

## **Orbilia jesu-laurae (Ascomycota, Orbiliomycetes), a new species of neotropical nematode-trapping fungus from Puerto Rico, supported by morphology and molecular phylogenetics**

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## *Orbilina jesu-laurae* (Ascomycota, Orbiliomycetes), a new species of neotropical nematode-trapping fungus from Puerto Rico, supported by morphology and molecular phylogenetics

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**Abstract:** *Orbilina jesu-laurae* is a new species of nematode-trapping fungus found on decorticated angiosperm wood in a tropical rainforest in Puerto Rico. The single specimen was studied from fresh apothecia and cultures. Morphology was studied and phylogenetic analysis (rDNA: ITS and LSU) was conducted using both sexual and asexual morphs. Nematodes were added to cultures to verify the formation and morphology of the trapping structures. Our results show that the species is in the *Arthrobotrys* clade, the phylogenetically closest relative being a possibly Mexican genotype with unknown morphology, erroneously referred to as *Arthrobotrys musiformis* in GenBank. Macro- and micromorphological, ecological and biogeographic data are provided along with a discussion of closely related species.

**Key words:** adhesive networks, *Arthrobotrys*, *Ascomycota*, Caribbean, ITS, LSU, morphology, nematodes, Neotropics, new species, *Orbilina*, *Orbiliomycetes*, phylogeny, Puerto Rico

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## Introduction

Fungi are recognized worldwide as saprobes, parasites and mutualists, but a small percentage (0.5%) of *Mucoromycota*, *Basidiomycota* and *Ascomycota* are carnivorous (Yang & al. 2012; Spatafora & al. 2016). Fungi with this lifestyle often occur in microhabitats with low concentrations of available nitrogen. In such situations they capture and consume small animals and thus can colonize available substrates (Yang & al. 2012). Carnivorous *Ascomycota* produce complex trapping structures such as constricting rings, sessile adhesive knobs and adhesive networks (Yang & al. 2012). Examples of all these trapping structures are found in association with the asexual

states of the genus *Orbilina* Fr. Based on their conidial and trapping states, all those taxa that produce adhesive networks were previously referred to *Arthrobotrys* Corda, originally described in 1839, three years after Fries published the genus *Orbilina*. Baral & al. (2018) treated these fungi as an unpublished series within the genus *Orbilina*. Because life cycles in some fungi are polymorphic and include both meiosporic and mitosporic structures (teleomorphs and anamorphs), occurring separately in space and time, the same species may have been studied and named independently. In the present case of *Arthrobotrys*, the first teleomorphic state was elucidated by Pfister (1997). The genus *Arthrobotrys*, originally characterized by 1-septate conidia produced in single or superposed

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clusters on mononematous conidiophores, is a good example of the problem of dual names. If one is recognizing the genus *Orbilina* in a wide sense, then under the one-fungus-one-name rule the name *Orbilina* takes precedence over *Arthrobotrys*. Members of *Orbilina* with predacious capabilities together with some non-predacious taxa form a monophyletic group, here referred to as the *Arthrobotrys* clade. A majority of species in the *Arthrobotrys* clade capture and consume copepods, mites, collembola, dipterans, but primarily nematodes (Baral & al. 2018). They have been tested for possible use in biocontrol of pests (Niu & Zhang 2011). The *Arthrobotrys* clade as treated by Baral & al. (2018) includes five unpublished infrageneric taxa, which are basically equivalent to the five previously described genera *Arthrobotrys*, *Dactylella* Grove, *Dactylellina* Morelet, *Drechslerella* Subram. and *Gamsyella* Scholler & al. Of these genera, four are predacious and monophyletic, whereas *Dactylella*, which comprises all non-predacious members of the group, is paraphyletic according to Baral & al. (2018). More than 100 species have been published in the genus *Arthrobotrys*. However, Scholler & al. (1999) recognized only 46 species in the genus when newly circumscribed to include those species with three-dimensional adhesive networks. Teleomorphs of the *Arthrobotrys* clade are mostly characterized by narrow, subulate and curved, falcate ascospores with a small, apical spore body (Baral & al. in press).

