Five new species of entomopathogenic fungi from the Amazon and evolution of neotropical Ophiocordyceps

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ABSTRACT

The neotropical biogeographic zone is a 'hot spot' of global biodiversity, especially for insects. Fungal pathogens of insects appear to track this diversity. However, the integration of this unique component of fungal diversity into molecular phylogenetic analyses remains sparse. The entomopathogenic fungal genus *Ophiocordyceps* is species rich in this region with the first descriptions dating to the early nineteenth century. In this study, material from various ecosystems throughout Colombia and Ecuador was examined. Molecular phylogenetic analyses of five nuclear loci including *SSU*, *LSU*, *TEF*, *RPB1*, and *RPB2* were conducted alongside a morphological evaluation. Thirty-five specimens were examined representing fifteen different species of *Ophiocordyceps*, and five new species, *Ophiocordyceps blattarioides*, *Ophiocordyceps tiputini*, *Ophiocordyceps araracuarensis*, *Ophiocordyceps fulgoromorphila*, and *Ophiocordyceps evansii*, were described. An accurate identification of the host allowed us to conclude that host identity and host habitat are positively correlated with phylogenetic species of *Ophiocordyceps* and are probably strong drivers for speciation of neotropical entomopathogenic fungi.

Introduction

The entomopathogenic genus *Ophiocordyceps* was established by Petch (1931) for fungi possessing clavate asci with gradually thickening apices and elongate, fusiform ascospores that do not disarticulate into part-spores. The type species, *Ophiocordyceps blattae* Petch, from Sri Lanka, is rarely collected, but other more commonly collected fungi include *Ophiocordyceps unilateralis* (Tul.) Petch on *Hymenoptera* adults (worker ants) and *Ophiocordyceps sinensis* (Berk.) G.H. Sung, J.M. Sung,

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Hywel-Jones & Spatafora on *Lepidoptera* larvae. In other major taxonomic studies of the genus *Cordyceps* (L.) Link, Kobayasi (1941, 1982) and Mains (1958) both treated *Ophiocordyceps* as a subgenus of *Cordyceps*. With advances in the genetic characterization of fungi, a phylogenetic framework for *Cordyceps* (*Ascomycota: Hypocreales*) derived from molecular data has greatly clarified taxonomic systems previously based on morphology alone (Sung *et al.* 2007). *Ophiocordyceps* sensu Petch was resurrected as a genus, and expanded to include the majority of taxa with firm, darkly pigmented stromata based on the placement of *O. unilateralis*, but with a diversity of ascus and ascospore morphologies. Currently there are 184 species recognized in *Ophiocordyceps*, with many additional species described as asexual taxa (Quandt *et al.* 2014), making it the largest of the entomopathogenic genera in *Hypocreales* (Sung *et al.* 2007; Johnson *et al.* 2009). Molecular phylogenetic analyses have incorporated material from Asia, Europe, and North America, highlighting phylogenetic relationships for members of genus *Ophiocordyceps* in the Holarctic and Indo-Malaysian biogeographic zones, but relatively few neotropical specimens of the genus have been sampled (Sung *et al.* 2007). Based on work by mycologists such as Möller (1901), Henning (1904), and Spegazzini (reviewed in Kobayasi 1941), however, the neotropics are known to support a unique assemblage of *Ophiocordyceps*. More recently, Evans (1982) emphasized the importance of systematic collections of entomopathogenic fungi in the Amazon, a subregion within the neotropical zone. Producing a phylogeny of *Ophiocordyceps* inclusive of global diversity requires specimens from the neotropics, particularly considering the high diversity of entomofauna (Lopez & Miranda 2010).

In this study we sampled neotropical *Ophiocordyceps* species from Colombia and Ecuador, including the Imeri and Napo biogeographic provinces (Amazon subregion) and Magdalena province (Caribe subregion) proposed by Morrone (2006). Incorporation of these samples into existing molecular phylogenetic datasets, in combination with host identification to family and genus, allowed the description of new species and the development of hypotheses concerning the role of host association in the evolution and divergence of *Ophiocordyceps* species.

Material and methods

Material collected

Systematic collections were made in three sites of the Amazon region from Colombia with altitudes between 20 and 200 m above sea level, average temperatures of 28 °C, and relative humidity of 90 %. The first site was the Araracuara canyon of the Caquetá River (Puerto Santander, Amazonas) in May of 2009 and September of 2011. The second site was sampled in several expeditions to Uitoto San Francisco community along to the Igara Parana River (La Chorrera, Amazonas), in July of 2010 and March and September of 2011. The third site sampled was the scientific station El Zafire reserve in the Municipality of Leticia (Amazonas) in July of 2011 and January of 2012. Additionally, collections were made in Rio Claro (San Luis, Antioquia) in March of 2012 and

November of 2014, a region belonging to Magdalena biogeographic province. Some material collected in July 2004 in the Tiputini Research station (Napo, Ecuador) belonging to Napo biogeographic province was included in this study.

Collecting involved careful examination of leaf litter, downed wood, and elevated plant structures (e.g., leaves, twigs) to detect the emergence of stromata from insect cadavers. Dried specimens were placed in plastic bags with silica gel for transportation to the laboratory for identification. Collections were deposited in Antioquia University Herbarium (HUA), and the collection of Fungi of the Natural History Museum of Andes University (ANDES) in Colombia, and National Herbarium of Ecuador (QCNE). Additionally material provided by the National Herbarium of Colombia (COL) was revised. Fungal fruiting bodies were examined according to Sanjuan *et al.* (2014). Colour descriptions of stromata are according to Kornerup *et al.*, 1984. The host insects were identified to the extent possible (e.g., family, genus, etc.) as allowed by the condition of the specimen.

DNA extraction, PCR amplification, and sequencing

In the field, small pieces of fresh tissue from stromata were placed in 50 μL CTAB extraction buffer (1.4 M NaCl; 100 mM Tris−HCl pH 8.0; 20 mM EDTA pH 8.0; 2 % CTAB w/v) and the DNA extraction process was performed following the method in Kepler *et al.* (2012). Attempts were made to amplify six nuclear loci including the nuclear ribosomal internal transcribed spacer region (*ITS*), small subunit ribosomal RNA (*SSU*) and large subunit ribosomal RNA (*LSU*), elongation factor-1α (*TEF*), and the largest (*RPB1*) and second (*RPB2*) largest subunits of RNA polymerase II. The PCR reaction mixture consisted of 2.5 μL 10 × PCR buffer (Fermentas, Glen Burnie, MA), 2 μL MgCl$_2$ (25 mM), 0.5 μL each primer (10 μM), 0.5 μL dNTP (10 mM each), 1−2 μL template DNA, 0.2 μL *Taq* polymerase (5 U/μL) (Fermentas, Glen Burnie, MA) and dH$_2$O to a final volume of 25 μL.

Amplification of *ITS* was performed with primers ITS1f and ITS4 (White *et al.* 1990). *SSU* and *LSU* were amplified with NS1/SR7and NS3/NS4 (White *et al.* 1990) and LROR/LR5 primers (Vilgalys & Sun 1994), respectively. Amplification of *TEF* was performed with the primers 983F and 2218R (Rehner & Buckley 2005). Amplification of RPB1 was performed with primers cRPB1-1 aF and cRPB1-CaR (Castlebury *et al.* 2004). Amplification of RPB2 was performed with primers fRPB2-5f2 and fRPB2-7 cR (Liu *et al.* 1999). The PCR reactions were performed in a Thermocycler 1000 (BIORAD, Hercules, CA) programmed as follows: 94 °C for 3 min; ten cycles of 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 2 min; 35 cycles of 94 °C for 30 s, 50 °C for 1 min, and 72 °C for 2 min; one cycle of 72 °C for 3 min and 4 °C indefinitely (Kepler *et al.* 2011). Sequencing was performed with the amplification primers at the University of Washington (Seattle, USA) and Andes University (Bogotá, Colombia) sequencing centers. The *ITS1* and *LSU* of the several specimens from this study were submitted to the IBOL initiative (http://www.boldsystems.org/).

Sequence alignment and phylogenetic analysis

Sequences from this study were edited using Geneious Pro version 4.8.5 (Drummond *et al.* 2009). A BLAST query of the

GenBank database was used to confirm that these sequences belonged in *Ophiocordycipitaceae*. New DNA sequences of genes generated in this study were submitted to GenBank and are listed in Table 1. A dataset of taxa in *Ophiocordycipitaceae* was assembled from previously published studies (Sung *et al.* 2007; Schoch *et al.* 2012; Kepler *et al.* 2013; Wen *et al.* 2013; Quandt *et al.* 2014) using *SSU*, *LSU*, *TEF*, *RPB1*, and *RPB2* genes. Representatives from the genera *Metarhizium* and *Pochonia* (*Clavicipitaceae*) were used as the outgroup taxa. Alignment of *SSU* and *LSU* was performed using MUSCLE (Edgar 2004) with the default settings from Geneious Pro; alignment of protein coding genes *TEF*, *RPB1* and *RPB2* was performed with MAFFT (Katoh & Toh 2010) as part of the CIPRES gateway (Miller *et al.* 2010). Finally, each alignment was refined manually in BioEdit version 6.0.7. (Hall 1999) Alignments are deposited in TreeBase (accession number S15874).

Analyses for the best model of nucleotide substitution were performed using jModelTest version 2.1.3 (Posada 2008). The dataset was divided into 11 gene partitions, one each for *SSU* and *LSU* and one for each codon position for the three protein coding genes. We used the ML optimized base tree for likelihood calculations and used Bayesian information criterion (BIC) for model selection for each partition. Maximum Likelihood (ML) analyses were performed using RAXML-VI-HPC version 7.2 (Stamatakis 2006) with 1000 bootstrap replicates. All five genes were concatenated into a single dataset, and 11 data partitions were defined: one each for *SSU* and *LSU*, plus nine for each of the three codon positions for the protein coding genes *TEF*, *RPB1*, and *RPB2* (Kepler *et al.* 2012). Nodes were considered supported by bootstrap values of greater than 70 %. Bayesian Inference (BI) was performed using MrBayes version 3.1.2 (Huelsenbeck *et al.* 2001). Substitution models and partitions were specified as in RA×ML analyses. Ten million MCMCMC generations were performed, using a sample frequency of 1000 generations and a burn-in of 25 % of the total run. Two runs using four chains each (one cold and three heated chains) were performed, and each run was examined using Tracer version 1.5 (Drummond & Rambaut 2007) to verify burn-in parameters and convergence of individual chains. Nodes were considered supported by posterior probability values greater than or equal to 0.95.

