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PHYLOGENETIC SPECIES IDENTIFICATION OF *PILOBOLUS* ASSOCIATED WITH HORSES IN INDIANA AND OHIO

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ABSTRACT. *Pilobolus*, a coprophilous zygomycete, is associated with herbivores. The various species of this fungus have been collected from a wide range of hosts. Most species have been isolated from multiple hosts. However, *P. longipes* has been reported exclusively associated with *Equus* spp. This study was undertaken to examine the species specific relationship of *P. longipes* and members of *Equus,* specifically *E. caballus* (horses). Dung samples were collected from horses located within 25 miles of Richmond, Indiana for isolates of *Pilobolus*. Sporangiospores from these fungal isolates were used as the source of DNA. Sequences of taxonomically informative 18S and ITS regions of rDNA were obtained using previously published protocols. DNA sequences were aligned with BLAST and compared with sequences deposited in GenBank. Sequences of DNA from the isolates in this study were examined using MEGA 4 with Clustal W. By comparing the sequences of DNA from isolates in this study with those in GenBank, it was determined that *P. longipes*, *P. sphaerosporus*, *P. kleinii* and *P. pullus* are associated with horses in Indiana and Ohio. All isolates recovered in this study are large-spore producing species of *Pilobolus*.

Keywords: *Pilobolus*, horses, DNA, ITS, 18S

INTRODUCTION

The coprophilous fungus, *Pilobolus*, is a well known, but little studied organism. Because of its dramatic ballistic spore discharge mechanism and its photogenic qualities, *Pilobolus* is mentioned in many general biology texts and is often given a lot of space in mycology texts (Buller 1934, Yafetto et al. 2008, Page 1962, 1964). However, other than these characteristics, little is known about the environmental conditions most conducive to its growth, and while having been collected and described from many locations worldwide, few correlations have been drawn about the growth of the fungus in relationship to its hosts, climate, nutrition, economic value, parasitic or symbiotic relationships with other organisms.

Pilobolus has been reported associated with many herbivores including virtually all ungulates, a large number of rodents, and various other mammals (Hu et al. 1989, Santiago et al. 2008). However, there seems to be almost no correlation between most species of *Pilobolus* and a particular species of host. However, *P. longipes* has been reported only associated with the genus *Equus* (horses, zebras, donkeys). This study was designed to examine the specificity of this relationship of the various species of *Pilobolus* and *Equus caballus* (horses) and to determine whether *P. longipes* is the most frequently occurring species found in association with horses. *Pilobolus* isolates associated with each of the horses in this study were identified to species using molecular techniques.

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METHODS AND MATERIALS

Isolates of *Pilobolus* were collected from the dung of horses in Ohio and Indiana and cultivated in microcosms until sporangia were produced. Mature sporangia were collected and maintained in collecting water using techniques described previously (Foos 1989, Foos & Royer 1989, Foos et al. 2001).

It has been shown that multiple species of *Pilobolus* exist simultaneously in dung (Foos 1997), so multiple sporangia were collected from each dung sample. When two or more isolates from the same dung sample were morphologically identical, only one was maintained for further study.

Pure cultures were obtained using single sporangium transfers to plastic Petri dishes containing dung agar or synthetic hemin medium (SHM) (Levetin &Caroselli 1976). Cultures arising from sporangial transfers were

maintained on SHM or dung agar in disposable plastic Petri dishes sealed with parafilm at 22 ± 2 °C with alternating 12 h light and dark periods of 2000 lux, cool white fluorescent illumination. Sporangia from pure culture isolates were examined microscopically and tentatively identified to species using morphological characteristics.

Individual sporangia which adhered to the lids of the Petri dishes were collected using sterile inoculating needles and placed in 0.2 ml microcentrifuge tubes containing 20 µl sterile collecting water (with 3% penicillin, 3% streptomycin, and 1% Tween 20), labeled and stored at 4 °C. DNA was extracted from the sporangiospores using techniques described previously (Foos et al. 2011).

The primers designed specifically to amplify and sequence taxonomically significant DNA fragments were used (White et al. 1990). Primers NS1 – NS8 were used to amplify the nuclear 18S small subunit (SSU) of rRNA and primers ITS5 and ITS4 were used to amplify the entire ITS region of nuclear rRNA, specifically the 5.8S region, the associated internal transcribed spacers (ITS1 and ITS2), with terminal portions of the 18S and 28S regions of rRNA.

