

Phylogeny of *Hirsutella* species (*Ophiocordycipitaceae*) from the USA: remedying the paucity of *Hirsutella* sequence data

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Abstract: *Hirsutella* (*Ophiocordycipitaceae*: *Hypocreales*) is a genus of insect, mite, and nematode pathogens with an asexual morph, which generally produce a mucilaginous cluster of one or several conidia on phialides that are basally subulate and taper to a fine neck. The generic name *Hirsutella* has been proposed for suppression in favour of *Ophiocordyceps* as a consequence of the ending of dual nomenclature for different morphs of pleomorphic fungi in 2011. Though the generic name is well established, geographically dispersed, and speciose, exceptionally few sequences are available in online databases. We examined 46 isolates of 23 *Hirsutella* species from the USA, curated by the USDA-ARS Collection of Entomopathogenic Fungal Cultures (ARSEF Culture Collection), that previously had not been molecularly characterized and produced a phylogeny of these organisms; we included previously published *Hirsutella* and *Ophiocordyceps* taxa. In producing the largest phylogeny of *Hirsutella* isolates so far, we provide: (1) context for discussing previously-hypothesized relationships; (2) evidence for revisions as taxonomic transitions move forward; and (3) available molecular data to be incorporated into further evolutionary studies of *Ophiocordycipitaceae*.

Key words:

18S rDNA
biocontrol
entomopathogenic fungi
Hypocreales
pleomorphic fungi
rpb1
tef1

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INTRODUCTION

Hirsutella (Patouillard 1892) contains the names of over 70 species of asexually-reproducing pathogens of insects, mites, and nematodes that are distributed mainly within *Ophiocordycipitaceae* (Kepler *et al.* 2013, Quandt *et al.* 2014), though the genus is usually considered to be associated with the genus *Ophiocordyceps* typified by a sexual morph (Sung *et al.* 2007). *Hirsutella* species are distinguished from other asexually typified genera by the basally swollen or subulate phialides that taper to an apex, where a mucilaginous packet of one or several conidia forms. Since being described, the morphological concept of the genus has expanded to include species with traits either varying or lacking from a general suite of characters (e.g. phialides without basal inflation, conidia unbounded by a mucilaginous sheath, polyphialidic conidiogenous cells), thereby making morphological inferences of interspecific relationships confusing, if not impossible.

Hirsutella is one of the many fungal taxa typified by asexual morphs affected by the ending of dual nomenclature for pleomorphic fungi in 2011 (McNeill *et al.* 2012). Quandt *et al.* (2014) argued that *Hirsutella* should be suppressed in favour of *Ophiocordyceps* because of a lack of type material for clarification and the larger task of new combinations to be made if *Hirsutella* were selected. Also, the desire to preserve the term 'cordyceps' within the genus to reflect the cultural

and economic importance of *O. sinensis* (Sung *et al.* 2007) was expressed. Indeed, the adoption of *Ophiocordyceps* for a new species only known from a *Hirsutella* morph already has been put to practice. Simmons *et al.* (2015) described *Ophiocordyceps myrmicarum* from asexually reproducing "*Hirsutella*" cultures, isolated from European fire ant (*Myrmica rubra*) populations in Maine, for which the sexual morph was not observed. The suppression of the generic name, however, is not to imply a lack of knowledge or importance of these fungi, but instead should act to propel research into the phylogeny of these organisms so that they may be incorporated into the larger evolutionary history and impending taxonomic revisions of the family *Ophiocordycipitaceae*.

Hodge (1998) produced the only monograph of *Hirsutella*, and was among the first to produce a molecular phylogeny of this genus to determine interspecific relationships that could not be deduced through morphological comparisons. Hodge examined 13 isolates and determined that *Hirsutella* was monophyletic with three *Harposporium* isolates under parsimony analyses of nuclear ITS rDNA sequences. Hodge (1998: 210) stated, however, that ". . . analyses that include more data from different areas of the genome and more taxon sampling are in order before nomenclatural changes can be suggested." Indeed, Chaverri *et al.* (2005) described *Podocrella peltata* with supporting data from the 28S rDNA region from a *Harposporium* sp. (ARSEF 5410), which Hodge (1998) had found belonged within the *Hirsutella* monophyletic

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group. Quandt *et al.* (2014) concurred with the synonymy of *Harposporium* and *Podocrella*, but placed these taxa in a clade disparate from *Ophiocordyceps*, and thus *Hirsutella s. str.*; the authors also argued for the suppression of the generic name *Podocrella* in favour of *Harposporium*, citing the nomenclatural precedence of *Harposporium* (i.e. priority of publication), and the shared conidia morphology within the clade.

Since the study of Hodge (1998), little phylogenetic work has been conducted with *Hirsutella*, except for limited discussion in descriptions of new species (Seifert & Boulay 2004, Evans *et al.* 2010, Ciancio *et al.* 2013, Simmons *et al.* 2015). The lack of available molecular data, however, has made the task of species designation difficult and potentially misleading, in that by limiting the taxa to which an investigator compares a new species risks falsely inflating the genus with a species that has already been described, but for which molecular data have not been generated.

To populate online databases with sequence data from a well-curated and accessible fungal collection, we examined isolates named as belonging to *Hirsutella* in the USDA-ARS Collection of Entomopathogenic Fungal Cultures (ARSEF Culture Collection). We produced a phylogeny of these taxa based on three genetic loci, and we assembled morphological data of these species, or specific isolates from the literature. We discuss the relationships of these species hypothesized in Hodge's monograph and other studies, provide evidence for revisions as taxonomic transitions move forward under the new rules (McNeill *et al.* 2012), and make available molecular data to facilitate the use of these fungi and their associated data in further evolutionary studies of *Hirsutella*, *Ophiocordyceps*, and *Ophiocordycipitaceae*.

MATERIALS AND METHODS

DNA extraction, PCR amplification and sequencing

Cultures of ARSEF isolates were provided by Richard A. Humber (ARSEF Culture Collection, Ithaca, NY), and tissues were lyophilized at the USDA-ARS Systematic Mycology and Microbiology Laboratory (Beltsville, MD), before being sent to the University of Maine (Orono, ME) for DNA extraction. Genomic DNA was extracted from 47 ARSEF *Hirsutella* isolates (Table 1) with MO BIO UltraClean® Microbial DNA isolation kits (MO BIO Laboratories, Carlsbad, CA) following the manufacturer's protocol, with further disruption of the tissue with a Mini-BeadBeater 16 (Biospec Products, Bartlesville, OK) for 3 min. Stock extracts were diluted before amplifications to decrease inhibitory contaminants from the extraction kit spin filters.