Fungal diversity has only rudimentary documentation for the Caribbean Islands, although the region is one of the world's biodiversity hotspots (Myers & al. 2000). Fungal species richness in Puerto Rico has been better explored in some localities compared to other islands. According to the locality index (<http://www.cybertruffle.org.uk/>), no orbiliaceous fungi have been reported in a preserved area near San Juan National Park (Julio Enrique Monagas National Park). Little is known about the diversity of *Orbiliomycetes* from Puerto Rico. Cantrell & Lodge (2008) compiled a list of the fungi from Puerto Rico, and only mentioned four species of *Orbilina*: *O. andina* Pat., *O. chrysocoma* (Bull.) Sacc., *O. delicatula* (P. Karst.) P. Karst. (as "*O. delica*" in error) and *O. cf. gailardii* Sacc. Up to now, 14 species of *Orbiliomycetes* have been verified from Puerto Rico by Baral & al. (in press) in their monographic work on the class. The names listed by Cantrell & Lodge (2008) are to be considered as doubtful due to the lack of any morphological or specimen data. Some occurrences were missed in their list, for example *O. pilosa* (Dennis) Baral [Pfister 1997, as "*Orbilina* sp./*Monacrosporium* ? *doedycoides*" (Drechsler) R. C. Cooke & C. H. Dickinson)]. On the other hand, Pfister collected some other species that he did not publish, such as *Orbilina* sp. (PR-98-20, culture #256; Pfister pers. comm.) and *O. tenuissima* Speg. (PR-11), ascospore isolates from which were produced *Arthrobotrys*-like anamorphs. However, these collections were not studied in detail (except for the teleomorph of PR-11). PR-98-20 was identified as *Arthrobotrys musiformis* Drechsler by Pfister, but cultures

are now unavailable. These three species represent the only known Puerto Rican reports of species belonging to the nematode-trapping *Arthrobotrys* clade.

During a field trip of the ascomycete workshop arranged in connection with the 11<sup>th</sup> International Mycological Congress held in Puerto Rico in 2018, several collections of *Orbiliomycetes* were made in the Julio Enrique Monagas National Park. One of them caught the attention of the first author due to its distinctive morphological features. This species was obviously not reported by Cantrell & Lodge (2008) or Pfister (1997). The morphology of the asci and ascospores clearly indicates a relationship to *Orbilina auricolor* (A. Bloxam) Sacc. and related species of the *Arthrobotrys* clade. The aim of this investigation is to describe this apparently new species and to provide morphological, biogeographical and phylogenetic evidence for its distinction.

## Material and methods

The specimen was collected in Bayamón, Puerto Rico, on 15 July 2015 in the Julio Enrique Monagas National Park. The collection was air-dried and subsequently deposited in the Farlow Herbarium, Harvard University (FH). Months later, pieces of wood with the apothecia were placed on a black matboard and rehydrated with a spray bottle for macrophotography. This was done with a Canon EOS 60d digital SLR camera using a Canon EF-S 60 mm macro lens. An Olympus SZX9 stereomicroscope was employed to observe and characterize macromorphology and to perform hand-sectioning. For micromorphological observations a Motic B1 light microscope was used. Digital images were taken with a USB Moticam 2500 camera and biometry was done with the software Motic images Plus 2.0. For each informative morphological feature, 10–30 photographs were taken prior to biometric analysis. The living or dead state of the cells was determined based on the findings of Baral (1992). Mounting media employed were tap water (H<sub>2</sub>O) for observing living cells, Congo red (CR) to raise wall contrast, particularly of dead cells, potassium hydroxide 5% (KOH) for killing cells or rehydrating dead specimens, and Melzer's reagent (MLZ) for exploring amyloid or dextrinoid reactions. The symbols and abbreviations were adopted from Baral (1992): \* = living state; † = dead state; \*† = living and dead state (no difference noted); SCBs = KOH-soluble cytoplasmic bodies; SBs = spore bodies; VBs = refractive vacuolar bodies; LBs = lipid bodies. Colour coding refers to Anonymous (1976).

