Sloth Hair as a Novel Source of Fungi with Potent Anti-Parasitic, Anti-Cancer and Anti-Bacterial Bioactivity

Sarah Higginbotham1*, Weng Ruh Wong2, Roger G. Linington2, Carmenza Spadafora3, Liliana Iturrado1, A. Elizabeth Arnold4

1 Smithsonian Tropical Research Institute, Panama, Republic of Panama, 2 Department of Chemistry and Biochemistry, University of California Santa Cruz, Santa Cruz, California, United States of America, 3 Instituto de Investigaciones Científicas y Servicios de Alta Tecnología, Panama, Republic of Panama, 4 School of Plant Sciences, University of Arizona, Tucson, Arizona, United States of America

Abstract

The extraordinary biological diversity of tropical forests harbors a rich chemical diversity with enormous potential as a source of novel bioactive compounds. Of particular interest are new environments for microbial discovery. Sloths – arboreal mammals commonly found in the lowland forests of Panama – carry a wide variety of micro- and macro-organisms on their coarse outer hair. Here we report for the first time the isolation of diverse and bioactive strains of fungi from sloth hair, and their taxonomic placement. Eighty-four isolates of fungi were obtained in culture from the surface of hair that was collected from living three-toed sloths (Bradypus variegatus, Bradypodidae) in Soberanía National Park, Republic of Panama. Phylogenetic analyses revealed a diverse group of Ascomycota belonging to 28 distinct operational taxonomic units (OTUs), several of which are divergent from previously known taxa. Seventy-four isolates were cultivated in liquid broth and crude extracts were tested for bioactivity in vitro. We found a broad range of activities against strains of the parasites that cause malaria (Plasmodium falciparum) and Chagas disease (Trypanosoma cruzi), and against the human breast cancer cell line MCF-7. Fifty fungal extracts were tested for antibacterial activity in a new antibiotic profile screen called BioMAP; of these, 20 were active against at least one bacterial strain, and one had an unusual pattern of bioactivity against Gram-negative bacteria that suggests a potentially new mode of action. Together our results reveal the importance of exploring novel environments for bioactive fungi, and demonstrate for the first time the taxonomic composition and bioactivity of fungi from sloth hair.

Introduction

Despite vast increases in spending on international healthcare over the last 20 years, communicable diseases continue to represent an enormous burden to global health [1]. Chronic infectious illnesses such as malaria and diverse neglected tropical diseases (NTDs) affect millions of people each year, mainly women and children in developing countries [2]. The rapid spread of antibiotic resistance has decreased the arsenal available to treat many infectious diseases, with recent emergence of pandrug resistance yielding illnesses that are recalcitrant to treatment with any known drug [3]. Concurrently non-communicable diseases are rising in incidence compared to communicable diseases; in 2008 cancer was responsible for about 7.8 million deaths (about 13% of all deaths worldwide; [4]).

Natural products represent one of the most significant sources of new drugs today. Approximately 50% of all medicines introduced between 1981 and 2006 were of natural product origin [5], with an additional 34 natural product-based drugs launched between 1997 and 2007 [6]. Since the discovery of penicillin over 80 years ago, fungi have contributed enormously to natural product drug discovery, providing a host of invaluable medicines including the β-lactam antibiotics, griseofulvin, cyclosporine, fusidic acid, and lovastatin [7]. However, in recent years the hit-rate for bioactivity from fungi has slowed, possibly pointing to the impending exhaustion of ‘low hanging fruit’ – the easily and frequently accessed fungi such as soil microfungi – as sources of new bioactive metabolites.

Conservative estimates suggest that the total number of fungal species in existence exceeds 5 million, yet fewer than 100,000 fungal species have been described [8][9]. This suggests that exploring new environments that may be home to previously undescribed fungi could be extremely productive. For example, recent examination of tropical fungal endophytes has described tropical leaves as a ‘biodiversity hotspot’ [10][11][12] that is proving fruitful as a source of new bioactive metabolites.

Gloer [14] proposed that production of secondary metabolites by fungi may be influenced by the selective pressures imparted by other organisms. This suggests that environments where many different species exist in close proximity may have particular potential as sources of bioactive metabolites. One such unexplored environment is the fungal microbiome associated with the coarse,
sponge-like hair of the three-toed sloth (*Bradypus variegatus*), an arboreal mammal commonly found in the lowland tropical forests of Central America.

The coat of three-toed sloths consists of two distinct layers: an inner layer of fine, soft hair close to the skin, and an outer layer of coarse hairs that are oval in cross section and are approximately 0.4 mm wide [13]. These outer hairs carry transverse cracks that are home to an apparently ubiquitous green alga, most commonly of the genus *Trichophyton* [16]. The alga is popularly thought to provide camouflage for sloths against the mottled background of their habitat, but this has not been confirmed with data. Simos et al. [16] suggested that exopolymeric substances produced by the alga might encourage the growth of beneficial bacteria. Sloth hair is commonly home to cyanobacteria and diatoms, as well as a variety of macro-organisms (e.g., cockroaches, roundworms, and moth larvae; [16]). However, little is known about communities of fungi on sloth hair.

