MP, the SSU, ITS and TEF-1 $\alpha$  datasets had tree lengths of 181, 631 and 565, consistency indexes (CI) of 0.906, 0.729 and 0.789 and retention indexes (RI) of 0.953, 0.906 and 0.903 respectively. Maximum likelihood trees are displayed (Fig. 3–5) with bootstrap values for MP in italics and ML roman (*MP/ML*).

Posterior probability values higher than 0.95 are indicated with bold lines.

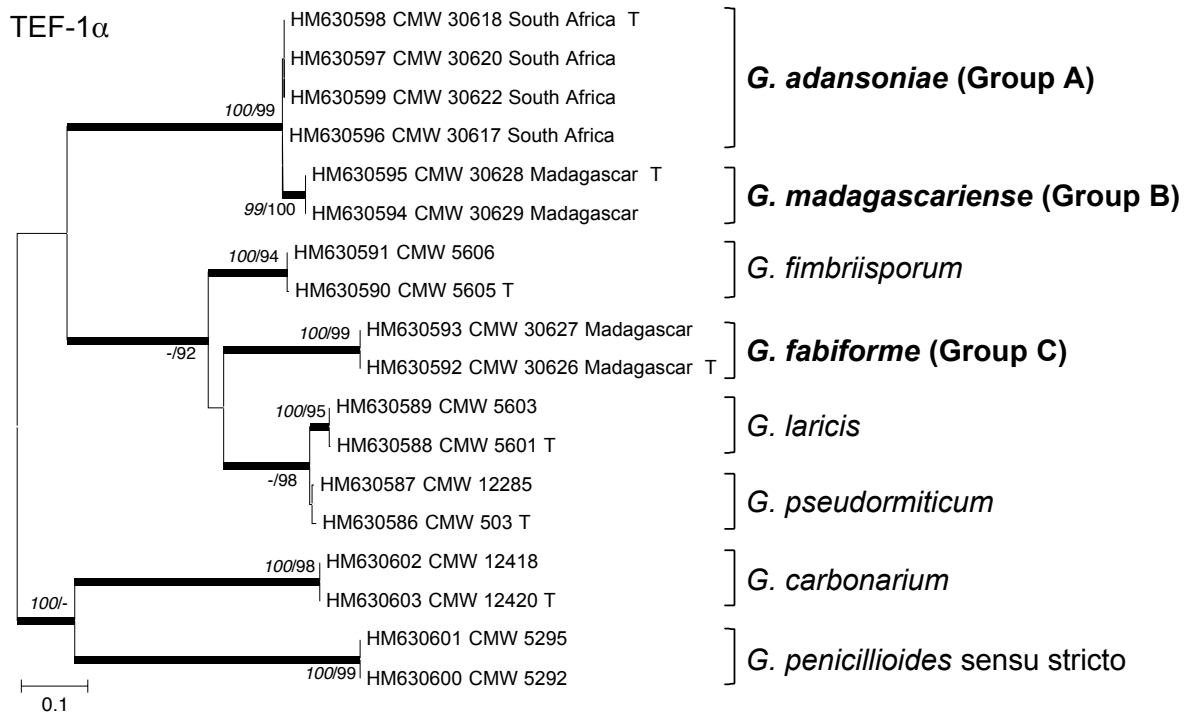
Based on the SSU gene sequences (Fig. 3) the isolates obtained from baobab trees formed a monophyletic lineage in the *G. penicillioides* complex, with *G. penicillioides*, *G. basitruncatum* and two undescribed *Graphium* species, one from Ecuador (Geldenhuis et al. 2004) and one from Costa Rica (Hulcr et al. 2007), as the closest phylogenetic neighbours. The isolates from South Africa were identical in sequence to the Madagascar isolates, except for the 372 bp group I intron that was absent in the isolates from Madagascar.

Data from the ITS gene (Fig. 4) distinguished the six known species in the *G. penicillioides* complex, and also five of the seven species from previous studies yet to be described, including *Graphium* sp. 1 and sp. 2 (Massoumi Alamouti et al. 2007), *Graphium* sp. 4 (Okada et al. 2000), *Graphium* sp. 6 (Hulcr et al. 2007) and *Graphium* sp. 7 (GQ266157, GQ266158;

ITS



**Fig. 4** Unrooted ML tree of the *Graphium penicillioides* complex based on ITS sequences. Bootstrap values for MP (*italics*) and ML (roman) above 75 % are given at the nodes. Branches with Bayesian posterior probabilities of more than 0.95 are printed in bold. GenBank accession numbers are shown in bold for isolates newly sequenced in this study.