DNA was amplified using AmpliTaq® Gold DNA polymerase (Applied Biosystems, Foster City, California) and a dNTP mix (Promega Corporation, Fitchburg, Wisconsin). Thermal cycling was conducted in a Perkin Elmer GeneAmp® PCR System 2400. PCR reaction conditions for thermal cycling were 94 °C for 5 min, followed by 36 cycles of 94 °C for 1 min, 50 °C for 1 min 30 sec, 72 °C for 2 min, followed by an extension at 72 °C for 7 min. PCR products were purified with QIAquick® PCR Purification Kit (Qiagen, Inc., Valencia, California).

PCR amplified DNA fragments were electrophoresed prior to and following the clean up process, on 1% agarose gels in 1X TBE buffer (50 mM Tris-HCl, 50 mM boric acid, and 1 mM EDTA) containing ethidium bromide and visualized using a ChemiImager™ 4400 Imaging System (Alpha Innotech, San Leandro, California). A 100 bp DNA ladder (Takara Mirus Bio, Madison, Wisconsin) was used as a size marker.

PCR products were sequenced with BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California) and a sequence reaction mix comprised of 2 µl H_2O, 3 µl 5X buffer, 1 µl BigDye, 2 µl 10 mM primer, and 2 µL fungal DNA. Thermal cycling conditions for sequencing were 25 cycles of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. Sequences were analyzed using an Applied Biosystems 3700 automated fluorescence system at the Indiana Molecular Biology Institute.

DNA sequences were examined and compared using CodonCode Aligner (CodonCode Corp., Dedham, Massachusetts) containing PHRED and PHRAP (Ewing &Green 1998, Ewing et al. 1998) for base calling, sequence comparisons and sequence assembly. Contigs created in CodonCode were oriented with BLAST (Altschul et al. 1990) and aligned using Clustal W (Thompson et al. 1994, 1997). Phylogenetic and molecular evolutionary analyses were conducted and trees constructed using MEGA version 4 (Tamura et al. 2007).

Evolutionary histories of both 18S and ITS rDNA sequences were inferred using the neighbor-joining method (Saitou &Nei 1987). The bootstrap consensus tree inferred from 2000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test were shown next to the branches (Felsenstein 1985). The trees were drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using maximum composite likelihood (Tamura et al. 2004) and are in the units of the number of base substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons.

RESULTS

Fifteen isolates of *Pilobolus* were obtained from horse dung in Ohio and Indiana. Locations, collection dates, and voucher numbers of these isolates are listed in Table 1. *Pilobolus kleinii* Tiegh., *P. longipes* Tiegh., *P. pullus* Massee and *P. shpaerosporus* Palla were isolated from this dung. DNA sequences of both 18S and ITS regions were analyzed from each isolate, except that the sequences from the 18S region of IUE 0018 and the ITS regions of IUE 0006 and IUE 0021 could not be recovered.

Table 1.—Specimens of *Pilobolus* isolated from horse dung in Ohio and Indiana listed by IUE voucher numbers, collection dates and locations.

Voucher Number	Date	Location	GPS-N	GPS-W	Species
IUE0002	5/2/2004	Fayette Co., IN	39° 38.798′	85° 12.860′	*P. sphaerosporus*
IUE0004	5/8/2004	Union Co., IN	39° 38.570′	84° 49.920′	*P. kleinii*
IUE0005	5/8/2004	Union Co., IN	39° 36.709′	84° 53.225′	*P. kleinii*
IUE0006	5/8/2004	Union Co., IN	39° 38.815′	84° 54.507′	*P. kleinii*
IUE0007	5/22/2004	Wayne Co., IN	39° 52.008′	85° 09.714′	*P. sphaerosporus*
IUE0009	5/24/2004	Preble Co., OH	39° 51.976′	84° 30.283′	*P. kleinii*
IUE0010	6/14/2004	Wayne Co., IN	39° 57.501′	85° 06.160′	*P. pullus*
IUE0013	6/14/2004	Wayne Co., IN	39° 57.869′	85° 05.983′	*P. sphaerosporus*
IUE0014	6/14/2004	Wayne Co., IN	39° 59.574′	85° 05.329′	*P. pullus*
IUE0015	6/14/2004	Wayne Co., IN	39° 56.831′	85° 04.044′	*P. sphaerosporus*
IUE0016	7/12/2004	Darke Co., OH	39° 56.079′	84° 30.305′	*P. longipes*
IUE0017	7/12/2004	Darke Co., OH	39° 55.947′	84° 29.859′	*P. pullus*
IUE0018	7/12/2004	Darke Co., OH	39° 58.577′	84° 32.364′	*P. sphaerosporus*
IUE0020	7/12/2004	Darke Co., OH	39° 57.774′	84° 33.122′	*P. longipes*
IUE0021	7/12/2004	Preble Co., OH	39° 52.144′	84° 43.396′	*P. sphaerosporus*