In an effort to uncover new sources of drugs for treating vector-borne diseases, cancer, and bacterial infections, we used a culture-based approach to examine fungal communities associated with the coarse, outer hair of *Bradypus variegatus* in Panama. Here we report the isolation of fungi with bioactivity against *Trypanosoma cruzi*, the causal agent of Chagas disease; *Plasmodium falciparum*, the causal agent of malaria; the human breast cancer cell line MCF-7; and a range of Gram-negative and Gram-positive human pathogenic bacteria. Phylogenetic analyses were used to identify these fungi at a finer and more robust level than is possible using typical database-matching methods alone. Our results suggest that sloth hair is an interesting new source of important bioactive fungi with much scope for exploration of the five other extant sloth species found across the neotropics.

**Materials and Methods**

Approval for sampling of sloth hair was obtained from the Institutional Animal Care and Use Committee (IACUC) of the Smithsonian Tropical Research Institute and collection permits were obtained from Panama's Autoridad Nacional del Ambiente (ANAM). A single sample of coarse outer hair was collected from around the lower back of each of nine living three-toed sloths encountered in February 2011 along Pipeline Road in Soberanía National Park, Republic of Panama (N9°44’; W79°44’; [17]). Hair samples were placed in sterile Falcon tubes half filled with silica gel and stored at ambient temperature until processed.

**Isolation and cultivation of fungi**

Each hair was rinsed with sterile water to remove debris and loosely associated microorganisms. Under aseptic conditions hairs were cut into pieces approximately 2.5 cm long and placed on the surface of potato dextrose agar (PDA) or 2% malt extract agar (MEA) in sterile Petri plates. Plates were sealed with Parafilm, incubated at room temperature, and checked for new fungal growth daily for 2 weeks. Hyphae were cut aseptically from each plate and transferred to axenic culture on the same medium. Growth was observed around the adjacent portion of the nuclear ribosomal large subunit (LSU rDNA) were amplified as a single fragment using primers ITS1F or ITS5 and LR3 following Hoffman et al. [18]. PCR products were visualized using SYBR green following electrophoresis on a 1% agarose gel and submitted to the University of Arizona Genetics Core for cleanup, normalization, and bidirectional Sanger sequencing. Sequences were assembled automatically and bases called using *phred* and *phrap* [19][20] with orchestration by Mesquite [21], followed by manual editing in Sequencer 4.5 (GeneCodes Corp.). Sequences were compared against the NCBI non-redundant database using BLASTn [22] to estimate taxonomic placement and were submitted to GenBank under accession nos. KF746076-KF746159 (Table S1).

Because identification based only on BLAST matches must be treated with caution (e.g., [23]) we used phylogenetic analyses to provide stronger inference regarding taxonomic affiliation. Sequence data for 84 fungal isolates were loaded as one group into Mesquite [21] and aligned by MUSCLE [24]. The resulting alignment, which identified clusters of easily alignable sequences, was inappropriate for a single phylogenetic analysis due to the prevalence of ambiguous and unalignable regions. Sequences that aligned coherently to one another were partitioned into groups of apparently closely related strains. Resulting groups were then analyzed as separate data sets as follows: All sequences in each group were compared against the NCBI database using BLASTn. The top 50 hits for each sequence were downloaded and filtered to remove (1) redundant sequences, and (2) sequences from potentially mis-identified strains and unvoucherized specimens (see [23]). At least one sequence from a reliable culture collection was included in each data set, and one or two outgroups were selected from species closely related to named sequences in the dataset (chosen by literature review).

Each resulting data set was aligned separately in MUSCLE. Sequences in each alignment were trimmed to consistent starting and ending points, and alignment quality was verified by visual inspection in Mesquite. All alignments were submitted to TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2: S14945). Models of evolution were inferred using *jModeltest* [25][26]. Phylogenetic trees were inferred by maximum likelihood in GARLI [27], with support determined by 100 bootstrap replicates implemented in the CIPRES web portal [28], and using Bayesian methods in MrBayes (accessed via CIPRES [29]). For the latter, analyses consisted of 5 million generations, with four chains, random starting trees, and sampling every 1000th tree. Completeness was assessed based on asymptotes of the −ln li values and evaluation of standard deviations of split frequencies. Posterior probabilities provided inferentially independent values to support the topological relationships and associated support values from maximum likelihood analyses. Results of each phylogenetic analysis were compared against the existing literature for each taxonomic group to ensure that relationships of known taxa were appropriate, thus providing confidence with regard to our placement of sloth hair fungi.

**Preparation of fungal extracts**

A single agar plug of each strain selected for further analysis was transferred under sterile conditions to a fresh plate of 2% MEA and incubated on the benchtop at room temperature until approximately 50% of the plate was covered with mycelial growth. Fifteen agar plugs (each 5 mm in diameter) were cut with a sterile cork borer under sterile conditions and transferred to flasks containing 37 ml of 2% malt extract broth (MEB). Flasks were incubated on an orbital shaker (28°C, 125 rpm) for 2 weeks.
## Table 1. Eighty-four fungal isolates from the coarse outer hair of nine individuals sloths (*B. variegatus*), their top BLAST matches, and maximum identity value from BLAST searches; group ID and tree (Fig. 1) revealing phylogenetic placement; and taxonomic placement based on phylogenetic analyses at the family (order) and genus levels (Fig. 1).