Amplifications were conducted in Eppendorf Mastercycler® gradient (Eppendorf Nother America, Westbury, NY) or Bio-Rad T100 (Bio-Rad Laboratories, Hercules, CA) thermal cyclers. Final concentrations of PCR reagent solutions in 25 µL were: (1) 1× Promega GoTaq PCR buffer (Promega, Madison, WI); (2) 1.5 mM MgCl₂; (3) 0.2 mM dNTPs; (4) 0.5 µM of each primer; (5) 1 U GoTaq DNA polymerase; and (6) 0.02–0.2 ng extracted DNA. PCR conditions for *tef1*, *rpb1*, 18S rDNA, and 28S rDNA were as by Johnson *et al.* (2009) and for nuclear ITS rDNA as by Simmons *et al.* (2015).

Primer sequences were obtained from previous works or designed in Geneious (Geneious version 7.1.8). Primer combinations used for amplifications were: (1) 983F and 2218R (Carbone & Kohn 1999, Rehner & Buckley 2005) for *tef1*; (2) RPB1-313F (5'-YTGGARATTGTCTGCCAYAAY-3') and RPB1-1149R (5'-RCGCTTDCCATCAAGTTGC-3') or RPB1CrW (5'-CCNGCDATNCRTRTRCCATRWA-3'), which was derived from RPB1Cr (Castlebury *et al.* 2004), for *rpb1*; (3) NS1 and NS4 (White *et al.* 1990) for 18S rDNA; (4) LR0R and LR5 (Vilgalys & Hester 1990, Rehner & Samuels 1994) for 28S rDNA; and (5) ITS1F or ITS5 and ITS4 (White *et al.* 1990, Gardes & Bruns 1993) for nuclear ITS rDNA. Amplified products were visualized and gel extracted as by Simmons (2011) and sequenced by the University of Maine Sequencing Facility. All products were sequenced with the same primers by which they were amplified, except some *tef1* sequences, which were additionally sequenced with primers 1577F and 1567R (Rehner & Buckley 2005).

Phylogenetic analyses

Chromatograms were manually inspected for ambiguous nucleotides, assembled, and aligned with *tef1*, *rpb1*, and 18S rDNA sequences of *Ophiocordycipitaceae* (Table 1) in Geneious (Geneious version 7.1.8). PartitionFinder v1.1.1 (Lanfear *et al.* 2012) recommended that the super-matrix of the three loci alignments be divided into seven partitions for phylogenetic consideration: one partition for the 18S rDNA alignment and for each of the three codon positions in both protein encoding genes *tef1* and *rpb1*. The Akaike information criterion (AIC) in jModeltest 0.1.1 (Guindon & Gascuel 2003, Posada 2008) was used to select the nucleotide substitution model for each partition. Maximum likelihood (ML) phylogenetic analyses were conducted in GARLI 2.01 (Zwickl 2006) with the recommended partition parameters to determine the best tree topology (Fig. 1) and bootstrap support values from 500 search replicates, which were summarized in SumTrees (Sukumaran & Holder 2010). Bayesian posterior probabilities (BPP) were estimated with the same partition parameters in an analysis conducted in MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003), in which two runs of four chains each were executed simultaneously for 5 000 000 generations, with sampling every 500 generations. SumTrees was used to compute BPP from a summary of 7501 trees retained after a burn-in of the first 2500 trees collected.

RESULTS

The super-matrix phylogenetic analyses included *tef1*, *rpb1*, and 18S rDNA sequences from 47 ARSEF isolates referred to *Hirsutella*, 46 of which were represented by novel sequence data from these genetic loci (Table 1). One isolate, *Hirsutella gigantea* (ARSEF 30), had been investigated previously for a phylogeny with *tef1* sequence data (Simmons *et al.* 2015), but additional data for *rpb1* from this culture are provided in the present study. We excluded alignments of nuclear ITS and 28S rDNA regions for combined phylogenetic analyses, because they generated incongruent tree topologies, but we report these sequences for further molecular identification of ARSEF cultures. The super-matrix of *tef1*, *rpb1*, and 18S rDNA was deposited in TreeBASE submission 17863.