Phylogenetic species identification using phylograms.—Phylograms were created using orthologous sequences from six species of *Pilobolus* obtained from GenBank as controls. These DNA sequences from these GenBank ex-type specimens were used as a phylogenetic species key.

The phylogram (Fig. 1) is inferred from the rDNA sequences that code for the 18S region of rRNA of the isolates. Sequences from the isolates in this study form a clade with large-spore producing species of *Pilobolus* from GenBank (*P. kleinii*, *P. longipes* and *P. sphaerosporus*), distinct from the clade with small-spore producing species from GenBank (*P. crystallinus*, *P. roridus* and *P. umbonatus*). Two subordinate or sister clades formed within the major clade of large-spore producing species. One contains *P. sphaerosporus* (DQ211052) from GenBank and isolates of *P. sphaerosporus* and *P. pullus* from this study. *Pilobolus pullus* forms a subordinate clade distinct from that of *P. sphaerosporus*. The other clade contains *P. longipes* (DQ211053), and *P. kleinii* (EU595656) from GenBank and specimens from this study. There is strong bootstrap support [100%] for these clades as represented in the phylogram.

The phylogram (Fig. 2) inferred from the rDNA sequences that code for the ITS region of rRNA is very similar to the phylogram inferred from the sequences for the 18S region (Fig. 1). One major clade includes all of the large-spore producing species from GenBank (*P. kleinii*, *P. sphaerosporus*, *P. longipes* and *P. heterosporus*) and sequences from specimens from this study. (The ITS sequence from *P. heterosporus* (HM049582) in GenBank is included. There is no 18S sequence of this species in GenBank or other DNA sequence repository.) The small spore-producing species from GenBank (*P. crystallinus*, *P. roridus* and *P. umbonatus*) are outside this clade. This major clade includes subordinate clades which contain all of the specimens from this study. *Pilobolus sphaerosporus* (DQ059382) from GenBank and specimens from this study form one subordinate clade. The isolates of *P. kleinii* and *P. pullus* are in a second subordinate clade, while *P. longipes* (FJ160950) and *P. heterosporus* (HM049582) from GenBank and specimens from this study form a third clade. The inferences from these distinct clades are supported by strong bootstrap values.

Phylogenetic species identification using homology.—Species identification using sequence identity of homologous regions was recently reported for *Pilobolus* (Foos & Sheehan 2011). Representative sequences of all species of *Pilobolus* deposited in GenBank were used as controls representing these species. Percentage identity of both the homologous 18S and ITS regions were examined.

When comparing specimens of various species of *Pilobolus*, homologous 18S regions of rDNA from GenBank representatives had $>$ 97% identity. Table 2 shows the percentage