Results

Phylogenetic analyses

We obtained 118 new sequences from 33 specimens. The combined dataset of 121 taxa and 5 genes consisted of 5427 bp (*nrSSU* 1496 bp, *nrLSU* 970 bp, *TEF1* 1073 bp, *RPB2* 1084 bp, *RPB1* 804 bp). Both ML and BI analyses displayed similar topologies resolving seven subclades of *Ophiocordycipitaceae* (Fig 1) including 1) 'gunnii' clade, 2) *Tolypocladium* (*Elaphocordyceps* sensu Sung *et al.* 2007), and the four subclades of *Ophiocordyceps*: 3)'ravenelii' (MLBS = 89; PP = 1.00), 4)'unilateralis' (MLBS = 62; PP = 1.00), 5) 'sobolifera' (MLBS = 100; PP = 1.00) and 6)'sphecocephala' (MLBS = 100; PP = 1.00). The genus *Polycephalomyces* sensu Kepler *et al.* (2013), recently accepted for inclusion in *Ophiocordycipitaceae* (Quandt *et al.* 2014), was also resolved in these analyses (MLBS = 88; PP = 0.94).

The 'ravenelii' clade included *Cordyceps gracillima* HUA 186132 (black circles in Fig 1) collected from *Scarabaeidae* (*Coleoptera*) larva in Puerto Santander, Amazonas (Colombia). For that reason *C. gracillima* was transferred to *Ophiocordyceps*. It was resolved as a sister species of *Ophiocordyceps melolonthae* which also infects *Scarabaeidae* larvae. In addition, in the 'gracilis' subclade the species *Ophiocordyceps heteropoda*, *Ophiocordyceps gracilis*, *Ophiocordyceps amazonica*, *Ophiocordyceps gracilioides*, and the new species described here, *Ophiocordyceps blattarioides*, were resolved as a well-supported clade (MLBS = 98; PP = 1.00). *Ophiocordyceps blattarioides* is pathogen of cockroaches, while the other species of the complex are pathogens of larvae of *Lepidoptera* (*O. gracilis*) and *Coleoptera* (*O. gracilioides*) and adult *Orthoptera* (*O. amazonica*).

The 'unilateralis' clade (MLBS = 62; PP = 1.00) is characterized by species typically possessing a *Hirsutella*-like asexual state and dark brown to black stromata that often produce an aperithecial tip. One new species described here, *Ophiocordyceps tiputini* (MLBS = 88; PP = 0.73), is a pathogen of *Megaloptera* larvae and is a member of this subclade, but represents a host affiliation that had not previously recorded for *Ophiocordyceps*. Although this clade contains numerous ant pathogens, they did not collectively form a monophyletic group. One group comprised *Ophiocordyceps pulvinata* and *Ophiocordyceps unilateralis*, which are pathogens of *Camponotus* ants (*Formicinae*). Meanwhile *Ophiocordyceps kniphofioides* var. *kniphofioides*, a pathogen of *Cephalotes atratus* (*Myrmicinae* tribe) and *O. kniphofioides* var. *ponerinarum*, a pathogen of *Paraponera clavata* (*Ponerinae* tribe) form a second ant clade that was inferred to share a more recent common ancestor with *O. tiputini*.

The 'sobolifera' clade (MLBS = 100; PP = 1.00) includes beetle pathogens, *Ophiocordyceps aphodii* and *Ophiocordyceps bruneipunctata*, and cicada (*Hemiptera*: *Cicadidae*) pathogens, including *Ophiocordyceps sobolifera*, *Ophiocordyceps yakusimensis*, *Ophiocordyceps longissima* and the new species described here, *Ophiocordyceps aracuanensis*.

The 'sphecocephala' clade is characterized by higher rates of nucleotide substitutions, which have complicated its inclusion in phylogenetic analyses (Sung *et al.* 2007, 2008). The 'sphecocephala' clade included 12 new specimens from the Amazon and displayed a topology concordant with previous studies (Sung *et al.* 2007, 2008; Kepler *et al.* 2013; Quandt *et al.* 2014). Although the internal nodes of the core were poorly resolved, species level clades (e.g., *Ophiocordyceps australis*, *Ophiocordyceps lloydii*, and *Ophiocordyceps dipterigena*) were strongly supported and two taxa *Ophiocordyceps fulgoromorphila* sp. nov., which attacks *Fulgoridae* (*Hemiptera*) (MLBS = 100; PP = 1.00), and *Ophiocordyceps evansii* sp. nov., which attacks *Pachycondyla* ants (MLBS = 100; PP = 1.00), are described as new species.

Taxonomy

Ophiocordyceps amazonica (Henn.) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora Stud. Mycol. 57: 40 (2007) (Fig 2A−C).

MycoBank No.: MB504219.

Basionym: *Cordyceps amazonica* Henn, Hedwigia 43: 247. (1904)

Taxon	Voucher#	Host/Substratum	ITS	nSSU	nLSU	TEF	RPB1	RPB2
Ha. balanoides	CBS 250.82	*Nematoda*	AJ292414	AF339588	AF339539	DQ522342	DQ522388	DQ522442
Ha. sinense	CBS 567.95	*Nematoda*	AJ292417	AF339594	AF339545	DQ522343	DQ522389	DQ522443
Ha. zeosporum	CBS 335.80	*Nematoda*	AJ292419	AF339589	AF339540	EF469062	EF469091	EF469109
Hy. aurantiaca	OSC 128578	*Hymenoptera* (adult ant)	JN049833	DQ522556	DQ518770	DQ522345	DQ522391	DQ522445
Hy. dipterigena	HUA 186134	*Diptera* (adult fly)			KJ917573			KC610712
Hy. dipterigena	NHJ 121702	*Diptera* (adult fly)	GU723771			GU797127		
Hy. muscaria	OSC 151902	*Diptera* (adult fly)		KJ878912	KJ878876	KJ878991	KJ878945	
Hirsutella sp.	OSC 128575	*Hemiptera*		EF469126	EF469079	EF469064	EF469093	EF469110
Hirsutella sp.	NHJ 12525	*Hemiptera*		EF469125	EF469078	EF469063	EF469092	EF469111
M. anisoplae	ARSEF 5714	*Lepidoptera*		AF543763	AF543787	AF543775	EF469092	EF469111
M. anisoplae	ARSEF 3145	*Coleoptera*		AF339579	AF339530	AF543774	DQ522399	DQ522453
N. atypicola	CBS 744.73	*Arachnida*		EF468987	EF468841	EF468786	EF468892	
O. acicularis	OSC 110987	*Coleoptera* (larva)		EF468950	EF468805	EF468744	EF468852	
O. acicularis	OSC 110988	*Coleoptera* (larva)		EF468951	EF468804	EF468745	EF468853	
O. acicularis	OSC 128580	*Coleoptera* (larva)	JN049820	DQ522543	DQ518757	DQ522326	DQ522371	DQ522423
O. agriotidis	ARSEF 5692	*Coleoptera* (larva)	JN049819	DQ522540	DQ518754	DQ522322	DQ522368	DQ522418
O. amazonica	HUA 186143	*Orthoptera* (Acrididae adult)		KJ917562	KJ917571	KM411989	KP212902	KM411982
O. amazonica	HUA 186107	*Orthoptera* (Acrididae imago)		KJ917561			KP212904	
O. amazonica	HUA 186113	*Orthoptera* (Acrididae imago)		KJ917566	KJ917571		KP212903	KM411980
O. amazonica	HUA 186136	*Orthoptera* (Acrididae imago)		KJ917560		KM411991	KP212905	
O. amazonica	HUA 186094	*Orthoptera* (Acrididae imago)		KJ917565		KM411986	KP212906	
O. amazonica	HUA 186100	*Orthoptera* (Acrididae imago)		KJ917564		KM411987	KP212907	
O. amazonica	HUA 186101	*Orthoptera* (Acrididae imago)				KM411988	KP212908	KM411981
O. amazonica	HUA 186106	*Orthoptera* (Acrididae imago)		KJ917563			KP212909	
O. aphodii	ARSEF 5498	*Coleoptera*		DQ522541	DQ518755	DQ522323		DQ522419
O. appendiculata	NBRC 100960	*Coleoptera* (larva)	JN943326	JN941728	JN941413		JN992462	
O. araracuarensis	HUA 186135	*Hemiptera* (cicada nymph)		KC610788	KC610769	KC610738	KF658665	KC610716
O. australis	HUA 186147	*Hymenoptera* (*Pachycondyla crassinoda* adult ant)	KF937351	KC610784	KC610764	KC610734	KF658678	
O. australis	HUA 186104	*Hymenoptera* (*Pachycondyla crassinoda* adult ant)	KC610783	KC610763	KC610733		KC610713	
O. australis	HUA 186097	*Hymenoptera* (*Pachycondyla crassinoda* adult ant)	KF937350	KC610786	KC610765	KC610735	KF658662	
O. barnesii	BCC 28560	*Coleoptera* (larva)		EU408776			EU408773	EU418599
O. barnesii	BCC 28561	*Coleoptera* (larva)		EU408775			EU408774	EU418572
O. blattarioides	HUA186093	*Dyctioptera* (imago Blattodea, imago)		KJ917559	KJ917570	KM411992	KP212910	
O. blattarioides	HUA 186108	*Dyctioptera* (Blattodea, imago)		KJ917558	KJ917569		KP212912	KM411984
O. blattarioides	HUA186093	*Dyctioptera* (Blattodea imago)		KJ917559	KJ917570	KM411992	KP212910	
O. barnesii	BCC 28560	*Coleoptera* (larva)		EU408776			EU408773	EU418599
O. barnesii	BCC 28561	*Coleoptera* (larva)		EU408775			EU408774	EU418572
O. brunneipunctata	OSC 128576	*Coleoptera* (Elateridae larva)		DQ522542	DQ518756	DQ522324	DQ522369	DQ522420
O. communis	NHJ 12581	*Isoptera* (adult termite)		EF468973	EF468831	EF468775		EF468930
O. communis	NHJ 12582	*Isoptera* (adult termite)		EF468975	EF468830	EF468771		EF468926
O. curculionum	OSC 151910	*Coleoptera* (adult Curculionidae)		KJ878918	KJ878885	KJ878999		
O. dipterigena	OSC 151911	*Diptera* (adult fly)		KJ878919	KJ878886	KJ878966	KJ879000	
O. dipterigena	OSC 151912	*Diptera* (adult fly)		KJ878920	KJ878887	KJ878967	KJ879001	
O. dipterigena	HUA 186102	*Diptera* (adult fly)		KC610787	KJ917568		KF658664	KC610715
O. dipterigena	MY 621	*Diptera* (adult fly)	GU723764			GU797126		
O. elongata	OSC 110989	*Lepidoptera* (larva)			EF468808	EF468748	EF468856	

