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### A new species of *Gloeandromyces* from Ecuador and Panama revealed by morphology and phylogenetic reconstruction, with a discussion of secondary barcodes in Laboulbeniomycetes taxonomy

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#### ABSTRACT

This paper describes and illustrates a new species of Laboulbeniales (Ascomycota, Laboulbeniomycetes) recovered from *Mastoptera guimaraesi* bat flies (Diptera, Streblidae) in Ecuador and Panama. Bat fly-associated Laboulbeniales are still unexplored in the Neotropics, with only four described species of *Gloeandromyces* and one species of *Nycteromyces* known. Morphological characteristics and phylogenetic analyses support placement of the new taxon in *Gloeandromyces* and its recognition as an undescribed species. *Gloeandromyces hilleri* sp. nov. is easily recognized by 2–3 longitudinal rows of undulations at its perithecial venter. Phylogenetic reconstructions of the large subunit (LSU) ribosomal DNA and the translation elongation factor 1a (*TEF1*) both resolve *G. hilleri* and *G. nycteribiidarum* as sister species. We discuss the utility of LSU and *TEF1* as secondary barcodes in Laboulbeniomycetes taxonomy.

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#### INTRODUCTION

Bats are the second-most diverse group of mammals on the planet. Their ecological abundance and colonial behavior-sometimes resulting in aggregations of up to thousands to millions of individuals-mean that bats are often parasitized by micro- and macroorganisms (Haelewaters et al. 2018b, 2020; Szentiványi et al. 2019). True insect ectoparasites of bats, those feeding on blood, are found among Diptera (true flies), Hemiptera (true bugs), and Siphonaptera (fleas). Of those, the bat flies are most conspicuous and speciose (Dick and Patterson 2006). An arsenal of organisms, including bacteria, blood parasites, viruses, and Laboulbeniales fungi, can be associated with bat flies themselves (Szentiványi et al. 2019). This observation points at their potential as vectors of pathogens such as Bartonella and Polychromophilus (Dick and Dittmar 2014; Obame-Nkoghe et al. 2016).

Laboulbeniales (Ascomycota, Laboulbeniomycetes) is an order of ectoparasitic fungi that exclusively occur on Arthropoda. Twelve bat fly–associated species are known from four genera: *Arthrorhynchus* (4 species), *Dimeromyces* (2 species), *Gloeandromyces* (4 species), and *Nycteromyces*  (2 species) (Haelewaters et al. 2020). Of these, *Dimeromyces* is the only genus with hosts other than bat flies; as many as 115 species are found on Acari (mites), Blattodea (termites), Coleoptera (beetles), Dermaptera (earwigs), Diptera (true flies), Hymenoptera (ants), Orthoptera (crickets), and Thysanoptera (thrips) (Rossi et al. 2016; Dogonniuck et al. 2019). Taxa in the genus *Gloeandromyces* have thus far been found on *Megistopoda aranea, Speiseria ambigua, Strebla wiedemanni*, and *Trichobius* spp. (Diptera, Streblidae) in the Neotropics (Haelewaters et al. 2018b; Haelewaters and Pfister 2019). Thus far, no reports of *Gloeandromyces* are known from bat flies in the genus *Mastoptera* (Streblidae).

Here, we describe a new species of *Gloeandromyces* from *Mastoptera guimaraesi* bat flies in Ecuador and Panama. We provide morphological, molecular phylogenetic, and ecological (host association) data to support the new species.

#### **MATERIALS AND METHODS**

**Examination of bat flies and morphological study of Laboulbeniales.**—Bats were captured using mist nets in Ecuador and Panama under the following permits: 023-IC-FAU-DNBAP/MA (Principal Investigator

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[PI]: Carl W. Dick) and SE/A-75-13 (PI: Rachel A. Page). Bat flies were removed from their bat hosts using featherweight forceps and preserved in 96% ethanol. Screening of bat flies was done under  $40-50\times$  magnification. Laboulbeniales thalli were removed from their bat fly host at the attachment region (foot or haustorium) using a Minuten Pin (BioQuip 1208SA; Rancho Dominguez, California) of which the tip was dipped in Hoyer's medium (30 g Arabic gum, 200 g chloral hydrate, 16 mL glycerol, 50 mL double-distilled water [ddH<sub>2</sub>O]) to prevent thalli from getting lost during transfer. Mounting procedures followed Benjamin (1971) with modifications as in Haelewaters et al. (2019).