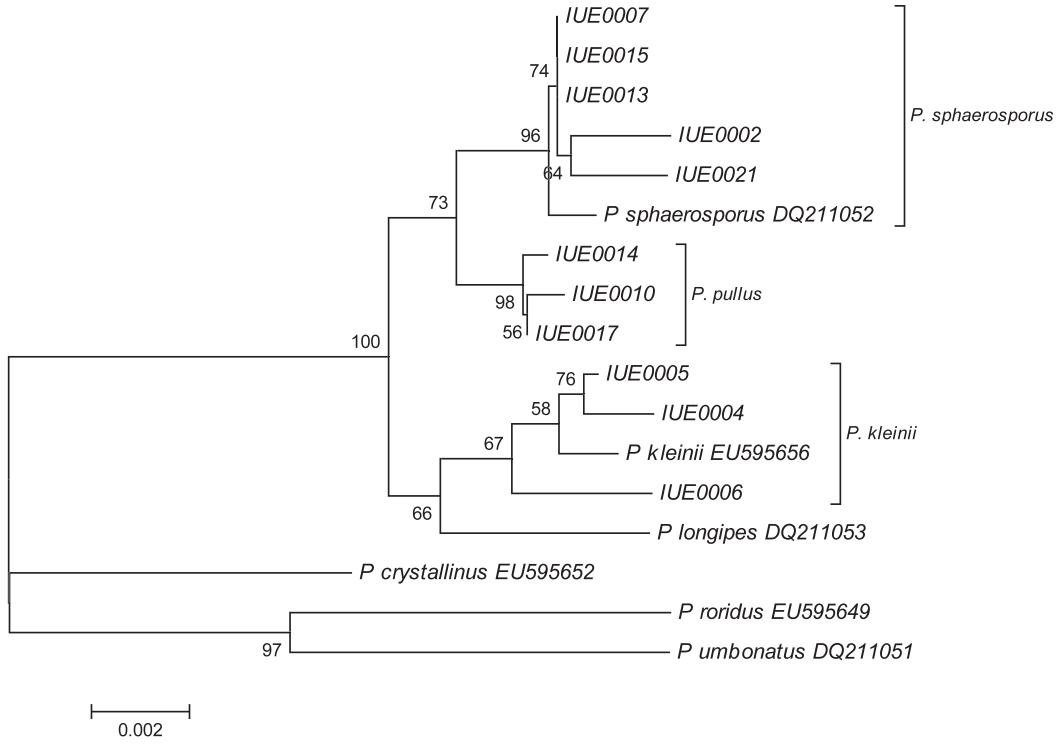

Figure 1.—Evolutionary relationships of *Pilobolus* based on 18S rDNA. The analysis involved 17 nucleotide sequences; ambiguous positions were removed for each sequence pair. There were a total of 1768 positions in the final dataset.

identities of the homologous 18S regions of rDNA for specimens from this study compared with the orthologous regions of GenBank specimens. The sequence homology of the three isolates of *P. kleinii* from this study and *P. kleinii* (EU595656) from GenBank displayed $>$ 99.6% identity. Some variation may be attributed to particular strains of the species or associated with the molecular techniques used to amplify and sequence the DNA.

The three isolates of *P. sphaerosporus* were identical to each other and had $>$ 99.8% identity with *P. sphaerosporus* (DQ211052) from GenBank. The other two isolates of this species were unique, yet had $\geq$ 99.6% identity with the GenBank control.

In all instances the identities of intraspecies homologous 18S rDNA regions from isolates from this study and from GenBank sequences were $\geq$ 99.6%. Interspecies homologies of the same 18S regions from the GenBank specimens had $<$ 99.3% identity.

Table 3 shows the percentage identities of the homologous ITS regions of rDNA for specimens from this study compared with the orthologous regions of GenBank specimens.

The identities of the homologous ITS regions of the three specimens of *P. kleinii* from this study and the GenBank control (FJ160957) were $>$ 95.4%. The homologous ITS regions of the two specimens of *P. longipes* from this study and GenBank (FJ160950) displayed $>$ 97.7% identity. The homologous ITS regions of *P. sphaerosporus* specimens from this study had $>$ 89.4% identity with GenBank (DQ059382). The homologous ITS regions of the three isolates of *P. pullus* had $>$ 99.8% identity to each other. No sequences from isolates of *P. pullus* are present in GenBank or other DNA sequence repository, so these isolates were compared with sequences from all species of *Pilobolus* for which sequences were available. When compared to the homologous ITS regions of the four large-spore producing species of *Pilobolus* in GenBank, including *P. heterosporus* (HM049582), the *P. pullus* sequences displayed 78.3 – 83.2% identity.