**Fig. 5** Midpoint rooted ML tree of the *Graphium penicillioides* complex based on TEF-1α gene sequences obtained in this study. Bootstrap values for MP (*italic*) and ML (roman) above 75 % are given at the nodes. Branches with Bayesian posterior probabilities of more than 0.95 are printed in **bold**.

unpubl.). The South African isolates (Group A) grouped in a monophyletic lineage with three of the Madagascar isolates (Group B). These two groups differed from each other in 7 bp. The other two Madagascar isolates (Group C) grouped closely with the undescribed *Graphium* sp. 4 (CBS 506.86) from the study of Okada et al. (2000).

In the tree obtained with TEF-1α gene sequences (Fig. 5), all the five known *Graphium* spp. that were included formed well-supported lineages. The isolates from baobabs formed clades similar to those obtained from the ITS tree, with two Madagascar isolates in a separate clade (Group C), not closely related to any other species. The remaining isolates again formed a larger monophyletic group, but with the Madagascar isolates forming a subgroup (Group B) based on 23 bp differences from the South African isolates (Group A).

### Morphology

Isolates representing Groups A (South Africa) and B (Madagascar) had similar growth form in culture (Fig. 6, 7), but slight differences in conidial shape and size. Group B had conidia of variable size including smaller or larger conidia than those in Group A. The conidial morphology was more variable in Group B than in Group A.

Group C isolates were very different to those in Group A and B based on culture (Fig. 6i), and micromorphology. This group of isolates at first produced conidia that were cylindrical to obovoid (Fig. 6k) when just beginning to sporulate, but reniform conidia (Fig. 6l) were abundant in cultures older than four weeks. No teleomorph structures were found to be associated with any of the isolates, neither in culture nor host tissue from which the isolations were made.

The growth rates and optimal growth temperature in culture for isolates representing Group A and B differed substantially (Fig. 7). In this regard, Group A isolates all grew faster than Group B isolates at all temperatures tested, while isolates representing Group C grew the slowest (Fig. 7).

### Taxonomy

Based on DNA sequence data and morphology, the isolates from baobabs in South Africa and Madagascar could be separated into three distinct groups (Group A–C). These three groups represent distinct taxa, described here as new species in the genus *Graphium*.

