Unravelling the diversity behind the *Ophiocordyceps unilateralis* complex: Three new species of zombie-ant fungi from the Brazilian Amazon

João P. M. Araújo[a], Harry C. Evans[b], David M. Geiser[c] & David P. Hughes[d]

[a]Department of Biology, Penn State University, University Park, Pennsylvania, United States of America.

[b]CAB International, E-UK, Egham, Surrey, United Kingdom

[c]Department of Plant Pathology, Penn State University, University Park, Pennsylvania, United States of America.

[d]Department of Entomology and Department of Biology, Penn State University, University Park, Pennsylvania, United States of America.

Correspondence authors: joaofungo@gmail.com;

Abstract

In tropical forests, one of the most common relationships between parasites and insects is that between the fungus *Ophiocordyceps* (Ophiocordycipitaceae, Hypocreales, Ascomycota) and ants, especially within the tribe Camponotini. These fungi have the ability to penetrate the exoskeleton of the ant and to manipulate the behavior of the host, making it leave the nest and ascend understorey shrubs, to die biting onto the vegetation: hence, the term zombie-ant fungi to describe this behavioral changes on the host. It is posited that this behavioral change aids spore dispersal and thus increases the chances of infection. Despite their undoubted importance for ecosystem functioning, these fungal pathogens are still poorly documented, especially regarding their diversity, ecology and evolutionary relationships. Here, we describe three new and host-specific species of the genus *Ophiocordyceps* on *Camponotus* ants from the central Amazonian region of Brazil which can readily be separated using classic taxonomic criteria, in particular ascospore morphology. In addition, we also employed molecular techniques to show for the first time the phylogenetic relationships between these taxa and closely related species within the *Ophiocordyceps unilateralis* complex, as well as with other members of the family *Ophiocordycipitaceae*.

Introduction

In tropical forests, social insects (ants, bees, termites and wasps) are the most abundant land-dwelling arthropods. Although they represent only 2% of the nearly 900,000 known insect species on Earth, they are estimated to compose more than half the biomass (Fittkau & Klinge 1973; Höldobler & Wilson, 2009). One of the better known members within this group are the ants, which form a single family (Formicidae), with close to 13,000 species described (Agosti & Johnson 2009). Ants occupy a large range of habitats from high canopy to the leaf litter, forming huge colonies commonly comprising hundreds of thousands to millions of individuals.

Ants are susceptible to a wide variety of parasites. Amongst these, one group is particularly well adapted to live in tropical forests and to exploit the ant's abundance, the entomopathogenic fungus *Ophiocordyceps*. The genus currently comprises around 160 species (Robert et al. 2005; Sung et al. 2007), infecting many different insects with a wide range of ecologies – from solitary wandering beetles to highly-organized ant societies – in the Blattaria, Coleoptera, Dermaptera, Diptera, Hemiptera, Hymenoptera, Isoptera, Lepidoptera, Mantodea, Odonata and Orthoptera (Araújo & Hughes, in prep.). The functional morphology of *Ophiocordyceps* is equally diverse and has been linked to the host's ecology and biology (Evans et al. 2011).

Species of *Ophiocordyceps* were placed originally within *Cordyceps*, a genus established to accommodate fungal pathogens of arthropods bearing the sexual spore-producing structures on conspicuous stalks, arising from the host cadaver (Evans et al. 2011). However, due to the polyphyletic nature of *Cordyceps* – as evidenced by recent phylogenetic studies – species formerly assigned to the genus have now been reorganized into four genera (*Cordyceps*, *Elaphocordyceps*, *Metacordyceps* and *Ophiocordyceps*), within three families (*Cordycipitaceae*, *Clavicipitaceae* and *Ophiocordycipitaceae*) (Sung et al. 2007).

Within the Formicidae, *Ophiocordyceps* infections have been reported from the basal primitive groups (Ponerines) through to modern genera, such as *Camponotus* (Evans & Samson, 1982; Sanjuan et al. 2001; Evans et al. 2011), and often occur in epizootic proportions, killing large number of ants in small patches of forest (Andersen et al. 2009; Pontoppidan et al. 2009). Such events are pan-tropical with records from Asia, Australasia, Africa and the Americas (Evans 1974; Andrade 1980; Evans & Samson 1982, 1984; Evans 1988a, 2001; Kepler et al. 2011; Luangsa-ard et al. 2011; Kobmoo et al. 2012).

Entomopathogenic fungi infect their hosts following spore contact and subsequent germination on the cuticle – typically, an adhesive pad (appressorium) is formed – whereby, the germ tube penetrates the host's exoskeleton and, on reaching the haemocoel, the fungus proliferates as yeast-like cells (Evans 1988b). In the *O. unilateralis* complex, a series of synchronized events are triggered within the ant host in order to make it leave the colony, climb understorey shrubs to die in an elevated position – characteristically, biting the underside or edge of a leaf – and, under favorable environmental conditions, a spore-producing structure arises from the back of its head (Andersen et al. 2009). This phenomenon is called "extended

phenotype", where the parasite genes are expressed in the host phenotype, for the exclusive purpose of increasing parasite fitness (Dawkins 1982).