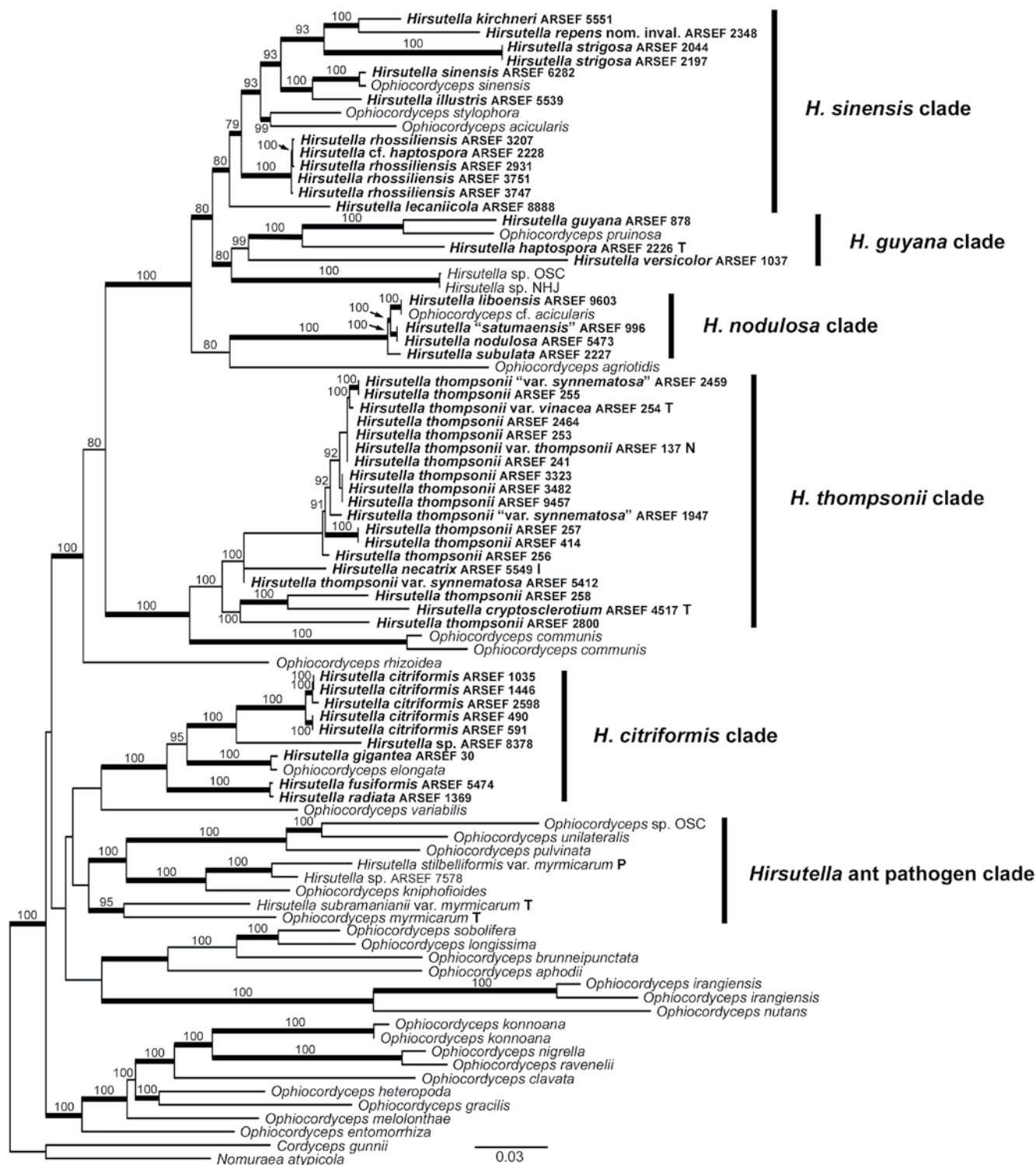


Fig. 1. Best ML tree from GARLI analysis of combined *tef1*, *rpb1*, and 18S rDNA datasets, with taxa represented by sequences obtained in this study in bold. Culture designations of *Hirsutella* species and *Ophiocordyceps myrmicarum* indicated as: I, ex-isotype; N, ex-neotype; P, ex-paratype; T, ex-type. Values at nodes represent ML bootstrap percentages $\geq 70\%$ from a summary of 500 replicates, and branches in bold represent BPP $\geq 95\%$.

The best ML tree from GARLI analysis (Fig. 1) grouped *Hirsutella* and *Ophiocordyceps* taxa monophyletic to *Purpureocillium* (*Nomuraea atypicola*) and *Drechmeria* (*Cordyceps gunnii*) outgroup taxa, which are also classified within *Ophiocordycipitaceae* (Quandt *et al.* 2014). We

recognize six distinct groups to facilitate discussion below, though support values for these groups vary. These six groups include *H. citriformis*, *H. thompsonii*, *H. nodulosa*, *H. guyana*, *H. sinensis*, and the *Hirsutella* ant pathogen clade.

Table 1. Species and culture information of *Ophiocoryzicaceae* used for phylogenetic analyses, and sequences of ARSEF *Hirsutella* cultures additionally obtained in this study, with typification of relevant taxa. Sequences obtained in this study in **bold**.

Species	Isolate no.	Host	GenBank accession no.				
			<i>tefi</i>	<i>rpbl</i>	18S rDNA	ITS rDNA'	28S rDNA'
<i>Hirsutella cf. haptospora</i>	ARSEF 2228	Diptera: Itonididae	KM652001	KM652041	KM652075	KM652166	KM652118
<i>Hirsutella citriformis</i>	ARSEF 490	Hemiptera: Delphacidae	KM651987			KM652151	KM652103
<i>Hirsutella citriformis</i>	ARSEF 591	Hemiptera: Delphacidae	KM651988			KM652152	KM652104
<i>Hirsutella citriformis</i>	ARSEF 1035	Hemiptera: Cixiidae	KM651989	KM652030	KM652064	KM652153	KM652105
<i>Hirsutella citriformis</i>	ARSEF 1446	Hemiptera: Cixiidae	KM651990	KM652031	KM652065	KM652154	KM652106
<i>Hirsutella citriformis</i>	ARSEF 2598	Hemiptera: Psyllidae	KM651991			KM652155	KM652107
<i>Hirsutella citriformis</i>	ARSEF 9180	Hemiptera: Psyllidae				KM652156	KM652108
<i>Hirsutella cryptosclerotium</i> ^T	ARSEF 4517	Hemiptera: Pseudococcidae	KM651992	KM652032	KM652066	KM652157	KM652109
<i>Hirsutella fusiformis</i>	ARSEF 5474	Coleoptera: Curculionidae	KM651993	KM652033	KM652067		KM652110
<i>Hirsutella gigantea</i>	ARSEF 30	Hymenoptera: Pamphiliidae	JX566980	KM652034			
<i>Hirsutella guyana</i>	ARSEF 878	Hemiptera: Cicadellidae	KM651994	KM652035	KM652068	KM652158	KM652111
<i>Hirsutella haptospora</i> ^T	ARSEF 2226	Acar: Uropodina	KM651995	KM652036		KM652159	
<i>Hirsutella illustris</i>	ARSEF 5539	Hemiptera: Aphididae	KM651996	KM652037	KM652069	KM652160	KM652112
<i>Hirsutella kirchneri</i>	ARSEF 5551	Acar: Eriophyidae	KM651997		KM652070	KM652161	KM652113
<i>Hirsutella lecanicola</i>	ARSEF 8888	Hemiptera: Coccidae	KM651998	KM652038	KM652071	KM652162	KM652114
<i>Hirsutella liboensis</i>	ARSEF 9603	Lepidoptera: Cossidae			KM652072	KM652163	KM652115
<i>Hirsutella necatrix</i> ^T	ARSEF 5549	Acar	KM651999	KM652039	KM652073	KM652164	KM652116
<i>Hirsutella nodulosa</i>	ARSEF 5473	Lepidoptera: Pyralidae	KM652000	KM652040	KM652074	KM652165	KM652117
<i>Hirsutella radiata</i>	ARSEF 1369	Diptera	KM652002	KM652042	KM652076		KM652119
<i>Hirsutella repens</i> nom. inval.	ARSEF 2348	Hemiptera: Delphacidae	KM652003		KM652077	KM652167	KM652120
<i>Hirsutella rhossiliensis</i>	ARSEF 2931	Tylenchida: Heteroderidae	KM652004	KM652043	KM652078	KM652168	KM652121
<i>Hirsutella rhossiliensis</i>	ARSEF 3207	Tylenchida: Criconematidae	KM652005	KM652044	KM652079	KM652169	KM652122
<i>Hirsutella rhossiliensis</i>	ARSEF 3747	Lepidoptera: Pyralidae	KM652006	KM652045	KM652080	KM652170	KM652123
<i>Hirsutella rhossiliensis</i>	ARSEF 3751	Lepidoptera: Pyralidae	KM652007	KM652046	KM652081	KM652171	KM652124
<i>Hirsutella "satumaensis"</i>	ARSEF 996	Lepidoptera: Hepialidae	KM652008	KM652047	KM652082	KM652172	KM652125
<i>Hirsutella sinensis</i>	ARSEF 6282	Hymenoptera: Formicidae	KM652009	KM652048	KM652083	KM652173	KM652126
<i>Hirsutella sp.</i> ARSEF 7578	ARSEF 7578	Hymenoptera: Formicidae	JX566981	KJ680152			
<i>Hirsutella sp.</i> ARSEF 8378	ARSEF 8378	Hemiptera: Cixiidae	KM652010	KM652049	KM652084		KM652127
<i>Hirsutella sp.</i> NHJ	NHJ 12525	Hemiptera	EF469063	EF469092	EF469125		
<i>Hirsutella sp.</i> OSC	OSC 128575	Hemiptera	EF469064	EF469093	EF469126		
<i>Hirsutella stibelliformis</i> var. <i>myrmicarum</i> ^T	IMI 396397	Hymenoptera: Formicidae	GQ866964				
<i>Hirsutella strigosa</i>	ARSEF 2044	Hemiptera: Delphacidae	KM652011			KM652174	KM652128
<i>Hirsutella strigosa</i>	ARSEF 2197	Hemiptera: Cicadellidae	KM652012	KM652050	KM652085	KM652175	KM652129