For slides deposited at PUL, we applied a double-slide mounting technique: Thalli were placed in a droplet of Hoyer's medium on a  $22 \times 22$  mm coverslip. Next, thalli were arranged in one or two rows, depending on the number. An 18 × 18 mm coverslip with a drop of Amann's medium was flipped upside down and gently dropped sideways on top of the larger coverslip. With a Kimwipe, excess mounting medium was removed from the edges of the small coverslip. The corners of the small coverslip were sealed to the larger one with nail polish or B-72 in acetone (Gaylord AB72; Syracuse, New York). Both coverslips, now sealed together at the corners, were placed on a flat surface with the smaller coverslip facing up. Solakryl BMX (Ento Sphinx, Pardubice, Czech Republic) was applied onto the smaller coverslip, and the slips were then gently placed sideways on a clean microscope slide. The Solakryl BMX medium was given time to spread, filling up the area in between the larger coverslip and the microscope slide, with the smaller coverslip in between. When necessary, additional Solakryl BMX was applied to fill up any air bubbles that appeared under the edges of the slips. The resulting slide consists of the  $22 \times 22$  mm coverslip on top, then the  $18 \times 18$  mm coverslip, and the slide at the bottom; the Amann's medium with thalli in between the two coverslips is separated from the air by a layer of Solakryl BMX.

Mounted specimens were viewed at 200–1000× magnification. Photomicrographs were taken with an Olympus SC30 camera mounted on an Olympus BH2 bright-field compound microscope using cellSens 1.18 imaging software (Olympus, Tokyo, Japan). Line drawings of thalli were made with PITT artist pens (Faber-Castell, Nürnberg, Germany) based on images, then scanned using an HP Scanjet G5040 scanner (Palo Alto, California) and edited with Photopea (https:// www.photopea.com/). For morphological comparison with previously described species of *Gloeandromyces*, we used relevant systematic and taxonomic papers (Thaxter 1917, 1931; Haelewaters and Pfister 2019). Slides are deposited at the Farlow Herbarium (FH; Harvard University, Cambridge, Massachusetts) and the Kriebel Herbarium (PUL; Purdue University, West Lafayette, Indiana).

Extraction of Laboulbeniales DNA.—Bat flies found to be infected with Gloeandromyces were placed on a microscope slide. A single Gloeandromyces thallus was carefully removed from the host and placed into a droplet of glycerin using a Minuten Pin under an Olympus SZH10 dissecting microscope. A coverslip was gently placed on top of the thallus. The position of the thallus on the slide was marked with a permanent ink marker to help with locating the specimen under the microscope. Thalli were examined under the Olympus BH2 compound microscope at 200–1000×. Images were taken with an Olympus SC30 camera for record keeping and to confirm morphological identity. The slide was placed back under the dissecting microscope, and the coverslip was carefully removed to expose the thallus for isolation of DNA using the REPLI-g Single Cell Kit (Qiagen, Valencia, California) with modifications (Haelewaters et al. 2019). A no. 10 surgical blade on disposable Bard-Parker handle (Aspen Surgical, Caledonia, Michigan) was used to cut the thallus in half through the perithecium to ensure successful DNA extraction. Each thallus half was carefully picked up and placed in a 0.2-mL polymerase chain reaction (PCR) tube with 2 µL of phosphate-buffered saline (PBS) solution. After addition of 1.5 µL of prepared D2 buffer, the PCR tube was incubated at 65 C for 30 min to maximize cell lysis. Subsequent steps followed the manufacturer's instructions, but using half the amount of reagents listed. DNA extracts were stored at -20 C until PCR amplification.