| Fungus ID | Sloth hair Sample # | Top BLAST Hit, (Accession Number) | Max ID (%) | Group ID | Tree | Family (Order) | Genus | OTU |
|-----------|---------------------|-----------------------------------|------------|----------|------|----------------|-------|-----|
| F4847     | S006                | Fungal sp., (HQ832955.1)^a         | 99         | 18       | B    | Apiosporaceae (Incetae sedis) | Arthrinium | S*  |
| F4821     | S007                | Bionectria cf. ochroleuca, (EJ52110.1)^a | 99         | 3        | P    | Bionectriaceae (Hypocreales) | Bionectria | I   |
| F4889     | S008                | Bionectria cf. ochroleuca, (EJ52111.1)^a | 99         | 3        | P    | Bionectriaceae (Hypocreales) | Bionectria | I   |
| F4913     | S009                | Bionectria cf. ochroleuca, (EJ52110.1)^a | 99         | 3        | P    | Bionectriaceae (Hypocreales) | Bionectria | I   |
| F4874     | S005                | Bionectria pityrodes, (UQ411387.1)^a | 99         | 3        | P    | Bionectriaceae (Hypocreales) | Bionectria | W*  |
| F4806     | S008                | Colletotrichum sp., (DQ300348.1)^a | 99         | 7        | F    | Glomerellaceae (Glomerellales) | Colletotrichum | R*  |
| F4839     | S002                | Ascomycota sp., (HQ60154.1) | 99         | 10       | K    | Valsaceae (Diaporthales) | Cytospora | D   |
| F4818     | S002                | Sordariomycetes sp., (JX174122.1)^a | 99         | 10       | K    | Valsaceae (Diaporthales) | Cytospora | D   |
| F4801     | S003                | Endomelanconiopsis endophytica, (GQ46968.1)^a | 100        | 5        | E    | Botryosphaeriaceae (Botryosphaerales) | Endomelanconiopsis | U*  |
| F5071     | S003                | Fungal sp., (FJ612981.1) | 99         | 15       | H    | Xylariaceae (Xylariales) | Entonema | N*  |
| F4876     | S006                | Fungal sp., (EU563515.1) | 99         | 13       | N    | Nectriaceae (Hypocreales) | Fusarium | X   |
| F4828     | S005                | Fungal sp., (FJ612934.1) | 99         | 12       | M    | Nectriaceae (Hypocreales) | Fusarium | O*  |
| F4898     | S003                | Fungal sp., (FJ61205.1) | 99         | 12       | M    | Nectriaceae (Hypocreales) | Fusarium | Y*  |
| F4881     | S009                | Fusarium concolor, (GQ505763.1) | 99         | 13       | N    | Nectriaceae (Hypocreales) | Fusarium | X   |
| F4897     | S003                | Uncultured soil fungus clone, (DQ420777.1) | 98         | 11       | L    | Nectriaceae (Hypocreales) | Fusarium | AA* |
| F4877     | S003                | Uncultured soil fungus clone, (DQ420802.1) | 98         | 11       | L    | Nectriaceae (Hypocreales) | Fusarium | K   |
| F4890     | S008                | Uncultured soil fungus clone, (DQ420802.1) | 98         | 11       | L    | Nectriaceae (Hypocreales) | Fusarium | K   |
| F4900     | S004                | Fungal sp., (FJ612088.1) | 100        | 1        | I    | Hypocreaceae (Hypocreales) | Hypocrea | E   |
| F4812     | S001                | Hypocrea jecorina, (AF510497.1)^a | 99         | 1        | I    | Hypocreaceae (Hypocreales) | Hypocrea | A   |
| F4813     | S001                | Hypocrea jecorina, (EU280094.1) | 100        | 1        | I    | Hypocreaceae (Hypocreales) | Hypocrea | A   |
| F4815     | S001                | Hypocrea jecorina, (EU280094.1) | 100        | 1        | I    | Hypocreaceae (Hypocreales) | Hypocrea | A   |
| F4814     | S001                | Hypocrea jecorina, (JQ070073.1)^a | 100        | 1        | I    | Hypocreaceae (Hypocreales) | Hypocrea | A   |
| F4852     | S001                | Hypocrea jecorina, (JQ070073.1)^a | 100        | 1        | I    | Hypocreaceae (Hypocreales) | Hypocrea | A   |
| F4853     | S001                | Hypocrea jecorina, (JQ070073.1)^a | 100        | 1        | I    | Hypocreaceae (Hypocreales) | Hypocrea | A   |
| F4855     | S001                | Hypocrea jecorina, (JQ070073.1)^a | 100        | 1        | I    | Hypocreaceae (Hypocreales) | Hypocrea | A   |
| F4856     | S001                | Hypocrea jecorina, (JQ070073.1)^a | 100        | 1        | I    | Hypocreaceae (Hypocreales) | Hypocrea | A   |
| F4854     | S001                | Hypocrea jecorina, (JQ411369.1)^a | 100        | 1        | I    | Hypocreaceae (Hypocreales) | Hypocrea | A   |
| F4882     | S009                | Hypocrea jecorina, (JQ411369.1)^a | 100        | 1        | I    | Hypocreaceae (Hypocreales) | Hypocrea | A   |
| F4901     | S004                | Hypocrea lilii, (AY605743.1)^a | 100        | 1        | I    | Hypocreaceae (Hypocreales) | Hypocrea | E   |
| F4831     | S005                | Hypocrea lilii, (UF923806.1)^a | 100        | 1        | I    | Hypocreaceae (Hypocreales) | Hypocrea | E   |
| F4902     | S005                | Hypocrea lilii, (UF923806.1)^a | 100        | 1        | I    | Hypocreaceae (Hypocreales) | Hypocrea | E   |
| F4873     | S005                | Hypocrea muroaiana, (JX943367.1) | 99         | 1        | I    | Hypocreaceae (Hypocreales) | Hypocrea | H   |
| F4883     | S006                | Hypocrea nigricans, (JN943369.1) | 99         | 1        | I    | Hypocreaceae (Hypocreales) | Hypocrea | E   |
| F4816     | S001                | Trichoderma reesi, (UQ979435.1)^a | 99         | 1        | I    | Hypocreaceae (Hypocreales) | Hypocrea | A   |
| F4830     | S005                | Uncultured Trichoderma clone, (JX317342.1)^a | 100        | 1        | I    | Hypocreaceae (Hypocreales) | Hypocrea | H   |
| F4845     | S005                | Uncultured Trichoderma clone, (JX317342.1)^a | 99         | 1        | I    | Hypocreaceae (Hypocreales) | Hypocrea | H   |
| F4860     | S005                | Uncultured Trichoderma clone, (JX317342.1)^a | 99         | 1        | I    | Hypocreaceae (Hypocreales) | Hypocrea | H   |
| F4825     | S004                | Lasiodiplodia parva, (GQ469961.1)^a | 100        | 4        | Q    | Botryosphaeriaceae (Botryosphaerales) | Lasiodiplodia | C   |
| F4844     | S004                | Lasiodiplodia parva, (GQ469961.1)^a | 100        | 4        | Q    | Botryosphaeriaceae (Botryosphaerales) | Lasiodiplodia | C   |
| F4802     | S004                | Lasiodiplodia parva, (GQ469964.1)^a | 100        | 4        | Q    | Botryosphaeriaceae (Botryosphaerales) | Lasiodiplodia | C   |
| F5068     | S009                | Lasiodiplodia theobromae, (FJ478103.1)^a | 99         | 4        | Q    | Botryosphaeriaceae (Botryosphaerales) | Lasiodiplodia | C   |
### Table 1. Cont.

