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Multiple new species of *Ophiocordyceps* fungus on ants

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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.

Material and methods

Sampling

Surveys were undertaken in the central Amazonian region of Brazil at Reserva Adolpho Ducke. The reserve is comprised by ca. 10,000 ha (02˚55'S, 59˚59'W), adjacent to Manaus (Amazonas state) and composed of terra-firme forest,

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with plateaus, lowlands and campinarana vegetation, characterized to occur in patches of sandy soil across the Rio Negro basin.

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.

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 ascoma, 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.

Results

Taxonomic treatment

Ophiocordyceps briophyticola Araújo & D. P. Hughes sp. nov.

External mycelium covering most of the host, produced from all orifices and sutures, brown at maturity. Stroma single, rarely branched, produced from dorsal pronotum, averaging 15-20 mm, up to 30 mm, cylindrical, velvety and dark brown, tapering towards the apex; Fertile region (ascoma) of lateral cushion, 1-2, hemispherical to globose, dark-brown to black, variable in size, averaging 1-1.5 x 0.8-1 mm. Perithecia immersed to partially erumpent, flask-shaped, 220–250 x 100–165 µm, pronounced ostiole. Asci 8-spored, hyaline, cylindrical, (110-) 130–145 x 8 – 10µm; prominent cap, 7–8 x 3 µm. Ascospores hyaline, thin walled, vermiform 90 – 120 x 4 µm, 5–8-septate, straight to sinuous, round to slightly tapered apex.

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Asexual-morph. Hirsutella A-type not observed. Hirsutella C-type, produced from brown cushions (sporodochia) on leg and antennal joints; phialides subulate at base, 40–60 x 3–5 µm long, tapering to a long, hyaline neck. Conidia not observed.

Germination process. All the ascospores remained unchanged after five days on water-agar plate. We observed several different round spores and *Verticillium*-like growing among the ascospores. Further studies will address more attempts to incubate the ascospores for more days, aiming hyphal or capilliconidiophore germination.

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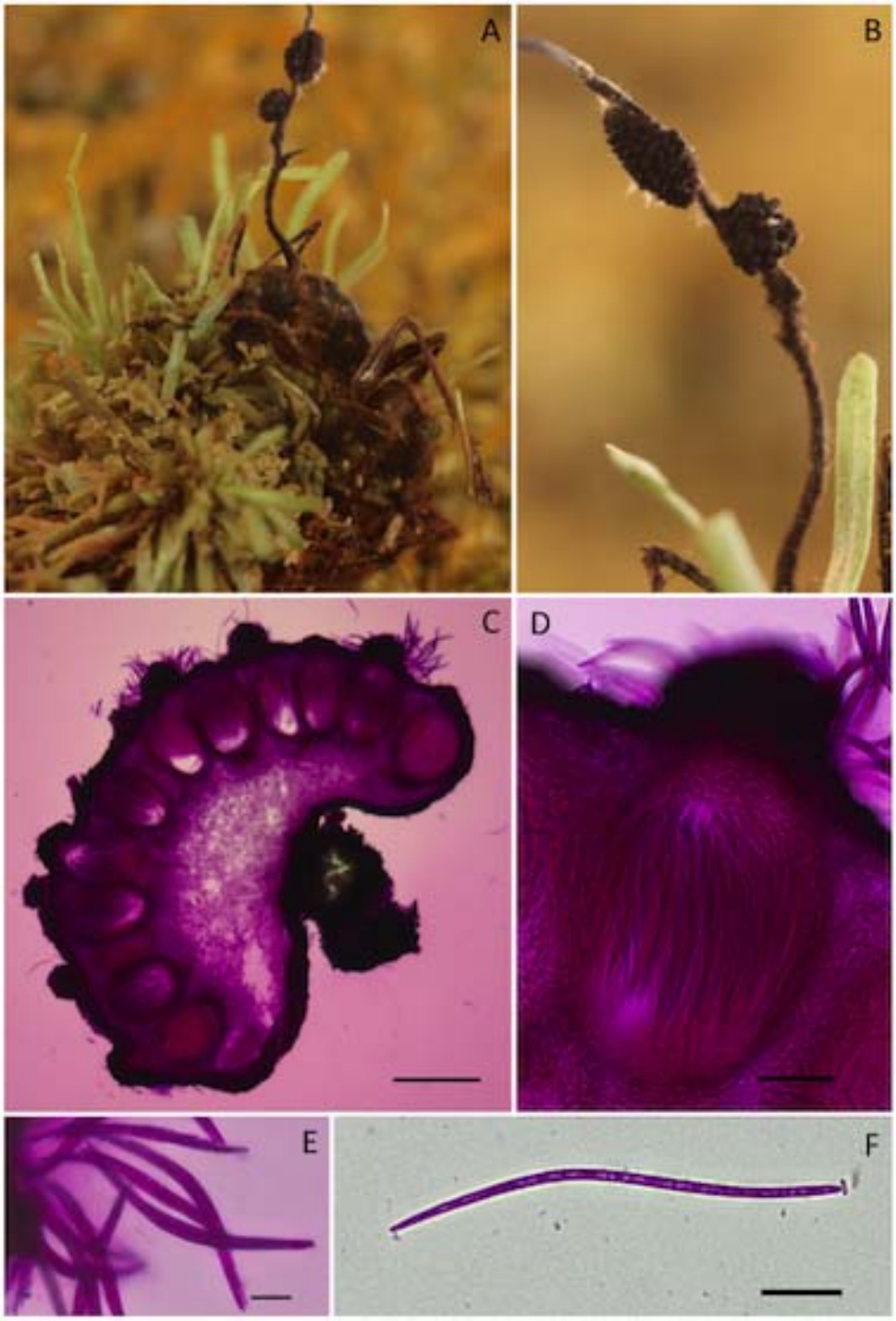