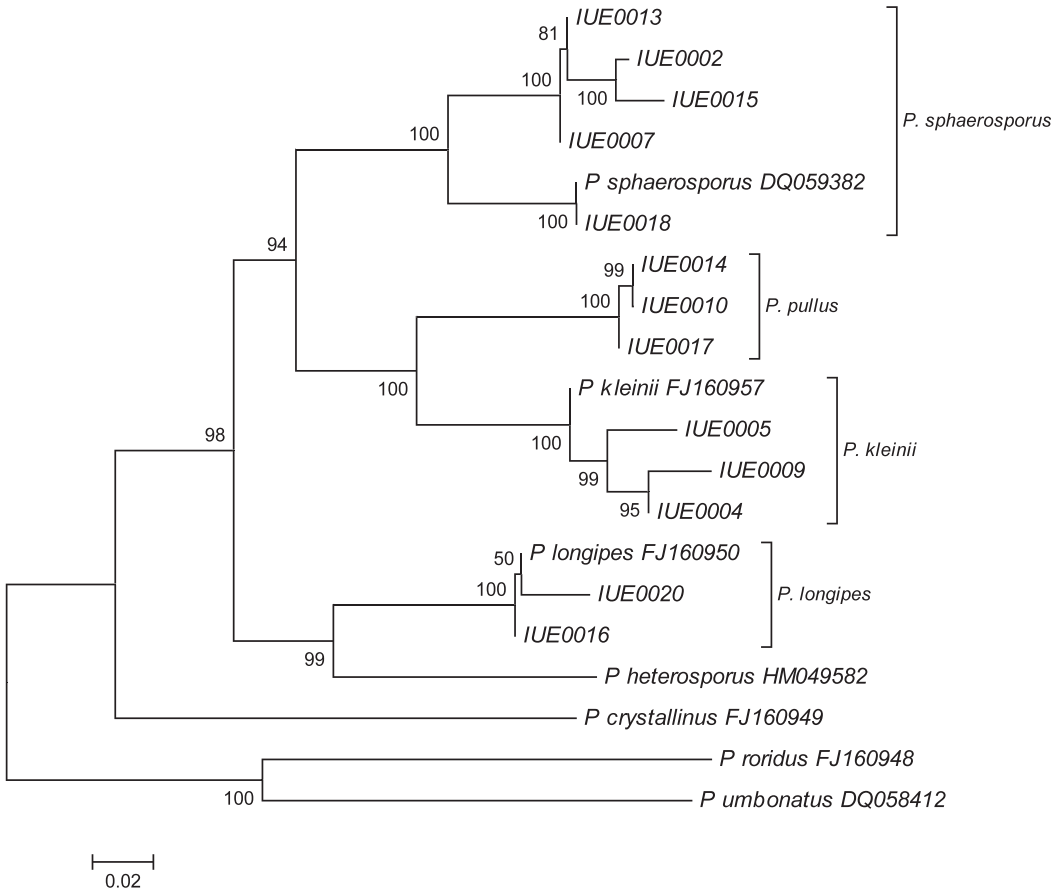

Figure 2.—Evolutionary relationships of *Pilobolus* based on ITS rDNA. The analysis involved 20 nucleotide sequences; ambiguous positions were removed for each sequence pair. There were a total of 811 positions in the final dataset.

DISCUSSION

Specimens of *Pilobolus* collected from horses in Indiana and Ohio were identified by phylogenetic species identification techniques using the 18S regions and the ITS regions of rRNA as taxonomic markers. Orthologous regions of DNA sequences from ex-type isolates of species of *Pilobolus* from GenBank were used for comparison.

Inferred evolutionary histories and taxonomically important characteristics used for identification do not always agree. However, the use of phylograms to show relationships among DNA sequences from various organisms permits an opportunity to distinguish among species and strains in which these DNA sequences differ. The phylogram in Figure 1 displays the inferred evolutionary distance based upon 18S rDNA sequences in this study and shows a great evolutionary distance between the major clade containing *P. sphaerosporus*, *P. pullus*, *P. kleinii* and *P. longipes*, all large-spore producing species, from *P. crystallinus*, *P. roridus* and *P. umbonatus*, all small-spore producing species. Figure 2, a phylogram illustrating the inferred evolutionary distance based upon ITS rDNA sequences shows the same pattern. The relationship of *P. pullus* to *P. kleinii* and *P. sphaerosporus* differs in the two inferred evolutionary histories and supports the concept of a "*Pilobolus kleinii* group" suggested earlier (Palla 1900, Massee 1901, Grove 1934).