| Fungus ID | Sloth hair Sample # | Top BLAST Hit, (Accession Number) | Max ID (%) | Group ID | Tree | Family (Order) | Genus | OTU |
|-----------|---------------------|-----------------------------------|------------|---------|------|----------------|-------|-----|
| F4807     | S008                | Lasiodiplodia theobromae, (GQ469934.1) | 100        | 4       | Q    | Botryosphaeriaceae (Botryosphaeriales) | Lasiodiplodia | C   |
| F4906     | S007                | Lasiodiplodia theobromae, (GQ469934.1) | 100        | 4       | Q    | Botryosphaeriaceae (Botryosphaeriales) | Lasiodiplodia | C   |
| F4904     | S006                | Fungal sp., (GU662656.1) [63]     | 99         | 14      | S    | Leptosphaeriaceae (Pleosporales) | Leptosphaeria | J   |
| F4905     | S006                | Fungal sp., (GU662656.1) [63]     | 100        | 14      | S    | Leptosphaeriaceae (Pleosporales) | Leptosphaeria | J   |
| F4819     | S006                | Fungal endophyte sp., (EU977287.1) [55] | 99         | 20      | D    | Lophiotomataceae (Pleosporales) | Unknown | P*  |
| F4831a    | S002                | Fungal endophyte sp., (EU561602.1) | 99         | 19      | C    | Montagnulaceae (Pleosporales) | Paraconiothyrium | A   |
| F4884     | S008                | Penicillium herquei, (EU833220.1) | 99         | 6       | O    | Trichocomaceae (Eurotiales) | Penicillium | V*  |
| F4863     | S009                | Penicillium sp., (JF288548.1) [64] | 99         | 6       | O    | Trichocomaceae (Eurotiales) | Penicillium | T*  |
| F4803     | S004                | Fungal endophyte sp., (EU561622.1) | 99         | 2       | J    | Amphisphaeriaceae (Xylariales) | Pestalotiopsis | G   |
| F4846     | S006                | Fungal endophyte sp., (EU561622.1) | 100        | 2       | J    | Amphisphaeriaceae (Xylariales) | Pestalotiopsis | G   |
| F4878     | S008                | Fungal endophyte sp., (EU561622.1) | 100        | 2       | J    | Amphisphaeriaceae (Xylariales) | Pestalotiopsis | G   |
| F4896     | S002                | Fungal endophyte sp., (EU561622.1) | 100        | 2       | J    | Amphisphaeriaceae (Xylariales) | Pestalotiopsis | G   |
| F4858     | S004                | Fungal endophyte sp., (EU561622.1) | 100        | 2       | J    | Amphisphaeriaceae (Xylariales) | Pestalotiopsis | B   |
| F4908     | S007                | Fungal endophyte sp., (EU561622.1) | 100        | 2       | J    | Amphisphaeriaceae (Xylariales) | Pestalotiopsis | B   |
| F4857     | S002                | Fungal endophyte sp., (EU561622.1) | 99         | 2       | J    | Amphisphaeriaceae (Xylariales) | Pestalotiopsis | Q*  |
| F4817     | S002                | Fungal endophyte sp., (HQ832816.1) | 100        | 2       | J    | Amphisphaeriaceae (Xylariales) | Pestalotiopsis | B   |
| F4826     | S004                | Fungal endophyte sp., (HQ832816.1) | 100        | 2       | J    | Amphisphaeriaceae (Xylariales) | Pestalotiopsis | B   |
| F4827     | S005                | Fungal endophyte sp., (HQ832816.1) | 100        | 2       | J    | Amphisphaeriaceae (Xylariales) | Pestalotiopsis | B   |
| F4837     | S002                | Fungal endophyte sp., (HQ832816.1) | 100        | 2       | J    | Amphisphaeriaceae (Xylariales) | Pestalotiopsis | B   |
| F4841     | S003                | Fungal endophyte sp., (HQ832816.1) | 100        | 2       | J    | Amphisphaeriaceae (Xylariales) | Pestalotiopsis | B   |
| F4861     | S005                | Fungal endophyte sp., (HQ832816.1) | 100        | 2       | J    | Amphisphaeriaceae (Xylariales) | Pestalotiopsis | B   |
| F4862     | S007                | Fungal endophyte sp., (HQ832816.1) | 100        | 2       | J    | Amphisphaeriaceae (Xylariales) | Pestalotiopsis | B   |
| F4895     | S002                | Fungal endophyte sp., (HQ832816.1) | 100        | 2       | J    | Amphisphaeriaceae (Xylariales) | Pestalotiopsis | B   |
| F4909     | S008                | Fungal endophyte sp., (HQ832816.1) | 100        | 2       | J    | Amphisphaeriaceae (Xylariales) | Pestalotiopsis | B   |
| F5074     | S003                | Fungal endophyte sp., (HQ832816.1) | 100        | 2       | J    | Amphisphaeriaceae (Xylariales) | Pestalotiopsis | B   |
| F4823     | S003                | Fungal endophyte sp., (HQ832816.1) | 100        | 2       | J    | Amphisphaeriaceae (Xylariales) | Pestalotiopsis | B   |
| F4894     | S002                | Fungal endophyte sp., (HQ832816.1) | 99         | 2       | J    | Amphisphaeriaceae (Xylariales) | Pestalotiopsis | G   |
| F4870     | S002                | Uncultured fungus clone, (IN890176.1) [65] | 99         | 2       | J    | Amphisphaeriaceae (Xylariales) | Pestalotiopsis | G   |
| F4875     | S005                | Uncultured fungus clone, (IN890258.1) [65] | 99         | 2       | J    | Amphisphaeriaceae (Xylariales) | Pestalotiopsis | B   |
| F4879     | S008                | Uncultured fungus clone, (IN890258.1) [65] | 99         | 2       | J    | Amphisphaeriaceae (Xylariales) | Pestalotiopsis | B   |
| F5069     | S006                | Uncultured fungus clone, (IN890258.1) [65] | 99         | 2       | J    | Amphisphaeriaceae (Xylariales) | Pestalotiopsis | B   |
| F5073     | S008                | Sordariomyces sp., (JQ760525.1) [65] | 99         | 17      | A    | Calosphaeriaceae (Calosphaeriales) | Phaeoacremonium | L*  |
| F5070     | S006                | Uncultured fungus clone, (GU721790.1) | 99         | 16      | T    | Cephalotheca (Sordariales) | Phialoconeum | F   |
| F5072     | S003                | Uncultured fungus clone, (GU721790.1) | 99         | 16      | T    | Cephalotheca (Sordariales) | Phialoconeum | F   |
| F4886     | S004                | Robillardia sessilis, (HQ608017.1) [58] | 99         | 8       | G    | Amphisphaeriaceae (Xylariales) | Robillardia | Z*  |
| F4891     | S009                | Phoma sp., (JQ621876.1) | 99         | 9       | R    | Pleosporales | Unknown | BB* |
| F4850     | S009                | Uncultured soil fungus clone, (DQ421180.1) [35] | 96         | 9       | R    | Unknown | Unknown | M*  |