Characters of the asexual morphs are presented in this study as well as those of the sexual morph. Difco potato dextrose agar (PDA) and corn meal agar (CMA) were prepared according to manufacturer's instructions and used to culture the spores from our specimen and obtain the asexual morph. The substratum bearing one apothecium was placed on the inner surface of the lid of a Petri

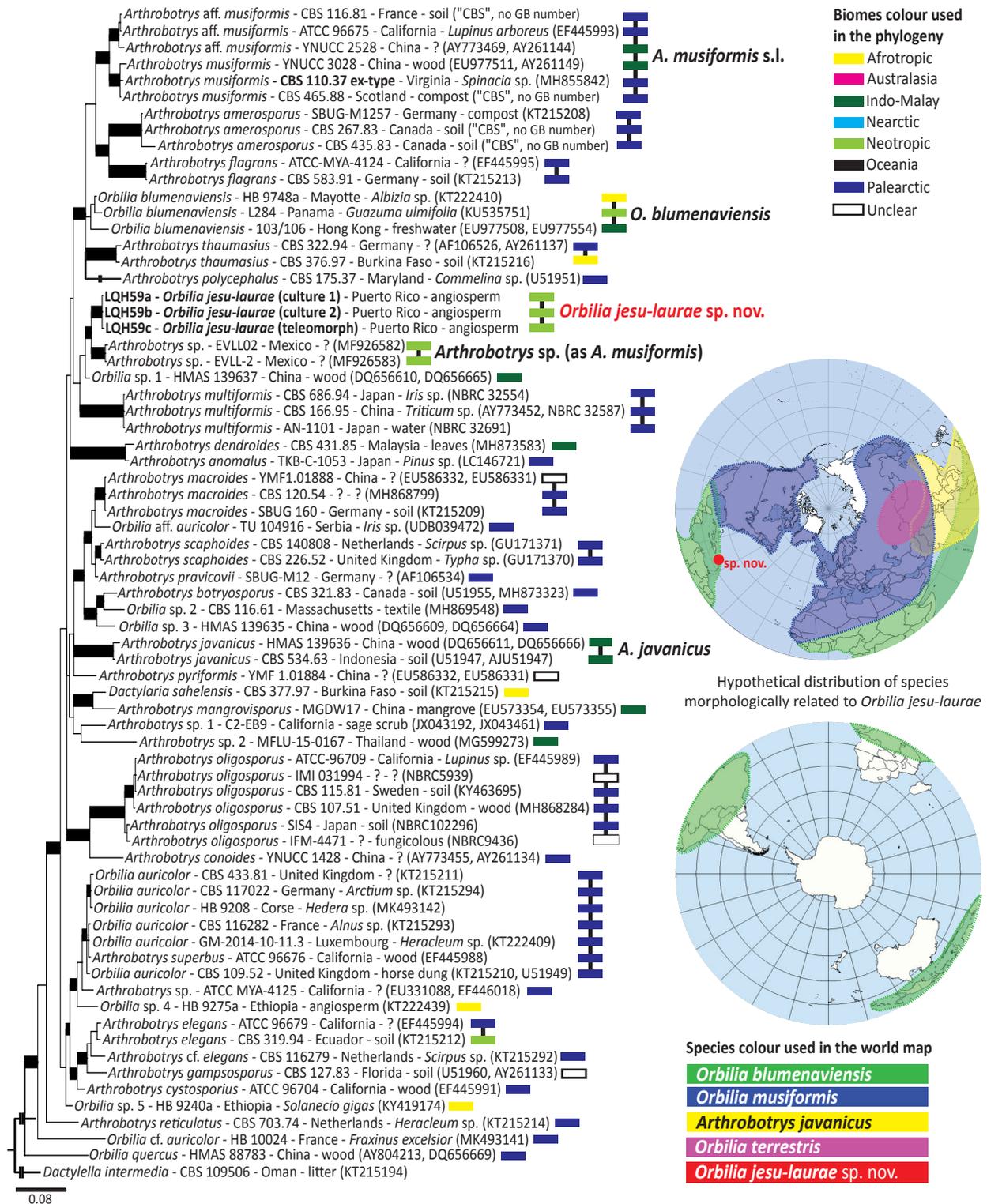


Fig. 1. Bayesian majority-rule consensus tree of the *Arthrobotrys* clade sensu Baral & al. (in press) based on ITS1-5.8S-ITS2 + LSU. Thickened branches are those with support above 0.95. Each taxon included in the phylogeny contains the following notation: species name + isolate number + geographical origin + substrate/host + GenBank accession numbers of sequences. Rectangles with colours to right of taxon information represent biomes (legend at upper right). Hypothetical distribution of taxa morphologically most similar to *Orbilbia jesu-laurae* in N and S hemisphere represented with colours (legend at bottom right).

dish on a small piece of dampened filter paper. The bottom of the Petri dish containing the medium was inverted, so the surface of the PDA or CMA was facing down. The

progress of ascospore discharge was checked under the stereomicroscope after several hours. Once spores were deposited on the medium, the lid was replaced with a new

one and the apothecia from which the spores were ejected were used for DNA extraction. Once mycelia covered the media, subcultures were made. DNA was also extracted from cultures. Nematodes (*Caenorhabditis elegans* Mau-pas) were added to some of the subcultures to observe the formation of traps. A block of PDA or CMA covered with hyphae and one from a nematode-infested culture were placed facing down and opposing each other at some distance in a new Petri dish of CMA to observe trap formation. The culture was monitored over 24 hours to observe the formation of the traps and capture of nematodes and to observe the different stages of the process. A series of images was made over two days at 40× magnification using the same tools explained above.