Figure 1. ***Ophiocordyceps bryophyticola*. A)** *Camponotus* sp. dying attached to bryophytes on the base of trees; **B)** Close-up of the fertile part (ascoma); **C)** Section through ascoma showing the perithecial arrangement; **D)** Close-up of perithecium; **E)** Asci; **F)** Ascospores

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Figure 2. ***Ophiocordyceps bryophyticola*. A)** Ant biting into the moss carpet with the antenna raised, showing the detail of **B)** phialides (Hirsutella B-type).

Ophiocordyceps chantifex Araújo & D. P. Hughes sp. nov.

Mycelium growing from all inter-segmental membranes, often covering the host body; initially white turning brown. Stroma single, produced from dorsal pronotum, averaging 10 mm, up to 15 mm in length, cylindrical, velvety and ginger brown, becoming cream-pinkish at the apical part; fertile region of lateral cushion, 1-2, hemispherical, chocolate brown, darkening with age, slightly variable in size, averaging 1.5 x 1 mm. Perithecia immersed to partially erumpent, globose-hemispherical shaped, 200-235 x 135-175, with short neck. Asci 8-spored, hyaline, cylindrical to clavate, 100-125 x 6 µm; prominent cap, 7-6 x 3-4 µm. Ascospore hyaline, thin-walled, vermiform 75-85 x 5 µm, 9–13-septated, sinuous to curved, never straight at maturity, rounded to acute apex.

Asexual-morph. Hirsutella-A type associated with apical region of stromata; phialides lageniform, 5-6 x 3 µm, tapering to a robust neck, 4-8 µm in length; conidia fusiform to limoniform, averaging 7 x 2.6 µm.

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Germination process. The released ascospores germinated within 24h to produce a single, long and extremely narrow hair-like capilliconidiophore; variable in length (65-) 75-90 (-95) µm; bearing a single terminal capilliconidium, hyaline, smooth-walled, uni or biguttulate, fusoid, narrowing apically.