OTU codes indicate operational taxonomic units based on 95% sequence similarity. Order and family level identifications were confidently assigned to 82 isolates and genus level identifications were confidently assigned to 80 isolates.

*Unpublished strains.

**OTUs occurring only once (singletons).

†Tentative phylogenetic placement.

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Figure 1. Results of maximum likelihood and Bayesian analyses of ITS rDNA data for fungi isolated in culture from coarse, outer hair of three-toed sloths in Soberanía National Park, Panama. Topology of each tree reflects ML analysis, and values above branches indicate ML bootstrap values and Bayesian posterior probabilities (≥0.50 and ≥0.75, respectively). Outgroups and taxon sampling for each tree were validated by literature surveys (see methods). Taxonomic conclusions are presented in Table 1. Figure 1(A): Placement of F5073 in group 17; (B) F4847 in group 18; (C) F4831a in group 19; (D) F4819 in group 20; (E) F4801 in group 5; (F) F4806 in group 7; (G) F4886 in group 8; (H) F5071 in group 15; (I)
Liquids cultures were mixed with an equal volume of ethyl acetate and blended for 2 minutes at 9000 rpm with a Polytron (Lauda-Brinkmann, Delran, NJ, USA). Fungal biomass was removed by vacuum filtration through Whatman filter paper (#1) and the filtrate was extracted twice with a 1:1 volume of ethyl acetate. The aqueous layer was discarded and the organic layer was dried and stored at \(-80^\circ\text{C}\).