Despite their recent iconic status (Evans et al. 2011), the taxonomy and evolutionary relationships of this important group of pathogens remain unclear. For many years, it was suspected that *O. unilateralis* – originally described as *Torrubia unilateralis* (Tulasne & Tulasne 1865) – represents a complex of species, based on macro-morphological variation within collections worldwide (Petch 1931; Kobayasi 1941; Mains 1958; Samson et al. 1982; Evans & Samson 1984). However, it was not until recently that species delimitation was proposed formally, when four new taxa were described from the Minas Gerais state of Brazil (Evans et al. 2011), and it was posited that each ant host within the Camponotini would have a different species of *Ophiocordyceps*. Subsequent descriptions of new taxa, from both Thailand and Japan on *Camponotus* and *Polyrhachis* hosts, are lending support to this hypothesis (Luangsa-ard et al. 2011; Kepler et al. 2011; Kobmoo et al. 2012), with the clear indication that there are still many species to be discovered.

During field surveys in the central Brazilian Amazon, we collected a range of *Camponotus* species killed by *Ophiocordyceps*, often in large numbers. Based on macro-morphological characters, all the specimens fell within *O. unilateralis sensu lato*: typically, comprising a stalk (stroma) arising from the dorsal neck (pronotum) with an asexual morph (*Hirsutella*) occupying the terminal region and the sexual morph (ascoma) occurring as lateral cushions or plates. Here, we describe and characterize three new species, which can readily be separated using traditional micro-morphology (Kobayasi 1941; Evans et al. 2011), as well as molecular phylogeny.

Materials and Methods

Sampling

Surveys were undertaken in the central Amazonian region of Brazil concentrating on four forest reserves (Figure 1): Reserva Adolpho Ducke, ca. 10,000 ha (02°55'S, 59°59'W), adjacent to Manaus (Amazonas state) and composed of terra-firme forest, with plateaus, lowlands and campinarana vegetation, characterized to occur in patches of sandy soil across the Rio Negro basin. Parque Nacional de Anavilhanas, an archipelago (2°23'S, 60°55'W) of more than 400 islands, 40 km up the Rio Negro from Manaus, colonised by Igapó forest (varzea), which is characterized by water-soaked soils with high acidity due to seasonal flooding. Parque Nacional do Viruá, ca. 227,000 ha (01°42' N, 61°10' W), located near Caracaraí city (Roraima state), based on sandy soils with many lagoons and lowland Amazonian forest. Estação Ecológica de Maracá, ca. 104,000 ha (3°22'N, 61°27'W), located 135 km from Boa Vista on an island of the Uraricoera River in Roraima state, presenting both savanna and a mix of humid lowlands and plateaus (terra firme).

Sampling protocol consisted of a careful inspection of soil, leaf litter, shrub leaves and tree trunks, up to *ca.* 2 m high. Infected ants – and the substrata they were attached to – were collected in plastic containers for transport to the laboratory and, wherever possible, examined the same day. During longer surveys,

the samples were air-dried overnight to prevent mold growth. All specimens were photographed individually, using a Canon 60D camera fitted with a MP-E 65mm (x5) macro lens, equipped with a MT-24EX macro lite flash.

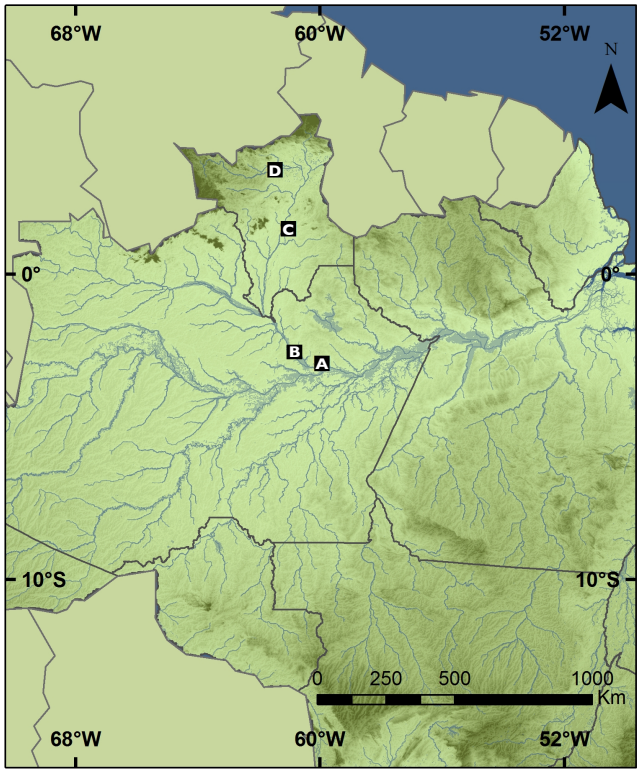

Figure 1: Map showing the forest reserves sampled in the central Brazilian Amazon: A) Reserva Adolpho Ducke; B) Parque Nacional de Anavilhanas; C) Parque Nacional do Viruá; D) Estação Ecologica de Maracá.

Morphological studies

Samples were screened using a stereoscopic microscope, and only mature fungal specimens were selected for further micro-morphological studies. In order to obtain ascospores, infected ants were attached to the lid of a plastic Petri dish using petroleum jelly, and suspended above a plate containing either distilled-water agar (DWA) or potato dextrose-agar (PDA). Plates containing the ants attached were maintained outside the lab at natural temperature and examined daily for the presence of ascospores, which, after ejection from the ascomata, formed sub-hyaline halos on the agar surface. Freshly-deposited ascospores were removed with a sterile hypodermic needle, with the aid of a stereoscopic microscope, and mounted on a slide in lacto-fuchsin (0.1g of acid fuchsin in 100 ml of lactic acid) for light microscopy (Olympus BX61). A minimum of 50 naturally-released ascospores were measured for morphological comparison (Table 1). The remaining ascospores were left *in situ* on the agar surface and examined over a number of days in order to

monitor germination events. For micro-morphology of the ascomata, either free-hand or cryo-sectioning (Leica CM1950 Cryostat) was used.