Table 1. (Continued).

Species	Isolate no.	Host	GenBank accession no.					
			<i>tef1</i>	<i>rbp1</i>	18S rDNA	ITS rDNA ¹	28S rDNA ¹	
<i>Hirsutella subramanianii</i> var. <i>myrmicarum</i> [†]	IMI 396400	Hymenoptera: Formicidae	EU797598					
<i>Hirsutella subulata</i>	ARSEF 2227	Lepidoptera: Microlepidoptea	KM652013	KM652051	KM652086	KM652176	KM652130	
<i>Hirsutella thompsonii</i>	ARSEF 241	Acari: Eriophyidae	KM652015			KM652178	KM652132	
<i>Hirsutella thompsonii</i>	ARSEF 253	Acari: Eriophyidae	KM652016		KM652088	KM652179	KM652133	
<i>Hirsutella thompsonii</i>	ARSEF 255	Acari: Eriophyidae	KM652017		KM652089	KM652180	KM652134	
<i>Hirsutella thompsonii</i>	ARSEF 256	Acari: Eriophyidae	KM652018	KM652053	KM652090	KM652181	KM652135	
<i>Hirsutella thompsonii</i>	ARSEF 257	Acari: Eriophyidae	KM652019	KM652054	KM652091	KM652182	KM652136	
<i>Hirsutella thompsonii</i>	ARSEF 258	Acari: Eriophyidae	KM652020	KM652055	KM652092		KM652137	
<i>Hirsutella thompsonii</i>	ARSEF 259	Acari: Eriophyidae				KM652183	KM652138	
<i>Hirsutella thompsonii</i>	ARSEF 414	Acari: Eriophyidae	KM652021	KM652056	KM652093	KM652184	KM652139	
<i>Hirsutella thompsonii</i>	ARSEF 2012	Acari: Eriophyidae				KM652185	KM652140	
<i>Hirsutella thompsonii</i>	ARSEF 2464	Acari: Eriophyidae	KM652022	KM652057	KM652094	KM652186	KM652141	
<i>Hirsutella thompsonii</i>	ARSEF 2800	Acari	KM652023	KM652058	KM652095	KM652187	KM652142	
<i>Hirsutella thompsonii</i>	ARSEF 3323	Acari: Tenuipalpidae	KM652024	KM652059	KM652096	KM652188	KM652143	
<i>Hirsutella thompsonii</i>	ARSEF 3482	Acari: Tetranychidae	KM652025	KM652060	KM652097	KM652189	KM652144	
<i>Hirsutella thompsonii</i>	ARSEF 9457	Acari: Tetranychidae				KM652190	KM652145	
<i>Hirsutella thompsonii</i> "var. <i>synnematos</i> "	ARSEF 1947	Acari: Tarsonemidae	KM652026			KM652191	KM652146	
<i>Hirsutella thompsonii</i> "var. <i>synnematos</i> "	ARSEF 2459	Acari: Eriophyidae	KM652027	KM652061	KM652099	KM652192	KM652147	
<i>Hirsutella thompsonii</i> var. <i>synnematos</i>	ARSEF 5412	Acari: Tetranychidae				KM652193	KM652148	
<i>Hirsutella thompsonii</i> var. <i>thompsonii</i> ^N	ARSEF 137	Acari: Eriophyidae	KM652014	KM652052	KM652087	KM652177	KM652131	
<i>Hirsutella thompsonii</i> var. <i>vinacea</i> [†]	ARSEF 254	Acari: Eriophyidae	KM652028	KM652062	KM652101	KM652194	KM652149	
<i>Hirsutella versicolor</i>	ARSEF 1037	Hemiptera: Membracidae	KM652029	KM652063	KM652102		KM652150	
<i>Ophiocordyceps acicularis</i>	OSC 110987	Coleoptera	EF468852	EF468852	EF468950			
<i>Ophiocordyceps</i> cf. <i>acicularis</i>	OSC 128580	Coleoptera	DQ522326	DQ522371	DQ522543			
<i>Ophiocordyceps agriotidis</i>	ARSEF 5692	Arthropoda	DQ522322	DQ522368	DQ522540			
<i>Ophiocordyceps aphodii</i>	ARSEF 5498	Coleoptera: Scarabaeidae	DQ522323		DQ522541			
<i>Ophiocordyceps brunneipunctata</i>	OSC 128576	Coleoptera: Elateridae	DQ522324	DQ522369	DQ522542			
<i>Ophiocordyceps clavata</i>	NBRC 106961			JN992461	JN941727			
<i>Ophiocordyceps communis</i>	NHJ 12581	Isoptera	EF468775		EF468973			
<i>Ophiocordyceps communis</i>	NHJ 12582	Isoptera	EF468771		EF468975			
<i>Ophiocordyceps elongata</i>	OSC 110989	Lepidoptera	EF468748	EF468856				
<i>Ophiocordyceps entomorrhiza</i>	KEW 53484	Coleoptera	EF468749	EF468857	EF468954			
<i>Ophiocordyceps gracilis</i>	EFCC 8572	Lepidoptera	EF468751	EF468859	EF468956			

Table 1. (Continued).

