Unravelling the diversity behind *Ophiocordyceps unilateralis* complex: Three new species of Zombie-Ant fungus from Brazilian Amazon

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Abstract

In Tropical forests, one of the most common relationships between parasites and insects occur between the parasitic fungus *Ophiocordyceps* (Ophiocordycipitaceae, Hypocreales, Ascomycota) and ants, especially attacking species within the tribe Camponotini. These fungus have the ability to penetrate through the host's cuticle and manipulate their behavior, making them leave their nests to die biting onto an elevated leaf, in order to facilitate the fungus development and further spore discharge. Despite their importance for the ecosystem maintenance, this group of fungal pathogens is still poorly documented regarding their diversity, ecology and evolutionary relationships. The three species documented here could be readily separate based in macroscopic features such as ascospores, external morphology and hosts. In addition, we provide the phylogenetic relationships between them, close related species and members of the Ophiocordycipitaceae family.

Introduction

In tropical forests, social insects (bees, wasps, ants and termites) are the most abundant land-dwelling arthropods. Although they represent only 2% of the nearly 900,000 known insects species on Earth, they likely compose more than half the biomass (Höldobler & Wilson, 2009; Fittkau & Klinge 1973). One of the most

well known members within this group are the ants, which comprises a single family (Formicidae) with close to 13,000 species described (Agosti & Johnson 2009). They occupy a large range of habitats from high canopy to the leaf litter, forming huge colonies commonly presenting hundreds of thousands to millions of members.

Ants are susceptible to many parasite and pathogens. Among these parasites one group specifically is also very well adapted to live in tropical forests and exploit the insects abundance, the entomopathogenic fungus *Ophiocordyceps*. The genus currently comprises around 160 species (Sung et al. 2007; Robert et al. 2005) infecting many different groups of insects, from solitary wandering beetles to members of the well-organized ant societies.

These parasites are known to infect many groups of insects presenting a wide range of ecologies: Odonata, Dermaptera, Orthoptera, Blattaria, Isoptera, Mantodea, Hemiptera, Coleoptera, Hymenoptera, Diptera and Lepidoptera (Araújo & Hughes, in prep.). The functional morphology of *Ophiocordyceps* is also diverse and was suggested to be related to the host's ecology and biology (Evans et al 2011).

Species within *Ophiocordyceps* were originally placed among the genus *Cordyceps* (Cordycipitaceae - Hypocreales – Ascomycota), a genus established to accommodate fungal pathogens of arthropods bearing sexual spore producing structures elevated by conspicuous stalk, which arises from host cadaver (Evans et al. 2011). However, due to the polyphyletic nature of *Cordyceps*, species formerly allocated to the genus were reorganized into four genera (*Cordyceps*, *Metacordyceps*, *Elaphocordyceps*, and *Ophiocordyceps*) in three families (Cordycipitaceae, Clavicipitaceae and Ophiocordycipitaceae) (Sung et al. 2007).

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As an ant pathogen, some species of *Ophiocordyceps* are known to infect from basal groups like Ponerines, i.e. *O. australis* and *O. kniphofioides* var. *ponerinarum*, to modern genera like *Camponotus*, i.e. *O. unilateralis* s.l. (Evans & Samson, 1982; Sanjuan et al. 2001; Evans et al. 2011). These infections occur frequently as an epizootic phenomenon killing a large number of ants in small patches of forest (Pontoppidan et al. 2009; Andersen et al. 2009). Such event is not restricted to a single region but presents a pan-tropical distribution with records from many different countries like Thailand, Philippines, Japan, Australia, China, Ghana, Costa Rica, Ecuador, Peru, Brazil and others (Evans & Samson 1982, 1984; Kepler et al. 2011, Luangsa-ard 2011).

The most sophisticated example occurs in infections caused by members of *O. unilateralis* complex infecting the sister groups of ants *Camponotus* and *Polyrhachis* (Kobmoo et al. 2012). These fungi infect their hosts by spore contact, which penetrate the host's cuticle and proliferate as yeast-like cells. After that, a series of synchronized events are triggered within the host in order to make it leave the colony to die in an elevated position, biting the underside or edge of the leaf, further growing a spore-producing structure 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 to increase parasite fitness (Dawkins, 1982).