Permanent slides were deposited in the Entomological Collection at INPA (Instituto Nacional de Pesquisas da Amazônia), with isotype collections held in ???. Permits for collecting and export were provided by INPA to DPH (PUT THE AUTHORIZATION #).

DNA extraction, PCR, sequencing and phylogenetic analyses

DNA extractions were made using a tiny piece of the ascoma. The tissue was placed with two metal balls (1/8") in a 2 ml Eppendorf tube, frozen in liquid nitrogen and broken into powder using Tissuelyzer (Qiagen) for 1 min. Afterwards, with the fungal tissue still frozen, 0.8 ml of DNA extraction buffer (1% SDS, 0.024 g/ml PAS, 0.2 ml/ml RNB, Distilled water) and 0.8 ml phenol-chloroform was added, incubated at 60˚C for 10 min and centrifuged for 10 min at 10,000 rpm. The water phase was transferred to a new 2 ml tube (about 0.8 ml), added 0.5 ml of chloroform, mixed by inverting and centrifuged for 10 min at 10,000 rpm. The supernatant was transferred to a new tube, added 80% of the volume of isopropanol, mixed by inverting and centrifuged for 10 min at max speed (14,000 rpm). The supernatant was removed and the pellet formed at the bottom washed with 0.5 ml of 70% ethanol 200 proof pure ethanol (Koptec), centrifuged for 5 min at 14,000 rpm, discarding the supernatant afterwards. The tube with the pellet was air-dried and dissolved in 30 µl of TE buffer.

Approximately 1,030 bp of nu-SSU, 770 bp of nu-LSU and 550 bp of ITS were amplified by PCR. The nu-SSU amplification was performed using two overlapping sets of primers, NS1/ SR7 (R. Vilgalys, 5'-GTTCAACTACGAGCTTTTTAA-3') and NS4/ NS3 (White et al. 1990). The nu-LSU amplification was amplified with the primers LR5/LR0R (Vilgalys and Sun, 1994) and for ITS amplification, ITS1F (Gardes & Bruns, 1993) and ITS4 (White et al. 1990) were used. PCR was performed in a T-3000 Thermocycler (Biometra GmbH, Göttingen, Germany) with the following mix: 2.5 µl PCR buffer, 0.5 µl dNTP, 1.5 MgCl, 0.5 each primer, 1 µl template, O.1 Invitrogen Taq Platinum Polymerase and 18.4 µl Gibco UltraPure Distilled Water. PCR products were cleaned using the Qiagen PCR Purification Kit following the manufacturer's instructions and sequenced using the Genomics Core Facility service at Penn State University.

Sequences were manually edited using Sequencher version 4.7 (Gene Codes Corp., Ann Arbor, MI, USA). Sequences generated for this study were compared with close related species sequences available on Genbank. Herbarium numbers and Genbank accession numbers are provided in table 1. The alignment was obtained by MUSCLE algorithms using Mega 5 (Kumar et al. 2012) and gaps were treated as missing data. Neighbor-joining analyses were conducted using Mega 5 (Kumar et al. 2012) with 1,000 bootstrap replicates on a concatenated dataset containing all three genes.

RESULTS

Ophiocordyceps atriceps Araújo, H.C. Evans & D.P. Hughes sp. nov.

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Type: Brazil. Amazonas: Reserva Adolpho Ducke, 100 m, 10 Jan 2014, *Araújo* & H.C. Evans, A-35, on *Camponotus atriceps* Smith (holotype INPA #; isotype INPA #, FROST #).

External mycelium abundant, covering most of the host, produced from all orifices and sutures; initially white, turning light-brown. Stromata single, produced from dorsal pronotum, averaging 15-20 mm, up to 25 mm in length, cylindrical, velvety and ginger brown at the base, becoming cream-pinkish towards apex; fertile region of lateral cushions, 1-2, hemispherical, chocolate brown, darkening with age, variable in size, averaging 1.5 x 0.5-0.8 mm. Perithecia immersed to partially erumpent, flask-shaped, 240-280 x 100-150 µm, with short, exposed neck or ostiole. Asci 8-spored, hyaline, cylindrical to clavate, 110-140 x (4.5-) 6-6.5 (-8) µm; prominent cap, (3.5-) 5 x 5.5 (-6.5) µm. Ascospores hyaline, thin-walled, vermiform (75-) 80-85 (-100) x (2-) 3 (-3.5) µm, 5 septate, sinuous to curved, never straight at maturity, rounded to acute apex.

Asexual morph. *Hirsutella*-A type only, produced on the upper stromatal surface; phialides cylindrical to lageniform, 5-7 x 2-3 µm, tapering to a long neck, 5-11 µm; conidia not seen. This asexual morph occurs in all the species included here and is not considered to be critical for species separation. Hence, it is not analyzed in detail.

Additional specimens examined:

Germination process

The released ascospores germinated within 24-48 h, producing 1-2, uniformly straight, thread-like structures (capillicondiophores); typically, of uniform length and averaging 55 µm; bearing a single terminal spore (capilliconidiospore), hyaline, smooth-walled, curved and fusarium-like in shape at maturity, 10-11 x 2-2.5 µm, narrowing apically.