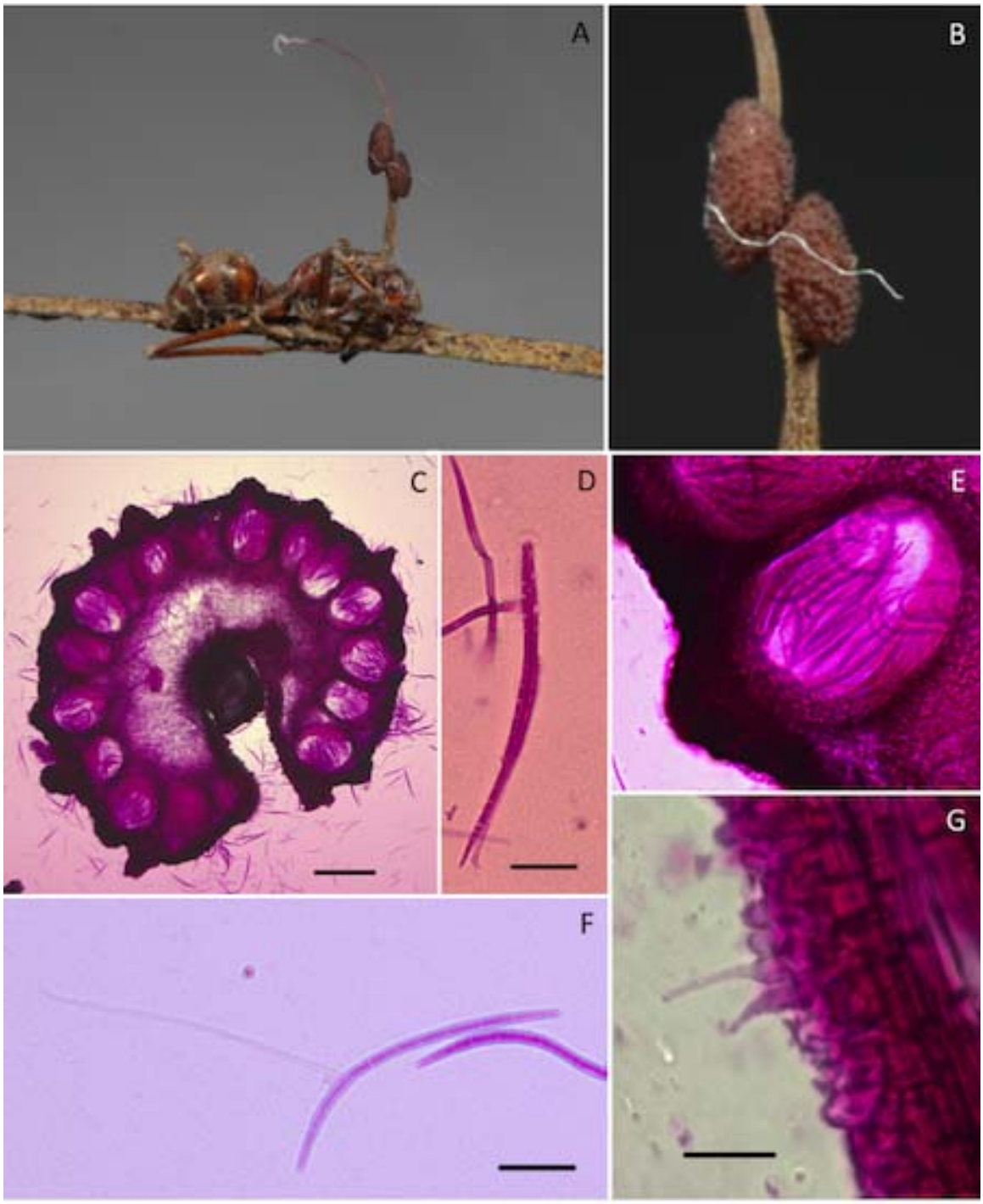

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Figure 3. ***Ophiocordyceps chantifex*. A)** Camponotus chantifex biting onto a palm leaf; **B)** Close-up of the ascoma; **C)** Cross section of the ascoma showing the perithecial arrangement; **D)** Ascus with prominent cap; **E)** Close-up of the perithecium; **F)** Multiseptated ascospore with long capilliconidiophorel; **G)** Hirsutella A-type phialide on the stroma.

Ophiocordyceps elegans Araújo & D. P. Hughes sp. nov.