However, the taxonomy of this important group of pathogens remains unclear. It is known that *O. unilateralis*, originally described as *Torrubia unilateralis* (Tulasne & Tulasne 1865) is a complex of multiple species based in years of collections worldwide (Petch 1931; Kobayasi 1941; Mains 1958; Evans & Samson 1984; Evans et al. 2011; Luangsa ard 2011). Especially after Evans et al. (2011), it was clear that there are still many species to be discovered and described within the complex *O. unilateralis* that was overlooked by decades.

Throughout field collections in four Reserves and National Parks along the Brazilian Amazon, we found an impressive number of ants killed by *Ophiocordyceps*. Based on macromorphology characters we could place the species we found among *Ophiocordyceps unilateralis*, which are a stalk (stromata) arising from the back of the ant's head presenting an anamorph hymenium occupying the terminal region and the sexual spore-producing structure (ascomata) occurring as lateral cushions or plates. The three different species presented here were readily separate based in traditional micromorphology used for the taxonomy of the group (Kobayasi 1941; Evans et al 2011).

Materials and Methods

Sampling

Collections of specimens were held along Brazilian Amazon in four different reserves (figure 4): Reserva Adolpho Ducke, a forest reserve of around 10.000 ha (02°55'S, 59°59'W), being composed by terra-firme forest, with plateaus, lowlands and campinarana vegetation. Estação Ecológica de Maracá is a reserve of around 104.000 ha (3°22'N, 61°27'W), located 135 km from Boa Vista, located in an island of the Uraricoera river ar Roraima state, presenting savanna and amazonic forests. Viruá National Park present more than 227.00 ha (01°42' N, 61°10' W), located at Caracaraí city, presenting sandy soil, many lagoons and also amazonic forest with high variation of around 100 m. The Anavilhanas National Park is an archipelago (2°23'S, 60°55'W) comprising more than 400 islands, 40 km up to Rio Negro from Manaus, presenting Igapó forest, which is characterized by soaked soils with high acidity. The islands vegetation physiognomy varies to each island, according to its size. The biggest islands present high canopy with approximately 25 m high with some emergent ones above this limit, the smallest present plants with lower size, which area subject to the flood season.

Samplings were performed by a careful inspection of soil, leaf litter, shrubs, leaves and tree trunks up to *ca.* 2 m high and infected ants and the substratum they were attached collected and transferred to plastic boxes for further examination. All developmental stages were collected, but just mature ones were used for morphological examination. When possible, the macroscopic features were recorded at the same day the materials were collected. To record the samples, all

specimens were individually photographed using camera Canon 60D coupled with MP-E 65mm 5X Macro lens equipped with MT-24EX Macro Lite Flash.