The high percentage identities of the homologous 18S regions of rDNA make these good sequences to use to identify specimens of *Pilobolus* to genus. Intraspecies identities of the homologous 18S region of rDNA from specimens of *Pilobolus* were $> 99\%$. The most

Table 2.—Comparison of 18S homologous rDNA sequences from known species of *Pilobolus* in GenBank with sequences from isolates collected in this study in percent identity. No GenBank representative of *P. pullus* existed prior to this study. Here *P. pullus* is compared with representatives of all *Pilobolus* species from GenBank.

GenBank Control Species	bp	GenBank Number	This Study Isolate	bp	IUE ID	GenBank Number	Identity %
P. kleinii	1763	EU595656	*P. kleinii*	1763	0004	HQ682649	100.00
			P. kleinii	1763	0005	AY823738	99.98
			P. kleinii	1764	0006	DQ363379	99.60
			P. pullus	1764	0017	HQ877880	99.32
P. longipes	1763	DQ211053	*P. pullus*	1764	0017	HQ877880	99.21
P. sphaerosporus	1764	DQ211052	*P. sphaerosporus*	1764	0002	HQ682648	99.89
			P. sphaerosporus	1764	0007	HQ682650	99.89
			P. sphaerosporus	1764	0013	HQ682651	99.89
			P. sphaerosporus	1764	0015	HQ682652	99.89
			P. sphaerosporus	1763	0021	HQ682653	99.60
			P. pullus	1764	0017	HQ877880	99.55
P. pullus	1764	HQ877880	*P. pullus*	1764	0014	HQ877879	99.94
			P. pullus	1674	0010	HQ877878	99.76
P. crystallinus	1763	EU595652	*P. pullus*	1764	0017	HQ877880	98.13
P. roridus	1762	EU595649	*P. pullus*	1764	0017	HQ877880	97.34
P. umbonatus	1764	DQ211051	*P. pullus*	1764	0017	HQ877880	97.51

similar orthologous 18S sequence from a genus other than *Pilobolus* found in GenBank was 94.18% identity from *Pilaira anomala* (EU-595659). From this study we can infer that homologous identities of the 18S region between isolates of *Pilobolus* > 99% indicate the same species, identities of 95 – 99% indicate both are the same genus, but different species.

The rDNA that codes for the complete ITS region of rRNA contains two non-coding internal transcribed spacers that are more variable than coding regions (Nilsson et al. 2008). Because of this homologous identities within ITS regions of rDNA are much lower than those of the 18S regions.

Intraspecies identities of homologous ITS regions from the specimens collected in this study and the GenBank controls ranged from 95.4 – 100% for *P. kleinii*, 97.7 – 100% for *P. longipes*, 89.4 – 100% for *P. sphaerosporus* and > 99.8% among the specimens of *P. pullus*. The relative low percentage identity (89.4%) of intraspecies homologies of the ITS region of *Pilobolus sphaerosporus* isolates in this study indicates multiple strains.

Homologous identities of ITS regions of rDNA from isolates of different species of *Pilobolus* deposited in GenBank range from 59.7 – 83%. The most similar orthologous ITS sequence from a genus other than *Pilobolus* found in GenBank was from *Mucor heimalis* (DQ888726) with 62.0% identity. Interspecies homologous identities of the ITS regions of rDNA of isolates are more variable than those of 18S regions. Two isolates of *Pilobolus* having homologous identities of > 85% would indicate that they are the same species.

Variability of taxonomically valuable morphological characteristics is at the core of the difficulty identifying cryptic species of *Pilobolus*, thus requiring the use of phylogenetic species identification. The ephemeral nature of specimens, the propensity for multiple species of the same genus to grow in the same habitat, and the difficulty in culturing cryptic organisms have caused multiple field studies to use phylogenetic species identifications to report the presence of species that have not been isolated or cultured (Smit et al. 1999, Anderson & Cairney 2004, Bonito et al. 2010, Poitelon et al. 2009). This creates a taxonomic problem. If DNA from all taxa were to have been sequenced and reported, then the discovery of a new DNA sequence would indicate the presence of an undescribed organism. However, DNA sequences from most organisms, particularly cryptic organisms and microorganisms that were collected and identified many years ago, have not been identified. So, when new phylogenetic species are identified using DNA

Table 3.—Comparison of ITS homologous rDNA sequences from known species of *Pilobolus* in GenBank with sequences from isolates collected in this study in percent identity. No GenBank representative of *P. pullus* existed prior to this study. Here *P. pullus* is compared with representatives of all *Pilobolus* species from GenBank.