Mycelium produced sparsely from joints, not covering the host body, dense when touching the substrate, dark brown. Stroma single, arising from the dorsal pronotum, never branching, averaging 1.8–2 cm in length, 0.2 mm thick, dark brown at the base turning into lighter brown into the apex; fertile part of a single lateral cushion, disc-shaped, chestnut-brown, averaging 6.5 x 6.3 mm. Perithecia immersed to partially erumpent, flask-shaped, (205-) 225-230 (-265) x 135 (-180) µm with short neck. Asci 8-spored, hyaline, cylindrical, 150-160 x 8-9 µm; apical cap prominent, 6 x 3 µm. Ascospores hyaline, thin-walled, multiguttulate, cylindrical, 120-140 x 3 µm, 7-septate, straight or curved tapering to the apex.

Anamorph. Hirsutella A-type associated with apical region of stroma; phialides lageniform, 5-8 x 3-4 µm, tapering to a long neck, 8-12 µm; conidia hyaline, limoniform, 5x2 µm.

Germination process. Ascospores released on agar germinated after 72h to produce a single, straight capillicondiophore; 25-30 µm, bearing a terminal capilliconidium, hyaline, smooth-walled, guttulate, 5-9 x 2 µm, narrowing apically.

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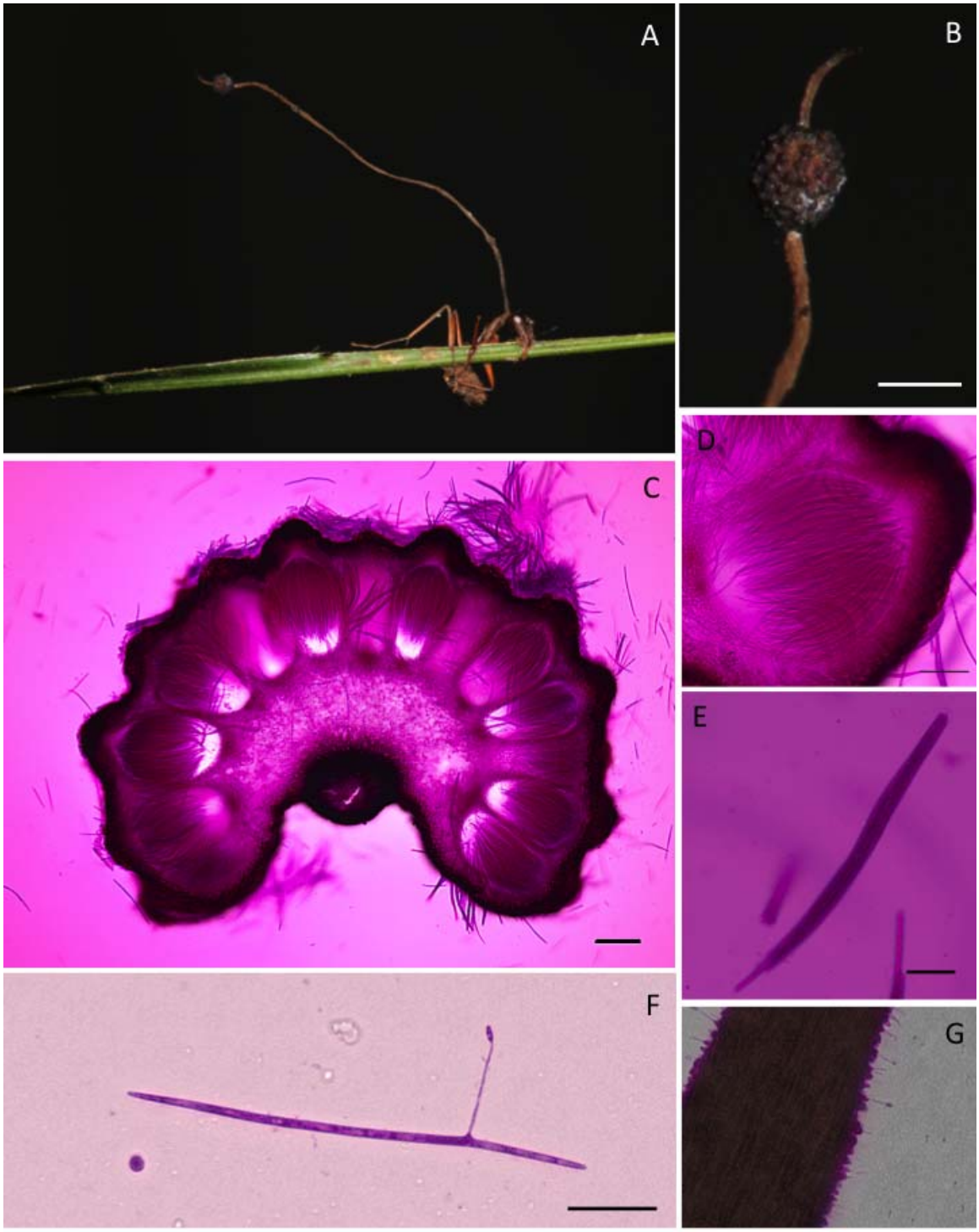