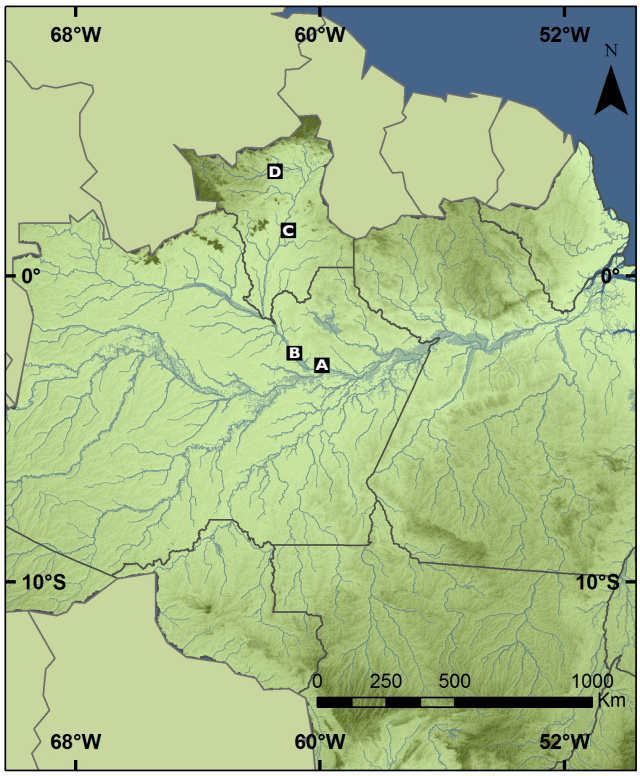

Figure 1: Map showing the sampled areas: A) Adolpho Ducke Reserve; B) Anavilhanas National Park; C) National Park of Viruá; D) Maracá Ecological Station.

Morphological studies

To obtain ascospores, the infected ants were attached to a plastic Petri dish lid and suspended above a dish containing either Water-Agar or PDA (Potato-dextrose-agar). These were observed daily for the presence of ascospores on media, which is easily recognizable by a cloudy mass on the media. The ascospores when released were transferred to microscopy slides, which were mounted in Lacto-fuchsin (0.1g of acid fuchsin in 100 ml of lactic acid). In the laboratory, either free-hand or by cryosectioning (Leica CM1950 Cryostat) of ascoma and stroma were made and mounted in slides for micro morphological examination using Olympus BX61 light microscope. At least, 50 naturally released ascospores were measured for morphological comparison (table 1).

Specimens with permanent slides containing morphological features used in this study were deposited in Entomological collection at INPA (Instituto Nacional de Pesquisas da Amazônia) with isotype collections held in ???. Permits for export and collecting were provided by INPA to DPH (PUT THE AUTHORIZATION #).

RESULTS

The species names are as *Ophiocordyceps* sp.1, sp.2 and sp.3 because this is not an official publication and new species names should be used just after that.

***Ophiocordyceps* sp.1** JPM Araújo, HC Evans & D.P. Hughes sp. nov.

IF XXXXXXX

Type: Brazil. Amazonas: Adolpho Ducke Reserve, 10 Jan. 2014, A35, on *Camponotus atriceps* Smith 1858.

External mycelium covering most of the host, produced form all orifices and sutures; initially white, turning into light-brown. Stromata single, produced form dorsal pronotum, commonly 15-20 mm, up to 25 mm in length, cylindrical, velvety, ginger brown at the base, becoming cream-pinkish at terminal part; 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 X-spores, hyaline, cylindrical to clavate, 110-140x(4.5-) 6-6.5 (-8) µm; prominent cap, (3.5-) 5x5.5 (-6.5) µm. Ascospores hyaline, thin-walled, vermiform (75-)80-85(-100) x (2-)3(-3.5) µm, 5 septa, sinuous to curved, never straight at maturity, rounded to acute apex.

Anamorph

Hirsutella-A type only; produced on the upper stromata (stalk) surface, phialides cylindrical to lageniform, 5-7x2-3 µm, tapering to a long neck, 5-11 µm; conidia not observed. The Hirsutella-A occurred in all species included in this study and were not deeply analyzed for species separation since other unique sexual (teleomorph) structures were present for each species.

Germination process

The released ascospores germinated withtin 24-48h, producing 1-2, uniformly straight, thread-like structures (capillicondiophore); roughly uniform length, averaging 55 µm, bearing a single terminal spore (capilliconidiospore) connected by an apical nub, hyaline, smooth-walled, fusarium-like in shape at maturity, 10-11x2-2.5 µm, narrowing apically.

Host attachment

Hosts commonly found biting tightly the edge of palm-leaves, some times the edge of dicot leaves, rarely palm-spines. Abundant mycelium growth from mouth and parts touching the substrate, sticking the host to the leaf in addition to the locked jaws.