**PCR amplification and sequencing.**—Two ribosomal loci (small subunit [SSU], large subunit [LSU]) and one protein-coding locus (translation elongation factor 1a [*TEF1*]) were amplified using both 1:1 and 1:10 diluted DNA extracts. Primers pairs included NSL1/NSL2 for SSU (Haelewaters et al. 2015), LIC24R/LR3 (Vilgalys and Hester 1990; Miadlikowska and Lutzoni 2000) and LR0R/LR5 (Vilgalys and Hester 1990; Moncalvo et al. 2000) for LSU, and EF1-1018F/EF1-1620R and Al33\_alternative\_f/EF1-1620R (Stielow et al. 2015) for *TEF1*. Amplifications were performed on a pro S Mastercycler (Eppendorf, Hauppauge, New York) in 25-µL reactions containing 12.5 µl of 2× MyTaq Mix (Bioline, Swedesboro, New Jersey), 9.5 µL of ddH<sub>2</sub>O, 1.0 µL for each primer, and 1.0 µL of DNA extract. Cycling

conditions for SSU were as follows: initial denaturation at 95 C for 5 min; then 40 cycles of denaturation at 95 C for 30 s, annealing at 55 C for 45 s, and extension at 72 C for 45 s; and final extension at 72 C for 1 min. For LSU: initial denaturation at 94 C for 5 min; then 35 cycles of denaturation at 94 C for 30 s, annealing at 50 C for 45 s, and extension at 72 C for 1 min; and final extension at 72 C for 7 min. For *TEF1*: initial denaturation at 94 C for 5 min; then 10 cycles of denaturation at 94 C for 50 s, annealing at 54 C for 50 s with 1 C decrease per cycle, and extension at 72 C for 50 s; followed by 40 cycles of denaturation at 94 C for 50 s, annealing at 48 C for 50 s, and extension at 72 C for 50 s; and final extension at 72 C for 7 min.

Gel electrophoresis was performed to check the success of PCR amplifications. PCR products were loaded onto Tris-acetate-EDTA (TAE) 1% agarose gels for electrophoresis at 130 V for 30 min. Ultraviolet (UV) transillumination was used to evaluate product size (Gel Doc EZ Imager; Bio-Rad, Hercules, California). Purification of successful PCR products and sequencing were outsourced to Genewiz (South Plainfield, New Jersey).

#### Sequence alignment and phylogenetic analyses.—

First, we used T-BAS 2.1 (Carbone et al. 2019) and the "Place Unknowns" tool to place newly generated SSU and LSU sequences onto the Laboulbeniomycetes reference tree version 2 (Blackwell et al. 2020). Two FASTA files of the newly generated, unaligned SSU and LSU sequences of *Gloeandromyces* were uploaded to the T-BAS interface. We selected the "de novo" option for the RAxML placement, with 500 bootstrap replicates and *Rhizopus oryzae* as outgroup.

Next, we constructed two single-locus data sets (LSU, TEF1) of Gloeandromyces sequences to investigate phylogenetic structure within the genus. For the LSU data set, 26 Gloeandromyces sequences were downloaded from National Center for Biotechnology Information (NCBI) GenBank (https://www.ncbi.nlm. nih.gov/genbank/) and complemented with newly generated sequences of two isolates (D. Haelew. 1722a, D. Haelew. 1738b) and a sequence of Stigmatomyces ceratophorus as outgroup. For the TEF1 data set, 11 Gloeandromyces sequences and three Nycteromyces streblidinus sequences (outgroup) were newly generated during this study. Sequences were aligned using MUSCLE 3.7 (Edgar 2004) on the CIPRES Science Gateway 3.3 (Miller et al. 2010). Appropriate models of nucleotide substitution were selected using jModelTest2 (Darriba et al. 2012) on CIPRES (Miller et al. 2010), under the Akaike information criterion corrected for

small sample size (AICc). We performed Bayesian analyses using a Markov chain Monte Carlo (MCMC) coalescent approach. The following priors were entered in BEAUti 1.8.4 (Drummond et al. 2012): TIM2+G (LSU) or GTR+G (TEF1) as substitution model, strict molecular clock, speciation: Birth-Death Incomplete Sampling (Stadler 2009) as tree prior, random starting tree, 40 million generations, and 4000 as sampling frequency. The generated XML files were run using BEAST on XSEDE in CIPRES (4 runs) and with BEAST 1.8.4 from the command line (1 run). Resulting log files were opened in Tracer 1.6 (Rambaut et al. 2014) to assess MCMC trace plots and effective sample sizes (ESS). For the TEF1 analysis, one run failed to reach convergence and was excluded in subsequent steps. A standard burn-in of 10% was used for all included runs, resulting in combined ESS values of well above 200 for all sampled statistics. Trees files were combined in LogCombiner 1.8.4 after removal of 10% burn-in. TreeAnnotator 1.8.4 was used to generate consensus trees with 0% burn-in and infer the maximum clade credibility tree (with highest product of individual clade posterior probabilities) for both data sets. Final LSU and TEF1 trees, with posterior probabilities, were visualized in FigTree 1.4.3 (http://tree.bio.ed.ac.uk/soft ware/figtree/) and edited using Photopea (https://www. photopea.com/).

