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Epitypification and re-description of the zombie-ant fungus, *Ophiocordyceps unilateralis* (*Ophiocordycipitaceae*)

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Abstract: The type of *Ophiocordyceps unilateralis* (*Ophiocordycipitaceae*, *Hypocreales*, *Ascomycota*) is based on an immature specimen collected on an ant in Brazil. The host was identified initially as a leaf-cutting ant (*Atta cephalotes*, Attini, Myrmicinae). However, a critical examination of the original illustration reveals that the host is the golden carpenter ant, *Camponotus sericeiventris* (Camponotini, Formicinae). Because the holotype is no longer extant and the original diagnosis lacks critical taxonomic information – specifically, on ascus and ascospore morphology – a new type from Minas Gerais State of south-east Brazil is designated herein. A re-description of the fungus is provided and a new phylogenetic tree of the *O. unilateralis* clade is presented. It is predicted that many more species of zombie-ant fungi remain to be delimited within the *O. unilateralis* complex worldwide, on ants of the tribe Camponotini.

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INTRODUCTION

Ophiocordyceps unilateralis (*Ophiocordycipitaceae*: *Hypocrea-les*) is a fungal pathogen of ants belonging to the tribe Camponotini (Formicinae: Formicidae) with a pantropical distribution (Evans 2001). The fungus alters the behaviour of the ant host causing it to move and die away from the nest, often in an exposed position and, typically, clinging onto and biting into vegetation in a "death-grip" (Hughes *et al*. 2011). This host manipulation by *O. unilateralis* is a particularly spectacular and complex example of the extended-phenotype paradigm (Dawkins 1982, Andersen *et al*. 2009, Hughes 2013, Hughes *et al*. 2016), which duly garnered the epithet, the zombie-ant fungus (Evans *et al*. 2011a), and spawned considerable media coverage by the popular press and scientific magazines alike (Kaplan 2011, Costandi 2012, Boddy 2014, Pennisi 2014). In addition, it stimulated on-going research on the nature of the ant-fungal association, as well as on fungal phylogeny, that has generated a wealth of information (reviewed in Hughes *et al*. 2016). Significant advancement has been made in understanding the mechanisms involved at the molecular level: thus, manipulation of the ant brain by the fungus has been ascribed to two candidate metabolites – guanobutyric acid and sphingosine – previously implicated in neurological diseases and cancer (de Bekker *et al*. 2014). Using comparative genomics and a mixed transcriptomics approach, it has also been shown that genes unique to the fungus are up-regulated that encode for proteins known to cause neurological and behavioural changes (de Bekker *et al*. 2015, de Bekker *et al*. 2017).

Contemporary studies have tended to use the over-arching term *O. unilateralis sensu lato* for the zombie-ant fungus since it has long been suspected, but only recently established, that this is a species complex. In fact, morphological variations had been noted in collections from around the world from a very early stage (Petch 1924, 1931, 1933, 1935, 1937, Kobayasi 1941, Mains 1958, Evans 1974, 1982, Evans & Samson 1984), but it was concluded that "whilst it is tempting to separate geographic isolates (ecotypes), there is not enough evidence at the moment to conveniently divide the species into varietal units: more information is needed concerning host specificity and the range of variation in temperate, subtropical and tropical specimens" (Evans & Samson 1984). Some three decades later, Evans *et al*. (2011a) set out to uncover the taxonomic diversity of the newly-termed zombie or brain-manipulating fungus, based on an examination of fresh material collected on infected carpenter ants within a fragment of Atlantic rainforest in Brazil. Four *Camponotus* species were identified and, following a critical morphological comparison of the freshly-released (mature) ascospores – as well as of the germination process – and of the associated asexual morphs, four *Ophiocordyceps* species were delimited; leading to the supposition that "each species of the tribe Camponotini may be attacked by a distinct species of *Ophiocordyceps*" (Evans *et al*. 2011a), and "that there may be hundreds of species within the complex parasitising formicine ants worldwide" (Evans *et al*. 2011b). This hypothesis would appear to be holding true based on subsequent publications involving both morphological and molecular evidence, with six new species being described from Thailand (Luangsa-ard *et al*. 2011, Kobmoo *et al*. 2012, Kobmoo *et al*. 2015), one from Japan (Kepler *et al*. 2010), three from the Brazilian Amazon (Araújo *et al*. 2015) and another 14 in the pipeline (Araújo *et al*. 2018).

Significantly, however, only Kobayasi (1941) appears to have examined the type specimen – named as *Torrubia unilateralis*

on the Brazilian ant *Atta cephalotes* (Tulasne & Tulasne 1865) – and he noted that it "is now preserved in [the] Paris Entomological Museum [and] is immature". Unfortunately, repeated attempts to obtain the type for examination of the fungus and identification of the ant host were unsuccessful and it was concluded that the specimen was lost, leading to speculation that this may have gone missing during the Second World War (Evans *et al*. 2011a). From the latter study, and the confirmation that *O. unilateralis* represents a species complex, it became necessary to designate a new type, especially since *Ophiocordyceps* is the type genus of the recently-recognised family *Ophiocordycipitaceae* which is based on the placement of *O. unilateralis* within a Bayesian consensus tree (Sung *et al*. 2007). *Ophiocordyceps* is a highly diverse genus, with considerable pharmaceutical potential (Berenbaum & Eisner 2008, Paterson 2008, Molnár *et al*. 2010, Zhang *et al*. 2012) – species of which have also been identified recently as primary endosymbionts in certain insect hosts (Nishino *et al*. 2016, Gomez-Polo *et al*. 2017) – and thus *O. unilateralis* is central to our understanding of this medically-important group, as well as being considered as a keystone species for unravelling ecosystem functioning and biodiversity of fungi in tropical forests (Evans *et al*. 2011b).