Ecology

Hosts commonly found biting the apical part of palm-leaves, occasionally, on dicot leaves, rarely on palm-spines: abundant mycelial growth from the mouthparts helping to stick the host to the leaf, in addition to the locked jaws.

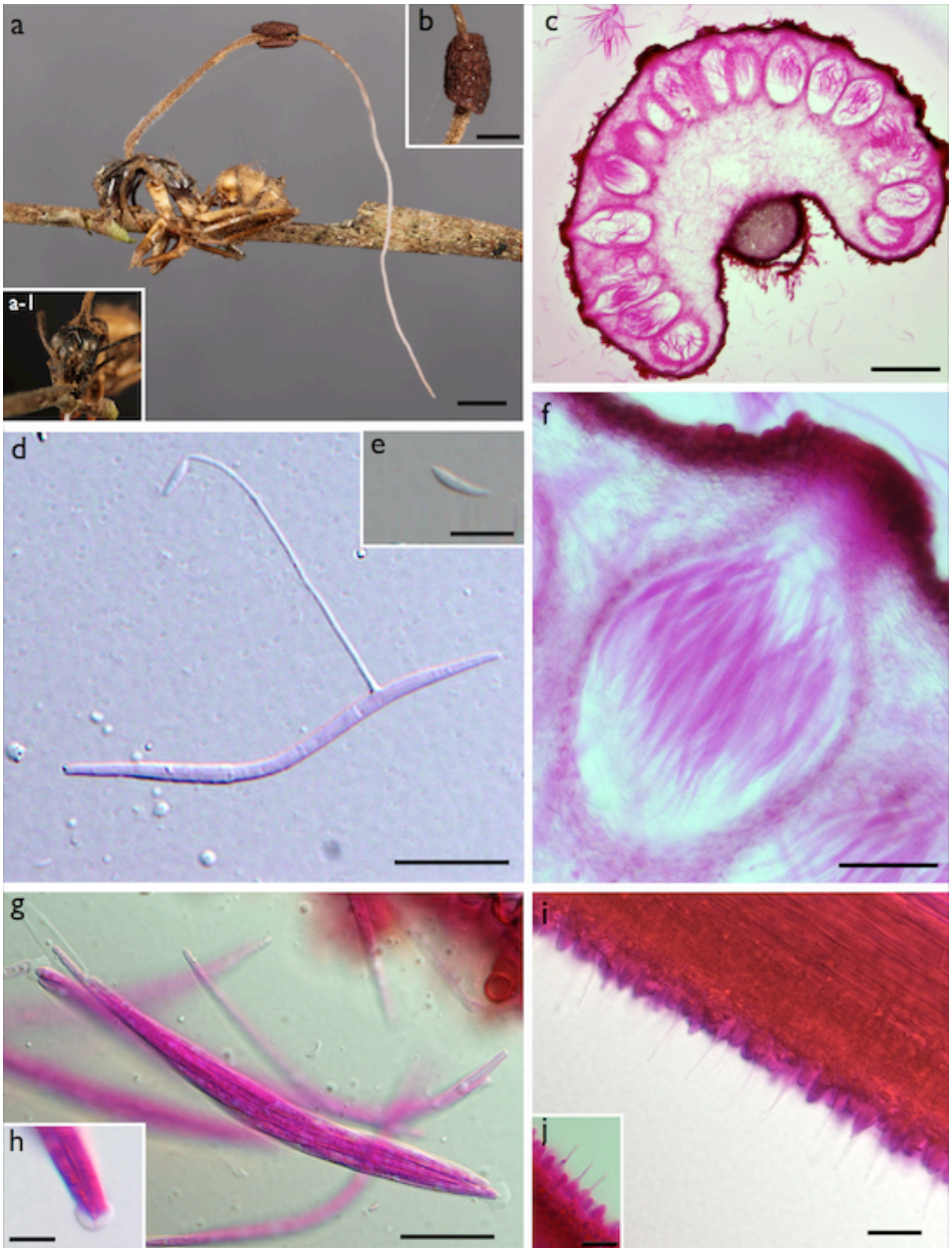

Figure 2. *Ophiocordyceps* atriceps a) Single stroma, characteristic of *Ophiocordyceps unilateralis sensu lato*, with a single lateral ascoma, arising from dorsal pronotum of *Camponotus atriceps*, firmly attached to a palm leaf (bar = 1.5 mm); **a-1**) ant displaying a behavior manipulation of biting the leaf; **b)** Detail of fertile region (ascoma) (bar = 0.8 mm); **c)** Section through ascoma showing the mainly immersed perithecial arrangement (bar = 200 µm); d) Ascospore with a needle-like outgrowth (capilliconidiophore) producing terminal conidium (bar = 20 µm); e) Close-up of conidium (bar = 10 µm); f) Close-up of perithecium (bar = 50 µm); g) Ascus, clavate in shape and with a prominent cap (**arrow?**, bar = 20 µm); h) detail of the ascal cap (bar = 5 µm); i) Section of upper part of stroma showing asexual morph (*Hirsutella*-A type), with a palisade of subulate phialides (bar = 10 µm); j) Close up (bar = 10 µm).

Ophiocordyceps bidens Araújo, H.C. Evans & D.P. Hughes sp. nov.

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Type: Brazil. Amazonas: Reserva Adolpho Ducke, 100m, 15 Jan 2014, Araújo & H. C. Evans, B-66, on *Camponotus bidens* Mayr (holotype: INPA #; isotype FROST #).