DNA was extracted using Qiagen QIAamp DNA Micro Kit from mycelia from cultures and from apothecia according to the manufacturer's instructions adding a period of 24 hours of incubation in the lysis buffer at 56°C. DNA extract dilutions of 1/10 were used to perform PCR amplification of the entire internal transcribed spacer region (ITS1-5.8S-ITS2) and partial nuclear large subunit (LSU). Primers, TAQ and information on quantities for PCR followed Karekehian & al. (2019). PCR products were sequenced by GENEWIZ (Cambridge, Massachusetts) using the same primer pairs. Quality of the sequences was checked in Geneious v.6.1.7. and they were checked for contamination using BLASTn (Altschul & al. 1990). Phylogenetic analyses were done using two rDNA regions (ITS and LSU) for species of the *Arthrobotrys* clade. Sequences were taken from GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>). For details about species and GenBank accession numbers see Fig. 1. The constructed alignment (see Supplemental content online) included sequences of 71 isolates in the *Arthrobotrys* clade. The sequences were aligned using Mafft v7.017 (Katoh & al. 2002). Gblocks v. 0.91 was used to identify and eliminate ambiguously aligned regions (Castresana 2000). The analyses were performed using the optimal model of nucleotide substitution (Jmodeltest; Posada 2008). Bayesian inference (BI) was done using Geneious v. 6.1.7. For more details about phylogenetic methods see Quijada & al. (2014, 2017). Adobe Illustrator CS5 was used to assemble figures. Names of the biomes for the biogeographic analyses follow Olson & al. (2001).

## Results

*Orbilina jesu-laurae* Quijada, Beltrán-Tej., Pfister & Baral, **sp. nov.** – Fig. 2, 3, 4, 5.  
Mycobank: MB 833713.

Holotype: Puerto Rico, Julio Enrique Monagas National Park, Bayamón, 18.4093°N, 66.1408°W, 15 m a.s.l., on decorticated branch of unidentified angiosperm on ground, 15 Jul 2018, *L. Quijada LQH-59c* (FH! [sexual morph]). Ex-type cultures were dried down and accessioned as specimens in FH.

GenBank numbers of sequences from sexual morph: ITS = MN816818, LSU = MN816821.

GenBank numbers of sequences from asexual morph: obtained on PDA (*LQH-59a!*) ITS = MN816816, LSU = MN816819; obtained on CMA (*LQH-59b!*) ITS = MN816817, LSU = MN816820.