In his diagnosis, Louis Tulasne described the unilateral position of the fleshy, hemispherical, fertile stroma on the stipe, but failed to provide details of the asci or ascospores, nor did these structures appear in the accompanying illustration by his brother, Charles (Tulasne & Tulasne 1865). This supports the statement of Kobayasi (1941) that the type was immature. Theoretically, the illustration could still stand as the holotype but, because there is no extant material, this would serve as the lectotype and a suitable epitype should be designated (Ariyawansa *et al*. 2014), not a neotype as Evans *et al*. (2011a) had originally and mistakenly proposed. The resultant search for a suitable epitype was based on the evidence from the illustration that the host representing the type is a *Camponotus* ant (Samson *et al*. 1982): specifically, the golden carpenter ant *Camponotus sericeiventris*, with its distinctive pronotal plate, and not the leafcutter *Atta cephalotes*, which is a myrmicine ant having no historical association with *O. unilateralis* (Evans & Samson 1984, Evans 2001). Cooke (1892) used the same Tulasne illustration to re-describe the so-called "one-sided ant club", with additional information that the fungus had been "collected by Trail in Brazil". This specimen is in the RBG Kew fungarium and was found by the English naturalist J.W.H. Trail in 1874 in the Brazilian Amazon, which was examined by Massee (1895) who reported it to be on the same ant species as the type. However, we consider that the type specimen of *O. unilateralis* was more likely to have originated in the Atlantic rainforest region of south-east Brazil – where several European naturalists were based in the 1860s – and from where the type of *Camponotus sericeiventris* was collected (Rio de Janeiro) during a series of French expeditions (Guérin-Menéville 1838); specimens from which were deposited in the Paris Entomological Museum, where the type of *O. unilateralis* was also deposited (Tulasne & Tulasne 1865).

Epitypification has been delayed until now because all the targeted collections of infected *C. sericeiventris* ants from Atlantic rainforest in south-east Brazil proved to be immature (Evans *et al*. 2011a). In fact, some newly-infected specimens were marked *in situ* – whilst others were harvested and incubated in the laboratory – to monitor progress, but none developed to maturity. The present paper is the result of the discovery of specimens with fertile stromata, from the same region of Brazil (Zona da Mata Mineira), enabling a full description of the species, as well as a phylogenetic analysis.

MATERIALS AND METHODS

Field collection

Collecting was concentrated in a vestige of secondary Atlantic rainforest near Viçosa, Minas Gerais, in the Zona da Mata Mineira of south-east Brazil – belonging to the Universidade Federal de Viçosa (UFV) – where *ad hoc* surveys for zombie-ant fungi had been carried out previously (Evans *et al*. 2011a, b). Although *Camponotus sericeiventris* is relatively common in this habitat, it is confined mainly to open, heavily-disturbed areas and the incidence of infected ants was found to be low. All the initial collections proved to be immature and it was decided to follow progress *in situ* by flagging specimens and monitoring development of the ascostromata through weekly observation. However, none of the five tagged specimens survived, due to predation and loss through heavy rain. Subsequently, additional specimens were bagged but were spoiled by run-off water following storms. Finally, several more immature specimens were harvested together with the vegetation, transferred to a humid chamber in a greenhouse at UFV – with an 8 h misting/16 h dry regime – and monitored. Asexual morphs developed successfully but, because ascostromatal development was slow, the specimens were overgrown by opportunistic fungi before maturation was complete. The taxonomy of the asexual morphs is based on these paratype specimens. The mature epitype was collected by one of us (VRH) from another forest reserve in the Zona da Mata Mineira, some 150 km from the main study site, in the municipality of Juiz de Fora. These specimens were deposited in the fungarium of the Universidade Federal de Viçosa (VIC).

DNA extraction and PCR

We used a BLAST search in the GenBank nucleotide database to ensure the quality of the sequences generated in this study. Sequences that were identified as species not closely related to the species treated in this study were discarded and interpreted to be from a contaminant. All the sequences included here passed the above quality control checks.

The molecular studies were conducted according to Araújo *et al*. (2018), described below. The DNA templates were obtained directly from two specimens of *O. unilateralis* infecting *Camponotus sericeiventris* from the type locality in Minas Gerais (Brazil) that were collected in the field and dried *in silica* to avoid overgrowth by opportunistic fungi. For DNA extraction, the ants were dissected and the fungal contents (mummified mycelium and hyphal bodies) within their abdomens were placed in 1.5 mL Eppendorf tubes with 100–200 µL of CTAB (2 % CTAB powder, 100 mM Tris pH8, 20 mM EDTA, 1.4 M NaCl) and ground mechanically; 400 µL of CTAB were then added and the tubes were incubated at 60 °C for 20 min and centrifuged for 10 min at 14 000 rpm. The supernatant (approx. 400 µL) was transferred to a new 1.5 mL Eppendorf tube, mixed with 500 µL of 24:1 chloroform: isoamyl-alcohol (Sigma) and mixed by inverting. The mix was then centrifuged for 20 min at 14 000 rpm and the

supernatant transferred to a new 1.5 mL Eppendorf tube and further cleaned using the GeneCleanIII kit (MP Biomedicals), following Araújo *et al*. (2018) modifications.