***Graphium adansoniae*** Cruywagen, Z.W. de Beer & Jol. Roux, *sp. nov.* — MycoBank MB518674; Fig. 6a–d

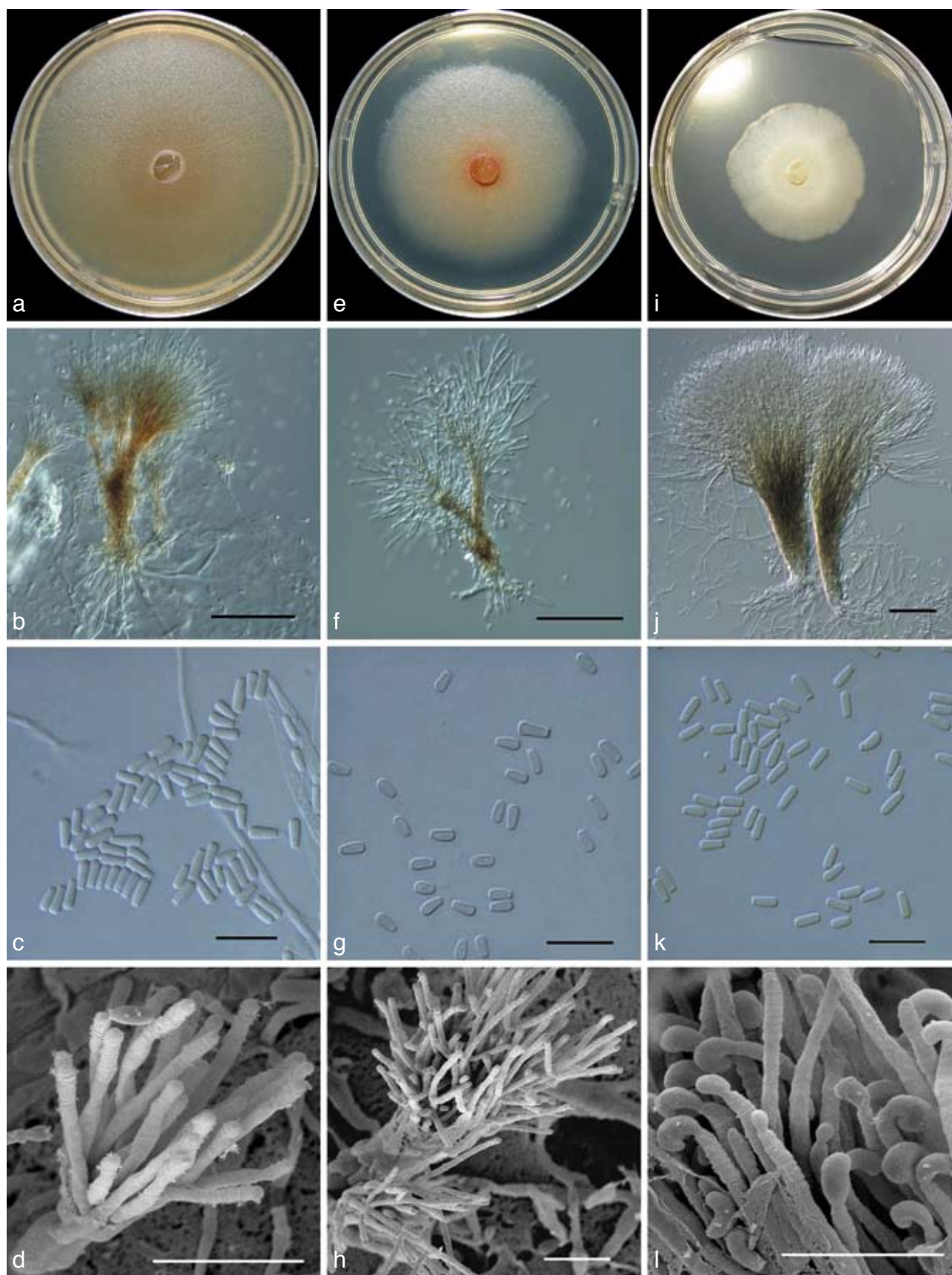
*Teleomorph.* Unknown.

Coloniae 61 mm diametro post 7 dies in 25 °C in MEA, crescunt optime in 35 °C. Coloniae mellinae, infra fulvae. Synnemata abundantia sed dispersa, ex agar orientia, plerumque singula, interdum aggregata. Stipites laete vel atrobrunnei, (68–)88–140(–151) μm longi, apice (23–)60–97 μm lati. Conidia non septata, hyalina cylindrica vel obovoidea basi truncata, (3.5–)4–5(–6) × 1.5–2.5 μm, in massa hyalina mucosa facta, primo alba, cum aetate brunnescentia.

*Etymology.* Name reflects the African baobab tree *Adansonia digitata* from which the fungus was isolated.

Colonies on MEA 61 mm diam after 7 d at 25 °C in the dark, with optimal growth at 35 °C; hazel on OA and honey on MEA; reverse buff. Mycelium mostly aerial, flat, hyphae hyaline, septate, 1–3.5 μm wide. Synnemata scattered but abundant, arising from the agar, mostly single, sometimes in groups. Stipes pale to dark brown, (68–)88–140(–151) μm long and (23–)60–97 μm wide at the apex. Conidiophores with 2–4 branches, conidiogenous cells annellated. Conidia aseptate, hyaline, cylindrical to obovoid, (3.5–)4–5(–6) × 1.5–2.5 μm, congregating in a hyaline mucilaginous mass on a synnemata; conidial mass bright transparent white when young, becoming brown with age.

*Specimens examined.* SOUTH AFRICA, Limpopo, Pafuri, on elephant damaged African baobab tree (*Adansonia digitata*), 14 Feb. 2008, E.M. Cruywagen, holotype PREM 60308, culture ex-type CMW 30618 = CBS 124917; Limpopo, Musina, on *A. digitata* tree damaged by a motor vehicle, 20 Jun. 2007, E.M. Cruywagen & J. Roux, PREM 60309, culture CMW 30620 = CBS 124915.