Figure 4. ***Ophiocordyceps elegans.*** **A)** *Camponotus* sp. biting into vegetation with the long stroma arising from its dorsal pronotum; **B)** Close-up of the ascoma; **C)** Section through ascoma showinh the perithecial arrangement; **D)** Close-up of perithecium; **E)** Ascus; **F)** Long ascospores with the straight capilliconidiophore bearing an apical capilliconidium; **G)** Hirsutella A-type layer on the apical part of the stroma.

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Ophiocordyceps sporodochialis –nigrans Araújo & D. P. Hughes sp. nov.

External mycelium produced from all orifices and sutures; initially white, becoming ginger brown, covering the host body, notably the abdominal part. Stroma single, produced from dorsal pronotum, 10–15 x 0.2 mm, cylindrical, black, covered with ginger velvety hyphae fading away towards the apex; fertile region of lateral cushions, 1–2, disc-shaped to hemispherical, light brown, darkening with age, averaging 1.5 x 1 mm. Perithecia immersed to partially erumpent, flask-shaped, 215–240 x 120–150 (-180) µm, with short, exposed neck or ostiole. Asci 8-spored, hyaline, thin-walled, vermiform to clavate, 120–145 x 8 (-10) µm; cap prominent, 8 x 4 µm; Ascospores hyaline, thin-walled, vermiform, 90–105 (-115) x 3-4 µm, 5-septate, gently curved, rarely straight; tapering to a round apex.

Anamorph. Hirsutella A-type associated with the apical part of stroma. Hirsutella C-type, produced from light brown cushions on leg and antennal joints: phialides subulate, robust, 85–120 x 4–6 (-8) µm, Look for conidia on antenna.

Germination process. Ascospores germinating after 24–72h to produce 1 (–2), uniformly straight, extremely narrow hair-like capilliconidiophores, 50–60 µm; bearing a single terminal capilliconidium, hyaline, smooth-walled, biguttulate, clavate, 9 x 2 µm, narrowing apically.

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Figure 5. ***Ophiocordyceps sporodochialis-nigrans*****. A)** *Camponotus* sp. infected biting into a leaf; **B)** Close-up of the ascoma; **C)** Section through ascoma showing the perithecia arrangement; **D)** Close-up of perithecium; **E)** Asci; **F)** Ascospore with capilliconidium.