Four loci were used in the analyses, i.e. small subunit nuclear ribosomal DNA (SSU), large subunit nuclear ribosomal DNA (LSU), translation elongation factor 1-α (*tef1*) and the largest subunit of RNA polymerase II (*rpb1*). The final concatenated dataset consisted of 3 795 bp. The primers used were, SSU: NS1 (GTAGTCATATGCTTGTCTC) and NS4 (CTTCCGTCAATTCCTTTAAG) (White *et al*. 1990); LSU: LR0R (5'-ACCCGCTGAACTTAAGC-3') and LR5 (5'-TCCTGAGGGAAACTTCG-3') (Vilgalys & Hester 1990); *tef1*: EF1-983F (5'-GCYCCYGGHCAYCGTGAYTTYAT-3') and EF1-2218R (5'- ATGACACCRACRGCRACRGTYTG-3') (Rehner & Buckley 2005); CRPB1: (5'-CCWGGYTTYATCAAGAARGT-3') (Castlebury *et al*. 2004) and RPB1Cr_oph: (5'-CTGVCCMGCRATGTCGTTGTCCAT-3') (Araújo *et al*. 2018).

To amplify the target loci, each 25 µL PCR amplification mix contained 4.5 µL of buffer E (Premix E – Epicentre) 0.5 µL of each forward and reverse primers (10 mM), 1 µL of DNA template, 0.1 Platinum Taq polymerase (Invitrogen) and 18.4 µL of ultra-pure distilled water (Gibco). The amplification reactions were placed in a Biometra T300 thermocycler under the following conditions: for SSU and LSU (1) 2 min at 94 °C, (2) 4 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 2 min, followed by (3) 35 cycles of denaturation at 94 °C for 30 s, annealing at 50.5 °C for 1 min, and extension at 72 °C for 2 min and (4) 3 min at 72 °C. For *tef1* and *rpb1* (1) 2 min at 94 °C, (2) 10 cycles of denaturation at 94 °C for 30 s, annealing at 64 °C for 1 min, and extension at 72 °C for 1 min, followed by (3) 35 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 1 min, and extension at 72 °C for 1 min and (4) 3 min at 72 °C. Each 25 µL amplification reaction was cleaned by adding 3.75 µL of Illustra ExoProStar enzymatic PCR clean up (1:1 mix of Exonuclease I and alkaline phosphatase, GE Healthcare Life Sciences), incubated at 37 °C for 1 h and 80 °C for 15 min in the thermocycler. The purified PCR products were sequenced by Sanger DNA sequencing [Applied Biosystems 3730xl DNA Analyzer (Life Technologies, Carlsbad, CA, USA)] at the Genomics Core Facility service at Penn State University.

Phylogenetic analyses

The raw sequence reads were edited manually using Geneious v. 8.1.8 (Kearse *et al*. 2012). Individual gene alignments were generated by MUSCLE (Edgar 2004), implemented in Geneious v. 8.1.8 (Kearse *et al*. 2012). The alignment of every gene was improved manually, annotated and concatenated into a single combined dataset using Geneious v. 8.1.8 (Kearse *et al*. 2012). Ambiguously aligned regions were manually excluded from phylogenetic analysis and gaps were treated as missing data. The final alignment length was 3 795 bp – 1 071 for SSU, 961 for LSU, 1 011 for *tef1* and 752 for *rpb1*. A Maximum likelihood (ML) analysis was performed with RAxML v. 8.2.4 (Stamatakis 2006) on a concatenated dataset containing all four genes. The dataset consisted of eight data partitions. These included one each for SSU and LSU, and three for each of the three codon positions of the protein coding genes, *tef1* and *RPB1*. The GTRGAMMA model of nucleotide substitution was employed during the generation of 1 000 bootstrap (bs) replicates. The sequences generated for this study were deposited in GenBank (Table 1).

RESULTS

Taxonomy

Ophiocordyceps unilateralis (Tul.) Petch, *Trans. Br. Mycol. Soc*. **16**: 74.1933. *emend.* H.C. Evans, D.P. Hughes & Araújo. Figs 1–2. *Basionym*: *Torrubia unilateralis* Tul., *Sel. Fung. Carp.* **III**: 18. 1865. *Synonym*: *Cordyceps unilateralis* (Tul.) Sacc. *Syll. Fung*. **2**: 570. 1883.

Description on host: *External mycelium* sparse, pale brown; emerging from sutures on body and legs. *Clava* stromatal, solitary, arising from the dorsal pronotum; cylindrical, brown and hirsute at the base. *Ascostroma* produced unilaterally, almost encircling the clava; hemisphaerical, 1.5–1.7 × 0.8 µm, dark brown, with roughened surface due to prominent perithecial necks. *Ascomata* (*perithecia*) partially erumpent, flask-shaped, 200–250 × 140–160 µm. *Asci* 8-spored, hyaline, cylindrical, (90–)95–125 × 6–8(–9) µm, swollen centrally tapering to a distinct foot and apical cap region (5–6 × 4–5 µm). *Ascospores* multiserriate, hyaline, thin-walled, filiform, (70–)75–85 × 2–2.5 µm, 4–5-septate; curved, tapering at both ends.

Lectotype designated here: holotype **Brazil**, *"Atta cephalotes"*, Tulasne (1865) *Sel. Fung. Carp.* **III**, plate I, fig. 3–4, MBT379723.

Epitype designated here: **Brazil**, *Minas Gerais*, Juiz de Fora, Paraibuna river (700–800 m a.s.l.), on *Camponotus sericeiventris* (Camponotini: Formicinae: Formicidae), on shrub leaf, 10 Aug. 2014, *V.R. Halfeld*, I14-1369A (epitype VIC 44303, MBT379722).

Additional materials examined: **Brazil**, *Minas Gerais*, Viçosa, Mata do Paraíso (700 m a.s.l.), on *Camponotus sericeiventris*, on shrub leaf, 26 Apr. 2010, *H.C. Evans*, MAP-61 (paratype VIC 44354); 12 Aug. 2012, *H.C. Evans*, MP-426 (paratype VIC 44349); 7 Feb. 2013, *H.C. Evans*, MP-502 (paratype VIC 44350).

Asexual morph: The asexual morph of the epitype proved to be in poor condition and the diagnosis below is based on the paratype collections.