Species	Isolate no.	Host	GenBank accession no.				
			<i>tef1</i>	<i>rpb1</i>	18S rDNA	ITS rDNA ¹	28S rDNA ¹
<i>Ophiocordyceps heteropoda</i>	EFCC 10125	Hemiptera	EF468752	EF468860	EF468957		
<i>Ophiocordyceps irangiensis</i>	OSC 128578	Hymenoptera: Formicidae	DQ522345	DQ522391	DQ522556		
<i>Ophiocordyceps irangiensis</i>	OSC 128579	Hymenoptera: Formicidae	EF469060	EF469089	EF469123		
<i>Ophiocordyceps kniphofioidea</i>		Hymenoptera: Formicidae	KC610739		KC610790		
<i>Ophiocordyceps konnoana</i>	EFCC 7295	Coleoptera		EF468862	EF468958		
<i>Ophiocordyceps konnoana</i>	EFCC 7315	Coleoptera	EF468753	EF468861	EF468959		
<i>Ophiocordyceps longissima</i>	EFCC 6814	Hemiptera: Cicadidae	EF468757	EF468865			
<i>Ophiocordyceps melolonthae</i>	OSC 110993	Coleoptera: Scarabaeidae	DQ522331	DQ522376	DQ522548		
<i>Ophiocordyceps myrmicarum</i> ^{1,2}	ARSEF 11864	Hymenoptera: Formicidae	JX566973	KJ680151	KJ680150		
<i>Ophiocordyceps nigrella</i>	EFCC 9247	Lepidoptera	EF468758	EF468866	EF468963		
<i>Ophiocordyceps nutans</i>	OSC 110994	Hemiptera: Pentatomidae	DQ522333	DQ522378	DQ522549		
<i>Ophiocordyceps pruinosa</i>	NHJ 12994	Hemiptera	EU369024	EU369063	EU369106		
<i>Ophiocordyceps pulvinata</i>	TNS F30044	Hymenoptera: Formicidae	GU904209	GU904210	GU904208		
<i>Ophiocordyceps ravenelli</i>	OSC 110995	Coleoptera	DQ522334	DQ522379	DQ522550		
<i>Ophiocordyceps rhizoidea</i>	NHJ 12522	Isoptera	EF468764	EF468873	EF468970		
<i>Ophiocordyceps sinensis</i>	EFCC 7287	Lepidoptera	EF468767	EF468874	EF468971		
<i>Ophiocordyceps sobolifera</i>	KEW 78842	Hemiptera: Cicadidae		EF468875	EF468972		
<i>Ophiocordyceps</i> sp. OSC	OSC 110997	Hymenoptera: Formicidae	EF468774	EF468879	EF468976		
<i>Ophiocordyceps stylophora</i>	OSC 111000	Coleoptera: Elateridae	DQ522337	DQ522382	DQ522552		
<i>Ophiocordyceps unilateralis</i>	OSC 128574	Hymenoptera: Formicidae	DQ522339	DQ522385	DQ522554		
<i>Ophiocordyceps variabilis</i>	ARSEF 5365	Diptera: Xylophagidae	DQ522340		DQ522555		
Outgroup:							
<i>Cordyceps gunnii</i>	OSC 76404	Lepidoptera	AY489616	AY489650	AF339572		
<i>Normuraea atypicola</i>	CBS 7444.73	Arachnida	EF468786	EF468892	EF468987		

¹Indicates ex-isotype culture.

²Indicates ex-neotype culture.

³Indicated ex-paratype culture.

⁴Indicates ex-holotype culture.

⁵Sequences were not used for phylogenetic analyses.

⁶*Ophiocordyceps myrmicarum* is noted, because it is only known from its asexual life stage.

DISCUSSION

Morphology

Morphological characters of mitosporic reproductive structures (presence of synnemata; phialide appearance; conidia shape and condition) traditionally have been used as a basis for species description and hypothesized relatedness within *Hirsutella*. However, our data support Hodge's (1998) contention that the morphological features of *Hirsutella* are insufficient for determining interspecific relationships. Her subsequent phylogeny of the genus from nuclear ITS rDNA sequences (~600 bp each), including those obtained from eleven ARSEF isolates, seven of which are in this study, supported the monophyly of *Hirsutella*, so long as it includes three isolates of *Harposporium*, two of which would produce synanamorphs in culture studies. Based on our molecular phylogeny (Fig. 1), few trends in host taxa or morphological characters appear phylogenetically informative (Table 2). Some host based groupings can be inferred: the majority of the isolates from ant hosts cluster in one lineage, and species in the *H. nodulosa* clade primarily are associated with *Lepidoptera* hosts. However, other isolates originating from *Lepidoptera* are distributed within other clades, and isolates from *Hemiptera* hosts are distributed throughout all clades except one (*H. nodulosa*). Additional sampling of species from additional geographic regions or rare *Hirsutella* taxa, however, may expand these clades and make more relevant traits apparent.

Hirsutella thompsonii clade

Hirsutella thompsonii is the most widely studied of the *Hirsutella* species, being an important biocontrol agent for mite pests in agriculture. Isolates considered in this study, all originating from *Acari* hosts, form a clade including the morphologically similar species *H. necatrix* (Minter *et al.* 1983), another mite pathogen, and *H. cryptosclerotium* (Fernández-García *et al.* 1990), which originated from a different host taxon in *Hemiptera*. The monophyletic, core clade of *H. thompsonii* is composed of *Hirsutella thompsonii* var. *thompsonii* (ARSEF 137 – ex-neotype culture), most other *H. thompsonii* cultures, and *H. thompsonii* var. *vinacea* (ARSEF 254 – ex-holotype culture). Isolates ARSEF 1947 and 2459, which are included in the core clade, may form synnemata in culture (Humber *et al.* 2014), but they are not monophyletic with *H. thompsonii* var. *synnematosus* (ARSEF 5412), which is in a polytomy with *H. necatrix* and the core clade. Bayesian posterior probability support values deteriorate in the core clade because of differences in the placement of ARSEF 9457 by our analyses; Bayesian phylogenetic reconstruction removed ARSEF 9457 from the core clade and placed it in the polytomy alongside *H. necatrix* (ARSEF 5549 – ex-isotype culture) and *H. thompsonii* var. *synnematosus*. Additionally, ARSEF 258 and 2800, also considered *H. thompsonii*, lie outside of the core clade, grouping with *H. cryptosclerotium* (ARSEF 4517 – ex-holotype culture). In our phylogeny isolates of *Ophiocordyceps communis* are sister to the taxa described above, most likely because of long-branch attraction instead of true phylogenetic relatedness; regardless, Sung *et al.* (2007) describe the asexual morph of *O. communis* to be transitional between *Hymenostilbe* and *Hirsutella*.

