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First genetic identification of *Pilobolus* (Mucoromycotina, Mucorales) from Africa (Nairobi National Park, Kenya)

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ABSTRACT

Pilobolus are abundant in herbivore dung. Although they are non-pathogenic to herbivores, they are vectors to lungworms present in the dung that cause bronchitis if ingested. Thus, determining the presence of *Pilobolus* in the field might prove useful to assess a link between *Pilobolus* and lungworm infections, as well as the areas where they occur. Species identification within the genus has mostly relied on morphological data, which lacks accuracy due to the overlap of morphological characters. In this study, we applied genetic identification to assess the presence of *Pilobolus* species in Nairobi National Park, Kenya. This method is more reliable than the more commonly used morphometric analyses, and the generated sequences are useful for future reference. In this study, we collected dung samples from different herbivores in Nairobi National Park, and sporangium isolates were obtained for pure cultures. DNA was extracted from the pure cultures and fungal barcode primers were employed for PCR amplification and sequencing. Two species of *Pilobolus* were identified, *Pilobolus pullus*, with a high genetic affinity, and a second cryptic species, morphologically identified as *Pilobolus crystallinus*, but did not have a close genetic match to any species in Genbank. Lack of identification of other *Pilobolus* species, known as key species in lungworm transmission, may suggest the absence of such in the sampled area.

1. Introduction

Pilobolus (Wigg, 1784) is a globally widespread genus of coprophilous fungi from the order Mucorales. Coprophilous fungi play a significant role in the decomposition of herbivore dung and are therefore vital in nutrient recycling in ecosystems (Richardson, 2008). Coprophilous fungi form an important food source for mycophagous arthropods that thrive on herbivore dung and, therefore, form an integral component of the detritus food webs (Wicklow and Angel, 1974; Shaw, 1992).

Some *Pilobolus* species are implicated in the spread of equine and bovine lungworms (*Dictyocaulus* sp.), causative agents for parasitic bronchitis of herbivores (Jørgensen et al., 1982). *Pilobolus kleinii* (Van Tieghem, 1878) and *Pilobolus crystallinus* (Wigg, 1784) have specifically been identified as the major species involved in the spread of lungworms in animals. Lungworms live in the lungs of infected animals although some lungworm eggs are swallowed by animals and pass through the gastrointestinal tract and are secreted along with faeces. The eggs in the faeces hatch to produce larvae, which are infective when ingested. Herbivores tend to avoid foraging near faeces of conspecifics (Cooper et al., 2000; Walsh et al., 2013). Because larvae are not capable of moving far on their own, they attach to *Pilobolus* sporangia to disperse with the fungal spores using the "projectile" dispersal system characteristic of this genus (Yafetto et al., 2008). Consequently, lungworm larvae are then dispersed away from the dung where they are ingested by foraging herbivores (Eysker, 1991).

Identification of *Pilobolus* species in the park is also essential to indicate some environmental factors present throughout the park. High species diversity shows that there is environmental stability while low species diversity could indicate ecosystem stress (Ebersohn and Eicker,

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1992). More importantly, Nairobi National Park is only 7 km away from the centre of Nairobi City and thus the presence of lungworm carrying species in the area may suggest the presence of lungworms being transmitted from wild to livestock animals. Furthermore, previous research on species characterization and diversity of *Pilobolus* species has concentrated on sources from domestic herbivores with very limited attention to sources from wild herbivores (Richardson, 2008; Pierce and Foos, 2011). Extensive research on the diversity of *Pilobolus* has been carried out in temperate regions (Foos and Sheehan, 2011; Foos et al., 2011) whereas limited research has been conducted in Africa. Research on *Pilobolus* in the African tropics has mostly focused on morphological characterization of the genus (Caretta et al., 1998). To the best of our knowledge, there are no molecular genetic studies on the African *Pilobolus* species and yet such studies are useful in understanding the evolution and taxonomy of this genus in Africa. Genetic identification is needed to complement past and ongoing taxonomic descriptions, likely to be unreliable only on morphological characterization.