Apical region of the stromatal clava, smooth, pinkish-brown, tapering to an acute tip; covered by a loose to compact hymenium of scattered to dense phialides. *Phialides* of two types: with a prominent swollen base (10–12 × 3–3.5 µm), tapering abruptly to a thin neck region (12–15 × 0.5–1 µm), producing hyaline, guttulate, limoniform *conidia*, 6.5–8 × 2–2.5 µm, apically (= *Hirsutella* A-type, Evans & Samson 1984); with a cylindrical base (14–16 × 2.5–3 µm), tapering gradually to a long neck (45–50 µm), 1 µm at the tip, producing solitary, hyaline, cylindrical-fusoid *conidia*, 8–11 × 2.5–3 µm, with a rounded apex and truncate base (= *Hirsutella* B-type). *Hirsutella* B-type also produced separately in loose, brown sporodochia arising from the leg joints.

Notes: Other synonyms – *Torrubia formicivora*, *Cordyceps formicivora*, *C. ridleyi* and *C. subunilateralis* – have been listed by various authors (Petch 1933, Mains 1958, Evans & Samson 1984, Sung *et al*. 2007): however, because the ant hosts are not identified and the collecting localities of some are outside the geographic range of *Camponotus sericeiventris*, these can no longer be considered to be synonymous with *O. unilateralis s. str*. Examination of the types, as well as identification of the

Table 1. Specimen information and GenBank accession numbers for the sequences used in this study.

		GenBank Accession numbers[2]			
Species	**Voucher Information[1]**	**SSU**	**LSU**	***tef1***	***rpb1***
Ophiocordyceps acicularis	OSC 128580	DQ522543	DQ518757	DQ522326	DQ522371
Ophiocordyceps agriotidis	ARSEF 5692	DQ522540	DQ518754	DQ522322	DQ522368
Ophiocordyceps amazonica	HUA 186143	KJ917562	KJ917571	KM411989	KP212902
Ophiocordyceps annulata	CEM 303	KJ878915	KJ878881	KJ878962	KJ878995
Ophiocordyceps aphodii	ARSEF 5498	DQ522541	DQ518755	DQ522323	n/a
Ophiocordyceps araracuarensis	HUA 186135	KC610788	KC610769	KC610738	KF658665
Ophiocordyceps australis	HUA 186147	KC610784	KC610764	KC610734	KF658678
Ophiocordyceps blattarioides	HUA186093	KJ917559	KJ917570	KM411992	KP212910
Ophiocordyceps brunneipunctata	OSC 128576	DQ522542	DQ518756	DQ522324	DQ522369
Ophiocordyceps camponoti-atricipis	ATRI3	KX713666	n/a	KX713677	n/a
Ophiocordyceps camponoti-balzani	G104	KX713660	KX713593	KX713689	KX713703
	G143	KX713658	KX713595	KX713690	KX713705
Ophiocordyceps camponoti-bispinosi	BISPI2	KX713665	KX713588	n/a	KX713700
	OBIS	KX713639	KX713612	KX713694	KX713718
	OBIS3	KX713638	KX713614	KX713695	n/a
	OBIS4	KX713637	KX713615	KX713692	KX713720
Ophiocordyceps camponoti-leonardi	TL1	KJ201515	n/a	KJ201526	n/a
	C36	KJ201512	n/a	JN819013	n/a
Ophiocordyceps camponoti-rufipedis	G108	KX713659	KX713594	KX713679	KX713704
	G177	KX713657	KX713596	KX713680	n/a
Ophiocordyceps camponoti-saundersi	C19	n/a	n/a	JN819042	n/a
	C40	n/a	n/a	JN819044	n/a
Ophiocordyceps communis	NHJ 12582	EF468975	EF468830	EF468771	n/a
	NHJ 12581	EF468973	EF468831	EF468775	n/a
Ophiocordyceps curculionum	OSC 151910	KJ878918	KJ878885	n/a	KJ878999
Ophiocordyceps dipterigena	OSC 151911	KJ878919	KJ878886	KJ878966	KJ879000
Ophiocordyceps elongata	OSC 110989	n/a	EF468808	EF468748	EF468856
Ophiocordyceps evansii	HUA 186159	KC610796	KC610770	KC610736	KP212916
Ophiocordyceps formicarum	TNS F18565	KJ878921	KJ878888	KJ878968	KJ879002
Ophiocordyceps fulgoromorphila	HUA 186139	KC610794	KC610760	KC610729	KF658676
Ophiocordyceps gracilis	EFCC 3101	EF468955	EF468810	EF468750	EF468858
Ophiocordyceps halabalaensis	MY1308	n/a	n/a	GU797109	n/a
	MY5151	n/a	n/a	GU797110	n/a
Ophiocordyceps heteropoda	EFCC 10125	EF468957	EF468812	EF468752	EF468860
Ophiocordyceps irangiensis	OSC 128578	DQ522556	DQ518770	DQ522345	DQ522391
Ophiocordyceps kniphofioides	HUA 186148	KC610790	KF658679	KC610739	KF658667
Ophiocordyceps longissima	HMAS_199600	KJ878926	n/a	KJ878972	KJ879006
Ophiocordyceps lloydii	OSC 151913	KJ878924	KJ878891	KJ878970	KJ879004
Ophiocordyceps melolonthae	OSC 110993	DQ522548	DQ518762	DQ522331	DQ522376
Ophiocordyceps myrmecophila	CEM1710	KJ878928	KJ878894	KJ878974	KJ879008
Ophiocordyceps neovolkiana	OSC 151903	KJ878930	KJ878896	KJ878976	KJ879010
Ophiocordyceps nutans	OSC 110994	DQ522549	DQ518763	DQ522333	DQ522378
Ophiocordyceps polyrhachis-furcata	P39	KJ201504	n/a	JN819003	n/a
	P51	KJ201505	n/a	JN819000	n/a
Ophiocordyceps ponerinarum	HUA 186140	KC610789	KC610767	KC610740	KF658668
Ophiocordyceps pruinosa	NHJ 12994	EU369106	EU369041	EU369024	EU369063
Ophiocordyceps pulvinata	TNS-F 30044	GU904208	n/a	GU904209	GU904210

Table 1. (Ctd).