*Diagnosis* — *Orbilina jesu-laurae* differs from *O. blumenaviensis* (Henn.) Baral & E. Weber by mammiform paraphyses apices with abrupt beaks and often several beaks at each tip (terminal and lateral), and in thinner apothecia (0.1–0.2 mm) but of a similar size (1–2 mm). In the asexual morph, *O. jesu-laurae* has shorter and narrower conidiophores and consistently (0 or) 1-septate conidia of smaller size (mainly \*20–22 × 10–11 μm) compared to *O. blumenaviensis*.

*Description* — *Sexual morph*: apothecia rehydrated 1–1.5(–2) mm diam., 0.1–0.2 mm thick, light brown (57.1.Br) to medium orange-brown (53.m.O), round to somewhat undulating, scattered to subgregarious; disc flat, margin thin to thick, ± smooth, slightly protruding, sessile on a broad base, not erumpent. Asci \*(40.5–)45.5–51.5(–64) × 3–4 μm, †(35.5–)38.5–44(–47) × 2.5–3.5 μm, 8-spored, 2–4 lower spores inverted, pars sporifera \*(12–)21–25.5(–30) μm; apex †strongly truncate (not indented, not inflated), thin-walled; base with short to medium long stalks, h- or H-shaped. Ascospores \*(10–)10.6–11.4(–11.8) × 0.9–1.2 μm, †(8.8–)9.6–10.5(–12) × 0.9–1.1 μm, actual length 10–12.5 μm, narrowly subcylindric to subulate, slightly tapered above, more distinctly so below, with rounded to obtuse poles, medium to strongly curved (less curved in dead state); SBs \*1.8–2.3 × 0.4–0.8 μm, rod-shaped, attached to apex by a short filum. Paraphyses apically uninflated or often slightly to moderately thickened, lageniform-mammiform with cylindric beaks of \*2.7–4.5 × (1.5–)1.7–2(–2.5) μm with rounded tips, apically frequently bi- or trifurcate with 1 or 2 shorter lateral beaks, terminal cell \*(8.5–)18.5–22.5(–27.5) × (1.5–)2.5–3.5(–4) μm, protruding 3–5 μm beyond living asci, lower cells \*(6–)8–9.5(–11.5) × (1.5–)2–2.5 μm, sometimes branched. Medullary excipulum not differentiated from subhymenium, \*†8–25 μm thick, of dense *textura intricata*, medium to sharply delimited from ectal excipulum. Ectal excipulum composed of vertically oriented *t. globulosa-angularis-prismatica*, at base only of *t. globulosa*, \*†67–155 μm thick, differentiated into layers along flanks and margin. Inner layer of thin-walled *t. globulosa-angularis* extending from lower to upper flanks, \*†(9–)15–30(–64) μm thick, hyaline, cells at lower and upper flank \*(7.5–)13.5–17.5(–21.5) × (7–)10–13(–16.5) μm. Cortical layer of thick-walled (0.5–1.5 μm) *t. angularis-prismatica* extending from lower flank to margin, \*†(8–)12–16(–35) μm thick, deep yellow (88.d.Y) to light olive brown (94.1.OlBr), walls strongly stained red-brown in MLZ, of 2 or 3 rows of