**Bioassays**

Crude organic extracts of fungal cultures were used in bioassays against focal strains of the causal agents of malaria (\textit{Plasmodium falciparum}) and Chagas disease (\textit{Trypanosoma cruzi}), and against breast cancer cell line MCF-7, as described by Higginbotham et al. [29]. Bioactivity of extracts, which were diluted in DMSO (10 \(\mu\text{g}/\text{ml}\)), was measured as percent inhibition of growth (% IG) compared to the negative control (DMSO with no extract; 0% IG).

**BioMAP analyses**

An antibiotic activity profile screen, BioMAP (Antibiotic mode of action profile; [30]), was used to test activity of 50 crude organic extracts against 15 human pathogenic bacteria. Extracts with potent bioactivity were serially diluted (16 2-fold dilutions) and re-screened against the same panel of bacteria. Growth curves were plotted by recording OD\(_{600}\) values each hour for 24 hours and minimum inhibitory concentrations (MIC) were subsequently calculated for each extract. To establish concentration-independent bioactivity profiles for each extract, raw MIC values were normalized resulting in a range of values from 0 (inactive) to 1 (most bioactive). These activity profiles were then compared to profiles of known antibiotics belonging to all major structural classes. This method is effective in predicting the structural class of unknown antibiotic compounds from complex or crude mixtures [30].

**Results and Discussion**

A total of 84 fungal isolates was examined from the coarse outer hair of nine living individuals of the three-toed sloth (\textit{Bradypus variegatus}) encountered along Pipeline Road in Soberanía National Park, Republic of Panama. Hair samples were transported to the lab in sterile Falcon tubes which had been half filled with silica gel, a desiccant that is effective for storing fungal tissue [31][32]. Every piece of sloth hair placed on agar yielded multiple fungal isolates representing a variety of morphotypes. Phylogenetic analyses of axenic strains revealed a diverse group of fungi, some of which appear to be novel relative to previously observed or sequenced taxa (Table 1; Figure 1). Many of these isolates display bioactivity in vitro against parasites that cause malaria and Chagas disease, breast cancer cells, and both Gram-positive and Gram-negative human pathogenic bacteria (Table 2; Table 3).

**Phylogenetic analyses**

The 84 fungal isolates represented 28 operational taxonomic units (OTUs) based on 95% sequence similarity (Table 1). The two

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**Table 2.** Bioactivity of sloth hair surface associated fungi isolated on potato dextrose agar (PDA) or 2% malt extract agar (MEA) against causative agents of malaria (\textit{P. falciparum}) and Chagas disease (\textit{T. cruzi}), and against the MCF-7 breast cancer cell line.

| Fungus ID | OTU | Isolation Media | Putative Genus | \textit{P. falciparum} | \textit{T. cruzi} | MCF-7 |
|-----------|-----|-----------------|----------------|------------------------|-----------------|-------|
| F4813     | A   | PDA             | Hypocrea       |                        | A               | A     |
| F4814     | A   | MEA             | Hypocrea       |                        | A               | A     |
| F4815     | A   | PDA             | Hypocrea       |                        | A               | A     |
| F4816     | A   | PDA             | Hypocrea       |                        | A               | A     |
| F4818     | D   | PDA             | Cytospora      |                        | A               | A     |
| F4828     | O   | PDA             | Fusarium       |                        | A               | A     |
| F4853     | A   | MEA             | Hypocrea       |                        | A               | A     |
| F4854     | A   | MEA             | Hypocrea       |                        | A               | A     |
| F4855     | A   | PDA             | Hypocrea       |                        | A               | A     |
| F4863     | T   | PDA             | Penicillium    |                        | A               | A     |
| F4874     | W   | MEA             | Bionectria     |                        | A               | A     |
| F4881     | X   | MEA             | Fusarium       |                        | A               | A     |
| F4882     | A   | MEA             | Hypocrea       |                        | -               | A     |
| F4890     | K   | MEA             | Fusarium       |                        | -               | A     |
| F4894     | G   | MEA             | Pestalotiopsis |                        | A               |       |
| F4898     | Y   | PDA             | Fusarium       |                        | A               |       |

Bioactive fungi (A) are those causing \(\geq 50\%\) inhibition of growth of parasite or cancer cells in \textit{in vitro} assays.

- = extract not tested in this bioassay; empty cell = not highly active.

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most abundant OTUs represented 34 (40.5%) of the isolates. The remaining 50 isolates were represented by 26 distinct OTUs. The most abundant OTUs represented 34 (40.5%) of the isolates. The order, family could be confidently assigned to 82 fungi (Table 1; Figure 1). The representative 15 families and 10 orders (Table 1; Figure 1). The order, family) could be confidently assigned to 82 fungi (Table 1; Figure 1). The appropriate outgroups were added based on literature review. Each group to make non-redundant datasets to which one or two 18 groups contained between 1 and 6 sequences each. Between 34 and 165 sequences from closely related taxa were compiled for each group to make non-redundant datasets to which one or two appropriate outgroups were added based on literature review. Following phylogenetic analyses, high level identities (class, order, family) could be confidently assigned to 82 fungi representing 15 families and 10 orders (Table 1; Figure 1). The majority (81.7%) of these were Sordariomycetes. Fungi from this class are well documented sources of bioactive metabolites (e.g., [33][34]). The remaining isolates were Dothideomycetes (15.9%) and Eurotiomycetes (2.4%).