Hodge (1998: 154) considered *H. thompsonii* to be “. . . the core of a group of morphologically related species including *H. necatrix*, *H. gregis*, *H. cryptosclerotium*, *H. tydeicola*, and *H. sphaerospora*, pathogens of mites or mealybugs.” She examined the relationship of two *H. thompsonii* isolates, which are within our core clade, and *H. cryptosclerotium*, and she found *H. cryptosclerotium* to be sister to *H. thompsonii* with weak bootstrap support (67 %) in a nuclear ITS rDNA phylogeny. *Hirsutella cryptosclerotium* is distinguished by the production of pigmented sclerotia, but similar chlamydospore structures have been noted in other species, including *H. thompsonii* (Fernández-García *et al.* 1990). Hodge (1998) believed *Hirsutella necatrix* was more difficult to distinguish from *H. thompsonii*, except that the phialides were often formed in a verticillate arrangement and conidia were occasionally more ellipsoid.

Hodge (1998) posited that *H. kirchneri* and *H. gregis* (Minter *et al.* 1983), the latter of which we did not examine in this study, are variants from the same host and collection location. Similarly, Bałazy *et al.* (2008) produced phylogenies of nuclear ITS rDNA sequences that closely allied *H. gregis* and *H. kirchneri* isolates. A BLAST query of the nuclear ITS rDNA region generated from *H. kirchneri* (ARSEF 5551, GenBank KM652161) is 100 % and 99 % similar to the *H. gregis* and *H. kirchneri* isolates, respectively, as reported by Bałazy *et al.* (2008). The imperfect match of the two *H. kirchneri* sequences is explained by two ambiguous sites in the sequence determined by Bałazy *et al.* (2008) that are apparent in our ARSEF 5551 sequence. *Hirsutella kirchneri* and *H. thompsonii* are vastly disparate in our phylogeny, so the relationship of *H. gregis* to *H. thompsonii* within a morphologically defined ‘core clade’ as suggested by Hodge (1998) is not supported by our phylogeny.

Hirsutella tydeicola (Samson & McCoy 1982) is known only from the type collections, but Hodge (1998) considered *H. kirchneri* and *H. gregis* similar to this isolate in appearance aside from slight variations in conidia shape, suggesting that *H. tydeicola* may be more closely related to *H. kirchneri* in our phylogeny and therefore disparate from *H. thompsonii*. These morphological characters, however, may also prove misleading, and it could be that *H. tydeicola* resides in an unexpected location within the phylogeny; only a molecular characterization of this taxon will determine its phylogenetic position. Similarly, *Hirsutella sphaerospora* (Evans & Samson 1982), which we did not examine, was theorized to be closely related to *H. cryptosclerotium* (Fernández-García *et al.* 1990, Hodge 1998), which also exhibits polyphialidic conidiogenous cells. The morphological association of *H. sphaerospora* to both *H. cryptosclerotium* and *H. thompsonii* may provide additional support for this species' placement within a ‘core clade’, but its true position is questionable.

Hirsutella guyana clade

The *Hirsutella guyana* clade is represented by three *Hirsutella* isolates characterized in our study. The ex-type culture of *H. haptospora* (Bałazy & Wiśniewski 1986), which is considered in our phylogeny (ARSEF 2226), was isolated from mites in *Formica* nests from Poland. Hodge (1998) stated that this species was closely allied to *H. rhossiliensis* (Minter & Brady 1980), but our phylogeny supports the placement of

Table 2. Morphology of *Hirsutiella* species included in Fig. 1. Characters from Hodge (1998) unless specified.