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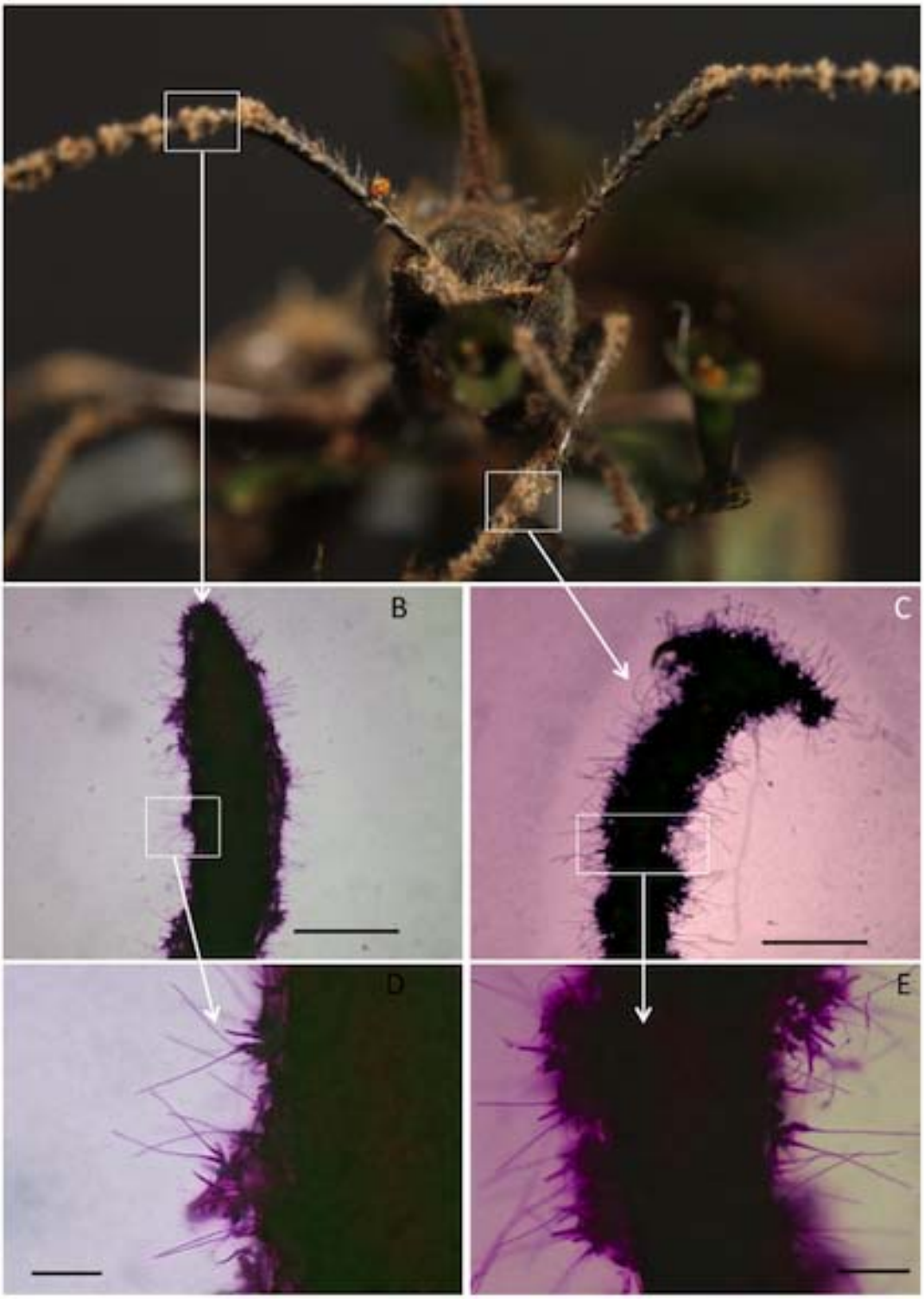

Figure 6. ***Ophiocordyceps sporodochialis-nigrans***. Plate showing the Hirsutella C-type growing on legs and antenna. A) Frontal view of the biting ant; B) Antenna covered with phialidesl C) eg covered with phialides; D-E) Close-up of the subulate phialides.

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Ophiocordyceps sporodochialis-brunneis Araújo & D. P. Hughes sp. nov.

External mycelium produced from all the orifices and sutures; initially white, becoming ginger brown, covering the host body with sparse hyphae. Stroma single, produced from dorsal pronotum, averaging 15 x 0.2 mm, cylindrical, black, covered with ginger velvety up to the ascoma, fading away towards the apex; fertile region of a single lateral cushion, disc-shaped, chestnut-brown, darkening with age, averaging 1 x 1.3 mm. Perithecia immersed to partially erumpent, flask-shaped, (170–) 200–215 x 100–130 µm, with short, exposed neck or ostiole. Asci 8-spored, hyaline, thin-walled, vermiform to clavate, 110–140 x 6-8 µm; cap prominent, 4 x 6 µm; Ascospores hyaline, thin-walled, vermiform, 75–90 x 3 µm, 5-septate, straight to gently curved, tapering to a round apex.

Anamorph. Hirsutella A-type associated with the apical part of stroma. Hirsutella C-type, produced from light brown cushions on leg and antennal joints: phialides subulate, robust, 70–95 x 5 µm. Look for conidia on antenna

Germination process. Ascospores germinating after 24–72h to produce 1–3, extremely narrow hair-like capilliconidiophores, 50–60 µm; bearing a single terminal capilliconidium, hyaline, smooth-walled, bi-pluri guttulate, clavate, 9–10 x 3 µm, narrowing apically.