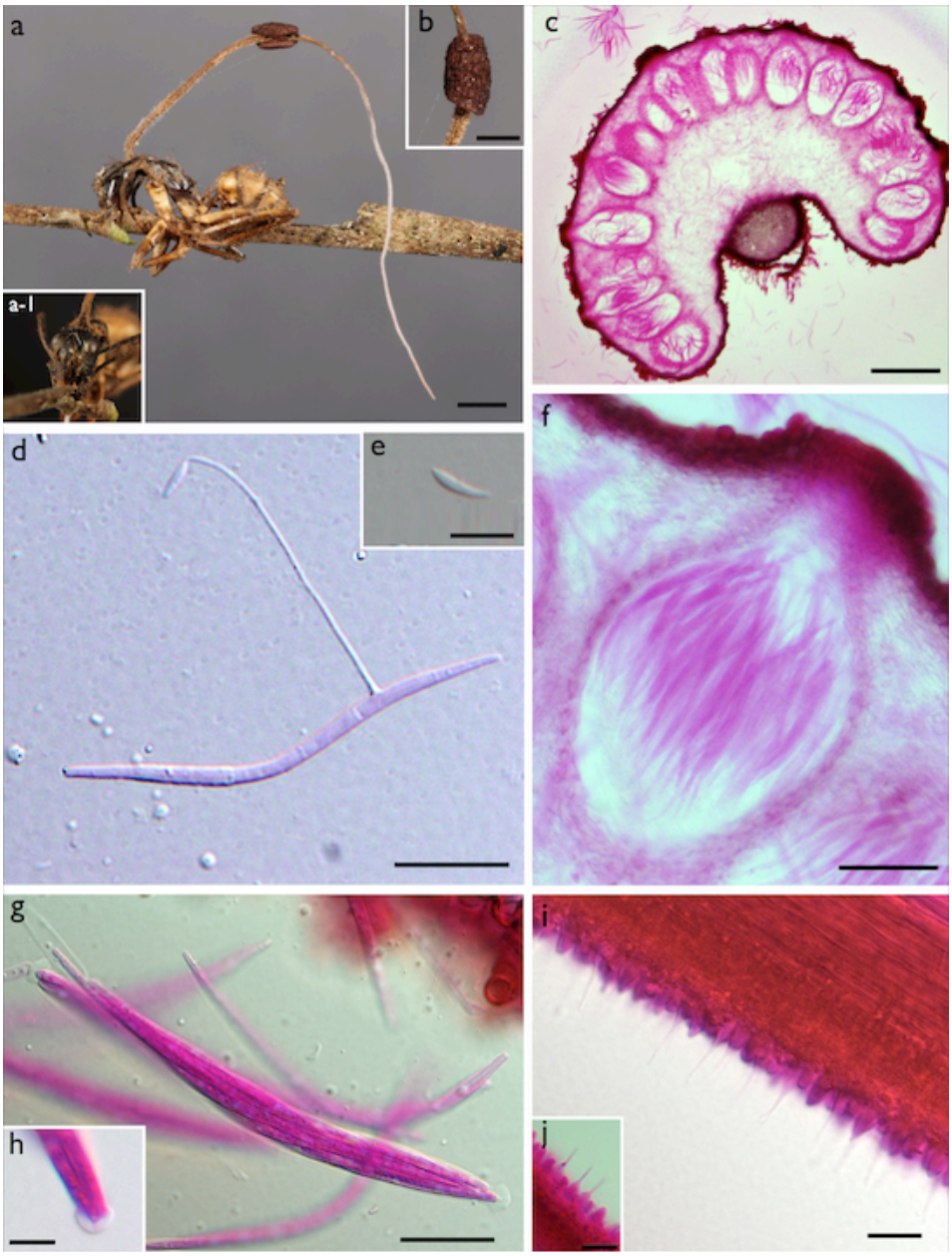

Figure 2. *Ophiocordyceps* sp1. a) Single stroma, characteristic of *Ophiocordyceps unilateralis sensu lato*, presenting a single ascoma arising from dorsal pronotum of *Camponotus atriceps*, firmly attached to a palm leaf (bar = 1.5 mm); **a-1**) ant displaying a behavior manipulation of biting the 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 perithecia (bar = 50 µm); g) Asci, clavate in shape and with a prominent cap (bar = 20 µm); h) detail of the asci cap (bar = 5 µm); i) Section of upper part of stroma showing anamorph phase (Hirsutella-A type), with a palisade of subulate phialides (bar = 10 µm); j) Close up of Hirsutella cells (bar = 10 µm).