O. evansii	HUA 186159	Hymenoptera (Pachycondyla harpax adult ant)	KP200889	KC610796	KC610770	KC610736	KP212916	
O. evansii	HUA 186163	Hymenoptera (Pachycondyla impresa adult ant)	KP200890	KC610797	KC610771	KC610737	KF658670	
O. forquignonii	OSC 151908	Diptera (adult fly)		KJ879003	KJ878947		KJ878922	KJ878889
O. fulgoromorphila	QCNE 186286	Hemiptera (Fulgoridae adult)		KC610793	KC610759			
O. fulgoromorphila	HUA 186139	Hemiptera (Fulgoridae adult)	KJ130991	KC610794	KC610760	KC610729	KF658676	KC610719
O. fulgoromorphila	HUA 186142	Hemiptera (Fulgoridae adult)		KC610795	KC610761	KC610730	KF658677	
O. gunni	OSC 76404	Lepidoptera (pupa)		AF339572	AF339522	AY489616	AY489650	DQ522426
O. gracilis	EFCC 3101	Lepidoptera (larva)		EF468955	EF468810	EF468750	EF468858	EF468913
O. gracilis	EFCC 8572	Lepidoptera (larva)	JN049851	EF468956	EF468811	EF468751	EF468859	EF468912
O. gracillima	HUA 186132	Coleoptera (Scarabeidae larva)	KJ130990		KC610768	KC610744	KF658666	
O. gracilioides	HUA 186095	Coleoptera (Elateridae larva)		KJ917556		KM411994	KP212914	
O. gracilioides	HUA 186092	Coleoptera (Elateridae larva)		KJ917555	KJ130992		KP212915	
O. heteropoda	EFCC 10125	Hemiptera (cicada nymph)	JN049852	EF468957	EF468812	EF468752	EF468860	EF468914
O. heteropoda	OSC 106404	Hemiptera (cicada nymph)		AY489690	AY489722	AY489617	AY489651	
O. irangiensis	OSC 128577	Hymenoptera (adult ant)	JN049823	DQ522546	DQ518760	DQ522329	DQ522374	DQ522427
O. irangiensis	OSC 128579	Hymenoptera (adult ant)		EF469123	EF469076	EF469060	EF469089	EF469107
O. irangiensis	NBRC 101400	Hymenoptera (adult ant)	JN943335	JN941715	JN941426		JN992449	
O. kniphofioides	HUA 186148	Hymenoptera (Cephalotes atratus adult ant)		KC610790	KF658679	KC610739	KF658667	KC610717
O. ponerinarum	HUA 186140	Hymenoptera (Paraponera clavata adult ant)	KJ130987	KC610789	KC610767	KC610740	KF658668	
O. poerinarum	HUA 186133	Hymenoptera (Paraponera clavata adult ant)					KF658669	
O. konnoana	EFCC 7295	Coleoptera (larva)		EF468958			EF468862	EF468915
O. konnoana	EFCC 7315	Coleoptera (larva)		EF468959		EF468753	EF468861	EF468916
O. lloydii	HUA 186164	Hymenoptera (Camponotus adult ant)	KP200892	KC610805		KC610741		
O. lloydii	HUA 186130	Hymenoptera (Camponotus adult ant)	KP200893	KC610791				
O. lloydii	OSC 151913	Hymenoptera (Camponotus adult ant)		KJ878924	KJ878891	KJ878970	KJ879004	KJ878948
O. longissima	EFCC 6814	Hemiptera (cicada nymph)			EF468817	EF468757	EF468865	
O. longissima	TNS F18448	Hemiptera (cicada nymph)		KJ878925	KJ878892	KJ878971	KJ879005	
O. longissima	HMAS 199600	Hemiptera (cicada nymph)		KJ878926		KJ878972	KJ879006	KJ878949
O. melolonthae	OSC 110993	Coleoptera (Scarabeidae larva)		DQ522548	DQ518762	DQ522331	DQ522376	
O. myrmecophila	MY 163	Hymenoptera (adult ant)	GU723759			GU797132		
O. myrmecophila	CEM 1710	Hymenoptera (adult ant)		KJ878927	KJ878893	KJ878973	KJ879007	
O. myrmecophila	HMAS 199620	Hymenoptera (adult ant)		KJ878929	KJ878895	KJ878975	KJ879009	
O. nigrella	EFCC 9247	Lepidoptera (larva)	JN049853	EF468963	EF468818	EF468758	EF468866	EF468920
O. nutans	OSC 110994	Hemiptera (Pentatomidae adult)		DQ522549	DQ518763	DQ522333	DQ522378	
O. pulvinata		Hymenoptera (Camponotus adult ant)		GU904208		GU904209	GU904210	
O. ravenelii	OSC 110995	Coleoptera (larva)		DQ522550	DQ518764	DQ522334	DQ522379	EF468923
O. rhizoidea	NHJ 12522	Isoptera (adult termite)	JN049857	EF468970	EF468825	EF468764	EF468873	EF468922
O. rhizoidea	NHJ 12529	Isoptera (adult termite)		EF468969	EF468824	EF468765	EF468872	
O. robertsii	KEW 27083	Lepidoptera (Hepialidae larva)			EF468826	EF468766		
O. rubiginosiperitheciata	NBRC 106966	Coleoptera (larva)	JN943344	JN941704	JN941437		JN992438	
O. sinensis	EFCC 7287	Lepidoptera (Hepialidae larva)	JN049854	EF468971	EF468827	EF468767	EF468874	EF468925
O. sobolifera	KEW 78842	Hemiptera (cicada nymph)	JN049855	EF468972	EF468828		EF468875	DQ522432
O. sobolifera		Hemiptera (cicada nymph)	AB027374	AB027328	AB027374			
O. sobolifera	TNS F18521	Hemiptera (cicada nymph)		KJ878933	KJ878898	KJ878979	KJ879013	
O. sphecocephala	OSC 110998	Hymenoptera (adult wasp)		DQ522551	DQ518765	DQ522336	DQ522381	
O. sphecocephala	NBRC 101416	Hymenoptera (adult wasp)	JN943348		JN941443		JN992432	EF468931
O. stylophora	OSC 111000	Coleoptera (Elateridae larva)	JN049828	DQ522552	DQ518766	DQ522337	DQ522382	
O. tiputinii	QCNE 186287	Megaloptera (larva)		KC610792	KC610773	KC610745	KF658671	

Table 1 – (continued)

| Taxon	Voucher#	Host/Substratum	ITS	nSSU	nLSU	TEF	RPB1	RPB2
O. tricentri		*Hemiptera* (*Cercopidae*)		AB027330	AB027376			DQ522436
O. unilateralis	HUA 186161	*Hymenoptera* (*Camponotus* adult ant)		KC610799		KC610742	KF658674	KC610718
O. unilateralis	HUA 186126	*Hymenoptera* (*Camponotus* adult ant)		KC610798			KF658673	
O. unilateralis	OSC 128574	*Hymenoptera* (*Camponotus* adult ant)		DQ522554	DQ518768	DQ522339	DQ522385	
O. variabilis	OSC 111003	*Diptera* (larva)		EF468985	EF468839	EF468779	EF468885	EF468933
O. variabilis	ARSEF 5365	*Diptera* (larva)		DQ522555	DQ518769	DQ522340	DQ522386	DQ522437
O. xuefengensis	GZUHHN 13	*Lepidoptera* (*Hepialidae*, *Phassus nodus*, larva)	KC631804	KC631785	KC631790	KC631795		
O. yakusimensis	HMAS 199604	*Hemiptera* (cicada nymph)		KJ878938	KJ878938		KJ879018	KJ878953
P. chlamydosporia	CBS 504.66	*Nematoda*	AJ292398	AF339593	AF339544	EF469069	EF469098	EF469120
P. chlamydosporia	CBS 101244	*Diplopoda*		DQ522544	DQ518758	DQ522327	DQ522372	DQ522424
Pol. cuboideus	NBRC100941	*Coleoptera* (larva)	JN943329	JN941725	JN941416		JN992459	
Pol. paracuboideus	NBRC 101742	*Coleopteran* (larva)	JN943338	JN941710	JN941431		JN992444	
Pol. prolifica	NBRC 101750	*Hemiptera* (*Tana japonensis*, cicada nymph)	JN943340	JN941708	JN941433		JN992442	DQ522430
Pol. formosus	ARSEF 1424	*Coleoptera*	KF049661	KF049615	KF049634	KF049689	KF049651	KF049671
Pol. nipponicus	BCC 1881	*Neuroptera*		KF049618	KF049636	KF049692		KF049674
Pol. ramosopulvinatus	EFCC 5566	*Hemiptera*	KF049658		KF049627	KF049682	KF049645	
Pol. ryogamiensis	NBRC 103842	*Coleoptera* (larva)	JN943345	JN941701	JN941440		JN992435	EF468924
Pu. lilacinum	ARSEF 2181	Soil		AF339583	AF339534	EF468790	EF468896	
Pu. lilacinum	CBS 431.87	*Nematoda*	AY624188	AY624188	EF468844	EF468791	EF468897	EF468940
Pu. lilacinum	CBS 284.36	Soil	AY624189	AY624189	AY624227	EF468792	EF468898	EF468941
T. capitatum	OSC 71233	*Fungi* (*Elaphomyces* sp.)		AY489689	AY489721	AY489615	AY489649	DQ522421
T. capitatum	NBRC 100997	*Fungi* (*Elaphomyces* sp.)	JN943313	JN941740	JN941401		JN992474	
T. fractum	OSC 110990	*Fungi* (*Elaphomyces* sp.)		DQ522545	DQ518759	DQ522328	DQ522373	DQ522425
T. subsessilis	OSC 71235	*Coleoptera* (*Scarabaeidae* larva)	JN049844	EF469124	EF469077	EF469061	EF469090	EF469108
T. japonicum	OSC 110991	*Fungi* (*Elaphomyces* sp.)	JN049824	DQ522547	DQ518761	DQ522330	DQ522375	DQ522428
T. ophioglossoides	OSC 106405	*Fungi* (*Elaphomyces* sp.)		AY489691	AY489723	AY489618	AY489652	DQ522429
T. ophoglossoides	NBRC 8992	*Fungi* (*Elaphomyces* sp.)	JN943316	JN941736	JN941405		JN992470	
T. paradoxum	NBRC 100945	*Hemiptera* (cicada nymph)	JN943323	JN941731	JN941410		JN992465	
C. pleuricapitata	NBCR 100745	*Hemiptera* (cicada nymph)		KF049606	KF049624	KF049679	KF049642	KF049667

Genus codes: *Ha. Haptocillium*; *Hy. Hymenostilbe*; *M. Metharizium*; *N. Nomuraea*; *P. Pochonia*; *Po. Polycephalomyces*; *Pu Purpureocillium*; *O. Ophiocordyceps*; *T. Tolypocladium*.