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Figure 7. ***Ophiocordyceps sporodochialis-brunneis.*** Camponotus sp. biting into a sapling's leaf edge. **B)** Close-up of the ascoma; **C)** Cross section of the ascoma, showing the perithecial arrangement; **D)** Close-up of ferithecium; **E)** Ascospores showing three capilliconidiophores; **F)** Ascus; **G)** Hirsutella A-type phialides.

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Figure 8. ***Ophiocordyceps sporodochialis-brunneis*. A)** Frontal view of the ant biting the edge of the leaf. B-C) Antenna covered with Hirsutella C-type phialides.

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Ophiocordyceps bicolor Araújo & D. P. Hughes sp. nov.

External mycelium produced from all the orifices and sutures; initially white, becoming ginger brown, covering the host body with sparse hyphae. Stroma single, produced from dorsal pronotum, averaging 3.5 x 0.25, up to 6 mm in length, cylindrical to laterally compressed, ginger to dark-brown; fertile part terminal of lateral cushions, 1–3, disc-shaped to hemispherical, chestnut-brown, darkening with age, 1.2 – 2.2 x 0.8–1.4 mm. Perithecia immersed to partially erumpent, flask-shaped, 200-230 (–250) x 135–165 µm, with short, exposed neck or ostiole. Asci 8-spored, hyaline, cylindrical to clavate, 110–130 x 8–9 µm; cap prominent, 6–3 µm; Ascsopores hyaline, sinuous to curved, rarely straight, 75–90 x 3 µm, X-septate; apex round to acute.

Anamorph. Hirsutella A-type only: produced laterally on upper stroma; phialides rare, cylindrical to lageniform, 7–10 x3–4 µm, tapering to a long neck, 10–15 µm; conidia limoniform, averaging 7–9 x 3 µm.

Germination process. Ascospores germinated in 24–48h to produce a single, narrow capilliconidiophore, 35–40 µm long; bearing a single capilliconidium, hyaline, smooth-walled, uni–biguttulate, clavate, 9–3 µm, narrowing apically.

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Figure 9. ***Ophiocordyceps bicolor*. A)** *Camponotus sp* biting a palm leaf; **B)** Close-up of the ascoma; **C)** Cross section show the perithecial arrangement; D) Close-up of the perithecium; E) Ascus; F) Capilliconidium; G) Sinuous ascospore with the capilliconidiophore bearing a capillicondium; H) Hirustella A-type phialide on the stroma.

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Ophiocordyceps triangularis Araújo & D. P. Hughes sp. nov.

External mycelium produced from all the orifices and sutures; initially white, becoming ginger brown, covering the host body with sparse hyphae. Stroma single, produced from dorsal pronotum, 5–7 x 0.15 µm, cylindrical ginger to dark-brown, swollen terminal part, clavate; fertile part constantly produced at the middle part of stroma, laterally attached, round-shaped, chestnut brown, darkening with age, averaging 2–2.5 x 0.25 µm, up to 4.2 µm in length; Perithecia immersed to partially erumpent, flask-shaped, averaging 225–250 x 135–165 µm, with short, exposed neck or ostiole. Asci 8-spored, cylindrical to clavate, 115–135 x 7–10 µm, cap prominent, 6–7 x 4 µm; Ascospores hyaline, cylindrical, robust, straight to gently curved, 75–85 x 4–5 µm, 5-septate, tapering to a round to slightly acute apex.

Anamorph. Hirsutella A-type only; produced on the clavate part of upper stromal phialides cylindrical to lageniform, 8–9 x 4 µm, tapering to long neck 9–10 µm.; conidia limoniform, averaging 5 x 2µm.

Germination process. Ascospores germinated 24–48h to produce a straight, robust capillicondiophore, often verrucose, 45–50 µm long; bearing a single capilliconidium, hyaline, smooth-walled, guttulate, 10-11 x 4 µm, truncate at base, narrowing apically.