#### RESULTS

Our newly generated SSU sequences of G. hilleri shared maximum identity, 99.46%, with G. nycteribiidarum (GenBank accession no. MH040533), followed by 97.11-97.12% identity with G. streblae (MH040551) and 97.11% identity with G. dickii (MH040546, paratype). Our LSU sequences were most similar to G. nycteribiidarum (MH040566, MH040567), sharing 99.42% identity. Our TEF1 sequences shared 97.88-98.05% with G. nycteribiidarum identity (MT197533, MT197540), followed by 88.31-88.76% identity with G. dickii (MT197539, MT197542).

Placement onto the Laboulbeniomycetes tree version 2 in T-BAS confirmed that newly generated SSU and LSU sequences for two isolates of the presumptive new taxon were placed in the genus *Gloeandromyces* with maximum support (FIG. 1). *Gloeandromyces hilleri* was retrieved as a separate species, positioned sister to *G. nycteribiidarum* with maximum support. Our single-locus LSU data set included 29 isolates (TABLE 1) representing six species and 939 characters, of which 773 were constant and 109 were parsimonyinformative. The *TEF1* data set included 14 isolates (TABLE 1) representing five species and 621



**Figure 1.** Placement of *Gloeandromyces hilleri* onto Laboulbeniomycetes reference tree version 2 in T-BAS. The tree is the result of a RAxML analysis with 500 bootstrap replicates. For each node, the maximum likelihood bootstrap ( $\geq$ 70) is presented above or below the branch leading to that node. Gray shading is added to indicate the new species.

characters, of which 442 were constant and 176 were parsimony-informative. All *TEF1* sequences were newly generated during this study. Phylogenetic

reconstructions of both respective data sets showed that *G. hilleri* is sister to *G. nycteribiidarum* with maximum support (FIGS. 2, 3).

	Table 1	1. Overview	of Laboulbeniale	s isolates	used in	phyloger	netic anal	vses
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			GenBank accession nos.	
D. Haelew.	Species	Host species	LSU	TEF1
1312b	Gloeandromyces dickii	Trichobius joblingi	MH040580	MT197539
1312c	Gloeandromyces dickii	Trichobius joblingi	MH040581	
1323b	Gloeandromyces dickii	Trichobius joblingi	MH040582	
1323c	Gloeandromyces dickii	Trichobius joblingi	MH040583	MT197542
1721a	Gloeandromyces hilleri	Mastoptera guimaraesi		MT197534
1722a	Gloeandromyces hilleri	Mastoptera guimaraesi	MT184892	MT197535
1738b	Gloeandromyces hilleri	Mastoptera guimaraesi	MT184893	MT197536
1319b	Gloeandromyces nycteribiidarum	Megistopoda aranea	MH040566	MT197540
1334c	Gloeandromyces nycteribiidarum	Megistopoda aranea	MH040567	MT197533
1306b	Gloeandromyces pageanus f. alarum	Trichobius joblingi	MH040574	
1322a	Gloeandromyces pageanus f. alarum	Trichobius joblingi	MH040577	
1327a	Gloeandromyces pageanus f. alarum	Trichobius joblingi	MH040578	
1091b	Gloeandromyces pageanus f. pageanus	Trichobius dugesioides	MG906798	
1367b	Gloeandromyces pageanus f. pageanus	Trichobius dugesioides	MH040568	
1425a	Gloeandromyces pageanus f. pageanus	Trichobius dugesioides	MH040569	
619a	Gloeandromyces pageanus f. polymorphus	Trichobius joblingi	KT800008	
1073b	Gloeandromyces pageanus f. polymorphus	Trichobius joblingi	MH040570	
1089a	Gloeandromyces pageanus f. polymorphus	Trichobius dugesioides	MH040571	
1100b	Gloeandromyces pageanus f. polymorphus	Trichobius joblingi	MH040572	
1272a	Gloeandromyces pageanus f. polymorphus	Trichobius dugesioides	MH040573	
1315a	Gloeandromyces pageanus f. polymorphus	Trichobius joblingi	MH040575	
1315b	Gloeandromyces pageanus f. polymorphus	Trichobius joblingi	MH040576	
1090a	Gloeandromyces streblae f. streblae	Trichobius dugesioides	MH040584	
1306c	Gloeandromyces streblae f. streblae	Trichobius joblingi	MH040585	MT197537
1308b	Gloeandromyces streblae f. streblae	Trichobius dugesioides	MH040586	
1309a	Gloeandromyces streblae f. streblae	Trichobius dugesioides	MH040587	MT197538
1317a	Gloeandromyces streblae f. streblae	Trichobius joblingi	MH040588	
1335c	Gloeandromyces streblae f. streblae	Trichobius joblingi	MH040589	MT197546
1320b	Gloeandromyces streblae f. sigmomorphus	Trichobius joblingi	MH040579	MT197541
1324b	Nycteromyces streblidinus*	Trichobius joblingi		MT197543
1324d	Nycteromyces streblidinus*	Trichobius joblingi		MT197544
1324e	Nycteromyces streblidinus*	Trichobius joblingi		MT197545
1136h	Stigmatomyces ceratophorus*	Fannia canicularis	MH145384	