Unlike morphological techniques, molecular taxonomy through the assessment of phylogenetic relationships is particularly useful in the identification of cryptic species (Bidochka et al., 2001). In addition to complementing morphological identification, molecular techniques may be the only method of choice when identification of species through chemical culture media or morphology is not feasible (Wu et al., 2003; Iotti et al., 2005; Schwarz et al., 2006; Herrera et al., 2011). Internal transcribed spacer (ITS) regions of fungal DNA is known to be suitable for species identification, distinguishing between intraspecific and interspecific variation in a broad spectrum of fungi (Schoch et al., 2012). The ITS region is taxonomically useful and, the sequences can be used to identify the *Pilobolus* species (Foos and Sheehan, 2011). Nairobi National Park is home to many host–herbivore species including the critically endangered Black Rhino (*Diceros bicornis*) and White Rhino (*Ceratotherium simum*), among many others. Thus, this study is important to ascertain whether possible vectors for lungworm disease are present in the park, ultimately identifying the possible threat of pulmonary bronchitis infecting herbivores.

2. Materials and methods

Sixty-five dung samples from 13 different herbivore species were collected from Nairobi National Park (Kenya) between July 2013 and May 2014 (Table 1). The Nairobi National Park central coordinates are 1°22′24″S 36°51′32″E and has a warm, temperate climate with average rainfall of 900 mm and average temperature of −18 °C. It consists of savannah ecosystems with scattered acacia and open grass plains. Wild herbivore dung samples were collected from different locations within the park (Fig. 1).

The samples were incubated in the laboratory in sterile Petri dishes lined with Whatman® cellulose filter paper at room temperature (23–25 °C) under natural light. The set-up was monitored daily for sporulation under a stereomicroscope (LEICA Microsystems). Upon sporulation, sporangiophores were picked using a sterile needle, placed on slides with a drop of water and examined under a light microscope (LEICA Microsystems). The morphological features such as shape, size, colour, height and width were observed and measured for each sporangiophore using LAS Suite software and used to assign species using the dichotomous key provided by Viriato (2008). Sporophores of *Pilobolus* species were also grown on dung agar prepared according to Swartz (1934). Isolates from the pure cultures were examined under a light microscope to confirm species identity morphologically. A sterile wire loop was used to collect spores that had shot to the opposite side of the flasks from the media.

Extraction of fungal DNA was done using the ZR Fungal/Bacterial DNA Miniprep™ kit (ZymoResearch, The Epigenetics Company™) according to the manufacturer's protocol. The PCR was carried out in a total volume of 25 μl containing 1× standard Taq reaction buffer, 200 μM of dNTPs, 0.2 μM of forward and reverse primer, ≥500 ng of template DNA and 0.7 units of Taq polymerase. The PCR ran in an Applied Biosystems thermocycler under the following cycling conditions: 3 min initial denaturation at 95 °C, 35 cycles of 30 second denaturation at 95 °C, 30 second primer annealing at temperatures specific for each of the primers, 1 min extension at 72 °C, and a final 10 min extension at 72 °C then maintained at 4 °C. Universal fungal primers ITS 4 and ITS 5 primers that target a short fraction of the 18S ribosomal RNA partial gene sequences, the internal transcribed spacer, 5.8S ribosomal gene, internal transcribed spacer 2 and a short partial gene sequence of the 28S ribosomal RNA, were used. The oligonucleotide sequences for the forward and reverse primers was 5′TCCTCCGCTTATTGATATGC-3′ and 5′-GGAAGTAAAAGTCGTAACAAGG-3′, respectively (Iotti et al., 2005).

The PCR product was visualized using agarose gel electrophoresis to determine whether PCR reactions were successful. The remaining PCR products were purified using the QIAquick PCR Purification kit (Qiagen Germany) according to the manufacturer's protocol. The purified PCR products were sequenced by direct cycle sequencing using an ABI PRISM DigDye Terminator v3.1 cycle sequencing kit. Sequence analysis was done on an AB1310 DNA (Applied Biosystems, CA).

Blast searches were conducted in Genbank with the recovered haplotypes to identify and assess the phylogenetic relationship of the sequenced *Pilobolus* species. All *Pilobolus* homologous sequences were downloaded from Genbank and included in the alignment, with only haplotypes in the final alignment. Preliminary alignments and exploratory analyses included *P. crystallinus*, *Pilobolus roridus* and *Pilobolus umbonatus* but these showed too high divergence to be aligned with the remaining taxa. Because of the morphological similarity encountered between 002, 008, 009, 010, 007 and 006 to *P. crystallinus*

Table 1
Dung piles collected per host species and *Pilobolus* species recovered from each using morphological means. (Note: *P. crystallinus* identified morphologically had the highest genetic affinity to *P. heterosporus*.)