**Fig. 6** a–d. *Graphium adansoniae*. a. A 14 d old culture on MEA; b. synnema; c. conidia; d. annelidic conidiogenesis, SEM. — e–h. *Graphium madagascariense*. e. A 14 d old culture on MEA; f. synnema; g. conidia; h. annelidic conidiogenesis, SEM. — i–l. *Graphium fabiforme*. i. A 14 d old culture on MEA; j. synnema; k. conidia; l. annelidic conidiogenesis, SEM (a–d: CMW 30618, Group A; e–h: CMW 30628, Group B; i–l, CMW 30626; Group C). — Scale bars: b, f, j = 50  $\mu$ m; in c, d, g, h, k, l = 10  $\mu$ m.



***Graphium madagascariense*** Cruywagen, Z.W. de Beer & Jol. Roux, *sp. nov.* — MycoBank MB518676; Fig. 6e–h

*Teleomorph.* Unknown.

Coloniae 50 mm diametro post 7 dies in 25 °C in MEA, crescunt optime in 30 °C. Coloniae mellinae, infra cinnamomeae. Synnemata dispersa sed abundantia, ex agaro orientia, plerumque singula, interdum aggregata. Stipites laete vel atrobrunnei, (58–)89–174(–211) µm longi, apice (10–)15–53(–85) µm lati. Conidia non septata, hyalina cylindrica vel obovoidea basi truncata, (2.5–)3–5(–7.5) × 1.5–2(–3) µm, in massa hyalina mucosa facta, primo alba, cum aetate brunnescentia.

*Etymology.* Name reflects the island of Madagascar where this species was found.

Colonies on MEA after 7 d in the dark at 25 °C attained 50 mm diam, with optimal growth observed at 30 °C. Colony colour on OA was hazel, and on MEA honey with reverse cinnamon. Mycelium mostly aerial, flat, hyphae hyaline, septate (1.5–)2–3(–3.5) µm wide. Synnemata scattered but abundant, arising from the agar, mostly single, sometimes in groups. Stipes are pale to dark brown, (58–)89–174(–211) µm long and (10–)15–53(–85) µm wide at the apex. Conidiophores with 2–4 branches, conidiogenous cells annellated. Conidia aseptate, hyaline, cylindrical to obovoid (2.5–)3–5(–7.5) × 1.5–2(–3) µm, produced in a hyaline mucilaginous mass on the synnemata, bright transparent when young, becoming brown with age.

*Specimens examined.* MADAGASCAR, Morondava, on dead baobab tree (*Adansonia rubrostipa*), 10 Oct. 2007, J. Roux & M.J. Wingfield, holotype PREM 60312, culture ex-type CMW 30628 = CBS 124919; Morondava, on dead *A. rubrostipa* tree, 10 Oct. 2007, J. Roux & M.J. Wingfield, culture CMW 30625 = CBS 124918, CMW 30629 = CBS 124920.

***Graphium fabiforme*** Cruywagen, Z.W. de Beer & M.J. Wingf., *sp. nov.* — MycoBank MB518678; Fig. 6i–l

*Teleomorph.* Unknown.

Coloniae 16 mm diametro post 7 dies in 25 °C in MEA, crescunt optime in 30 °C. Coloniae supra infraque fulvae. Synnemata in OA abundantia sed in medio culturarum congregata, ex agaro orientia, plerumque aggregata, interdum singula. Stipites atrobrunnei, (183–)319–496 µm longi, apice (82–)308–674 µm lati. Conidia non-septata, hyalina (3–)3.5–4.5(–5) × (1–)1.5–2 µm cylindrica vel obovoidea basi truncata juventute, etiam conidia reniformia in coloniis vetustioribus. Conidia in synnematis in massa hyalina mucosa facta, primo hyalino-alba, cum aetate atrobrunnescentia vel nigrescentia.

*Etymology.* Name reflects the reniform (fabiform) shape of the conidia formed in older cultures and also the institute, FABI, where the research to describe this species was conducted.