***Ophiocordyceps* sp.2** Araújo J.P.M., H.C. Evans & D.P. Hughes sp. nov.

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Type: Brazil. Amazonas: Adolpho Ducke Reserve, 15 Jan 2014, B66, on *Camponotus bidens* Mayr 1870

Mycelium dark-brown to black, forming aggregation of hyphae on the intersegmental membranes; stromata single, produced form dorsal pronotum, commonly 5-7 mm in length, cylindrical, black, presenting cream-white swollen terminal part; fertile region presenting always a single globose cushion, dark brown becoming black when old, averaging 0.8 x 04-07 mm. Perithecia immersed, slightly erumpent, globose to flask-shaped, 250-290 x 150-170 µm, with short neck. Asci X-spored, hyaline, cylindrical to clavate, (90-) 110-130 x (7-) 8-8.5 (-9.5) µm; small but prominent cap, 3.5 x 4.5 µm. Ascospore hyaline, thin-walled, cylindrical, (60-) 70-75 (-80) x 4.5-5 (-6), round and slightly narrow at the apexes.

Anamorph

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. Narrow limoniform conidia, averaging 6-7 x 2 µm, with a peculiar tail.

Germination process

Spores germinated 48-72h after released, producing always 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 side attached to the capilliconidiophore.

Host Attachment

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

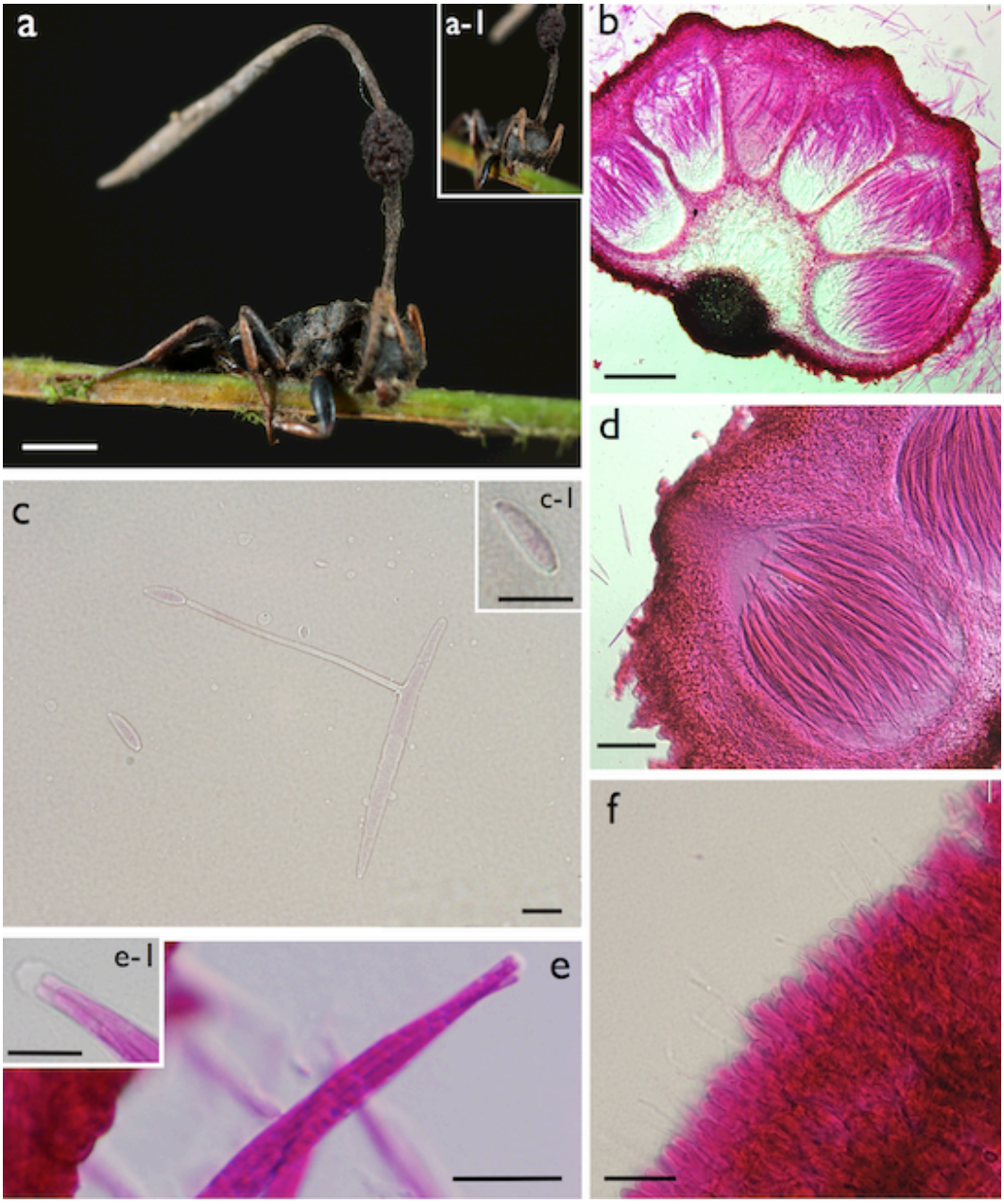

Figure 3. *Ophiocordyceps* sp 2. a) Infected *Camponotus bidens* biting into a palm leaf's tip (bar = ????); **a-1)** Detail showing a biting behavior and ascoma; **b)** Section through ascoma showing perithecial arrangement (bar = 100 µm); c) Ascospore after 48h germinating