Mycelium dark-brown to black; forming aggregations of hyphae on the intersegmental membranes. Stromata single, produced from dorsal pronotum, averaging 5-7 mm in length; cylindrical, black, with a cream-white swollen terminal part; fertile ascomatal region lateral, forming a single globose cushion, initially dark brown becoming black with age, averaging 0.8 x 0.4-0.7 mm. Perithecia immersed to slightly erumpent, globose to flask-shaped, 250-290 x 150-170 µm, with a short neck. Asci 8-spored, hyaline, cylindrical to clavate, (90-) 110-130 x (7-) 8-8.5 (-9.5) µm; with a small but prominent cap, 3.5 x 4.5 µm. Ascospores hyaline, thin-walled, cylindrical, (60-) 70-75 (-80) x 4.5-5 (-6), round and slightly narrow at the apices.

Asexual morph. *Hirsutella*-A type only, produced at the swollen upper part of stroma; phialides cylindrical to lageniform, 6 x 2.5-3µm, tapering to a long hair-like neck, 10-16 µm length; conidia narrow limoniform, averaging 6-7 x 2 µm, with a distinct tail.

Additional specimens examined:

Germination process

Spores germinated 48-72 h after release, consistently producing a single, straight, robust capilliconidiophore, (50-) 65 (-80) µm, bearing a single terminal capilliconidiospore, 10-11 x 3-4 µm, hyaline, smooth-walled, slightly truncate at the base.

Ecology

Often biting palm-tree parts, on spines or tip-edge of the leaf. Mycelium growing from mouth, attaching the ant to the substrate.

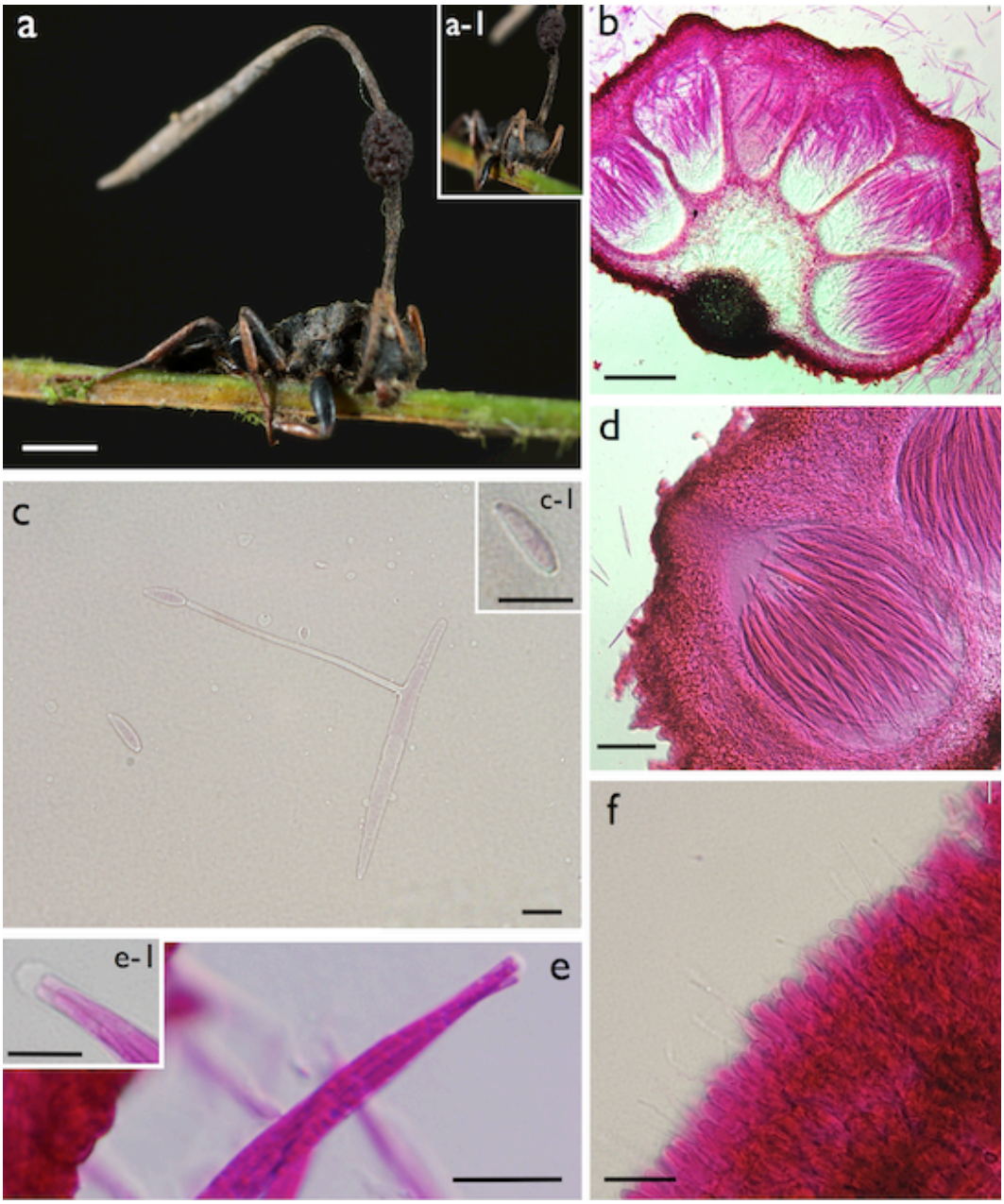

Figure 3. ***Ophiocordyceps bidens*** **a)** Infected *Camponotus bidens* biting into tip of palm leaf **a-1)** Detail showing biting behavior and ascoma; **b)** Section through ascoma showing perithecial arrangement (bar = 100 µm); c) Ascospore after 48 h germinating with a single capilliconidiophore with a capilliconidium at the tip (bar = 10 µm); c-1) Detail of the conidiospore (bar = 10 µm); d) Section of perithecium showing the arrangement of asci (bar = 50 µm); e) Ascus showing the ascospore arrangement NEED TO CHANGE THIS PICTURE (bar = 20 µm); e-1) Detail showing the prominent ascus cap (bar = 10 µm); f) Section of the swollen stromatal tip showing the asexual morph *(Hirsutella*-A)(bar = 10 µm).