		GenBank Accession numbers[2]			
Species	**Voucher Information[1]**	**SSU**	**LSU**	***tef1***	***rpb1***
Ophiocordyceps purpureostromata	TNS F18430	KJ878931	KJ878897	KJ878977	KJ879011
Ophiocordyceps rami	MY6736	KM655823	n/a	KJ201532	n/a
Ophiocordyceps rhizoidea	NHJ 12522	EF468970	EF468825	EF468764	EF468873
Ophiocordyceps septa	C41	KJ201525	n/a	JN819037	
Ophiocordyceps sobolifera	TNS F18521	KJ878933	KJ878898	KJ878979	KJ879013
Ophiocordyceps sphecocephala	OSC 110998	DQ522551	DQ518765	DQ522336	DQ522381
Ophiocordyceps stylophora	OSC 111000	DQ522552	DQ518766	DQ522337	DQ522382
Ophiocordyceps tiputini	QCNE 186287	KC610792	KC610773	KC610745	KF658671
Ophiocordyceps unilateralis s. str.	VIC 44303	KX713628	KX713626	KX713675	KX713730
	VIC 44354	KX713627	n/a	KX713676	KX713731
Ophiocordyceps unilateralis var. *clavata*	INPA 274589	KX713652	KX713600	KX713681	KX713708
	INPA 274590	KX713651	n/a	KX713682	KX713709
Ophiocordyceps variabilis	OSC 111003	EF468985	EF468839	EF468779	EF468885
Ophiocordyceps yakusimensis	HMAS_199604	KJ878938	KJ878902	n/a	KJ879018
Stilbella buquetii	HMAS_199617	KJ878940	KJ878905	KJ878985	KJ879020
Tolypocladium capitatum	OSC 71233	AY489689	AY489721	AY489615	AY489649
Tolypocladium japonicum	OSC 110991	DQ522547	DQ518761	DQ522330	DQ522375
Tolypocladium ophioglossoides	OSC 106405	AY489691	AY489723	AY489618	AY489652

[1]ARSEF, USDA-ARS Collection of Entomopathogenic Fungal Cultures, Ithaca, NY; ATR, BISP, G and OBIS abbreviations from D.P. Hughes personal collection, Penn State University, PA, USA; C, P and TL abbreviations follow those of Kobmoo *et al*. (2015); CEM from J. W. Spatafora lab collection, Oregon State University, OR, USA; EFCC, Entomopathogenic Fungal Culture Collection, Chuncheon, South Korea; HMAS, Chinese Academy of Sciences, Beijing, China; HUA, Herbarium Antioquia University, Medellin, Colombia; INPA, Herbarium of National Institute of Amazonian Research, Manaus, Brazil; MY, J.J. Luangsa-ard personal collection, BIOTEC, Thailand; NHJ, Nigel Hywel-Jones personal collection; OSC, Oregon State University Herbarium, Corvallis, OR; TNS, National Museum of Science and Nature, Tsukuba, Japan.

[2]SSU: partial small subunit (18S) nrRNA gene; LSU: partial large subunit (28S) nrRNA gene; *tef1*: partial translation elongation factor 1-α gene; *rpb1*: partial fragment of the largest subunit of the RNA polymerase II gene.

Camponotus species involved, will be necessary to clarify their taxonomic status.

The characteristic that distinguishes *O. unilateralis* from all the other zombie-ant species described, thus far, is the presence of both the A- and B-type phialides within the same hymenium of the stromatal clava (Fig. 2). Cylindrical, pinkish brown synnemata may also arise separately from the body and legs forming both phialide types. In other species, only the A-type phialides are produced in a compact hymenium at the tip of stromatal clava or on separate synnemata. This was named much later as *Hirsutella formicarum* on specimens from Guyana (Petch 1935): however, the description matches that of the *Hirsutella* B-type (conidia 9–11 × 2 µm), rather than the significantly smaller, limoniform conidia described by Kobayasi (1941), as well as by Petch (1924) from Asian collections. This led Mains (1951) to question the validity of *H. formicarum*: "it hardly seems possible that these are all conidial stages of *Cordyceps unilateralis*". We can now begin to understand why there was this disparity in the asexual morphs collected on different and geographically-separated ant hosts, as highlighted by subsequent publications (Evans & Samson 1984, Rombach & Roberts 1989). Evans & Samson (1984) also illustrated the asexual morph collected on *C. sericeiventris* in Honduras and showed that both A- and B-phialides occurred together on the same synnema; although the taxonomic significance of this character was overlooked at the time. The majority of this Honduran collection (~70 specimens) comprised infected ants exhibiting both the A- and B-asexual morphs described herein. These were found around the buttress base of tropical forest trees whilst others were located in the classic death-grip on nearby shrubs. The latter specimens were reported to have only the A-asexual morph, with morphologically distinct phialides and conidia (Evans & Samson 1984). The explanation for this variability of the fungus within a single ant species may lie in the recently-confirmed classification of *C. sericeiventris* into five subspecies, three of which have a purely Mesoamerican distribution (Bolton *et al*. 2007). Evidently, therefore, pathogen-host specificity may be even more complex than envisaged previously, but this will only be clarified by more comprehensive collections of infected *C. sericeiventris* from the Neotropics, specifically from Central America. We are confident, however, that the epitype named here is on the ant, *C. sericeiventris sericeiventris* (Bolton *et al*. 2007), whilst it is possible that novel taxa of *Ophiocordyceps* remain to be discovered on the other five ant subspecies. In addition, fresh material with mature ascostromata is still needed in order to determine the mode of ascospore germination in *O. unilateralis s. str.*, an overlooked but significant taxonomic trait in these fungi (Evans *et al*. 2011a, b).