Animal	Number of dung piles	*P. crystallinus*	*P. pullus*	*P. heterosporus*	Number of *Pilobolus* species recovered
Ceratotherium simum	7	–	–	–	0
Equus quagga	9	4	1	–	2
Kobus ellipsiprymnus	3	–	1	–	1
Aepyceros melampus	7	1	–	–	1
Syncerus caffer	9	1	3	–	2
Giraffa camelopardalis	8	–	2	–	1
Eudorcas thomsonii	2	–	2	–	2
Alcelaphus buselaphus	9	2	–	–	1
Madoqua kirkii	2	1	–	2	2
Lepus timidus	2	2	–	–	1
Nanger granti	3	1	–	–	1
Hippopotamus amphibius	1	–	–	–	0
Taurotragus oryx	3	2	2	–	2
Totals	65	14	11	2	

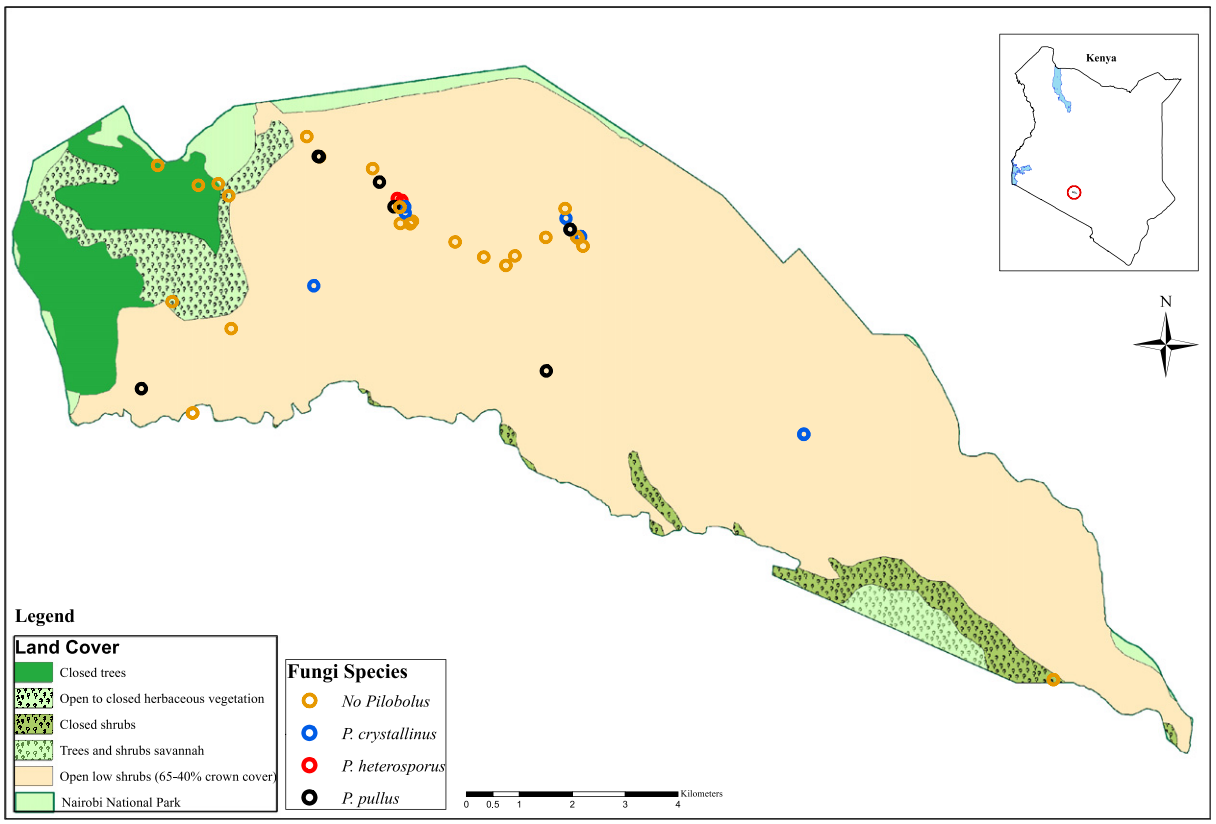

Fig. 1. Map of Nairobi National Park showing dung collection sites and presence or lack of *Pilobolus*.

(see Results section) we also attempted to run analyses with the less divergent *P. crystallinus* strain (FJ160949) alone but again alignment parameters could not be established with confidence.