Ophiocordyceps viruensis Araújo, H.C. Evans & D.P. Hughes sp. nov.

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Type: Brazil. Amazonas: Parque Nacional do Viruá, 200m, 15 Jan 2012, Araújo & F. B. Baccaro, V-25, on *Camponotus sp.* (holotype: INPA #; isotype FROST #).

Type: Brazil. Roraima: Parque Nacional do Viruá, 9 Feb 2012, V-15, on *Camponotus* sp.

Mycelium aggregations arising from all inter-segmental membranes (sutures), ginger in color. Stromata multiple, arising from dorsal, right and left sides of pronotum, and leg joints, (1.5-) 8-10 (16) x 0.3-0.4 (-1) mm, ginger at the base becoming purplish-cream towards the apex. Ascomata produced only on the pronotal stromata, never from those on legs; lateral cushions, 1-4, hemispherical, chocolate to dark brown with age. Perithecia immersed to semi-erumpent, ovoid to flask-shaped, 230-310 x 120-175 µm, with a short, exposed neck. Asci 8-spored, hyaline, cylindrical (135-) 170 (-190) x 8.5 (-11.5) µm; cap prominent, 4.5 x 5 µm; Ascospores hyaline, thin-walled, cylindrical, (60-) 75 (-80) x (3.5-) 4.5 (-5), occasionally swollen, narrowing to acute tips at both ends.

Asexual morph. *Hirsutella*-A type associated with the apical region of all stromata; phialides cylindrical to lageniform, 7.5 (-9.5) x 3.5 µm, tapering to a long hair-like neck, 6.5-11 µm in length, conidia not seen. *Hirsutella*-C type (= *H. sporodochialis*-type) produced from ginger cushions (sporodochia) on legs and antennal joints: phialides hyaline and subulate at the base, robust, XXX x XXX µm; no conidia observed.

Germination process

Ascospores germinated 48 h after release; typically, producing 2, rarely 3, hair-like capilliconidiophores, 120-150? µm in length; bearing a single terminal conidium, biguttulate, fusoid, narrowing apically.