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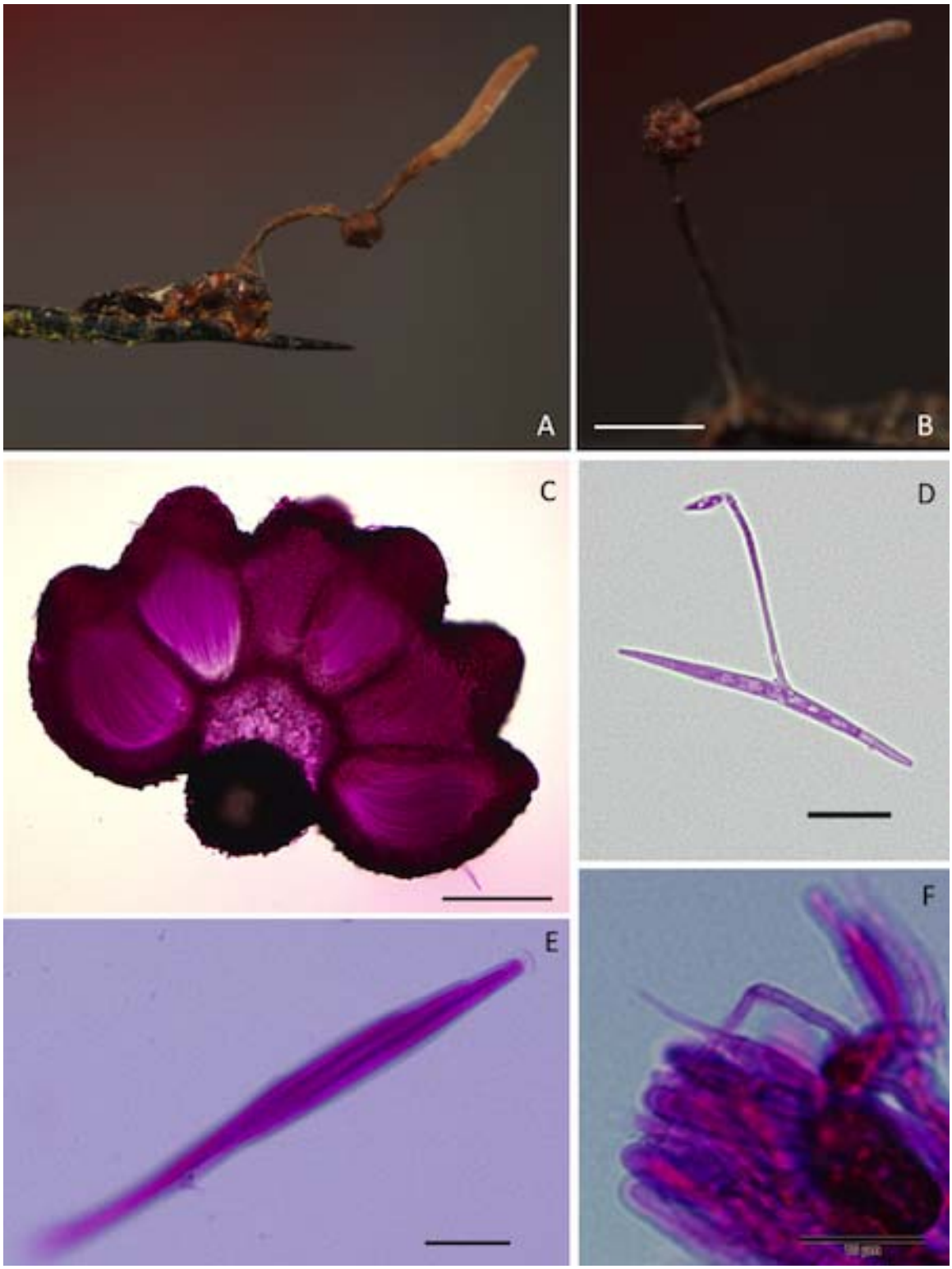

Figure 10. ***Ophiocordyceps triangularis.*** **A)** Tiny *Camponotus* sp biting onto a palm spine. **B)** Cloase-up of the ascoma; **C)** Cross section of the ascoma showing the perithecial arrangement; **D)** Ascospore with capilliconidiophore with verrucose apical portion; E) Ascus; F) Hirsutella A-type phialide on the stroma apex.