a single capilliconidiophore with a capilliconidia at the tip (bar = 10 µm); c-1) Detail of the conidiospore (bar = 10 µm); d) Detail of inside a perithecia showing the asci arrangement (bar = 50 µm); e) Asci showing the ascospores 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 tip of stalk (stroma), showing the anamorph (Hirsutella-A) cells (bar = 10 µm).

***Ophiocordyceps* sp.3** Araújo J.P.M., H.C. Evans & D.P. Hughes sp. nov.

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Type: Brazil. Roraima: National Park of Viruá, 9 Feb 2012, V-15, on *Camponotus* sp.

Mycelium aggregations arising from all intersegmental membranes (sutures), ginger in color. Multiple stromata produced 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 to the terminal part. Fertile region always produced on the pronotum stromata, never from legs; lateral cushions, 1-4, hemispherical, chocolate to dark brown with age. Perithecia immersed, semi-erumpent, ovoid to flask-shaped, 230-310 x 120-175 µm, with short, exposed neck. Asci 8-spored, hyaline, cylindrical (135-) 170 (-190) x 8.5 (-11.5) µm; prominent cap, 4.5 x 5 µm; Ascospore hyaline, thin-walled, cylindrical, (60-) 75 (-80) x (3.5-) 4.5 (-5), occasionally presenting swollen parts, narrowing in acute tips on both sides.

Anamorph

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, no conidia observed. *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 after 48h they were released, producing commonly 2, rarely 3, hair-like capilliconidiophores with 120-15 µm in length, bearing a single terminal conidia, biguttulate, fusoid, narrowing apically.