Phylogenetic relationships

The topology recovered in this study is in agreement with previous publications (Sung *et al*. 2007, Quandt *et al*. 2014, Sanjuan *et al*. 2015). The *Ophiocordyceps unilateralis s. lat.* clade

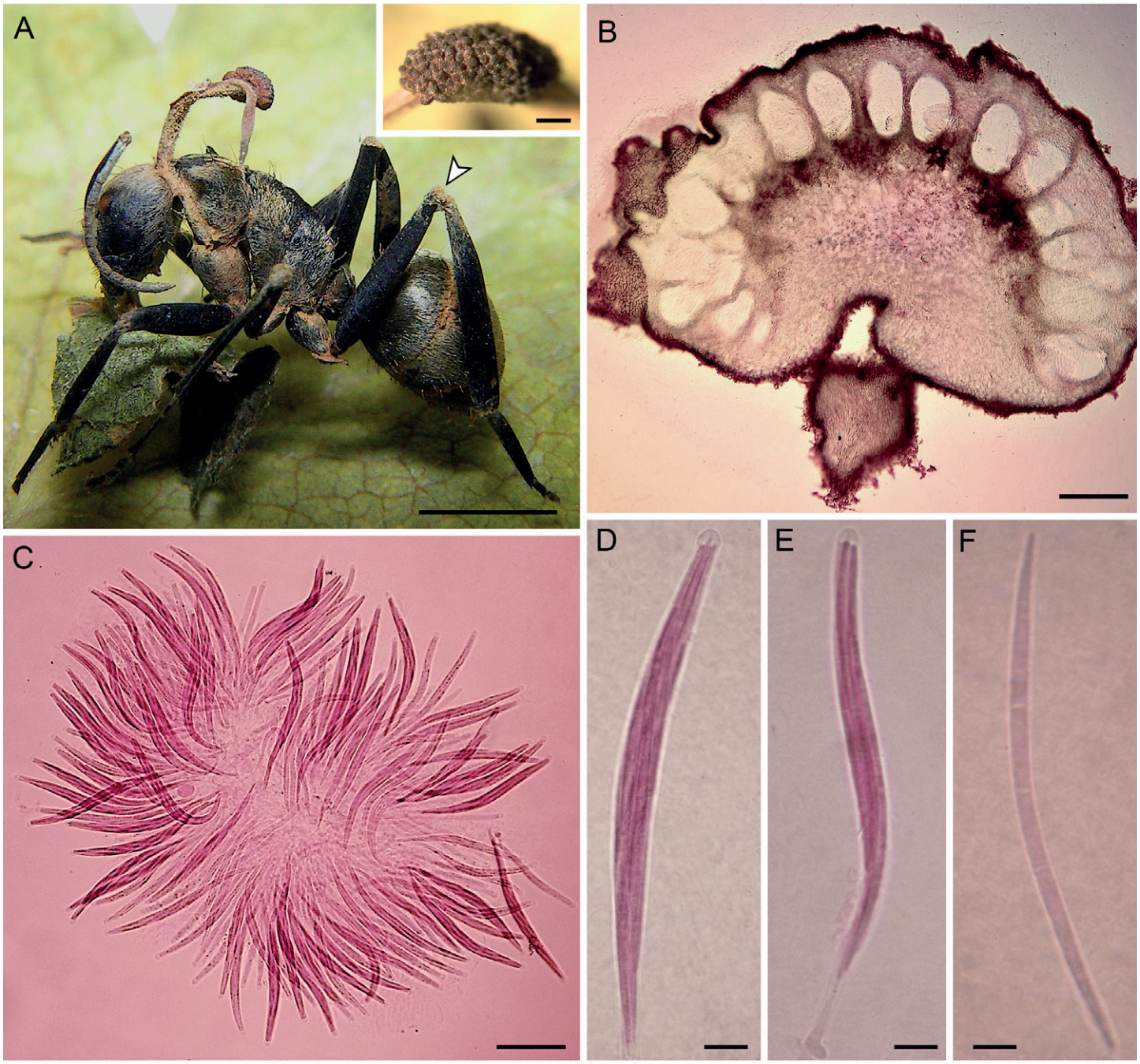

Fig. 1. *Ophiocordyceps unilateralis*, epitype (VIC 44303) on *Camponotus sericeiventris*. **A.** Golden carpenter ant biting into a leaf midrib, and the clava arising from the dorsal neck region with the unilateral ascostroma, arrow shows the sporodochium of the asexual morph (Bar = 3 mm); inset, showing details of the ascostroma (Bar = 0.8 mm). **B.** Section through the ascostroma, showing the crowded, partially erumpent ascomata (Bar = 200 µm). **C.** Asci en masse (Bar = 40 µm). **D–E.** Asci with the prominent apical cap and foot region (Bar = 10 µm). **F.** Ascospore (Bar = 8 µm).

was strongly supported (bs = 100 %). The proposed epitype – infecting *C. sericeiventris* – was strongly resolved, forming a sub-clade (bs = 75 %) with *O. camponoti-rufipedis*, which is a species native to the same geographic and ecological region as *O. unilateralis s. str.*, the Zona da Mata Mineira in the Atlantic rainforest of south-east Brazil.