Note. Species with an asterisk (\*) were selected as outgroup.

#### **TAXONOMY**

Gloeandromyces hilleri Haelew. & Pfliegler, sp. nov. FIG. 4

MycoBank MB834965

*Typification*: PANAMA. PANAMÁ OESTE PROVINCE: Isla de Barro Colorado, Lab Clearing, 9° 09'57.0"N, 79°50'15.6"W, 23 Jul 2013, on male *Mastoptera* cf. *guimaraesi* Wenzel, 1966 (Diptera, Streblidae), collected from male *Phyllostomus hastatus* (Chiroptera, Phyllostomidae), *Thomas Hiller P\_0075* (fly vial code) *BCA\_0120* (bat code), slide D. Haelew. 970b (**holotype** FH 00313744, 2 adult thalli from abdominal tergites). GenBank (isolate D. Haelew. 970a, 4 juvenile and 5 adult thalli from legs): SSU = MT184877.

*Etymology*: Named in honor of Dr. Thomas Hiller, bat fly specialist and collaborator of Danny Haelewaters in many projects.

*Diagnosis*: Different from *G. nycteribiidarum* by its perithecial venter with 2–3 longitudinal rows of undulations.

*Description*: Cell I short, slightly arcuated toward anterior side, carrying cells II and VI, proximally ending in an inconspicuous, slightly melanized foot. Cell II subequal or trapezoidal; septum II/III very oblique,

slightly arcuate. Cell III trapezoidal, dorsally slightly inflated. Basal cell of appendage pentagonal, narrower than cell III, colored darker yellowish brown in lower half, margins slightly broadening distally, giving rise to two short (up to 35 µm) branches of dichotomously dividing cells; outer suprabasal cell wider than inner one, both equally high; terminal cells antheridial. Cell VI obliquely positioned, irregularly sickle-shaped, sideways surrounded by cells II and VII. Cell VII irregular, usually anteriorly inflated. Perithecium with the venter slightly elongated, anterior margin nearly straight; posterior margin convex, with a longitudinal row of 7-9 roughly equal-sized triangular undulations rounded at the tip at the venter's entire length; 1-2 parallel rows with minor and often less undulations may be present; in addition, a conspicuous longitudinal ridge without undulations is present along the entire length of the perithecial venter; rows and ridge occasionally obliquely positioned. Perithecial neck abruptly distinguished, narrow, with subparallel margins; second tier of wall cells strongly curved; third tier of wall cells straight, ending with two rounded lobes, tapering to the tip, which is short and asymmetrical.