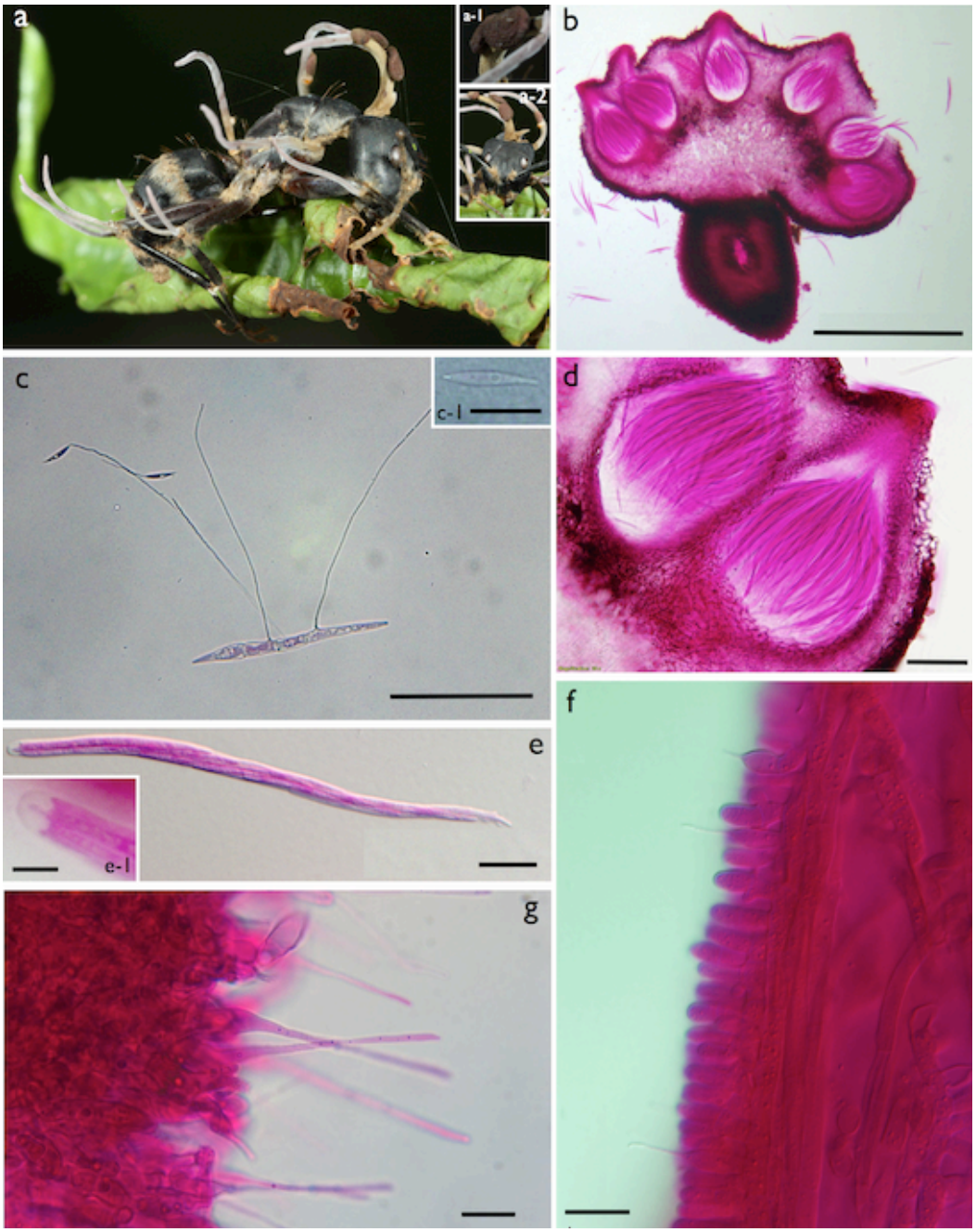

Figure 4. *Ophiocordyceps* sp3. a) *Camponotus* sp. biting into a leaf, several stroma arising from dorsal pronotum, mesonotum and leg joints, with a characteristic purplish coloration (bar = ???); **a-1)** fertile part (ascoma) (bar = ???); **a-2)** Close up of ant's head showing the biting behavior (bar = ???); **b)** Section through ascoma, with perithecial arrangement (bar = 500 µm); c) Ascospore after 24h, 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 anamorph (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)	Capilliconi dia	Capiliconidiophore (µm)	Ascospore size	A	B	C
O. sp1*	*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. sp2*	*Camponotus sp.*	8-10 (-16)	1.2 x 1	1-3	(115-) 120-125 (-130)	70-80 x 4.5	+	-	+
O. sp3*	*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 1: 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).

Phylogenetics Relationships

To be included….

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