Herbarium codes: ARSEF, USDA-ARS Collection of Entomopathogenic, Ithaca, NY, USA; BCC, BIOTEC Culture Collection, Klong Luang, Thailand; CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; EFCC, Entomopathogenic Fungal Culture Collection, Chuncheon, Korea; GZUH, Herbarium of Guizhou University, Guiyang, Guizhou, China; HUA, Herbarium Antioquia University, Medellin, COL; KEW, mycology collection of Royal Botanical Gardens, KEW, Surrey, UK; NHJ, Nigel Hywel-Jones personal collection; OSC Oregon State University Herbarium, Corvallis, OR, USA; QCNE, National Herbarium of Ecuador, Quito, ECU. MY, Luangsard et al.(2011); NBRC, Schoch et al. (2012).
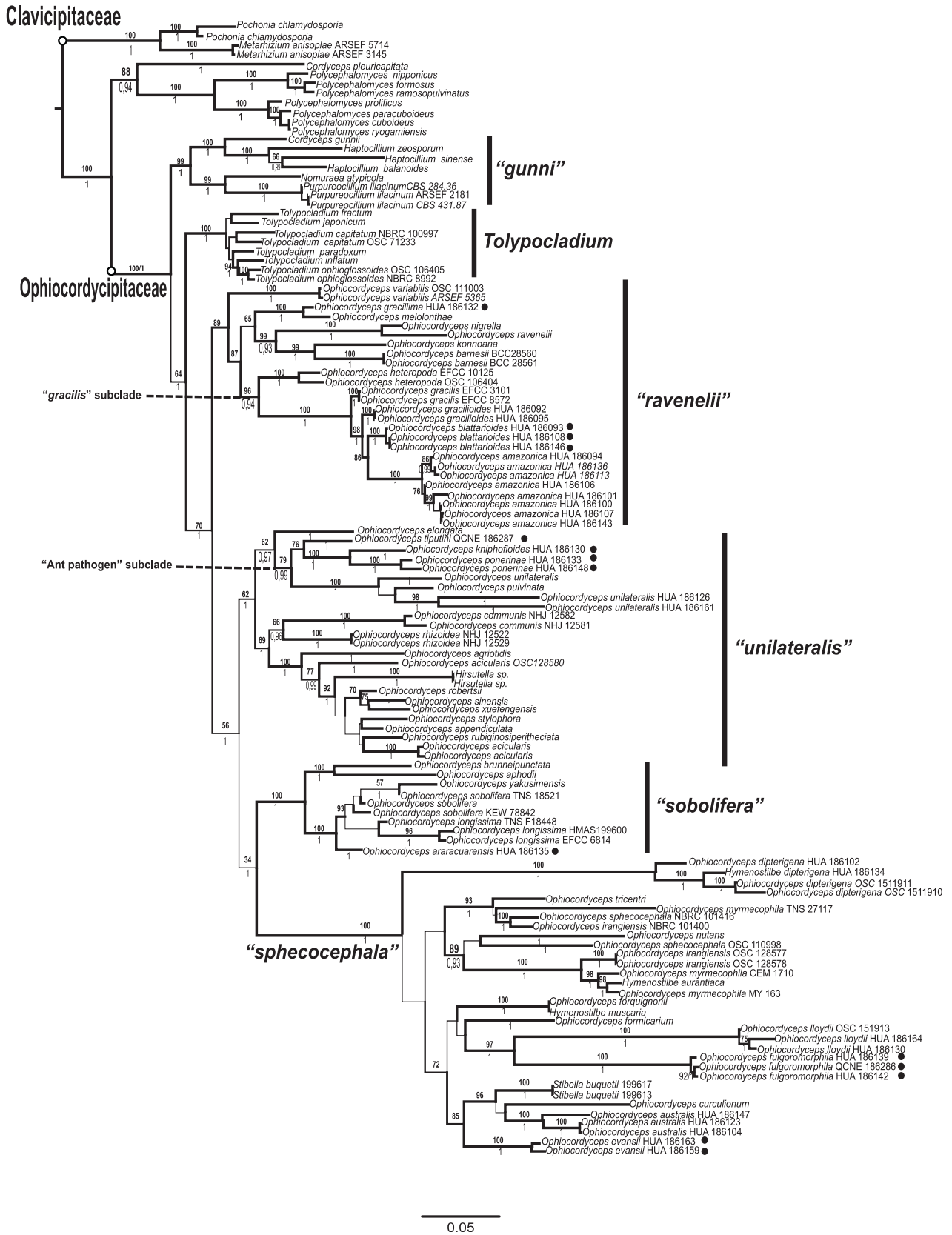
Fig 1 — The most likely tree from the maximum likelihood analysis of *Ophiocordycipitaceae* family including species collected in the Amazon region based on combined dataset of *SSU rRNA, LSU rRNA, TEF, RPB1* and *RPB2*. Numbers above branches indicate bootstrap support from 1000 maximum likelihood replicates. Numbers below branches indicate posterior probability support in Bayesian analyses. Thinned branches show low resolution in one of the two analyses. The subclades of *Ophiocordycipitaceae* are named as follow: 1. 'gunnii' clade, 2. *Tolypocladium* (*Elaphocordyceps* sensu Sung *et al.* 2007) and the four subclades of *Ophiocordyceps*: 3. 'ravenelii' 4. 'unilateralis', 5 'sobolifera' and 6'sphecocephala'. Black circles shown the new taxa proposed. The tree is rooted with *Metarhizium anisopliae* and *Pochonia chlamydosporia*.
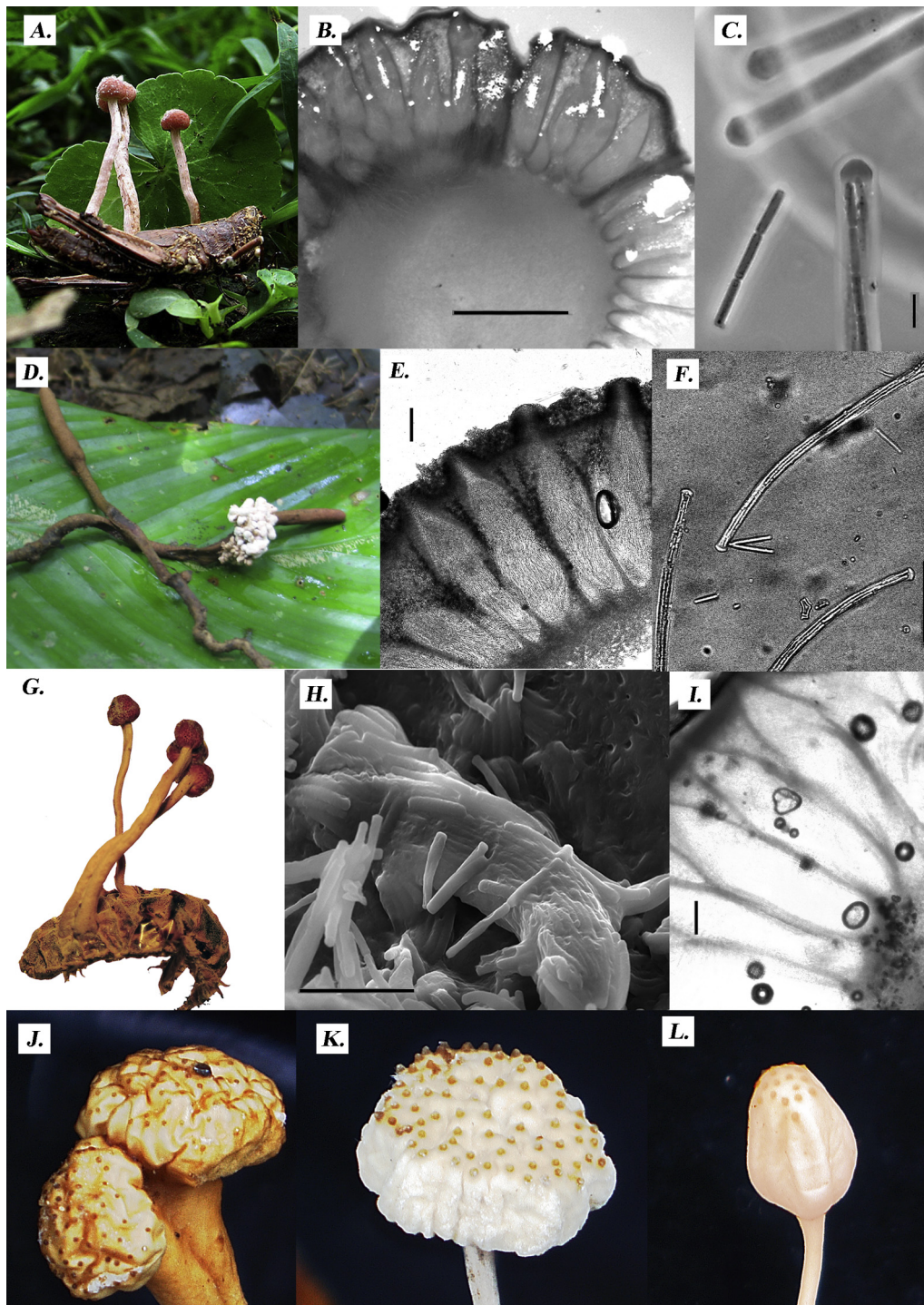
Fig 2 − **Amazonian species of *Ophiocordyceps*. (A–C) Epitype of *Ophiocordyceps amazonica* HUA 186143 (A) Stromata on *Prionacris* sp. adult. (B) Perithecia. (C) Asci and part-spores. (D–F) *Ophiocordyceps araracuarensis* sp. nov. HUA 186135 holotype. (D) Stromata and synnemata. (E) Perithecia on transversal arrangement. (F) Asci and part-spores. (G–I) *Ophiocordyceps blattarioides* sp. nov. HUA 186108 holotype. (G) Stromata on cockroach adult host. (H) Part-spores on SEM. (I) Perithecia arrangement. (J) Close up of *Ophiocordyceps dipterigena* HUA 186102. (K) Close up of *Ophiocordyceps lloydii* HUA 186164. (L) Close up of *Ophiocordyceps evansii* sp. nov. HUA 186163. Bars = 200 μm 5× (B), 10 μm 100× (C, E-F), 10 μm 2500× (H), 500 μm 10× (I).**