*Measurements: Thallus* 251–371  $\mu$ m in length from foot to perithecial tip. Cell I 51–122 × 19–51  $\mu$ m. Basal



**Figure 2.** Maximum clade credibility tree showing species in the genus *Gloeandromyces*, with *Stigmatomyces ceratophorus* as outgroup. The tree is the result of a Bayesian analysis of the LSU data set with 29 isolates. For each node, the Bayesian posterior probability ( $\geq$ 0.9) is presented above or below the branch leading to that node. Gray shading is added to indicate the new species.

cell of appendage 6–15 × 9–24  $\mu$ m. Perithecium 147–221 × 40–72  $\mu$ m. Ascospores 34–37 × 4.2–5.3  $\mu$ m (up to 9.6  $\mu$ m wide including slime sheath).

Additional specimens *examined*: PANAMA. PANAMÁ OESTE **PROVINCE:** de Isla Barro Colorado, shortcut to Fausto Trail (behind Smithsonian Tropical Research Institute Building F), 9° 09'57.0"N, 79°50'15.6"W, 22 Aug 2015, on female M. guimaraesi collected from female P. hastatus, Thomas Hiller P\_3133 (fly vial code) BCA\_6542 (bat code), slides D. Haelew. 1721b (paratype PUL F25939, 1 adult thallus from right-hand side of dorsal abdomen), D. Haelew. 1721c (paratype PUL F25940, 1 adult thallus from right-hand side of dorsal abdomen), D. Haelew. 1721d (paratype PUL F25941, 1 adult thallus from right metacoxa), and D. Haelew. 1721e (paratype PUL F25942, 1 adult thallus from right metacoxa), GenBank (isolate D. Haelew. 1721a, 1 adult thallus from dorsal abdomen): SSU = MT184878, LSU = MT184328, TEF1 = MT197534; ibid., on female M. guimaraesi collected from female P. hastatus, Thomas Hiller P\_3133 (fly vial code) BCA\_6542 (bat code), slide D. Haelew. 1722b (paratype PUL F25937, 1 adult thallus from dorsal abdomen, toward distal end), GenBank (isolate D. Haelew. 1722a, 1 adult thallus from dorsal abdomen): SSU = MT184879, LSU = MT184892, TEF1 = MT197535); ibid., on male M. guimaraesi collected from female P. hastatus, Thomas Hiller P\_3133\_c (fly vial code) BCA 6542 (bat code), slide D. Haelew. 1723a (paratype PUL F25936, juvenile thalli from left metafemur); ibid., on female M. guimaraesi collected from female P. hastatus, Thomas Hiller P\_3133d (fly vial code) BCA\_6542 (bat code), slide D. Haelew. 1724a (paratype PUL F25932, 1 adult thallus from dorsal abdomen, toward proximal end); ibid., on female M. guimaraesi collected from female P. hastatus, Thomas Hiller P\_3166 (fly vial code) BCA\_6541 (bat code), slide D. Haelew. 1738a (paratype PUL F25938, 1 adult thallus from dorsal abdomen), GenBank (isolate D. Haelew. 1738b, 1 adult thallus from dorsal abdomen):



**Figure 3.** Maximum clade credibility tree showing species in the genus *Gloeandromyces*, with *Stigmatomyces ceratophorus* as outgroup. The tree is the result of a Bayesian analysis of the *TEF1* data set with 14 isolates. For each node, the Bayesian posterior probability ( $\geq 0.9$ ) is presented above or below the branch leading to that node. Gray shading is added to indicate the new species.

SSU = MT184880, LSU = MT184893, *TEF1* = MT197536. ECUADOR. ESMERALDAS PROVINCE: San Francisco de Bogota, 1°05'15.72"N, 78°41'29.4"W, 74 m above sea level (a.s.l.), 6 Aug 2014, on female M. guimaraesi collected from female P. hastatus, Carl W. Dick TK 135532 (fly vial code) RMFN-588 (bat code), slides D. Haelew. 1021a (paratype FH, 1 adult thallus from dorsal abdominal tip), D. Haelew. 1021b (paratype FH, 1 adult thallus from left metacoxa), and D. Haelew. 1021c (paratype PUL F25935, juvenile thalli from dorsal abdominal tip); surroundings of San Francisco de Bogota, 1°04'21.36"N, 78°42'41.4"W, 86 m a.s.l., 9 Aug 2004, on male M. guimaraesi collected from female P. hastatus, Carl W. Dick TK 135881 (fly vial code) RMFN-588 (bat code), slide D. Haelew. 3024a (paratype PUL F25934, 1 juvenile thallus from left mesocoxa); GUAYAS PROVINCE: Manglares Churute, Cerro Cimalón, 2°25' 36.48"S, 79°33'40.68"W, 34 m a.s.l., 30 Jun 2004, on female M. guimaraesi collected from female P. hastatus, Carl W. Dick TK 134697 (fly vial code) SS-2342 (bat code), slides D. Haelew. 3023a (paratype PUL F25931, 5 adult thalli from right-hand side of dorsal abdomen) and D. Haelew. 3023b (paratype PUL F25933, 5 adult thalli from left-hand side of dorsal abdomen).