Eighty isolates could confidently be assigned to 15 genera (Table 1; Figure 1). Two isolates were given more tentative phylogenetic placements. F4886 (Figure 1(G)) was identified as Robillarda sp. or as a member of a closely related genus within the Amphiphilaceae. Isolate F4831a (Figure 1(C)) was identified as Paraconiothyrium sp. or as a member of a closely related genus within the Montagulaceae. The two most common genera, Pestalotiopsis sp. and Hypoaspis sp., were isolated from 7 of 9 and 5 of 9 sloth hair samples, respectively.

Isolate F4891 (Figure 1(R)) did not appear to be closely related to any named sequences in GenBank and could only be identified as possibly a member of the order Pleosporales (Dothideomycetes). Isolate F4850 (Figure 1(R)) had 96% sequence similarity to its closest match in GenBank, an uncultured soil fungus clone (Table 1; [35]). Further analyses are warranted in order to confirm whether isolates F4891 and F4850 are novel fungal species. In general, identifications assigned by phylogenetic analyses closely matched those of the top BLAST hits for each isolate (Table 1).
Bioactivity of sloth hair isolates

Sloth hair fungi that were cultivated in liquid medium and extracted with ethyl acetate were tested for bioactivity. We considered extracts to be highly bioactive if they caused at least 50% inhibition of the growth (i.e., ≥50% IG) of parasites or cancer cells in vitro.

Overall, two of 70 (2.5%) extracts tested were highly active against *P. falciparum*, eight of 62 (12.9%) extracts tested were highly active against *T. cruzi*, and 15 of 73 (20.6%) extracts tested were highly active against the MCF-7 breast cancer cell line (Table 2). Most extracts with bioactivity were active in only one assay (Table 2). Bioactive strains were isolated with equal frequency on MEA and PDA (Table 2). Notably, closely related isolates often differed in bioactivity: for example, three of 20 isolates identified as *Hypocreopsis* (Table 1) had bioactivity only against MCF-7 breast cancer cells; three were active against MCF-7 breast cancer cells and *T. cruzi* (Table 2); and one was active only against methicillin-resistant *S. aureus* (Table 3).

Of the 16 fungi that had high % IG in at least one *in vitro* assay, 13 belonged to the Hypocreales (Hypocreaceae, Bionectriaceae and Nectriaceae). The remaining three fungi belonged to the Valsaceae (Diaporthales), Botryosphaeraceae (Botryosphaeriales), and Amphisphaeriaceae (Xylariales). Activity against Gram-negative bacteria (Table 3).

Table 4. Minimum inhibitory concentrations (MIC) of 5 sloth hair associated fungal extracts in the BioMAP antibiotic profile screen [30].

| Fungus ID          | Minimum Inhibitory Concentration (Normalized 0–1) |
|--------------------|-----------------------------------------------|
|                    | F4806 | F4807 | F4818 | F4844 | F4898 |
| *Bacillus subtilis*| 1     | 0.85  | 1     | 1     | 1     |
| *Staphylococcus epidermis*| 0.46  | 0     | 0.88  | 0.87  | 0.69  |
| *Enterococcus faecium*| 0.6   | 0     | 0.52  | 0.6   | 0.53  |
| *Listeria ivanovi*| 0.6   | 0     | 0.4   | 0.46  | 0.53  |
| *Staphylococcus aureus*| 0.87  | 0     | 1     | 0.6   | 0.85  |
| MRSA               | -     | -     | -     | -     | -     |
| *Yersinia pseudotuberculosis*| 0     | 0.69  | 0     | 0     | 0     |
| *Pseudomonas aeruginosa*| 0     | 0     | 0     | 0     | 0     |
| *Salmonella typhimurium*| 0     | 0.53  | 0     | 0     | 0     |
| *Vibrio cholerae*| 0     | 0     | 0     | 0     | 0     |
| *Escherichia coli*| 0     | 0.69  | 0     | 0     | 0     |
| *Acinetobacter baumannii*| 0     | 1     | 0     | 0     | 0     |
| *Enterobacter aerogenes*| 0     | 0     | 0     | 0     | 0     |
| *Ochrobactrum anthropi*| 0     | 0.69  | 0     | 0     | 0     |
| *Providencia alcalifaciens*| 0     | 0.53  | 0     | 0     | 0     |

To generate a concentration-independent bioactivity profile, raw assay results were normalized giving a range of values from 0 (inactive) to 1 (most bioactive). These bioactivity fingerprints were then compared to fingerprints of known antibiotics from all the major structural classes, which had previously been tested in the BioMAP assay. The bioactivity fingerprint of F4807 was of particular interest as it did not match that of any antibiotic previously tested in the BioMAP screen.

- = Test failed, data not available.