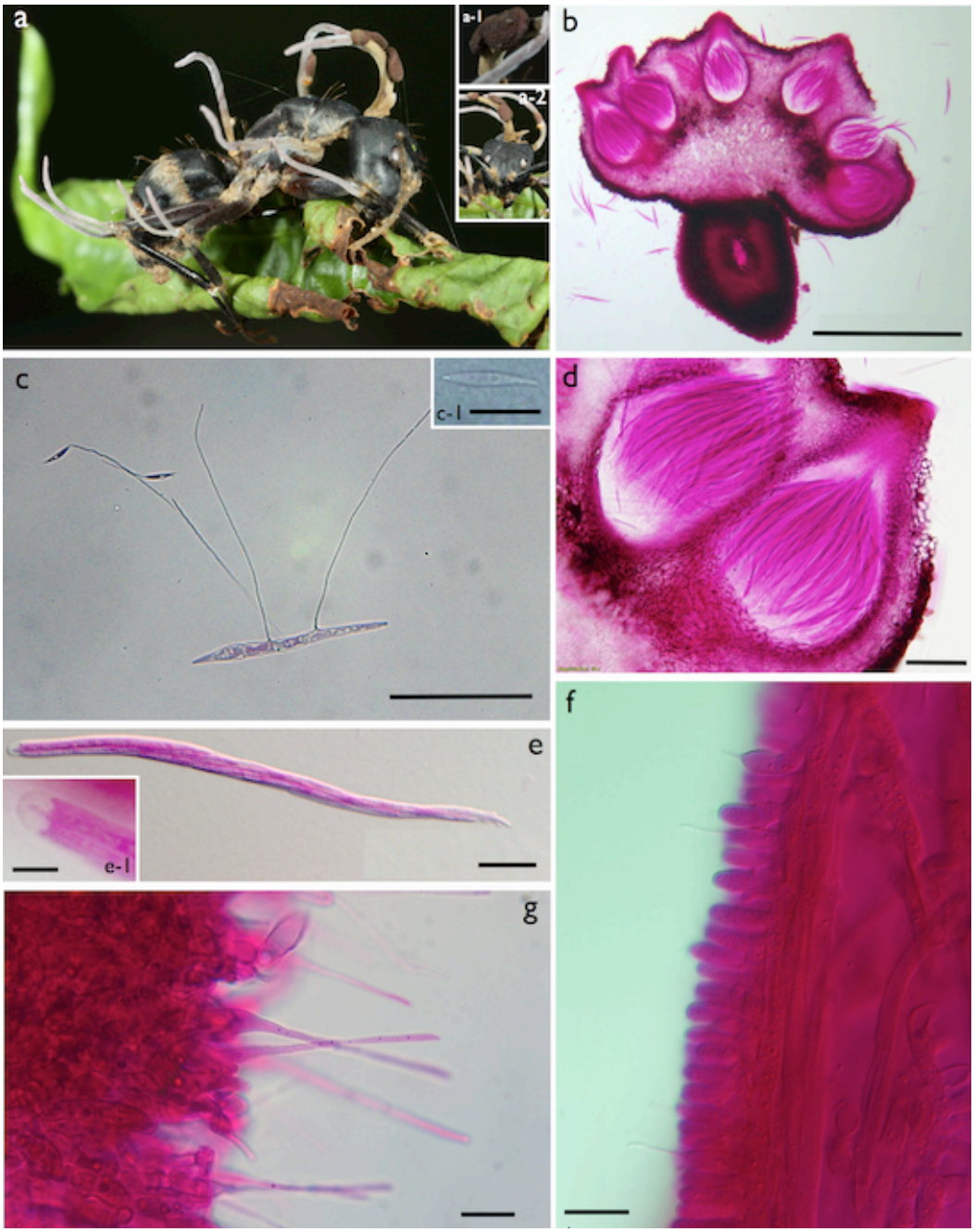

Figure 4. ***Ophiocordyceps camponoti-viruensi*** **a)** *Camponotus* sp. biting into a leaf, several stromata arising from dorsal pronotum, mesonotum and leg joints, with a characteristic purplish coloration (bar = ???); **a-1)** fertile cushion (ascoma) (bar = ???); **a-2)** Close up of ant's head showing the biting behavior (bar = ???); **b)** Section through ascoma showing perithecial arrangement (bar = 500 µm); c) Ascospore after 24 h, with very long capilliconidiophores (1-3) with capilliconidia at the tip (bar = 50 µm); c-1) Detail of capilliconidia MENTION THE SHAPE (bar = 10 µm); d) Close up of perithecia showing asci arrangement and the semi-erumpent ostiole (bar = 50 µm); e) Ascus showing the spiral arrangement of ascospores (bar = 20 µm); e-1) Ascus cap detail (bar = 5 µm); f) Section of upper part of stroma showing asexual morph (*Hirsutella*-A type), with long-necked phialides (bar = 10 µm); g) Phialides formed as mycelial cushions (sporodochia) on leg joints and MENTION THE PLACE WHERE ANTENNA ARE INSERTED (Hirsutella C-type) (bar = 10 µm).

		Macromorphology			Ascospore		Hirsutella type		
Fungus	Host	Stroma length (mm)	Fertile part (ascoma) (mm)	Capilliconidia	Capiliconidiophore (µm)	Ascospore size	A	B	C
O. atriceps	*C. bidens*	5-7.5	0.5-0.85 x 0.45-0.7	1	(50-) 65 (-80)	70-80 x 4.5-5	+	-	-
O. bidens	*Camponotus sp.*	8-10 (-16)	1.2 x 1	1-3	(115-) 120-125 (-130)	70-80 x 4.5	+	-	+
O. viruensis	*C. atricep*	10-20	1.5 x 0.75	1-2	(50-) 55 (-60)	80-100 x 3	+	-	-
O. camponoti-rufipedis	*C. rufipes*	8-10 (-15)	1 x 0.5	1-3	(45-) 60-70 (-80)	80-95 - 2-3	+	-	-
O. camponoti-balzani	*C. balzani*	8-10 (-15)	1.5 x 1	-	-	135-175 x 4-5	+	-	+
O. camponoti-melanotici	*C. melanoticus*	8-10 (-15)	1.3 x 0.8-1	-	-	170-210 x 4-5	+	-	-
O. camponoti-novogranadensis	*C. novogranadensis*	8-10 (-15)	0.8-1 x 0.5-0.6	1-4	20-25	75-95 x 2.5-3.5	+	+	-
O. halabalaensis	*C. gigas*	6.5-18	1.5 x 2	-	10-20	60-75 x 3-5	+	-	-

Table 2: Comparison of morphological characters for close related species attacking ants. Asterisks mean species described in this study. (Adapted from Evans et al. 2011; Luangsa-ard et al. 2011).