Notes: Currently, four species of *Gloeandromyces* have been formally described. These are *G. dickii* from Ecuador, Nicaragua, and Panama; *G. nycteribiidarum* from Costa Rica and Grenada; *G. pageanus* from Panama and Trinidad; and *G. streblae* from Panama and Venezuela (Thaxter 1917, 1931; Haelewaters et al. 2017; Haelewaters and Pfister 2019). Gloeandromyces hilleri is morphologically very distinctive by the longitudinal rows of undulations at the perithecial venter. However, we have observed some thalli on a single M. guimaraesi bat fly without the typical rows of undulations. We were doubtful about their identity until sequence data showed that they are representative of the same species (GenBank accession nos. MT184328 [LSU] and MT197534 [TEF1]). Also, thalli with the typical rows of undulations were present on this bat fly specimen (D. Haelew. 1721). This species exhibits intraspecific morphological plasticity, as previously reported for this genus (Haelewaters and Pfister 2019) as well as in another genus of bat flyassociated Laboulbeniales fungi, Arthrorhynchus (Blackwell 1980). It is also possible that the undulations only develop in adult specimens; more material is needed to understand the general developmental schema from ascospore to mature thallus.

#### DISCUSSION

**Secondary markers in Laboulbeniomycetes taxonomy.**—As we are discovering more and more cryptic fungal species, descriptions and identifications increasingly rely on good DNA barcode markers. The multicopy internal transcribed spacer (ITS) region was proposed as a universal barcode for Fungi (Schoch et al. 2012). For more than 70% of described fungi, the ITS



**Figure 4.** *Gloeandromyces hilleri*. Ascospores and adult thallus from the holotype slide (FH 00313744). Shown are cells I, II, III, VI, VII, the basal cell of the appendage (ba), and the four tiers of perithecial wall cells (w1 through w4). Bar = 50  $\mu$ m; del. Jingyu Liu.

region is effective in species recognition. Also in the Laboulbeniomycetes, ITS has been used for species delimitation, in Chitonomyces Peyr. (Goldmann and Weir 2012), Coreomyces Thaxt. (Sundberg et al. 2018), Herpomyces (Haelewaters et al. 2019; Gutierrez et al. 2020), and Hesperomyces Thaxt. (Goldmann et al. 2013; Haelewaters et al. 2018a). However, Walker et al. (2018) noted that the ITS region is hard to amplify for species of Laboulbeniomycetes using the standard universal or fungal primers such as ITS1f and ITS4 (White et al. 1990; Gardes and Bruns 1993). Both the ITS1 and ITS2 spacer regions are highly variable, and we do not know the extent of this variability in taxa for which no ITS sequence data exist, such as the vast majority of Laboulbeniales genera (Nilsson et al. 2008). An important result of this variability is primer mismatches, such as those identified for Archaeomycetes sequences in the binding site of the commonly used reverse primer ITS4 (Rosling et al. 2011), or for Ceraceosorales sequences in the ITS1 binding site (Kijpornyongpan and Aime 2016), which could have unforeseen consequences in underestimating diversity of fungi based on ITS sequencing only. In time, the design of specific primers (e.g., ITShespL and ITShespR specific for *Hesperomyces*; Haelewaters et al. 2018a) and next-generation sequencing approaches such as WideSeq allowing for longrange PCR amplification from the SSU to the LSU, thus avoiding that primers need to anneal to the variable ITS spacers (D. Haelewaters and M.C. Aime, unpubl. data), will help to generate ITS sequences for more Laboulbeniales taxa.