Description: Stromata solitary to single or gregarious, capitate 20–45 mm long. Fertile head spherical, 3–5.5 mm diam., pruinose when fresh, mammillate when dry, reddish brown (10D/8). Stipe rounded cylindrical to compressed, sometimes twisted, 17–40 × 1.0–2.0 mm, fleshy, glossy, pale red to dull red (9B4/9A3). Cortex composed of an ectal layer of longitudinal cells forming a pseudoparenchyma 25 μm thick, with perithecia arranged in a palisade. Perithecia ellipsoid, (760−)

800–1100 × (220–) 250–400 μm (n = 20), wall 15–25 μm. Asci cylindrical, 325–450 × 5 μm (n = 15), apex hemiglobose, 6–7 × 4–5 μm. Ascospores hyaline, filiform, multiseptate, breaking into regular cylindrical part-spores 9–15(–17) × 1.5–2 μm (n = 25). Not conidial state observed.

Lectotype: **Brazil**. Amazonas: Rio Jurua, Juruá, Miry, May 1901. Hennings, P. Fungi amazonici II. a cl. Ernesto Ule collecti. Tab IV f. 4a. (1904). 'lectotypus hic designatus'

Epitype: **Colombia**: Amazonas, Leticia, El Zafire research station, 4°0′21″S 69°53′55″W, alt 115 m, on adult of *Prionacris* sp. (*Orthoptera*: *Romelidae*) on the ground, 10 Jan 2012, *A. Vasco-P.* 2026 (HUA 186143).

Other specimens examined: **Colombia**: Amazonas, Leticia, El Zafire research station, 4°0′21″S 69°53′55″W, alt 115 m. 8 Mar 2012, *A. Vasco-P.* 1968 (HUA 186106), 18 Mar 2011, *A. Vasco-P.* 1856 (HUA 186100), 22 Mar 2011 *A. Vasco-P.* 1862 (HUA 186101), Peña Roja, 0°34′17.65″S 72° 8′24.55″W, alt 100 m, 09 Feb 2010, *A. Vasco-P.* 1781 (HUA 186084), Puerto Santander, Villa Azul, 0°34′17.65″S 72° 8′24.55″W, alt. 100 m, 9 May 2009, *T. Sanjuan* 697 (HUA 186136 – paratype); Antioquia: San Luis, Rio Claro 5°56′57.90″N 74°56′57.55″W, alt 250 m, 1 Sep 2012, *T. Sanjuan* 1001 (HUA 186113). **Ecuador**: Orellana, Yasuni National Park. Tiputini Research Station, 0°38′14.84″S 76°09′1.83″W, alt. 200 m.15 Jul 2004. *T. Sanjuan* 496b (QCNE186285); Pichincha, Pedro Vicente Maldonado, Finca La Nueva Esperanza, 00°0.5′58.87″N 79°05′41.45″ W, alt 800 m, 6 Nov 2005, *T. Sanjuan* 580 (QCNE186294).

Known distribution: Tropical lowlands forest in the Andes mountain of Colombia and Ecuador. Amazon region.

Notes about the host: On imagos and adults of grasshoppers from families *Acrididae* and *Romelidae* buried in the leaf litter. These families belong to the superfamily Acridomorpha easily distinguishable by its large, well-developed hind legs. Localities for all collected specimens of *Ophiocordyceps amazonica* are consistent with the dispersal in the neotropics for *Acridomorpha* reported by Amedegnato & Descamps (1982).

Ophiocordyceps araracuarensis T. Sanjuan & J.W. Spatafora, **sp. nov.** (Fig 2D–F).

MycoBank No.: MB805232.

Etymology: In reference to the Araracuara region in the locality where the type specimen was collected.

Holotype: **Colombia**: Amazonas, Puerto Santander, Villa Azul, Araracuara region 0°34′17.65″S 72° 8′24.55″W, alt 100 m, on nymph of *Cicadamorpha* buried in the ground, 8 May 2009, *T. Sanjuan* 696, (HUA 186135 – holotype).

Description: Stromata solitary, simple, cylindric, 83–97 mm long. Fertile head cylindrical, 25 × 4 mm, slightly punctate, dark brown (6E7) when fresh, dark brown (6F8) when dry. Stipe cylindrical, 62–78 × 2 mm, stiff and fibrous, dark brown (6F8), not straight, with scars where the conidial stage emerges. Perithecia distributed uniformly along of the fertile head, pyriform, 550–650 × 150–240 μm (n = 20), base with long neck of 8–10 μm wide, immersed in ordinal orientation. Cortex composed of indistinct branched, septate hyphae 3 μm diam. Asci cylindric 300 × 5–6 μm, apex ovoid to lenticular 3 × 5–6 μm (n = 20). Ascospores filiform, hyaline, breaking irregularly into truncate part-spores 9–12 (–16) × 1 μm (n = 50). Asexual state formed by a coraliform growth or soboles (conidial lateral branches), pinkish to whitish, emerging from

the stipe of the stroma, composed by hyaline conidial cells with ovoid to ellipsoid conidia, 6–8 × 2–4 μm (n = 50).

Other specimen examined: **Colombia**; Amazonas, Puerto Santander, Villa Azul, Araracuara region, 0°34′17.65″S 72° 8′24.55″W, alt 100 m, on nymph of *Cicadamorpha* buried in the ground, 8 May 2009, *T. Sanjuan* 695 (HUA 186136 – isotype).

Known distribution: Amazon region of Colombia.

Notes about the host: The hosts were in poor condition because they were mature specimens buried in floodplain soil; however, the pronotum with scutellar suture and anterior legs suited for excavation were preserved and are diagnostic of the family *Cicadidae*. This family of plant sap-sucking insects is distributed worldwide, mostly in Southeast Asia, but more recently new species had been found in the tropics suggesting that more than 90 % of tropical cicada species remain undescribed (Dietrich 2005).

Ophiocordyceps blattarioides T. Sanjuan & J.W. Spatafora, **sp. nov.** (Fig 2G–I).

MycoBank No.: MB805784.

Etymology: In reference to the suborder of the host *Blattaria* (Dictyoptera).

Holotype: **Colombia**: Amazonas, Leticia, El Zafire research station, 4°0′21″S 69°53′55″W, alt 115 m, on adult of cockroach (*Blattidae*, *Blattaria*: *Dictyoptera*) buried in leaf litter, 16 Jan 2012, *A. Vasco-P.* 2098 (HUA 186108 – holotype).

Description: Stromata solitary to gregarious, capitate, 10–20 mm long. Fertile head ovoid to subgloboid, 2–3 × 1.5–2.5 mm, pruinose when fresh, mammillate when dry, reddish brown (8/9D/E6). Stipe rounded cylindrical to compressed, sometimes twisted, 10–14 × 0.5–0.8 mm, fleshy, glossy, grayish red to dull red (7/8B4). Cortex composed of an ectal layer of longitudinal cells forming a pseudoparenchyma of 15 μm thick, with perithecia arranged in a palisade. Perithecia ellipsoid, (650–) 760–800 × 220–300 μm (n = 20), wall 15–25 μm. Asci cylindrical, 250–300 × 5 μm (n = 20), apex hemiglobose, 6–7 × 4–5 μm. Ascospores hyalines, filiform, multiseptate, breaking into regular cylindrical part-spores 6–12 (16) × 1.5 μm (n = 25). Not conidial state observed.

Other specimens examined: **Colombia**: Amazonas, La Chorrera. Vegsan, 1°41′38.58″S 72°28′44.25″W, alt160 m. 28 Jul 2010, *T. Sanjuan* 773 (HUA 186146 – paratype). Leticia, El Zafire research station, 4°0′21″S 69°53′55″W, alt 115 m. 16 Jan 2012, *A. Vasco-P.* 2099 (HUA 186109 – isotype); Puerto Santander, Villa Azul, 0°34′17.65″S 72° 8′24.55″W, alt 100 m. 16 Jun 2010, *A. Vasco-P.* 014 (HUA186093 – paratype).

*Known distribution*: Belize, tropical lowlands in the Andes mountain of Colombia and Ecuador. Amazon region.

Notes about the host: The triangular head, flattened-oval body and absence of ocelli are characteristics, which allow identification the host of specimens of HUA 186108–186109, 186146 as members of superorder *Dictyoptera*. Additionally the specimen HUA186093 has brown spots and short developed wings, which allow for identification as *Neostylopyga* (*Blattaria*: *Blattinae*).

Ophiocordyceps evansii T. Sanjuan, **sp. nov.** (Fig 2L; Fig 3A–B).

MycoBank No.: MB805413.