The high genetic variability in relation to the remaining taxa implied a high uncertainty in the alignment, resulting in large indel regions. In addition, high nucleotide dissimilarity of some regions meant that homology could not be inferred with confidence and slight modifications by eye changed the relationships between clades and overall tree topologies. In view of this, *P. crystallinus*, *P. roridus* and *P. umbonatus* were unsuitable for this particular analysis and were therefore excluded from the final alignment. Sequences were aligned in Seaview v.4.2.11 (Gouy et al., 2010) under ClustalW2 (Larkin et al., 2007) default settings but needed considerable editing and inclusion of indel regions. Overall the different alignments resulted in similar tree topologies.

The most appropriate substitution model for the Bayesian inference was determined by the Bayesian information criterion (BIC) in jModeltest v.0.1.1 (Posada, 2008). MrBayes v.3.1.2 (Huelsenbeck and Ronquist, 2001) was used with default priors and Markov chain settings and with random starting trees. The gamma shape parameter and proportion of invariant sites were estimated from the data. Each run consisted of four chains of 20,000,000 generations, sampled each 10,000 and posterior distributions of parameter estimates were visually inspected in Tracer v1.5 (Drummond and Rambaut, 2007). A plateau was reached after few generations with 10% of the trees resulting from the analyses discarded as burn in.

Phylogenetic relationships among haplotypes for each locus were estimated using a Maximum Likelihood (ML) approach, as implemented in the software RAxML v7.0.4 (Silvestro and Michalak, 2010), using the default settings. The ML 50% bootstrap consensus tree was built in PAUP 4 (Swofford, 2002). All analyses were performed through the CIPRES platform (Miller et al., 2010). Previous phylogenetic analyses on more conserved regions (Foos and Sheehan, 2011; Foos et al., 2011) have shown *P. kleinii* and *Pilobolus sphaerosporus* are sister clade to *Pilobolus longipes* and *Pilobolus heterosporus*. Thus, because of the uncertainty of the most suitable outgroup (*P. kleinii* vs *P. sphaerosporus*) we run all phylogenetic analyses unrooted.

3. Results

Pilobolus species identified through morphological characters were recorded for each dung pile from each host species (Table 1). However, during successive transfers to obtain pure cultures of single species for molecular analysis, the failure rate was high, with a total of 11 successful transfers. Eleven isolates of *Pilobolus* were obtained from the dung of 9 herbivore species. Sequences from two African buffalo (*Syncerus caffer*) cultures showed low amplification signals and were excluded from the final analyses.

3.1. Pilobolus sp (Fig. 2)

Trophocyst subglobose, up to 370 × 360 μm with rhizoidal extension up to 980 μm, yellowish pigmentation. Sporangiophore long-cylindrical, unbranched, phototrophic, up to 4 mm × 100 μm. Sporangia black, hemispherical to ovoid up to 480 × 250 μm. Columellae conical and smooth-walled. Subsporangial vesicle elliptical, smooth-walled, orange pigmentation up to 700 × 530 μm. Sporangiospores yellow, grainy content, smooth-walled, ellipsoid up to 8 × 5 μm. This species was collected from dung of the following hosts: buffalo (*S. caffer*), dikdik (*Madoqua kirkii*), eland (*Taurotragus oryx*), Grant's gazelle (*Nanger granti*), hartebeest (*Alcelaphus buselaphus*), impala (*Aepyceros melampus*), hare (*Lepus timidus*), and zebra (*Equus quagga*).

The described species (*Pilobolus* sp.) is morphologically similar to *P. crystallinus* var. *crystallinus* (F. H. Wigg.) Tode Schrift. Berl. Gesell. Naturf. Freunde 5: 47 (1784). Some of the isolates from this study produced sporangiospores bigger than those described by Tode.