Species	Clade	Conidioma form				Phialides				Conidia				Host
		Mononematous	Synnematous	Morphology	Verruculose	Morphology	Mucous sheath	Sclerotia	Host	Mononematous	Synnematous	Morphology	Mucous sheath	
<i>Hirsutiella citriformis</i>	<i>H. citriformis</i>	No	Yes	Ellipsoid base, tapering abruptly	No	Fusiform	Yes	No	Fusiform	Yes	No	Yes	No	Leaf- & planthoppers
<i>H. cryptosclerotium</i>	<i>H. thompsonii</i>	Yes	No	Basally inflated, inflated apices, sometimes polyphialidic	No	Globose	Yes	No	Globose	Yes	Yes	Yes	Yes	<i>Pseudococcidae</i>
<i>H. fusiformis</i>	<i>H. citriformis</i>	No	Yes	Ellipsoid base, tapering abruptly	No	Fusoid-Cylindric	Yes	No	Fusoid-Cylindric	Yes	No	Yes	No	Crickets
<i>H. gigantea</i>	<i>H. citriformis</i>	No	Yes	Basally inflated	No	Ellipsoid	Yes	No	Ellipsoid	Yes	No	Yes	No	<i>Lepidoptera</i>
<i>H. guyana</i>	<i>H. guyana</i>	Yes	No	Cylindric base, ortho- or phagiophialide, proximal restriction, sometimes polyphialidic	Neck, sometimes	Ellipsoid	Yes	No	Ellipsoid	Yes	No	Yes	No	Leafhoppers
<i>H. haptospora</i>	<i>H. guyana</i>	Yes	No	Basally inflated	No	Fusiform	Yes	No	Fusiform	Yes	No	Yes	No	Mites
<i>H. illustris</i>	<i>H. sinensis</i>	Yes	No	Tapering, elongated	Neck	Ellipsoid	Yes	No	Ellipsoid	Yes	No	Yes	No	<i>Lepidoptera</i> , Aphids
<i>H. kirchneri</i>	<i>H. sinensis</i>	Yes	In culture	Basally inflated, occasionally polyphialidic	Neck	Fusiform	Rare	No	Fusiform	Rare	No	Yes	No	Mites
<i>H. lecanicola</i>	<i>H. sinensis</i>	Yes	Yes	Basally inflated; polyphialidic & irregular when synnematosus	No	Ellipsoid	When mononematous	No	Ellipsoid	When mononematous	No	Yes	No	Scale insect
<i>H. liboensis</i> ²	<i>H. nodulosa</i>	No	Yes	Basally inflated, apical helix, often polyphialidic	No	Fusiform	Yes	No	Fusiform	Yes	No	Yes	No	<i>Cossidae</i>
<i>H. necatrix</i>	<i>H. thompsonii</i>	Yes	In culture	Basally inflated, verticillate arrangement	Neck, rarely	Subglobose	Occasional	No	Subglobose	Occasional	No	Yes	No	Mites
<i>H. nodulosa</i>	<i>H. nodulosa</i>	Yes	No	Tapering, apical helix, rarely polyphialidic	Yes	Fusiform	Yes	No	Fusiform	Yes	No	Yes	No	Mites, <i>Lepidoptera</i>
<i>H. radiata</i>	<i>H. citriformis</i>	No	Yes	Subulate	No	Ovoid	Yes	No	Ovoid	Yes	No	Yes	No	<i>Diptera</i>
<i>H. rhossiliensis</i>	<i>H. sinensis</i>	Yes	No	Basally inflated	No	Ovoid	Yes	No	Ovoid	Yes	No	Yes	No	Nematodes
<i>H. satumaensis</i>	<i>H. nodulosa</i>	No	Yes	Cylindric base	No	Fusiform	Yes	No	Fusiform	Yes	No	Yes	No	<i>Lepidoptera</i>
<i>H. sinensis</i> ¹	<i>H. sinensis</i>	Yes	No	Subulate, in verticils of 2-4, rarely polyphialidic	No	Ellipsoid	Yes	No	Ellipsoid	Yes	No	Yes	No	Caterpillar
<i>H. stilbelliformis</i> var. <i>myrmicarum</i> ³	Ant pathogen	Rarely	Yes	Subulate	Neck	Ovoid	Yes	No	Ovoid	Yes	No	Yes	No	<i>Myrmica</i>
<i>H. strigosa</i>	<i>H. sinensis</i>	Yes	Rarely	Subulate, elongated, occasionally polyphialidic	Neck	Ellipsoid	Yes	No	Ellipsoid	Yes	No	Yes	No	Leafhoppers
<i>H. subramaniiani</i> var. <i>myrmicarum</i> ³	Ant pathogen	Yes	No	Basally inflated	Neck	Obclavate	Yes	Yes	Obclavate	Yes	Yes	Yes	Yes	<i>Myrmica</i>
<i>H. subulata</i>	<i>H. nodulosa</i>	In culture	Yes	Tapering, short	No	Ellipsoid	Yes	No	Ellipsoid	Yes	No	Yes	No	<i>Lepidoptera</i>
<i>H. thompsonii</i>	<i>H. thompsonii</i>	Yes	Some varieties	Tapering, monosporic	No	Globose	Occasional	No	Globose	Occasional	No	Yes	No	Mites

Table 2. (Continued).

Species	Clade	Conidioma form			Phialides		Conidia				Host
		Mononematous	Synnematous	Morphology	Verruculose	Morphology	Mucous sheath	Sclerotia	Leafhoppers		
<i>H. versicolor</i>	<i>H. guyana</i>	Yes	No	Swollen, tapering abruptly, often polyphialidic	No	Ovoid	Yes	No	Leafhoppers		
<i>O. myrmicarum</i> ⁴	Ant pathogen	No	Yes	Subulate	No	Ovoid	Yes	No	<i>Myrmica</i>		

¹Liu *et al.* (2001).²Zou *et al.* (2010).³Evans *et al.* (2010).⁴Simmons *et al.* (2015). *Ophiocordyceps myrmicarum* is included, because it is only known from its asexual morph.

this species alongside *O. pruinosus* (Johnson *et al.* 2009) and a Brazilian isolate of *H. guyana* (Minter & Brady 1980). Interestingly, *H. cf. haptospora* (ARSEF 2228), which was also identified by Bałazy from gall midges in Poland (Humber *et al.* 2014), is monophyletic with isolates of *H. rhossiliensis*. *Hirsutella rhossiliensis* is a broad-range nematode pathogen, but Hodge (1998) noted the morphological similarity of this species to *H. haptospora*. It is possible, therefore, that ARSEF 2228 represents a host-jumping *H. rhossiliensis*.

Hirsutella nodulosa clade

Four *Hirsutella* isolates clustered in the *Hirsutella nodulosa* clade. *Hirsutella nodulosa* (Petch 1926) and *H. satumaensis* (Aoki *et al.* 1957) are monophyletic with little variation in our phylogeny, which is similar to the findings of Hodge (1998) from a nuclear ITS rDNA dataset. *Hirsutella nodulosa* was described from a lepidopteran in Sri Lanka, and ARSEF 5473 is from a lepidopteran in Michigan. Minter & Brady (1980) examined the type material and a culture from spider mites, and they suggested that the defining nodules described by Petch (1926) were conidia adhering by their mucilaginous sheath to hyphae, a condition that Hodge (1998) found to be a common occurrence in other *Hirsutella* species. Minter & Brady (1980) also documented helical twisting at the apex of phialides and warts on phialides and hyphae, features not originally described by Petch (1926). Other species also may exhibit warted phialides (e.g. *H. rhossiliensis*, Minter & Brady 1980) though not to the degree seen in *H. nodulosa*. *Hirsutella brownorum* (Minter & Brady 1980) and *H. liboensis* (Zou *et al.* 2010), the latter of which groups with *H. nodulosa* in our phylogeny, also possess phialides with apical helical twists. The determination of the phylogenetic position of *H. brownorum*, and additional taxa with apical helical twists (Zou *et al.* 2010), however, would be necessary before concluding that this trait evolved only once.

Hirsutella satumaensis was described from case-making clothes moths and silkworms, and ARSEF 996 is derived from one of Aoki's cultures of this species (Aoki *et al.* 1957). Hodge (1998), however, saw no evidence for this isolate to be defined as *H. satumaensis*, reported no other isolates or type material, and believed ARSEF 996 to be *H. nodulosa*. We support Hodge's hypothesis, and the phylogenetic position of *H. satumaensis* will remain unknown until a neotype fitting the description can be studied.