Etymology: This species is named in honour of Harold Charles Evans for his contributions to the knowledge of the entomogenous fungi parasitic on ants in the tropics.
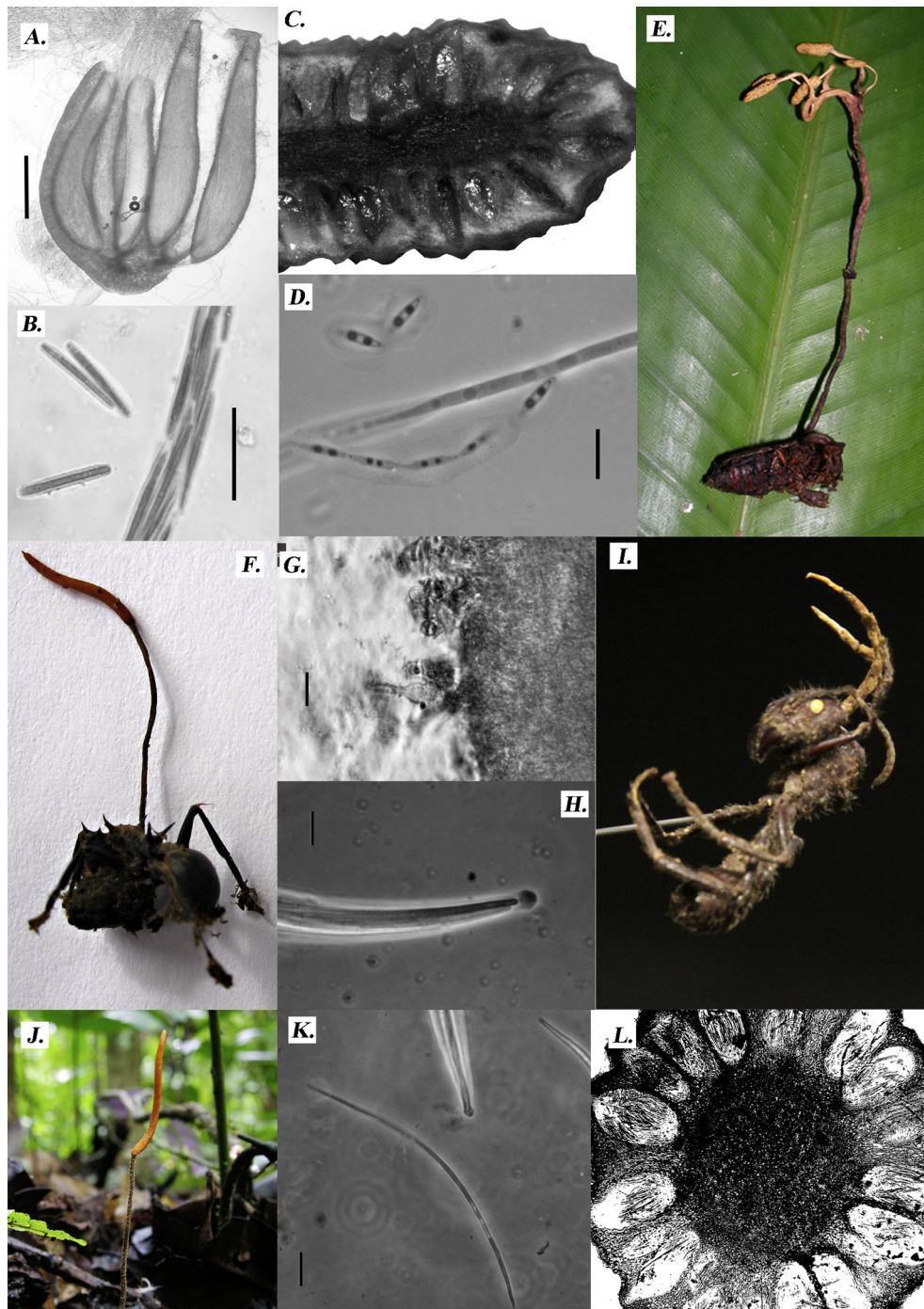
Fig 3 − Amazonian species of *Ophiocordyceps*. (A−B) *Ophiocordyceps evansii* sp. nov. HUA 186163 (A) Perithecia on longitudinal arrangement. (B) Part-spores. (C−E) *Ophiocordyceps fulgoromorphila* sp. nov. HUA 186139 holotype. (C) Perithecia on longitudinal arrangement. (D) Ascospores and part-spores. (E) Stromata on *Fulgoridae* adult. (F−H) *Ophiocordyceps kniphofioides* HUA 186130 (F) Stromata on *Cephalotes atratus* ants. (G) Conidial state *Hirsutella*-like shown bottle phialides. (H) Asci shown the tip. (I) Stromata of *Ophiocordyceps ponerinarum* HUA 186148 on *Paraponera clavata* ants. (J−L) *Ophiocordyceps tiputini* sp. nov. QCNE 186287 holotype. (J) Stromata. (K) Ascospores. (L) Asci. Bars = 300 μm 10× (A), 20 μm 100× (B), 10 μm 100× (D,G, H, K), 300 μm 10× (L).

Holotype: **Colombia**: Amazonas, La Chorrera, San Francisco, 1°21′40″S 72°51′48″W, alt 0 m, on the ant *Pachycondyla impressa* (*Ponerinae*) biting small roots in bark at 1 m from the ground, 18 May 2010, *T. Sanjuan* 858, (HUA 186159 − holotype).

Description: Stromata simple, capitate, slender, 20−30 mm long, filamentous, bicolored. Fertile head flattened obovoid in vertical profile with erumpent ostioles, 1.5−2 × 0.5−1 mm, dull red to brownish red (8B6/9C7). Stipe slender, cylindric,

18−28 × 0.7 mm glabrous, pliant, brownish black (7F8), concolorous with the head. Perithecia obclavate, 1600−1800 × 260−300 μm, with a prominent reddish brown ostiole, 60−80 × 80−100 μm, immersed vertically in clava consisting of very loosely interwoven hyphae with a very thick compact ectal layer, 40−50 μm wide of oblong or globoid cells. Asci cylindrical, 950−1200 × 8 μm when mature, apex hemispheric 8 × 10 μm. Ascospores arranged in parallel, filiform, easily breaking into part-spores, fusiform-ellipsoid, 17−20 × 1.5−2 μm. External mycelium brown dark, scarcely covering the host and attaching it sometimes to the substrate. Not conidial state observed.

Other specimens examined: **Colombia**: Amazonas: La Chorrera, San Francisco, primary forest, 1°21′40″S 72°51′48″W, alt 250 m, on the ant *Pachycondyla harpax* (*Ponerinae*), 22 Mar 2011, T. *Sanjuan* 912, (HUA 186163 − paratype).

Known distribution: In the Amazon region of Colombia, Ecuador, and Brazil.

Notes about the host: *Pachycondyla* is the second most diverse genus in the Ponerinae tribe of ants. They are terrestrial hunters with some species making their nest on the ground while others are arboreal. *Pachycondyla* is characterized by triangular mandibles without a circular orifice. The adhesive pads of the tarsus are considered an adaptation to an arboreal ecology (Orivel *et al.* 2001).

Ophiocordyceps fulgoromorphila T. Sanjuan, **sp. nov.** (Fig 3C−E).

MycoBank No.: MB805227.

Etymology: In reference to the host family *Fulgoridae* (*Hemiptera*: *Fulgoromorpha*).

Holotype: **Colombia**: Amazonas, Puerto Santander, Araracuara Canyon, 0°37′21″S 72°24′26″W, alt 115 m, on adult of *Fulgoridae* buried in leaf litter, 13 May 2009, T. *Sanjuan* 717, (HUA 186139 − holotype).

Description: Stromata solitary to gregarious, capitate, 87−110 mm long, perpendicularly branched in secondary bicolored stromata. Fertile head cylindrical (3.5−) 7−25 × 2−3.5 mm (n = 4), apex rounded, punctate, often rugose due to the ostioles, brownish yellow to brownish orange (4C5/7C4), ostioles yellow brown (5E6). Stipe slender, (57−) 80−120 × 0.5−2.5 mm, ligneous, grayish brown (5F3) becoming olivaceous buff (5C7). Secondary stipe glabrous, leathery, concolorous to the head above, 10−20 × 0.5−1.5 mm. The stipe extends to the fertile head as a columella composed by parallel, septate, brown, hyphae, 800 μm thick. Perithecia conical to ellipsoid, 780−1100 × 280−380 μm (n = 20), immersed in slightly oblique orientation in a cortex composed of brownish pseudoparenchyma with three layers of rounded cells and one of cylindrical base cells, 50 μm thick. Asci cylindrical 300−600 × 5−6 μm, (n = 20) with a 4 × 5 μm thick rounded cap. Ascospores hyaline, filiform, multiseptate, breaking irregularly into fusiform part-spores 9−12 (−16) × 1−2 μm (n = 30) with two oil drops. Not conidial state observed.

Other specimens examined: **Colombia**: Amazonas, Puerto Santander, Araracuara Canyon, 0°37′21″S 72°24′26″W, alt 115 m,14 May 2009, T. *Sanjuan* 729, (HUA 186142 − isotype), Villa Azul, 0°34′17.65″S 72° 8′24.55″W, alt 100 m, 8 May 2009, T. *Sanjuan* 689 (ANDES paratype); Antioquia, San Luis, refugio del cañon de Rio Claro, 5°56′57.90″N 74°56′57.55″W, alt

200 m, 9 Nov 2014, T. *Sanjuan* 1155 (ANDES). **Ecuador:** Orellana province, Yasuni National Park, Tiputini Research Station, 00°38′14,84″ S 76°09′01,83′ W, alt 150 m, 13 Jul 2004. T. *Læssøe* 11468 (QCNE 186286 − paratype).

Known distribution: Tropical lowlands in the Magdalena River basin in Colombia and Amazon region of Bolivia, Ecuador and Colombia.

Notes about the host: Despite the conditions of the specimens TS 689, 717, and 729, they all possess the conserved insertion of antenna and carina covering the eyes' fossa, as well as specimen TL 11468 having a longer prolongation and spines in its abdomen. This unique morphology of the head is the most diagnostic character of the *Fulgoridae*. This family is one of the most diverse on the neotropics. Of the 500 described species of *Fulgoridae*, only 17 species are recorded from Holoarctic (Urban & Cryan 2009).

Ophiocordyceps gracillima (Y. Kobayasi) T. Sanjuan & J.W. Spatafora, **comb. nov.**

Basionym: *Cordyceps gracillima* Y. Kobayasi Bull. Nat. Sci. Mus., Tokyo, Bot (7) 4: 126 (1981).