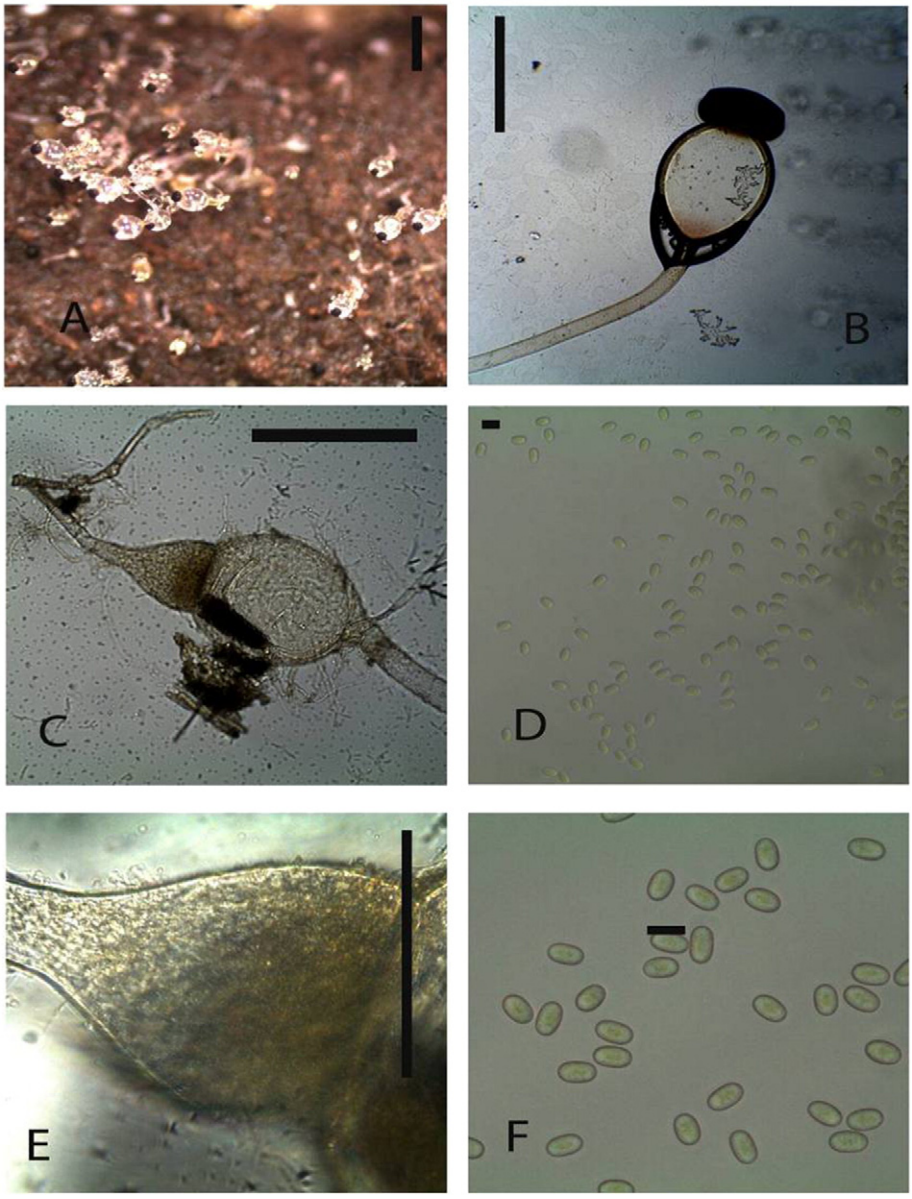

Fig. 2. *Pilobolus* sp. A: *Pilobolus* on substrate. B: Sporangiophore with sporangium and columellae. C: Trophocyst and rhizoidal extension. D, F: Sporangiospores. E: Trophocyst. *Bars*: A = 2000 μm; B = 500 μm; C = 200 μm; D–F = 50 μm.

3.2. *Pilobolus pullus* (Masse, 1899) (Fig. 3)

Trophocysts ovoid to globose, hyaline up to 180 μm diam. Sporangiophore long-cylindrical, up to 720 × 90 μm. Sporangia black, hemispherical up to 270 × 140 μm. Columellae conical and smooth-walled. Subsporangial vesicle hyaline, smooth-wall, slight yellow pigmentation, ovoid, up to 370 × 200 μm. Zygospores yellow, subcylindrical, homogenous content, up to 9 × 5 μm. This species was collected from buffalo (*S. caffer*), giraffe (*Giraffa camelopardalis*), eland (*T. oryx*), zebra (*E. quagga*), waterbuck (*Kobus ellipsiprymnus*) and Thomson's gazelle (*Eudorcas thomsonii*) dung.

The isolates from this species agrees with the description of Masse (1899) and Naumov and Pierre (1939). However, the rhizoidal extension for some of the fungal isolates was longer than 300 μm that has been previously described.