Colonies on MEA after 10 d in the dark at 25 °C attained 16 mm diam, with optimal growth observed at 30 °C. Colony colour on MEA buff, reverse also buff; on OA colour was grey-olivaceous. Mycelium mostly aerial, flat, hyphae hyaline, septate (1.5–)2–3(–3.5) µm wide. Synnemata abundant, concentrated in the centre of cultures, arising from the agar, mostly in groups, sometimes single. Stipes dark brown, (183–)319–496 µm long and (82–)308–674 µm wide at the apex. Conidiophores with 2–4 branches, conidiogenous cells annellated. Conidia aseptate, hyaline, (3–)3.5–4.5(–5) × (1–)1.5–2 µm, cylindrical to obovoid in young cultures, with reniform shaped conidia also present in cultures older than 3 wk and abundant in 4 wk old cultures. Conidia produced in a hyaline mucilaginous mass on the synnemata, bright transparent white when young, becoming dark brown to black with age.

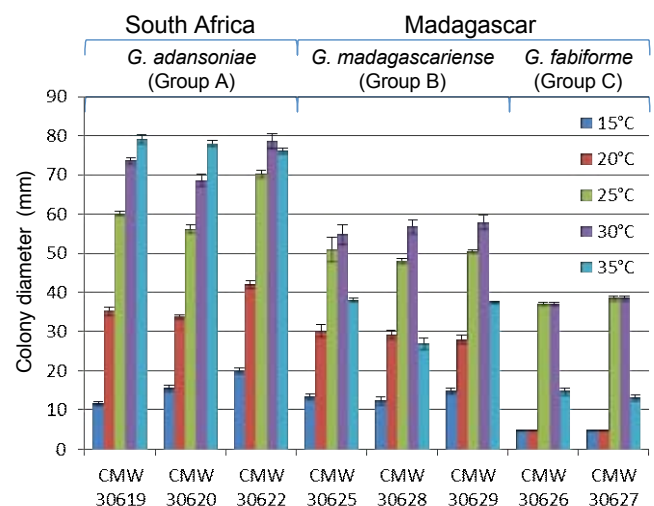
*Specimens examined.* MADAGASCAR, Morondava, on dead baobab tree (*Adansonia rubrostipa*), 10 Oct. 2007, J. Roux & M.J. Wingfield, holotype PREM 60310, culture ex-type CMW 30626 = CBS 124921; Morondava, on dead *A. rubrostipa* tree, 10 Oct. 2007, J. Roux & M.J. Wingfield, PREM 60311, culture CMW 30627 = CBS 127181.

## DISCUSSION

Three new species in the genus *Graphium* were isolated and described from baobab trees in South Africa and Madagascar. These included *G. adansoniae* from South Africa, and *G. madagascariense* and *G. fabiforme* from Madagascar. Isolates of all these species were collected from dead or wounded baobab trees and recognised based on DNA sequence and morphological comparisons. In addition to the SSU and ITS sequences used to delineate species in this group in the past (Okada et al. 2000, Jacobs et al. 2003, Geldenhuys et al. 2004, Massoumi Alamouti et al. 2007, Hulcr et al. 2007), the TEF-1α gene sequence data for species in the *G. penicillioides* complex, including the three new species, are presented for the first time in this study.

These three new species reside in *Graphium* established by Corda in 1837, which at present includes the *G. penicillioides* and *G. putredinis* complexes in the *Microascales* (cf., Okada et al. 1998). These two species complexes were shown by Issakainen et al. (1997, 1999) and Okada et al. (1998) to be phylogenetically very different from species in the *Ophiostomatales* that at one time accommodated the genus *Graphium* (Seifert & Okada 1993). Those species related to the *Ophiostomatales* with *Graphium* morphology in appearance now reside in *Pesotum*, reinstated for synnematos anamorphs in the *Ophiostomatales*. In contrast, *Graphium* was redefined to include all synnematos species in the *Microascales*, including those in the *G. penicillioides* and *G. putredinis* complexes (Okada et al. 1998, 2000). The *G. putredinis* aggregate is phylogenetically related to *Parascedosporium*, *Petriella*, *Pseudallescheria* and *Scedosporium* species that are pathogenic to humans (Issakainen et al. 1997, 1999, de Hoog et al. 2000, Okada et al. 2000, Gilgado et al. 2007).