MycoBank No.: 811229.

Specimens examined: Colombia: Amazonas, Puerto Santander, Villa Azul, 0°34′17.65″S 72°8′24.55″W, alt 100 m, 7 May 2009, on larva of *Scarabaeidae*, (*Coleoptera*) T. *Sanjuan* 679, (HUA 186132).

Known distribution: In the Amazon region of Colombia.

Ophiocordyceps kniphofioides *sensu stricto* H.C. Evans & Samson (1982). (Fig 3F−H).

MycoBank No.: 504288.

Synonym: *Cordyceps kniphofioides* var. *kniphofioides* H.C. Evans & Samson, Trans. Brit. Mycol. Soc. 79: 441. (1982).

Specimens examined: **Colombia:** Amazonas, La Chorrera, San Francisco, Chuquial forest, 1°21′40″S 72°51′48″W, alt 50 m, on adults of ant *Cephalotes atratus* biting and hidden in moss on branches and trunks of trees, 30 July 2009, T. *Sanjuan* 790, (HUA 186148); 11 September 2011, T. Sanjuan 975, (HUA 186124).

Known distribution: In the Amazon region of Colombia, Ecuador, and Brazil.

Notes about the host: *Cephalotes atratus* nests in the canopy and forages in the lower forest where it feeds on plant sucking homopterans (Evans & Samson 1982). The *Myrmicinae* tribe is considered an army group with a higher level of social organization. However *C. atratus* is an opportunistic predator closely related phylogenetically to *Paraponera clavata* (*Poneromorpha*) (Keller 2011).

Ophiocordyceps ponerinarum (H.C. Evans & Samson) T. Sanjuan & R.M. Kepler, **stat. nov.** (Fig 3I).

MycoBank No.: MB810374.

Synonym: *Cordyceps kniphofioides* var. *ponerinarum* H.C. Evans & Samson, Trans. Brit. Mycol. Soc. 79: 441. (1982).

Ophiocordyceps kniphofioides var. *ponerinarum* (H.C. Evans & Samson) G.H. Sung *et al.*, *Stud. Mycol.* **57**: 37 (2007).

Specimens examined: **Colombia:** Amazonas, Puerto Santander, Araracuara Canyon, 0°37′21″S 72°24′26″W, alt 115 m, on hunter ant *Paraponera clavata* and *Dinoponera longipes* on the ground, 13 May 2009, T. *Sanjuan* 727 (HUA 186140), Amazonas, La Chorrera, San Francisco, Chuquial forest, 1°21′40″S 72°51′48″W, alt 50 m, 11 September 2011, T. Sanjuan 986−987, (HUA 186133).

Known distribution: In the Amazon region of Colombia, Ecuador, and Brazil.

Notes about the host: *Paraponera clavata* and *Dinoponera longipes* are the largest Amazon ants with lengths of 26 mm and 30 mm, respectively (Kempf 1971). They are hunters and make their nests in the bases of trees, palms, and lianas. *P. clavata* initially was included in *Ponerinae* tribe, but with the molecular phylogenetic approach this single species was placed in the tribe called *Paraponerinae* and is more related to *Myrmicinae* which includes *C. atratus*, the host of *Ophiocordyceps kniphofioides* (Keller 2011).

Ophiocordyceps tiputini T. Sanjuan & R.M. Kepler, **sp. nov**. (Fig 3 J–L).

MycoBank No.: MB805228.

Etymology: Refers to the collection locality Tiputini River of the type.

Holotype **Ecuador:** Orellana province, Yasuni National Park, Tiputini Research Station, 00°38′14.84″ S 76°09′01.83′ W, alt 150 m, on larva of *Megaloptera* buried 10 cm in wet leaf litter, 13 Jul 2004. T. Læssøe 11465 (QCNE 186287 – holotype).

Description: Stromata single, simple or branched, capitate, slender, wiry, and stiff 150 mm long. Fertile head cylindric, dark orange to deep orange (5A8/6A8), 15–25 × 1.5–2.5 mm, having a central core and sterile acuminate tip. Stipe fibrillose, pliant, dark brown (7F8), 125–135 × 0.5 mm. Perithecia ovoid, 300 × 200–250 μm (n = 20), dark brown immersed, ordinal orientation. Cortex composed by a pseudoparenchyma with hexagonal cells. Asci broadly fusoid, (100–)125–200 × 5 μm, narrowing to 2.5 μm at the apex, cap prominent 4.5 × 2.5 μm. Ascospores elongate-fusiform with rounded apex 120–180 × 1–1.5 μm, not breaking into part-spores. Not conidial state observed.

Known distribution: In the Amazon region from Ecuador and Bolivia.

Notes about the host: The larva is slightly flattened with sclerotized head and conspicuous mouthparts. The mandibles have the typical chewing morphology with prominent maxillae characteristic of *Megaloptera*. The size of the larva, more than 6 cm, suggests that it probably belongs to the *Corydalidae* family (e.g., dobsonfly). In Bolivia, similar entomogenous fungi on the same host that have been photographed in cloud forest of Coroico and the low rain forest of Mashaquipe show the typical coiled worm of the *Megaloptera* larvae (Winkler, 2014).

Discussion

This study represents the first extensive phylogenetic investigation of *Ophiocordyceps* and the family *Ophiocordycipitaceae* in the neotropics. *Ophiocordyceps* in the Amazon subregion displays high morphological diversity and a broad range of host affiliations. Two main hypotheses, the 'host relatedness' and 'host habitat' hypotheses (Brooks & McLennan 1991) are frequently invoked to explain host affiliations observed among *Cordyceps* s.l. In the 'host relatedness' hypothesis, host speciation functions as an isolating mechanism that results in cospeciation of the symbiont and congruent host-symbiont phylogenies. The 'host habitat' hypothesis involves host shifts among often distantly related hosts that occupy a common ecological niche. This second hypothesis has been invoked

to explain host switching among closely related species of *Cordyceps* s.l. that are found associated with distantly related host species between cicadas and truffles (Nikoh & Fukatsu 2000; Spatafora *et al.* 2007) and between scale insects, grasses, and fungi (Kepler *et al.* 2012). Here we conducted molecular phylogenetic analyses of *Ophiocordyceps* and discuss their phylogenetic diversity and the relative importance of these two hypotheses as explanatory mechanisms for speciation of Amazonian entomopathogenic fungi.

New Amazonian species in the 'ravenelii' clade

In the 'ravenelii' clade (Fig 1), the species *Ophiocordyceps heteropoda*, *Ophiocordyceps gracilis*, *Ophiocordyceps gracilioides*, *Ophiocordyceps amazonica*, and *Ophiocordyceps blattarioides* form the well-supported 'gracilis' clade that is characterized by the production of dark red to brown to tan capitate stromata with similar macroscopic and microscopic characteristics (Fig1; Fig 2A,G; Table 2). *Ophiocordyceps heteropoda* attacks nymphs of *Hemiptera* (Cicadidae), *O. gracilis* attacks larval stages of *Lepidoptera* (mainly *Hepialidae*, *Nymphalidae*), *O. gracilioides* attacks larval stages of *Coleoptera* (Elateridae), *O. amazonica* attacks adult *Orthoptera* (Romaleidae, Acrididae) and *O. blattarioides* sp. nov. attacks adult *Dictyoptera* (Blattaria). Although Mains (1940) was unable to distinguish specimens collected on cockroaches in Belize from *O. amazonica* on grasshoppers based on morphological characteristics, our molecular phylogenetic analyses support the recognition of distinct phylogenetic species that are consistent with isolation by host within a common habitat, i.e., ecological niche. While *O. heteropoda* attacks cicada hosts buried in soil, the remainder of species in this clade infect hosts associated with the soil and leaf litter environments.

Despite the frequent collection of *O. amazonica* from the Amazon region, no type specimen exists owing to the destruction during WWII of the material on which the description published by Hennings (1904) was based. Here we conducted molecular and morphological analyses of 13 specimens of *O. amazonica*, and assigned an epitype from Amazonian material.

Ophiocordyceps gracillima is also located in the 'ravenelii' clade (Fig 1), in the 'ravenelii – scarabaeidae' subclade *sensu* Luangsa-ard *et al.* (2010), where most species parasitize beetle larvae. This species was described by Kobayasi (1981) from material collected in the Colombian Amazon region. It produces solitary cylindrical capitate stromata that are red-orange in colour and parasitize *Scarabaeidae* larvae buried in decaying, fallen tree trunks. In the phylogenetic analyses *O. gracillima* is a sibling species of an isolate of *Ophiocordyceps cf. melolonthae*. The specimen of *O. cf. melolonthae* analysed here was collected in the Appalachian Mountains of North America and occurred on a larva of *Scarabaeidae* buried in soil. This collection and others collected in the region (see http://cordyceps.us) were sulfur yellow in colour when fresh with a clavate stroma and irregular patches of perithecia. Differences in morphology and geography, along with nucleotide divergence are consistent with the recognition of *O. gracillima* as a distinct species, but the common host suggests a potential, larger 'scarabaeidae' clade of *Ophiocordyceps*. The close relationship between these two species and between their hosts, combined with their geographic separation, is consistent with the 'host-

Table 2 – A morphological comparison of Ophiocordyceps species belongs to 'gracilis' subclade.