Pilobolus ITS sequences varied from 604 to 684 base pairs in length (Genbank accessions: KP760860–68). The alignment resulted in a 772 bp consensus including indels. Blast searches matched *P. heterosporus* and *P. pullus*, which were included in the alignment. Six sequences (*Pilobolus* sp.) matched *P. heterosporus* (89 and 91%), while three sequences showed high similarity to *P. pullus*, with 99% identity. A total of seven haplotypes were obtained, with samples 002, 008 and 009 recovering the same haplotype (Table 1).

Genbank blasts matched the genomic regions to be 18S ribosomal RNA partial gene sequence (~40 bp), the internal transcribed spacer 1 (~230 bp), 5.8S ribosomal gene (~150 bp), internal transcribed spacer 2 (~230 bp) and 28S ribosomal RNA partial gene sequence (~45 bp), of other *Pilobolus* species, which varied slightly depending on the species compared due to the inclusion of indels.

The best fitting model for the BI tree was the TPM1uf + G (-lnL 3915.69392, BIC = 8296.997871). The Effective Sample Size (ESS) values for all runs were over 1800, thus confirming good convergence mixing of all mcmc runs. All analyses recovered a well-resolved monophyletic clade of strains 003, 004, and 005 (Genbank accessions KP760861–63) to with *P. pullus* (Bayesian posterior probability, BPP 1.00, ML bootstrap 100%) but the position of this clade within the tree recovered low support (BPP: 0.85, ML: 73%). The clade formed by samples 002, 008, 009, 010, 007 and 006 (Genbank accession KP760860,