The *G. penicillioides* species complex forms a distinct lineage in the *Microascales* and does not have known teleomorph connections. This group includes the type species for the genus, *G. penicillioides*, and thus represents *Graphium* s.str. In addition to *G. penicillioides*, six known and seven undescribed species were recognised in the complex based on ITS sequences (Okada et al. 2000, Geldenhuys et al. 2004, Hulcr et al. 2007, Massoumi Alamouti et al. 2007, unpublished data in GenBank (GQ266157, GQ266158)). Most of these species have been isolated from insect galleries or exposed sapwood of trees (Jacobs et al. 2003, Geldenhuys et al. 2004, Hulcr et al. 2007, Paciura et al. 2010). It is, therefore, not surprising that the



**Fig. 7** Average colony diameters (mm) of the selected *Graphium* isolates from baobabs, on MEA at 10 °C, 15 °C, 20 °C, 25 °C, 30 °C and 35 °C after 10 d.

newly described *G. adansoniae*, *G. madagascariense* and *G. fabiforme* from wounds on baobab trees, form part of this complex. Along with the undescribed *Graphium* species from native *Schizolobium parahybum* in Ecuador (Geldenhuis et al. 2004) and *Cecropia* in Costa Rica (Hulcr et al. 2007), these species from *Adansonia* are the first *Graphium* species to be reported from angiosperms in the tropics or subtropics. Apart from these three species, the only other species previously reported from Africa was *G. pseudormiticum*, from a pine-infesting bark beetle on non-native pine trees in South Africa and thus most likely a fungus native to Europe (Mouton et al. 1994).

An intriguing outcome of this study was that two of the most closely related new *Graphium* species were isolated from different *Adansonia* species on two very distinct land masses. *Graphium adansoniae* was found on *A. digitata* in Africa and *G. madagascariense* was isolated from *A. rubrostipa* in Madagascar. These results suggest that these fungi have some degree of host or substrate specificity. In a recent study, Pock Tsy et al. (2009) showed that baobab trees probably originated in West Africa and subsequently spread to other parts of the world where they speciated. The similarity in fungi isolated from different tree species on separate land masses, raises intriguing questions regarding the movement of fungi between these areas.

*Graphium* species are insect vectored (Jacobs et al. 2003, Geldenhuis et al. 2004, Massoumi Alamouti et al. 2007, Hulcr et al. 2007) and it is probable that there would be some relatedness among the insects that vector these fungi in Africa and in Madagascar. A study of these interactions would most likely lead to interesting observations about the biogeography and ecology of these microascalean fungi. This knowledge would add to intriguing patterns that are emerging relating to the occurrence of the sister species of *Gondwanamyces* in Africa (Marais & Wingfield 2001, Roets et al. 2009) and in Central America (Kolařík & Hulcr 2009). Similar to these two landmasses, Africa and Madagascar also share a common Gondwanaland ancestry which most likely played a role in the evolution of baobab species and their associated fungi and insects.

Another interesting discovery in the present study was that we found group I introns in the ribosomal SSU of one of the newly discovered species, *G. adansoniae*. These sequences corresponded well with the group I intron sequences previously found in *G. basitruncatum*, *G. putredinis*, and *Graphium* sp. 3 (Okada et al. 1998), that were later classified as group IC1 introns (<http://www.rna.cccb.utexas.edu>). Holst-Jensen et al. (1999) showed that group IC1 introns in several genera of the *Sclerotiniaceae* followed trends similar to what we observed for the *Graphium* isolates: 1) that introns were consistently present or absent at species level, but not at generic level; and 2) that introns were unique for each species. Our data also supported the suggestion by Haugen et al. (2005) that group I introns in the same insertion site are evolutionarily related. Holst-Jensen et al. (1999) found that their ITS and intron phylogenies were incongruent, leading them to conclude that introns and rDNA do not share a common evolutionary history. The reason for this is that introns are most likely transferred horizontally (Holst-Jensen et al. 1999, Keeling & Palmer 2008), and care should thus be taken in the interpretation of phylogenies constructed using intron sequences. However, our results and those of Holst-Jensen et al. (1999) and Haugen et al. (2005), suggest that group I intron sequences might be useful in addition to sequence data from other genes and biological data to distinguish between closely related taxa.

The discovery of three new species of *Graphium* adds knowledge to the poorly documented fungal biodiversity in Africa. In this respect, Crous et al. (2006) estimated that there could be around seven species of unique fungi on each native plant in

southern Africa. Clearly, only a fraction of these have been described and fewer have been studied in terms of their ecological roles. Surprisingly few fungi have been recorded from the iconic and biogeographically important baobabs. Future studies of the fungi on these trees will likely contribute interesting insights into the ecology, biodiversity and biogeography of fungi.

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