DISCUSSION

Our phylogenetic results corroborate previous studies regarding the monophyly of *Ophiocordyceps unilateralis* core clade (bs = 100 %) (Araújo *et al*. 2015, 2018, Sanjuán *et al*. 2015). The clade shares numerous apomorphic traits, including: having ants of the tribe Camponotini as hosts; the ability to manipulate host behaviour resulting in biting into subaxial surfaces of leaves or twigs; producing multiple asexual morphs and; forming capillisporophores and capillispores during ascospore germination (Evans *et al*. 2011a, b). Besides the morphological evidence that characterises the epitype proposed herein, *Ophiocordyceps unilateralis s. str*., we also demonstrate that this species is unique at the molecular level. Our analysis shows that *O. unilateralis s. str*. sits within the New World clade (Fig. 3) sister to another species from the Atlantic rainforest, *O. camponoti-rufipedis* (bs = 75 %). However, within the New World subclade – composed of species from Atlantic and Amazon rainforests –

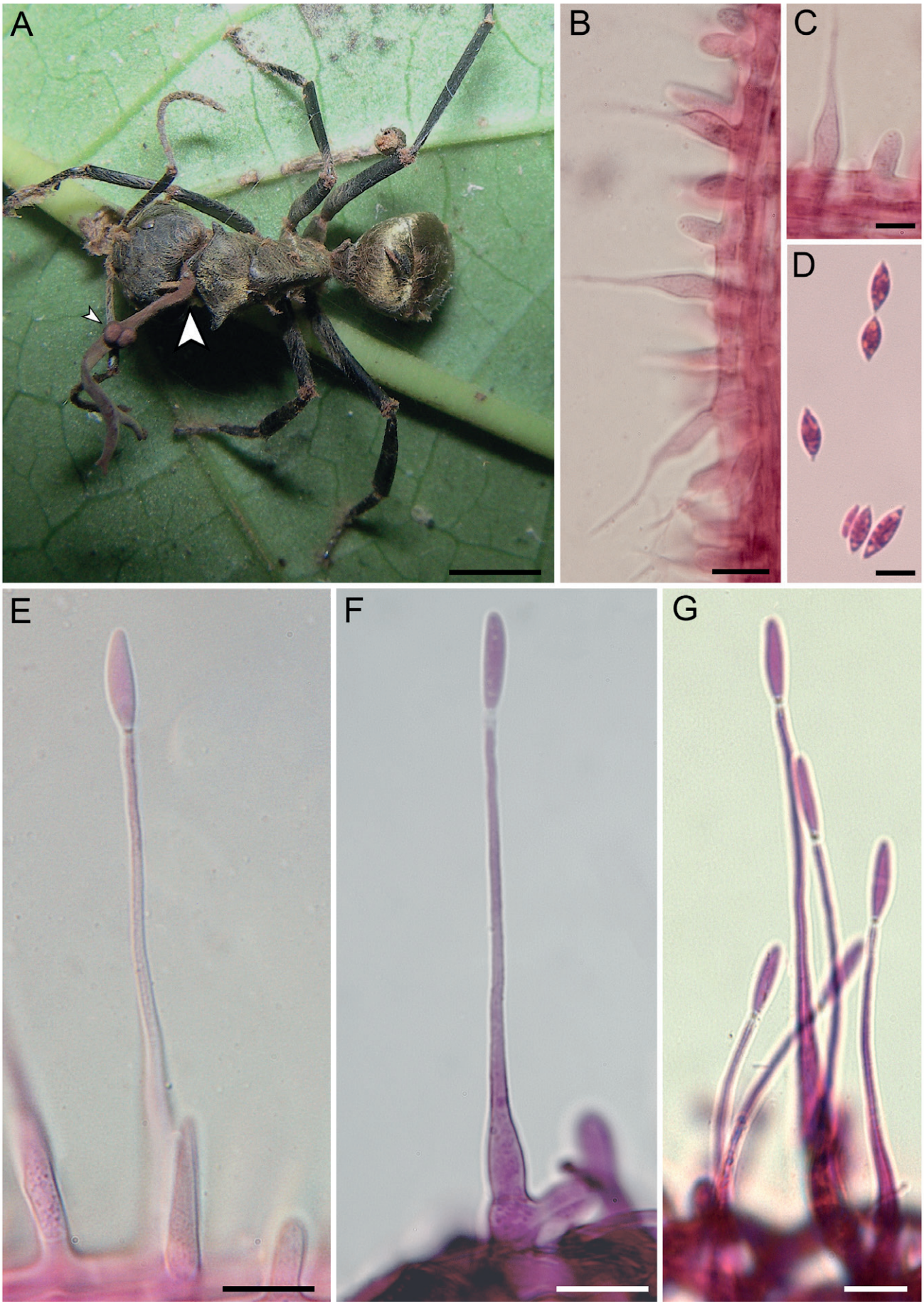

Fig. 2. Asexual morphs of *Ophiocordyceps unilateralis*, based on paratype (VIC 44350). **A.** *Camponotus sericeiventris* biting into midrib of shrub leaf, showing the clava emerging from the dorsal pronotum (large arrow) and the immature ascostromata forming laterally (small arrow) (Bar = 2.5 mm). **B–C.** Apical region of clava showing the A-phialides (Bar = 10 µm); **D**. Limoniform A-conidia (Bar = 7 µm). **E.** B-phialide from apical region of clava emerging from neck (Bar = 12 µm). **F–G.** B-phialides from sporodochium emerging from leg joint (Bars = 12 and 20 µm).