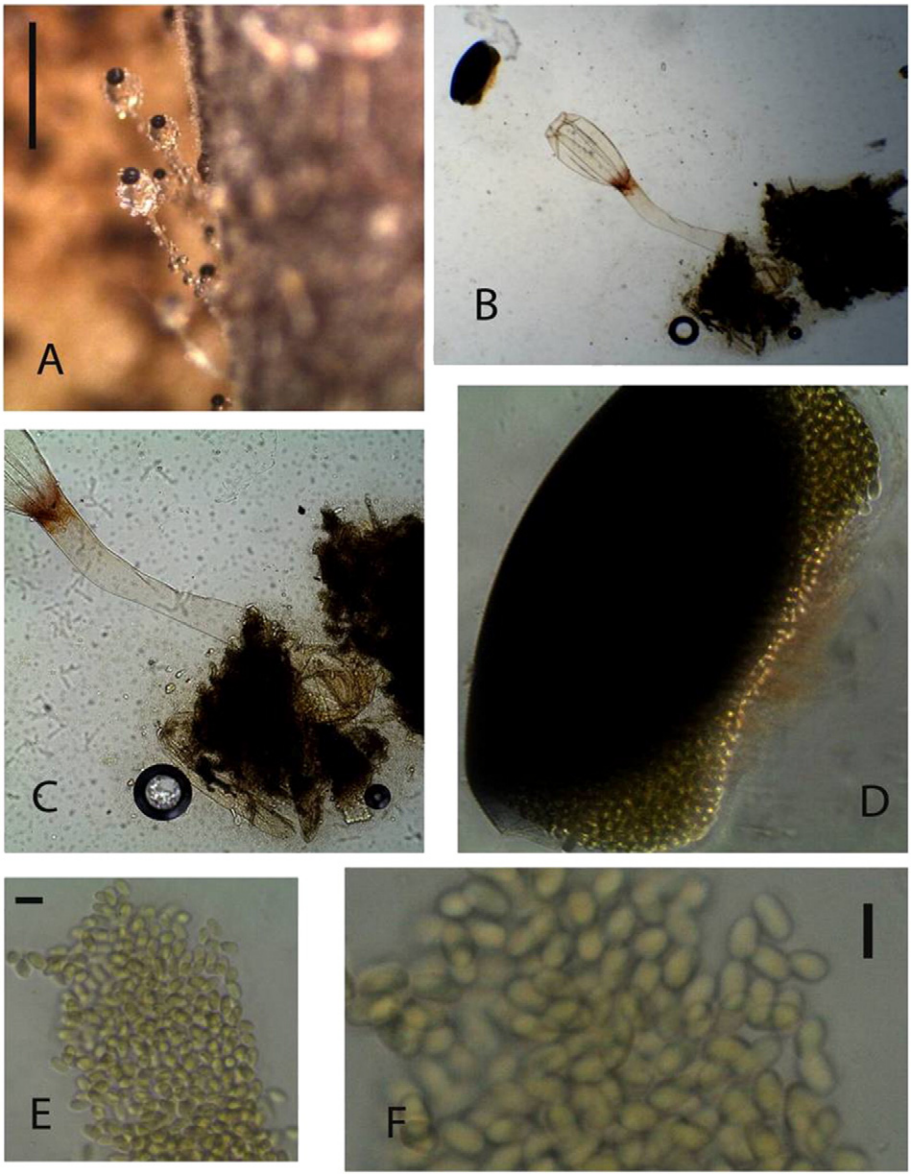

Fig. 3. *Pilobolus pullus* A: *Pilobolus* on substrate. B: *Pilobolus* squashed in glycerol. C: Columellae and sporangiophore. D: Sporangium. E–F: Sporangiospores. *Bars*: A = 2000 μm; B = 500 μm; C = 200 μm; D–E = 50 μm; F = 20 μm.

KP760864–68) is monophyletic (BPP 1.00, ML 100%) and sister taxa to *P. heterosporus*. All *Pilobolus* species are highly supported in all analyses. *P. longipes* is basal to the *P. heterosporus* and its sister clade *Pilobolus* sp.

4. Discussion

Taxonomic identification of *Pilobolus* species has mostly relied on morphological characters (Hu et al., 1989; Cavalcanti and Trufem, 2008; Viriato, 2008). However, the classification of such might be obscured by the presence of cryptic species, thus lacking the accuracy to assess differences within species and the assessment of genetic divergence (Pierce and Foos, 2011). This study sought to use the molecular identification of *Pilobolus* samples growing in herbivore dung in the large Kenyan savannah. Our results confirmed the validity of such markers at genus and intra-generic level (Schoch et al., 2012).

Pilobolus blast searches matched *P. pullus* closely and *P. heterosporus* more distantly, suggesting the identification of *P. pullus* and a new undescribed species, *Pilobolus* sp. Interestingly, sequences from our study identified morphologically as *P. crystallinus* (*Pilobolus* sp., this study) showed high genetic variation to all other available *Pilobolus* sequences in Genbank, with the closest match to *P. heterosporus* (89–91%). These findings are similar to those of Foos and Sheehan (2011) who reported varying levels of genetic identity (between 59.7 and 82%) of homologous ITS regions among species of *Pilobolus* in Genbank. We attribute this to possible genetic variation depending on location and hosts.

Morphological identification of *Pilobolus* sp. as *P. crystallinus* is questionable as supported by previous reports of errors in using morphological characteristics. For example, spore length that is one of the most important features used to identify species is given as a range, thus making an overlap of sizes between species highly likely (Foos and Jeffries, 1988; Foos et al., 2011). Furthermore, morphological characteristics of *Pilobolus* grown on artificial media on pure cultures have been shown to vary slightly compared to those growing on dung (Foos et al., 2011).

Past morphological identification studies on *Pilobolus* have clumped several species together, possibly obscuring the real species richness within the genus. For example, *Pilobolus hyalosporus*, *P. kleinii* and *P. crystallinus* only differ morphologically in spore size and colour and Hu et al. (1989) suggested collapsing the three into a single species:

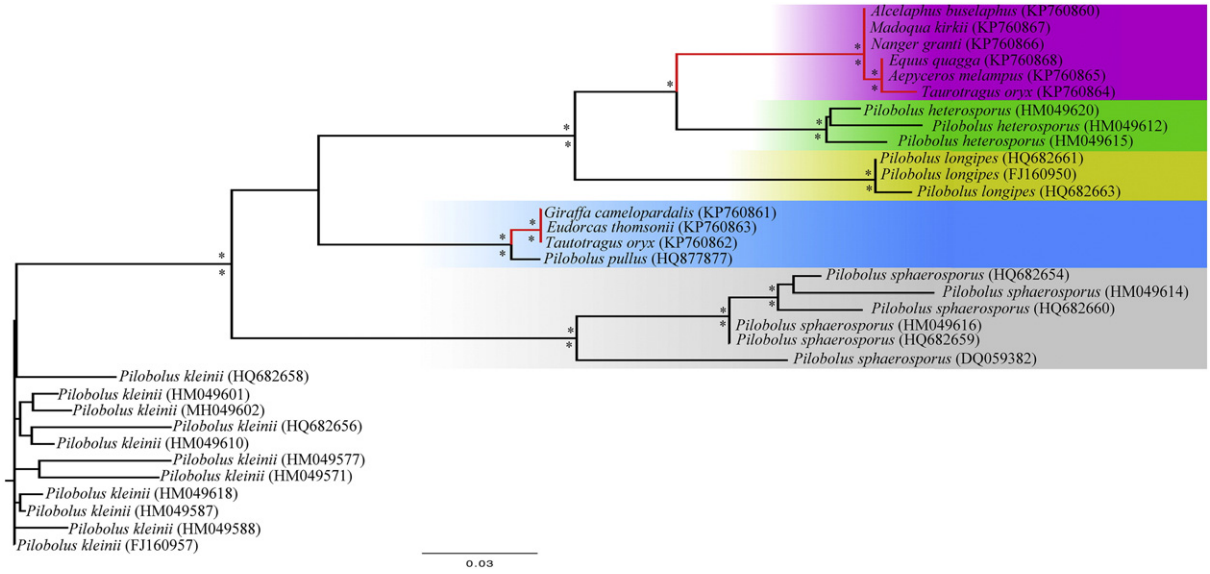

Fig. 4. Best ML tree for all *Pilobolus* sp. Asterisks (*) on and under nodes are posterior probabilities recovered from the Bayesian Inference analyses and bootstrap support from the ML 50% majority rule consensus tree (95% or above), respectively. Different clades are colour coded and nodes from this study are marked in red. Sequences from this study are labelled by their host species.

P. crystallinus with three varieties. However, after sequencing SSU and ITS rDNA sequences, it is clear that the three are distinct and should remain as separate species (Foos et al., 2011). Chances of misidentification while using morphological characters are high: consequently, it is pivotal to back such work through genetic identification. In addition, there are some species that need taxonomical revision through DNA data and are likely to represent several species that are currently classified under one species (O'Donnell et al., 2001; Stajich et al., 2009). Such an example is the species morphologically described in this study as *Pilobolus* sp. to *P. crystallinus*, which genetically is a highly divergent species.

Pilobolus samples identified morphologically as *P. pullus* showed a very high genetic affinity (99%) to others available in Genbank. Such genetic affinity and the well-supported monophyletic relationship (Fig. 4) to *P. pullus* from a different continent (North America) under different geographical and environmental conditions are surprising and may suggest that some *Pilobolus* lineages are very conserved. Further work on African *Pilobolus* species will elucidate important information on the genetic diversity of the genus.

P. crystallinus and *P. kleinii*, identified as the key species in the transmission of lungworms (*Dictyocaulus viviparus*) (Foos, 1997), were not identified through our genetic matches in this study. This may suggest a low transmission rate of the parasite in Nairobi National Park, indicating a low risk of bronchitis in the sampled areas through the lack of vectors to herbivores. Nevertheless, more research is needed to assess the full spectrum of species present in Nairobi National Park and other wildlife protected areas in Kenya to determine whether other *Pilobolus* species identified are also vectors for the parasites.

The presence of lungworms has been reported in wild animals such as hartebeest and wildebeest in Kenya (Spinage, 2012). Lungworm infection and clinical disease have also been confirmed in domestic animals in sub-Saharan Africa as well as neighbouring countries such as Tanzania and Uganda (Aruo, 1973; Over et al., 1992; Thamsborg et al., 1998) and thus we recommend future studies on the presence of lungworms in herbivore dung at Nairobi National Park (Jorgensen and Madsen, 1982). Comparison of such results with the findings of our study will help elucidate important information on the actual number of *Pilobolus* species implicated in the spread of the disease and the distribution of such. This will also give insight on the animals that are infected and provide a basis for intervention, especially for critically endangered species that could be eradicated by such illnesses.

Currently, there are only eight species of *Pilobolus* available in Genbank out of over sixty species described morphologically. This shows that there is little data to compare sequences and highlights the need to conduct further species genetic identification within the genus. To the best of our knowledge, apart from those generated in this study, there are no other *Pilobolus* sequences collected from Africa. More importantly, little information is available regarding the possible relationship between these fungi and its wildlife hosts (Pierce and Foos, 2011). This is further evidence of the lack of knowledge in this field and the need to further understand through genetic identification the biodiversity of the genus and the phylogenetic relationships to those already described.

Disclosures

The authors declare no conflict of interest.

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