GenBank Control Species	bp	GenBank Number	This Study Isolate	bp	IUE ID	GenBank Number	Identity %
P. kleinii	704	FJ160957	*P. kleinii*	613	0004	HQ682655	100.00
			P. kleinii	700	0005	HQ682656	95.43
			P. kleinii	690	0009	HQ682658	96.52
			P. pullus	689	0017	HQ877877	83.16
P. longipes	688	FJ160950	*P. longipes*	688	0016	HQ682661	100.00
			P. longipes	671	0020	HQ682663	97.76
			P. pullus	689	0017	HQ877877	78.37
P. sphaerosporus	694	DQ059382	*P. sphaerosporus*	618	0002	HQ682654	89.48
			P. sphaerosporus	695	0007	HQ682657	90.94
			P. sphaerosporus	694	0013	HQ682659	90.78
			P. sphaerosporus	696	0015	HQ682660	91.24
			P. sphaerosporus	694	0018	HQ682662	100.00
			P. pullus	689	0017	HQ877877	78.66
P. pullus	662	HQ877877	*P. pullus*	662	0014	HQ877876	100.00
		HQ877877	*P. pullus*	661	0010	HQ877875	99.85
P. heterosporus	698	HM049582	*P. pullus*	689	0017	HQ877877	82.50
P. crystallinus	657	FJ160949	*P. pullus*	689	0017	HQ877877	75.38
P. roridus	694	FJ160948	*P. pullus*	689	0017	HQ877877	70.42
P. umbonatus	707	DQ058412	*P. pullus*	689	0017	HQ877877	68.43

sequences, there is no way to know whether the organisms are undescribed morphological species, or the phylogenetic species identity of a morphological species described previously. As organisms for which we have phylogenetic species identifications are cultured and identified morphologically, we can reconcile the morphological species identity with the phylogenetic species identity of an organism.

In this study the specimens of *P. pullus* isolated at three locations are morphologically very similar to *P. kleinii*. However DNA sequences of taxonomically significant rRNA regions of *P. pullus* do not align with the homologous sequences of any known *Pilobolus* species. *Pilobolus pullus* from this study might be described as *P. kleinii* if only morphological characteristics were considered. However, the significant difference between the rDNA sequences of *P. pullus* and *P. kleinii* show them to be distinct cryptic species.

DNA sequences of only 7 of 59 species of *Pilobolus* described in the literature have been deposited in GenBank. Most of these species were originally described many years ago, the most recent by Buller (1934). Even though multiple species have been redescribed more recently (Hu et al. 1989), no molecular data were included. Specimens of *Pilobolus* are ephemeral, microscopic, and fragile. No type cultures exist. These conditions make the revision of this genus necessary. In fact, the very reasons that revisions are necessary make revisions very difficult.

The example of *P. pullus* from this study represents a chasm between phylogenetic species recognition (PSR) and morphological species recognition (MSR) and demonstrates the challenge represented the need MSR to be correlated with PSR (Taylor et al. 2006). When viable type cultures of species described in the nineteenth century and earlier are unavailable, and there is no specimen that can be examined using molecular methods, there is little likelihood that it will be possible to determine the PSR identity for most of the species that have been described.

We found four species of *Pilobolus* growing on dung of horses in Indiana and Ohio. *Pilobolus longipes*, often specifically associated with horses, was isolated. But, it did not constitute the only species nor was it the most commonly found species. The identities of the various species were supported by both

phylograms and sequence homology. These phylogenetic techniques correlated with tentative morphological species identifications. However, the phylogenetic techniques distinguished between specimens of cryptic species (*P. kleinii* and *P. pullus*) that would probably have been identified as a single species using morphological techniques.

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