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Of 50 fungal extracts screened by BioMAP, 20 were bioactive against at least one test organism (Table 3). The majority of isolates (16 of 20) active against bacteria in the BioMAP assay belonged to the families Lasiosphaeriaceae (Sordariales), Glomerellaceae (Glomerellales), Valsaceae (Diaporthales), Botryosphaeraceae (Botryosphaeriales), and Amphisphaeraceae (Xylariales). The remaining four fungi belonged to the Nectriaceae, Bionectriaceae and Hypocreaceae (Hypocreales). Activity against Gram-positive bacteria was detected far more frequently than against Gram-negative bacteria (Table 3).

Five isolates (*Colletotrichum* sp. F4806 (Figure 1(F)); *Cytospora* sp. 1, strain F4818 (Figure 1(K)); *Pseudomonas* sp. 1, strain F4898 (Figure 1(M)); *Lasiodiplodia* sp. 1, strain F4807 (Figure 1(Q)); and *Lasiodiplodia* sp. 1, strain F4844 (Figure 1(Q)) had particularly potent activity in the BioMAP assay and were selected for further testing. Serially diluted extracts were re-tested for activity against the panel of 15 bacteria and bioactivity profiles were constructed using normalized MIC values (Table 4).

The extract from *Lasiodiplodia* sp. 1 (strain F4807) was of particular interest as it had potent and specific activity against Gram-negative bacteria (Table 4). The bioactivity profile of the isolate did not match that of any of the known antibiotic classes previously tested in the BioMAP assay, suggesting a potentially novel mode of action (see [30]). Infections caused by emerging multidrug-resistant (MDR) Gram-negative bacteria are becoming a major clinical concern worldwide [45]. These infections most commonly include MDR *Pseudomonas aeruginosa*, extended-spectrum β-lactamase producing Enterobacteriaceae, and MDR *Acinetobacter baumannii* [46]. Recent data suggest that infections traced to *Staphylococcus aureus* bloodstream isolates are in decline,
but that infections by *Escherichia coli*, the most common Gram-negative species responsible for infections in human blood isolates, are increasing in frequency [47]. That report [47] also highlights the paucity of antibacterial agents under development for Gram-negative bacteria, with most effort being focused on Gram-positive MRSA. The increasing prevalence of MDR organisms is mainly due to overuse of broad-spectrum antibiotics and poor antibiotic stewardship [48]. Hence the specificity of the extract from strain F4807 for Gram-negative strains is highly valuable and worth pursuing for further analysis.

Fungi isolated in this study were taxonomically consistent with groups of fungi known to occur in soil and in plants as pathogens, saprotrophs, or endophytes [11][49]. Sloths may encounter such fungi incidentally from air spora, or via direct contact when they descend from trees in order to defecate and urinate, at which time they dig a hole in the soil that they subsequently cover with leaf litter [50]. Strikingly, comparison with a large collection of endophytes from terrestrial plants in Panama [29]; 1269 strains) revealed that sequences from 29 isolates from sloth hair were identical to strains obtained from plants (100% similarity over the full sequence length). Thus this study extends the known host range and ecological mode of putative endophytes. Some of these fungi may affiliate directly with the green alga that inhabits coarse hair of sloths, much like endolichenic fungi associate with green algae in lichen thalli and are often taxonomically similar to endophytes in the same environments [51][52].

Potential roles of these fungi in the health of sloths have not been investigated. Araújo Xavier et al. [50] reported fungal infections in *B. variegatus* caused by the fungal pathogen *Microsporum* (which also causes dermatitis in humans; [53]), but this genus was not recovered in the present study or in previous work by Suutari et al. [16]. In contrast to the human microbiome, where fungi comprise <0.01% of microbial communities on external surfaces such as skin [54], Suutari et al. [16] reported that fungi represented 8% of the flora in surveys of sloth-hair microbes. The high abundance and diversity of fungi associated with sloth hair, coupled with their bioactivity, may speak to a biological importance to sloths that is yet unexplored. The pressing need for new medications continues to represent one of humanity’s greatest challenges. It is commonly agreed that and newly discovered microbial taxa hold an important promise of novel chemistry. Abundant evidence points to novel environments as promising sources of as yet undescribed microorganisms [55]. Here we have demonstrated that hair of the three-toed sloth (*Bradypus variegatus*) in Panama is a rich source of diverse bioactive fungi. Strains isolated here included members of some well-studied lineages (e.g., *Hypocreales*, *Pleosporales*), but also members of understudied or potentially novel groups (e.g., *Endomelanconotis* sp. F4801; *Robillarda* sp. F4806; *Ascomycota* sp. F4891; *Ascomycota* sp. F4850). We anticipate that additional novel taxa could be isolated from sloth hair by expanding survey methods to diverse types of media and using high-throughput methods to assess community composition. We also anticipate that different taxa may be isolated from sloths in other regions, much as communities of endophytes in tropical plants differ markedly at a regional scale [56][57]. Thus our work suggests that fruitful exploration of the sloth microbiota is warranted for potential applications in drug discovery.

### Supporting Information

#### Table S1 Sloth hair fungus ID and Genbank accession numbers.

(DoCX)

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### Author Contributions

Conceived and designed the experiments: SH AEA CS WRW RL. Performed the experiments: SH AEA CS WRW RL LI. Analyzed the data: SH AEA WRW RL LI. Contributed reagents/materials/analysis tools: SH AEA WRW RL CS LI. Wrote the paper: SH AEA CS WRW RL.

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