Ciancio *et al.* (2013) described *H. tunicata* from Peruvian mites as being similar in appearance to *H. nodulosa*, but *H. tunicata* lacks the twisted apex of the phialides and possesses solitary conidia. In phylogenies from nuclear ITS rDNA and β -tubulin gene datasets produced by Ciancio *et al.* (2013), *H. tunicata* allied with an isolate of *H. nodulosa* from strawberry mites in Poland, but support values for this grouping were low, suggesting that morphological similarities between these species are not necessarily indicative of true relatedness. However, a BLAST query in GenBank of the nuclear ITS rDNA sequence obtained by Ciancio *et al.* (2013) from *H. tunicata* is best matched at 93% identity with the sequence we generated from *H. subulata* (Petch 1932) ARSEF 2227 (GenBank KM652176), which is also within the *H. nodulosa* clade. The relationship hypothesized by Ciancio *et al.* (2013), therefore, has yet to be disproven.

Our phylogeny also supports previous analyses (Simmons *et al.* 2015) in which comparable isolates of *O. acicularis* (Petch 1933) are not monophyletic, with representatives in both the *H. nodulosa* and *H. sinensis* clades. *Ophiocordyceps cf. acicularis* (OSC 128580), however, appears synonymous with *H. liboensis* in our phylogeny. Zou *et al.* (2010) believed *H. liboensis* could be the asexual morph of *Ophiocordyceps cochliidiicola* (Sung *et al.* 2007), the taxon for which *H. liboensis* was the closest relative in the nuclear ITS rDNA phylogeny produced by Zou *et al.* (2010), next only to an isolate of *H. nodulosa*. Additionally, Sung *et al.* (2007) placed *Cordyceps cf. acicularis* (as *O. cf. acicularis*) sister to an isolate of *O. cochliidiicola* in five-gene phylogenies with 100% support. The alliance of *H. liboensis* and *O. cochliidiicola*, in addition to their relatedness to *H. nodulosa*, seem highly likely, but we agree with Zou *et al.* (2010) that further study is necessary to confirm this relationship.

Hirsutella sinensis clade

The *Hirsutella sinensis* clade includes isolates originating from a variety of taxa including nematodes, mites, and both hemi- (*Hemiptera*) and holometabolist

(*Coleoptera*, *Lepidoptera*) insect hosts. Our data supports the evidence of Liu *et al.* (2001) that *H. sinensis* (Liu *et al.* 1989) is the anamorph of *Cordyceps sinensis* (Sung *et al.* 2007). Liu *et al.* (2001, as *C. sinensis*) used nuclear ITS rDNA sequences and morphological examination of ascospore microcyclic conidia of *C. sinensis* (Saccardo 1878) to argue that *H. sinensis* was the genuine asexual morph of *C. sinensis*, for which several names based on asexual morphs had been suggested.

Hirsutella strigosa (Petch 1939) forms a monophyletic group with *H. repens* (nom. inval.; Humber *et al.* 2014), for which no morphological data is published, and *H. kirchneri*, with which it shares some morphological features. Petch (1939) found *H. strigosa* with *H. versicolor*, and our phylogeny clearly differentiates these taxa. Hodge (1998) notes a distinguishing feature of this species is its exceptionally long and verruculose phialides, similar to *H. nodulosa*, but not apically twisted as in that species. Furthermore, Hodge noted that *H. illustris* and *H. rhossiliensis*, also within our *H. sinensis* clade, similarly share large phialides. *H. kirchneri* and *H. lecaniicola* (Petch 1933), however, do not share this characteristic.

***Hirsutella citriformis* clade**

Hirsutella citriformis (Speare 1920) is a widely distributed species infecting the Asian citrus psyllid *Diaphorina citri* (e.g. Subandiyah *et al.* 2000, Hall *et al.* 2012, Pérez-González *et al.* 2015) and is represented in our phylogeny by isolates collected in the Republic of Indonesia, Republic of the Philippines, and the USA. This species is monophyletic, indicating reliable morphological identification by different investigators, but Pérez-González *et al.* (2015) argued that morphologies of phialides and conidia of isolates vary from those of the original description and warn that new isolates could show increasing variability in these structures.

Other isolates in this clade originate from a diversity of insect taxa. *Hirsutella fusiformis* (Speare 1920) is poorly known from the type description and illustrations; Hodge (1998) examined the putative isolate of *H. fusiformis* we investigated in this study and found that it would not sporulate on tested media. In our phylogeny, *H. fusiformis* was associated with *H. radiata* (Petch 1935). Hodge (1998) postulated that *H. guignardii* (Samson *et al.* 1984) could be a synonym of *H. radiata*, based on the similarity of synnemata branching, though more delicate in the latter species. It is possible, based on the short branch lengths in our phylogeny comparable to other taxonomically identical taxa, that this putative *H. fusiformis* is instead one of these species but has degenerated in storage. Additionally in this clade, Simmons *et al.* (2015) previously confirmed the relationship of *Hirsutella gigantea* (Petch 1937) and *Ophiocordyceps elongata* (Sung *et al.* 2007) based on *tef1* data, and our wider phylogeny including an *rpb1* sequence obtained from this isolate provides further support for the association.

***Hirsutella* ant pathogen clade**

Our phylogeny places all *Hirsutella* isolates from *Formicidae* in a monophyletic clade. This clade, however, excludes isolates of *Ophiocordyceps irangiensis*, which, while also derived from *Formicidae* hosts, produce a typical *Hymenostilbe* asexual morph (Sung *et al.* 2007). Simmons *et al.* (2015)

noted similar phylogenetic relationships of these taxa in their ML analysis of *tef1*, *rpb1*, and 18S rDNA, but a Bayesian phylogeny of those same loci grouped *O. irangiensis* with isolates of *O. communis*, which produces a transitional *Hirsutella/Hymenostilbe* asexual morph (Sung *et al.* 2007).

An ongoing investigation

Hodge (1998) produced the only monographic work on *Hirsutella* that incorporated genetic sequences for an initial phylogenetic understanding of the genus. Our examination of ARSEF *Hirsutella* isolates from the USA sought to increase the sampling of these fungi in molecular databases, but a monographic revision of this genus would be incomplete if it did not consider the entirety of *Ophiocordyceps*, with which *Hirsutella* species are being synonymized. Indeed, more rigorous morphological examinations of the *Hirsutella* isolates in our molecular phylogeny are necessary before taxonomic revisions (combinations, typifications, etc.) can take place, but our molecular phylogeny provides an evolutionary context for morphological features and physiology and a measure of species' similarity that will facilitate the next steps in this process. Additionally, phylogenetic investigations into international or rare *Hirsutella* species and isolates, which we were unable to examine in this study, will likely lead to a deeper understanding of the evolution, taxonomy, and physiology of these fungi.

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