Phylogenetic Relationships

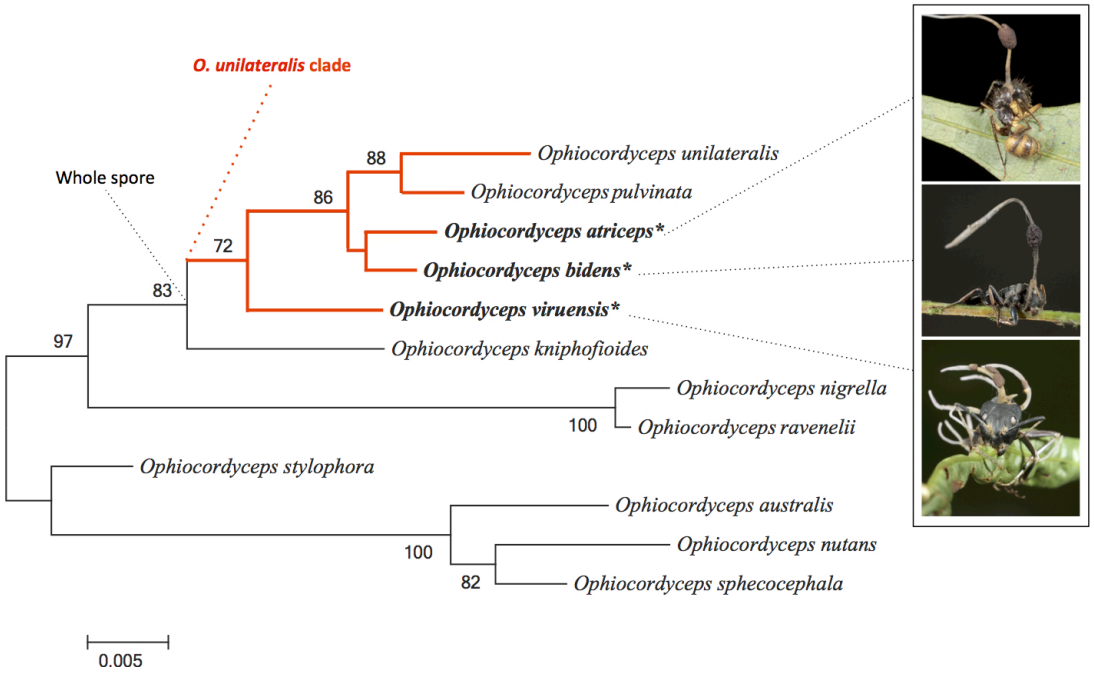

Figure 5. Neighbor-joining tree obtained from a concatenated dataset of three genes (nu-SSU, nu-LSU, ITS) showing the placement of *O. atriceps*, *O. bidens* and *O. viruensis* within *Ophiocordyceps unilateralis* complex and relative to other Ophiocordycipitaceae species. Numbers above branches indicate bootstrap scores >70.

Discussion

Here we describe three new species from Brazilian Amazon, which differ from all species described so far. We used morphology and genetic approaches, adding them to the 4 species previously described from Brazil (Evans *et al*. 2011). Our results confirm that it is justifiable to split *O. unilateralis s.l.* species into many more, unraveling a huge reservoir of undocumented species.

Ophiocordyceps unilateralis was originally described by Tulasne & Tulasne (1865) as *Torrubia unilateralis*. Eighteen years later, Saccardo (1883) transferred *T. unilateralis* to the genus *Cordyceps*, remaining this way until Petch (1931), which erects the genus *Ophiocordyceps*, and propose that four *Cordyceps* species (*C. blattae* Petch, *C. unilateralis* (Tul.), *C. peltata* (Wakef.) and *C. rhizoidea* (v. H.)) to be transferred to the new genus. Petch (1931) used microscopic characters such as clavate asci, hyaline and multi-septate spore, which do not disarticulate into partspores. More recently, with the advent of molecular techniques, Sung *et al*. (2007) confirmed the long suspicious that *Cordyceps* is a polyphyletic genus, thus rearranging the genus in four different ones: *Cordyceps* s.s., *Metacordyceps*, *Elaphocordyceps* and *Ophiocordyceps*.

Many *Ophiocordyceps* species has been recognized as important pathogens of ants, especially in tropical forests, with many species described so far. Some of them die on the ground among leaf litter (i.e. *O. myrmecophila* Ces., *O. australis* s.l. Speg., *O. irangiensis* Moureau), others biting twigs and leaves (i.e. *O. pulvinata* Kepler, Kaitsu & Spatafora, *O. camponoti-balzani* H. C. Evans & D. P. Hughes, *O. camponoti-rufipedis* H.C. Evans, *O. halabalaensis* Luangsa-ard, Ridkaew, Tasan. & Hywe-Jones), others dying on the base of trunks (i.e. *O. kniphofioides* var. dolichoderi H. C. Evans & Samson) and others are found attached to the leaves by mycelium growth (*O. lloydii* var. lloydii H. S. Fawk. and *O. lloydii* var. binata H. C. Evans and Samson).

Ophiocordyceps atriceps, *O. bidens* and *O. viruensis* shared common traits such as ant as the host, a stroma arising from the pronotum (dorsal neck region), bearing commonly one or more brown to blackish plates or cushions attached laterally. They were readily identified in the field as *Ophiocordyceps unilateralis* s.l., due these macro-morphological traits, which are exclusively shared by members of this complex of species. Further morphological analyses in the lab revealed clear differences between our collections and previous species belonging to *O. unilateralis* species complex. The spores (and its behavior) and host association were the most evident characteristics to distinguish them from other species, although other features such as ascoma, perithecia, asci, anamorphic morphology and other minor features were also used for comparison and further separation from species within the *O. unilateralis* complex (figures 1-3; table 1).

ECOLOGICAL DETAILS

The species described in the present study are currently known only from Brazilian amazon. The infected dead ants were collected biting mostly onto leaves, epiphytes and palm spines, occasionally on slender stems or lianas. We observed that early in the morning, even without rain, droplets of dew were formed at the very tip of the palm leaf, providing a daily supply of water for the fungus development. Thus, dying on this specific kind of leaf and position may be an advantage for the proper and faster fungus development. The same death position and substrate was also described for *O. halabalaensis* in Thailand (Luangsa-ard *et al*. 2011). The ants were found always in epizootic events, dying in clusters of dozens to up to hundreds. *O. atriceps* and *O. bidens* are commonly found by hundreds in all the four areas visited for this study and other areas previously visited across the Brazilian Amazon. In contrast, 19 *O. viruensis* were collected exclusively at Parque Nacional do Viruá only once, in a single small area with about 15m^2. Further expeditions will be addressed to locate more samples in order to better understand their morphological variation and ecological traits.

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