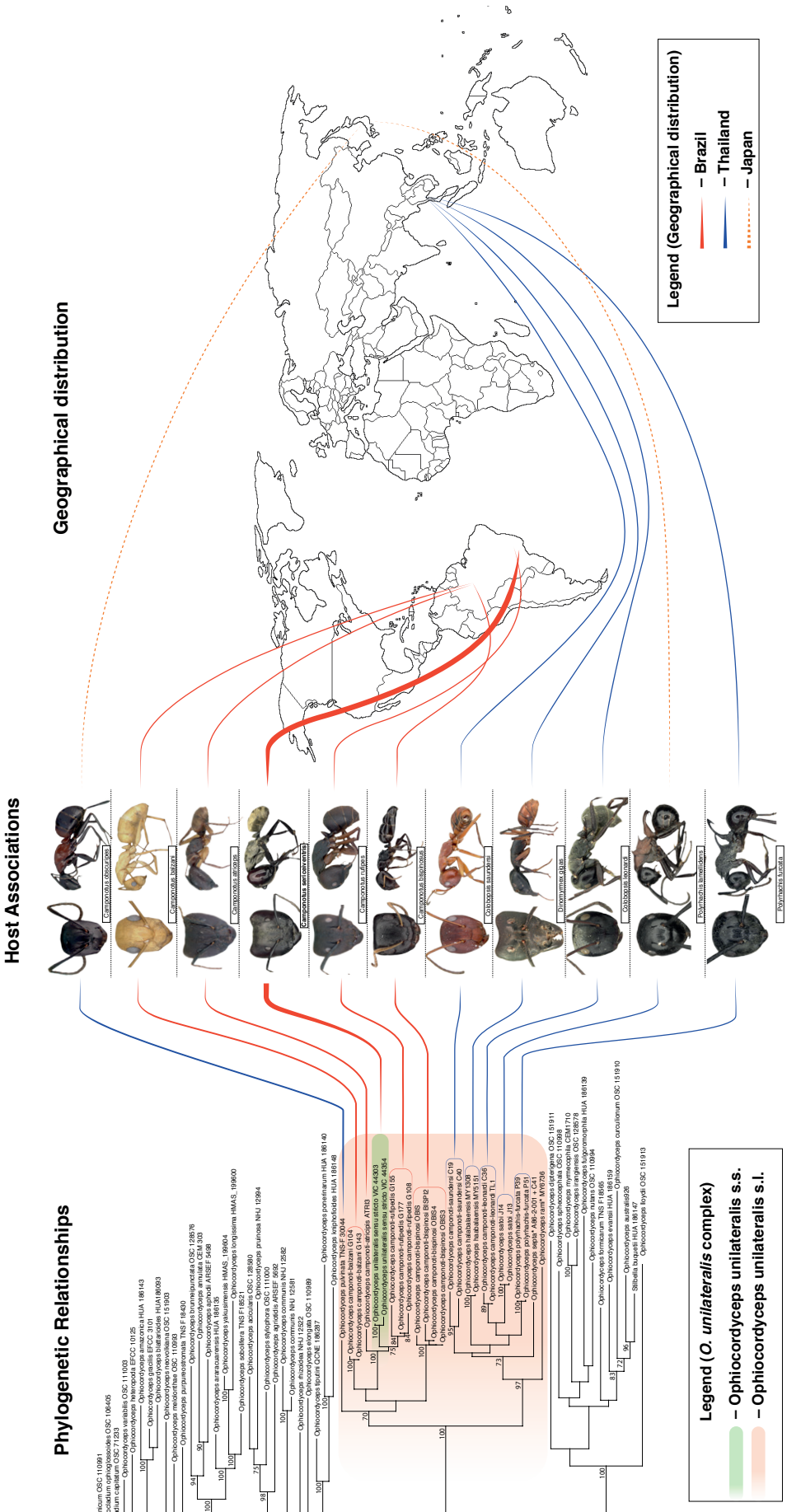

Fig. 3. Phylogeny, host association and geographic distribution of species within the *Ophiocordyceps unilateralis* complex. Phylogeny of *Ophiocordyceps* from the ML analysis obtained using RAxML to analyse a concatenated dataset of four loci (SSU, LSU, *tef1* and *rpb1*). The *O. unilateralis sensu lato* clade is highlighted in orange and the proposed epitype (*O. unilateralis sensu stricto*) line is bold. The host association and geographical distribution is also presented. Ant images from www.AntWeb.org and the photographers: April Nobile (*Camponotus obscuripes*, *Ca. balzani*, *Ca. atriceps*, *Ca. rufipes*, *Dinomyrmex gigas*), Will Ericson (*Ca. serviceiventris*, *Polyrhachis lamellidens*), Estella Ortega (*Ca. bispinosus*), Michael Branstetter (*Colobopsis saundersi*), Zach Lieberman (*Co. leonardi*, *Po. furcata*).

there is no clustering of species according to the region. Further studies, including more species from different continents, are helping to resolve the relationships within this clade (Araújo *et al*. 2018).

With the selection and re-description of the epitype of *Ophiocordyceps unilateralis*, it is now possible to construct a more meaningful phylogenetic tree for the *O. unilateralis* clade. Previously, trees were constructed using a sequence of the fungus from an unidentified ant in the herbarium of the Oregon State University (OSC 128574) (Sung *et al*. 2007, Kepler *et al*. 2010, Araújo *et al*. 2015, Kobmoo *et al*. 2015). This will be critical as more new species are identified within the *O. unilateralis* complex and we begin to understand more about the intricacies of the pathogen-host relationship. None more so than within the type of *O. unilateralis* on *Camponotus sericeiventris*, in which the evidence from Honduran collections suggests that different subspecies of the ant occur within the same forest habitat and that this is reflected in different death positions of the infected ants, as well as in morphological variation within the fungal pathogen. In order to coexist, the ant subspecies must occupy different niches within this ecosystem and, therefore, the fungus may also have evolved at the subspecies level with different morphological (spore forms) and physiological (neurotoxins) traits to maximize infection.

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