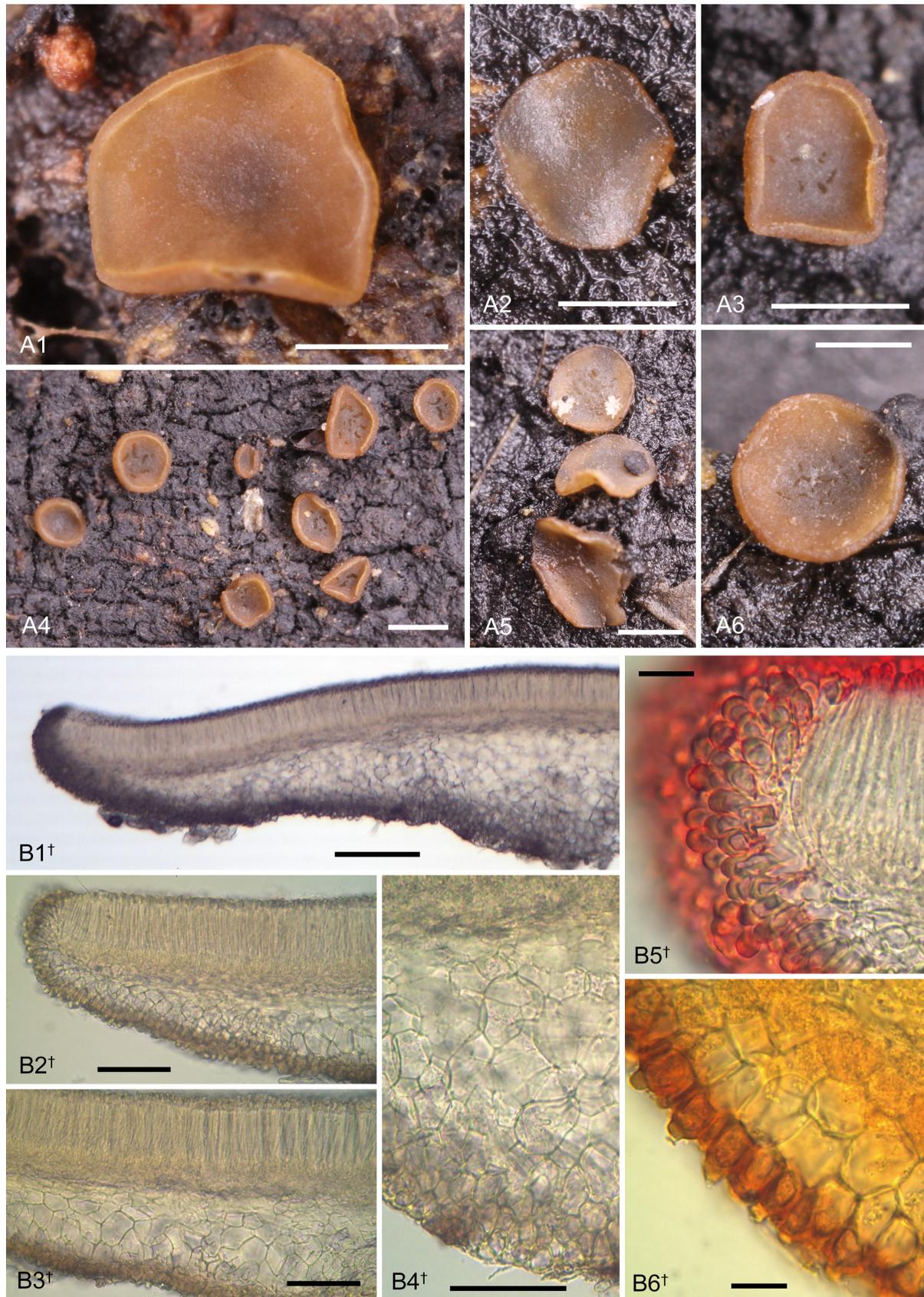


Fig. 2. Macromorphology and tissues in section of *Orbilia jesu-laurae* – A1–6: rehydrated apothecia on substrate; B1: transverse section of apothecia; B2–4: details of excipulum at margin, upper and lower flank; B5–B6: cells of cortical layer at margin and upper flank; B5: glassy processes stained in Congo red; B6: dextrinoid reaction or cortical cells in MLZ. – Reagents: B1–4 = H<sub>2</sub>O; B5 = CR; B6 = MLZ. – Scale bars: A1–6 = 500 µm; B1 = 100 µm; B2–4 = 50 µm; B5, 6 = 10 µm. – State: † = dead.