Species	Distribution	Host	Stromata	Fertil head	Ascomata	Asci (µm)	Reference
O. heteropoda	Japan, Australia	Nymphs of Hemiptera (Cicadidae)	Simple, solitary, 12 cm long	Ovoid, cinnamon buff, 7–9 × 6–7 mm	Immersed, ampullaceous, 610–660 × 210 µm, neck 160 µm, peridium 5–10 µm	Cylindrical, 250–300 × 5.2–7 µm Part-spores truncate 6–7.7 × 0.9–1 µm	Kobayasi (1941)
O. gracilis	Asia, Europe, America, Africa and Oceania	Larvae of Lepidoptera (Hepialidae, Nymphalidae)	Simple, usually solitary, 4–9 cm long	Globular ellipsoidal, red ochreous to pale orange, 4–9 × 4–7 mm	Immersed, elongate-oviform (320–)560–840 × 200–360 µm, peridium 20–40 µm	Cylindrical, (200–) 400–528 × 5–8 µm Part spores truncate 5–9 × 1.5–2 µm	Kobayasi (1941), Mains (1958), Samson & Brady (1983), Gorbunova et al. (2011)
O. gracillioides	Japan, China, Mexico, Colombia, Bolivia	Larvae of Coleoptera (Elateridae)	Simple, usually solitary, 2–5 (9) cm long	Spherical, pale rufous, 4–5.5 mm	Immersed, ellipsoidal to naviform, (680–)830–900 × 200–280 µm, peridium 50 µm	Cylindrical, 450–700 × 5–6.5 µm Part spores truncate 7–10(–12) × 1–2 µm	Kobayasi (1941), Fan et al. (2001), and this study.
O. amazonica	Argentina, Bolivia, Brazil, Colombia, Ecuador, Guyana, Peru	Imago and adults of Orthoptera (Romaleidae, Acrididae)	Simple, gregarious, 2–4.5 cm long	Subglobose, spherical, reddish brown, 2.5–5.5 mm	Immersed, ovoid-ellipsoid, (760–) 800–1100 × (220–) 250–400 µm, peridium 25 µm	Cylindrical, 325–450 × 5 µm Part spores truncate 9–15(–17) × (0.5–) 1.5–2 µm	Henning (1904), Mains (1959), and this study
O. blattarioides	Belize, Colombia, Ecuador	Adults of Blattaria (Dyctioptera)	Simple, gregarious, 1.4–2 long	Ovoid or subgloboid, chestnut brown, 2–3 × 1.5–2.5 mm	Immersed, ellipsoid, (650–)760–800 × 220–300 µm, peridium 15–25 µm	Cylindrical, (180–250 (–300) × 4–5 µm Part spores truncate 6–12 (–16) × 1.5 µm	Mains (1940), and this study

related' hypothesis of cospeciation with their hosts. However, sampling of additional isolates and species of both the fungi and hosts is necessary for a more definitive analysis. Further work is also necessary to determine the appropriate application of species names among members of this group, including examination of material collected from the type locality of *Ophiocordyceps melolonthae* by Tulasne in Boreal region of North America Boreal and both varieties mentioned by Mains (1958) *O. melolonthae* var. *melolonthae*, and *O. melolonthae* var. *rickii*.

A new species reveals a host-jumping event in the 'unilateralis' ant-pathogen subclade

In the '*unilateralis*' clade (Fig 1), the pathogens of ants include species that are characterized by the production of filiform stromata with aperithecial tips, asci that are more clavate than cylindrical, and ascospores that are more fusiform than filiform and that do not disarticulate into part-spores. Each species parasitizes one kind of ant with *Ophiocordyceps pulvinata*, *Ophiocordyceps halabalensis*, *Ophiocordyceps polyrhachis-furcata*, *Ophiocordyceps camponoti-leonardi*, *Ophiocordyceps camponoti-saundersi*, and *Ophiocordyceps unilateralis* parasitizing different species of *Camponotus* and *Polyrachis* (Formicidae: Formicinae) (Kepler et al. 2011; Kobmoo et al. 2012). Our phylogenetic analyses support the use of host-identity as criteria for species discrimination within this group (Fig 1). Therefore, we propose recognition of *Ophiocordyceps kniphofioides* varieties as an unique species; *O. kniphofiodes* is collected on adult *Cephalotes atratus* (Formicidae: Myrmicinae) and *Ophiocordyceps ponerinae* is collected on adults of *Paraponera clavata* and *Dinoponera longipes* (Formicidae: Ponerinae).

Despite its unique host affiliation, *Ophiocordyceps tiputini* is morphologically similar to species of the '*unilateralis*' clade (e.g., size and shape of its stroma; Fig 2J–L) and represents a host-shift from ants (Formicidae) to dobsonflies (Megaloptera) again to ants (Fig 1), within the ant-pathogen subclade. The ant *C. atratus* is an opportunist predator that nests in the canopy but is parasitized in the low level of the forest. Meanwhile *P. clavata* and *D. longipes* ants are generalist hunters that make their nests at the base of trees (Lattke 2003). *Megaloptera* larvae are generalist predators of small invertebrates. They live in freshwater streams with pupation occurring in soils immediate to streams (Grimaldi & Engel 2005). The specimen studied here was found in leaf litter of the floodplain or 'varzea' (Wittman et al. 2011) of the Tiputini River. The 'varzea' with a dense layer of wet leaf litter is an ecological niche shared by predaceous *Megaloptera* larvae (Contreras-Ramos 2004) and *Poneromorpha* ants (Keller 2011). We suggest that the host shifts of the '*unilateralis*' clade between *Megaloptera* and *Poneromorpha* occurred within a common habitat resulting in colonization of new host niches.

New cryptic species of Amazonian cicada pathogens in the "sobolifera" clade

The '*sobolifera*' clade comprises pathogens of cicada, including the best known and most frequently collected of these species of cicada pathogens, *Ophiocordyceps sobolifera*, also known in China as 'Chen chua' or 'flower of cicada'

(Kobayasi & Shimizu 1963). It produces a robust, fleshy, stipitate stroma with fertile clava. At the base of the fertile head are lateral, short branches, called soboles, which are the conidiogenous state of the fungus. *O. sobolifera* has been frequently recorded in China, Korea, Japan, and the Philippines as well as in the Caribbean islands, Florida, and Mexico (Petch 1942; Mains 1951). Also present in this clade is *Ophiocordyceps longissima*, another pathogen of cicadas; it is distinct from *O. sobolifera* in possessing a more elongated and cylindrical clava and typically in being attached to the host via a long tapering, rhizomorph-like stipe. A specimen identified as *O. sobolifera* was previously collected in Colombia (Kobayasi 1981) near the Caquetá River close to where our specimens (HUA 186135-136) were collected. The morphology of the Amazonian specimens studied here present some slight morphological differences with respect to *O. sobolifera*, including the shape of the fertile head, which is strictly cylindrical, and the measurement of the ascus, which is 100 μm smaller (Fig 3 A−C). Also, the perithecia of the Amazonian specimens have a pyriform shape with a short neck similar to *O. sobolifera*, rather than the ovoid perithecia of *O. longissima* (Fig 2 E).

Based upon these results, we describe *Ophiocordyceps araracuarensis* as a new cryptic species of cicada pathogen that was previously included in *O. sobolifera* based on the morphological species concept across a common host with a broad geographic distribution. Examples of cryptic species have been reported across fungi and their recognition has become common due to the increased use of molecular data and phylogenetic species concepts (e.g., Bickford *et al.* 2007; Le Gac *et al.* 2007; Bischoff *et al.* 2009; Rehner *et al.* 2011; Piątek *et al.* 2013; Sheedy *et al.* 2013; Sanjuan *et al.* 2014). Whether *O. sobolifera*, *O. longissima*, and *O. araracuarensis* occur on different subfamilies and genera of *Cicadidae* is difficult to ascertain owing to deterioration of host specimens. Dietrich (2005), however, affirmed that the diversity of cicadas in that region could be higher than in the rest of the world because of the diversity of arboreal plants on which they feed. An accurate assessment of the roles that biogeography and host association play in the diversification and speciation of this well identified hosts and a more intensive, global sampling.

New Amazonian species corroborate the adult host as an ecological characteristic of the 'sphecocephala' clade

The 'sphecocephala' clade is well supported (MLBS = 100; PP = 1.00) and is morphologically characterized by terminal fertile head with a peridium composed of well-defined pseudoparenchyma, perithecia that are mostly obliquely immersed, and filiform ascospores that disarticulate into fusiform part-spores. It is also ecologically unique in that the majority of species attack adult stages of insects from *Coleoptera*, *Hemiptera*, *Diptera*, and *Hymenoptera*. Numerous described species of Amazonian *Ophiocordyceps* were collected including *Ophiocordyceps dipterigena* on flies, and *Ophiocordyceps lloydii*, and *Ophiocordyceps australis* on ants. In addition to these taxa, two new species are described here: *Ophiocordyceps fulgoromorphila* is the first species reported as parasite of planthoppers in the *Fulgoromorpha*

(*Hemiptera*) (Fig 3 C−D) and is the first pathogen of *Fulgoridae* placed in the 'sphecocephala' clade. In addition to morphological characteristic of this clade, *O. fulgoromorphila* has the ability to produce secondary ramifications with new fertile heads after decay of the initial fertile head. *Ophiocordyceps fulgoromorphila* has yellow buff stromata and is resolved as a sibling species of *O. lloydii*. Combined morphological, ecological, and phylogenetic evidence support its recognition as a new species.

The other new species described in the 'sphecocephala' clade is *Ophiocordyceps evansii*. Initial observations of *O. evansii* led to its identification as an immature stage of *O. australis*, a parasite of the ant *Pachycondyla crassinoda* (Formicidae: Ponerinae). However, upon further examination of micromorphology and phylogenetic analyses, it is rejected as being conspecific with *O. australis*. With respect to some traits, *O. evansii* is morphologically more similar to *O. lloydii*, a parasite of *Camponotus* spp. ants (Formicidae: Formicinae), and *O. dipterigena*, a parasite of different dipteran families (Fig 2J−L). These species have narrowly ovoid perithecia that are longer than 900 μm and immersed perpendicularly to the surface at the top of the fertile 'head' (Fig 3I). But unlike the other species, the stroma of *O. evansii* has the appearance of a closed tulip flower and ellipsoid-fusiform part-spores greater than 10 μm (Fig 3B). In spite of the morphological similarities possessed by these species, they do not collectively form a monophyletic group within the 'sphecocephala' clade (Fig 1).

In conclusion, for *Ophiocordyceps*, as for many groups of fungi, morphological species concepts, when applied in absence of ecological and molecular data, are overly broad, resulting in the masking of evolutionarily unique taxa. Incorporation of new specimens of *Ophiocordyceps* from the neotropics within phylogenetic analyses revealed increased species diversity across all clades of *Ophiocordyceps*, supporting it as a diverse genus of neotropical entomopathogens. Moreover, the analysis revealed strong correlations between parasite and either host or habitat sufficient to distinguish morphologically cryptic phylogenetic species (e.g. new species in the 'unilateralis' subclade). Further investigation of this group will likely reveal many more undescribed species and parasite−host associations within the neotropics.

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