Recent work in Laboulbeniomycetes polyphasic taxonomy (Haelewaters et al. 2018a; Sundberg et al. 2018; Walker et al. 2018) has shown the potential of LSU as a secondary barcode, building on earlier reports of LSU and ITS performing equally for ascomycetous fungi in the subphylum Pezizomycotina (Schoch et al. 2012). The fact that LSU is also easy to amplify using universal primers makes this region a favorable marker in Laboulbeniomycetes taxonomy. Several studies have shown that the resolution of ITS is inferior to that of protein-coding genes. Lower taxon-specific techniques have been developed in the use of protein-coding secondary barcodes-calmodulin (CaM), RNA polymerase II largest and second largest subunits (RPB1, RPB2), and TEF1 (Nilsson et al. 2006; Seifert 2009; Schoch et al. 2012; Samson et al. 2014; Al-Hatmi et al. 2016).

In this paper, we have for the first time generated TEF1 sequences for any Laboulbeniomycetes taxa. Using general primers as provided by Stielow et al. (2015) resulted in the amplification of TEF1 for 14 Laboulbeniales isolates, including 11 isolates of Gloeandromyces spp. and 3 isolates of Nycteromyces streblidinus (TABLE 1). Successful primer combinations for our work were Al33\_alt\_f/EF1-1620R and EF1-1018F/EF1-1620R. The TEF1 data set did not include G. pageanus, but otherwise all other species of Gloeandromyces, including G. hilleri, were represented. The resulting TEF1 topology (FIG. 4) agrees with the LSU one (FIG. 3); G. hilleri is positioned as sister to G. nycteribiidarum, and the G. hilleri + G. nycteribiidarum clade is placed sister to G. dickii. The TEF1 topology also places the recently described positioninduced morphotype G. streblae f. sigmomorphus among isolates of "typical" G. streblae, confirming what Haelewaters and Pfister (2019) found in their LSU phylogeny.

As a final note, the SSU region also has been used for species delimitation purposes (see Goldmann and Weir 2012; Sundberg et al. 2018). However, compared with ITS and LSU trees, evolutionary distances among species are comparatively shorter in obtained SSU trees and nodes often lack support. In addition, in order for SSU to provide any resolution at species level, it is important to amplify and sequence large spans, preferably using NS1/NS4 (~1150 bp) or NS1/NS6 (~1450 bp) primer sets (White et al. 1990), to include phylogenetically informative regions. As a standard in our laboratory to increase amplification success and decrease costs of sequencing, however, we amplify the SSU region using Laboulbeniales-specific primers NSL1 and NSL2 (Haelewaters et al. 2015), resulting in a ~550 bp sequence. This sequence provides good resolution for higher-level molecular systematics (e.g., placement of taxa among other Laboulbeniomycetes) but is of little use for species-level phylogenetic analyses.

#### Biology and ecology of bat and bat fly hosts.-

Gloeandromyces hilleri is thus far only known from Mastoptera guimaraesi bat flies. This bat fly species is primarily found on Phyllostomus hastatus bats and has been reported from Colombia, Costa Rica, Ecuador, Honduras, Nicaragua, Panama (type), Paraguay, and Venezuela (Wenzel et al. 1966; Guerrero 1995; Dick and Gettinger 2005; Stamper 2012; Dick 2013). Phyllostomus hastatus is a relatively common and widespread phyllostomine bat species (Phyllostomidae subfamily Phyllostominae) that has been reported to roost in a variety of structures. Known roosting structures include caves, hollow logs and trees, hollow termite nests, tree leaves, and various man-made structures such as houses and other buildings (Tuttle 1976; Patterson 1992; Linares 1998). Moreover, and at least in some of these roost types, colonies consist of harems of up to 30 adult females plus a dominant male. Many of these harems roost together for many years, although turnover of males may be frequent (Nowak 1991).

These long-lived social groups, particularly if they occur in more permanent roost types (Patterson et al. 2007), allow for stable and predictable host associations of *P. hastatus* with its primary bat fly associates. These include M. guimaraesi as well as Strebla consocia Wenzel, 1966 and Trichobius longipes (Rudow, 1971) (Wenzel 1976). The co-occurrence of these three bat fly species on P. hastatus individuals and populations, coupled with predictable roosting associations, should increase the potential for host shifts of fungi such as G. hilleri from its type host M. guimaraesi to other co-occurring bat fly species. The potential for additional host associations of G. hilleri may be illuminated through further studies into the diversity and biology of Laboulbeniales on Neotropical bat flies.

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