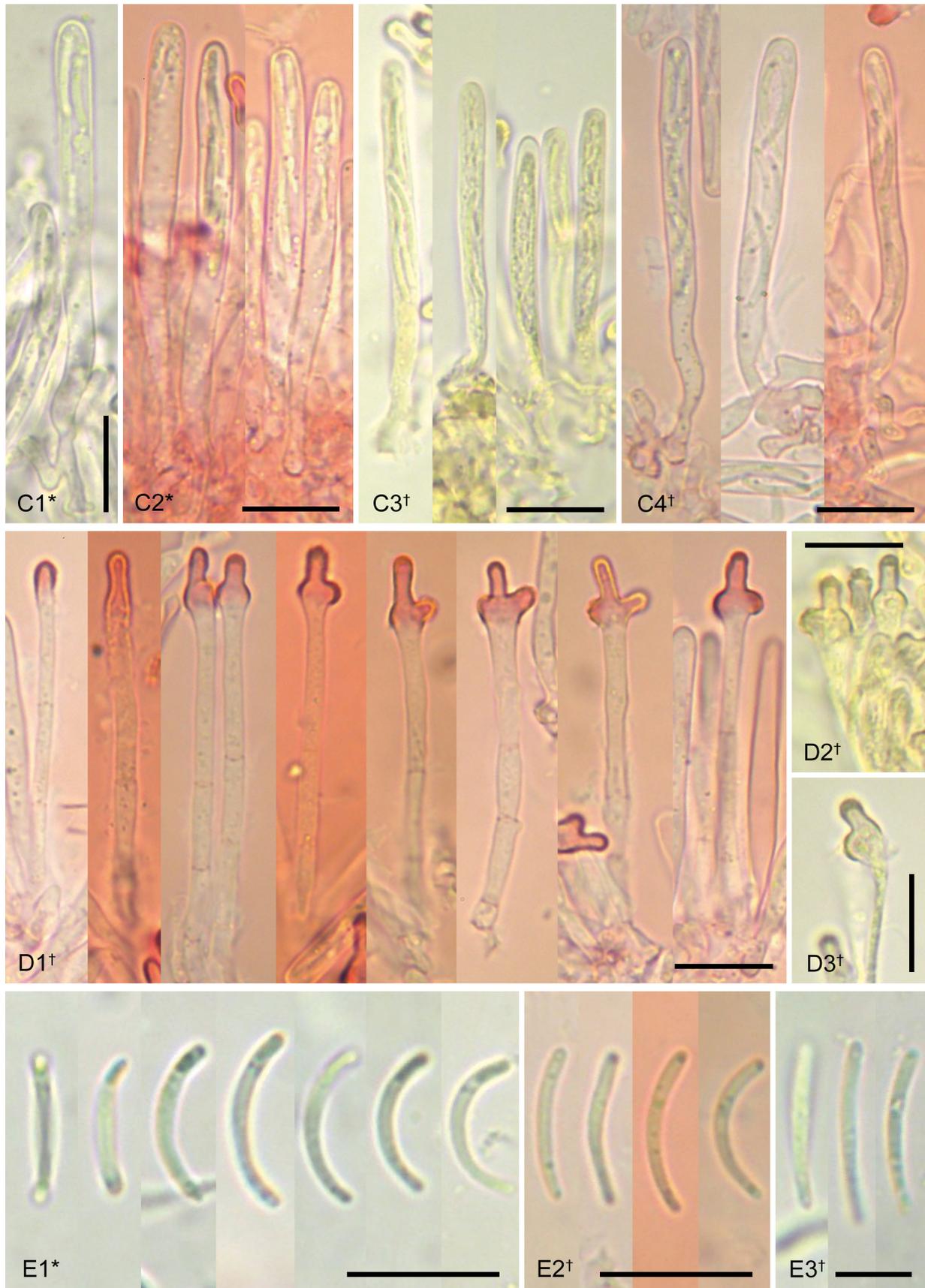


Fig. 3. Asci, paraphyses and ascospores of *Orbilia jesu-laurae* – C1–4: living and dead asci; D1–3: morphological variation of paraphyses, with one or three cylindrical beaks; E1–3: living and dead ascospores. – Reagents: C1, E1 =  $H_2O$ ; C2, C4, D1, E2 = CR; C3, D2, 3 = MLZ; E3 = KOH. – Scale bars: all = 10  $\mu m$ . – States: \* = living; † = dead.

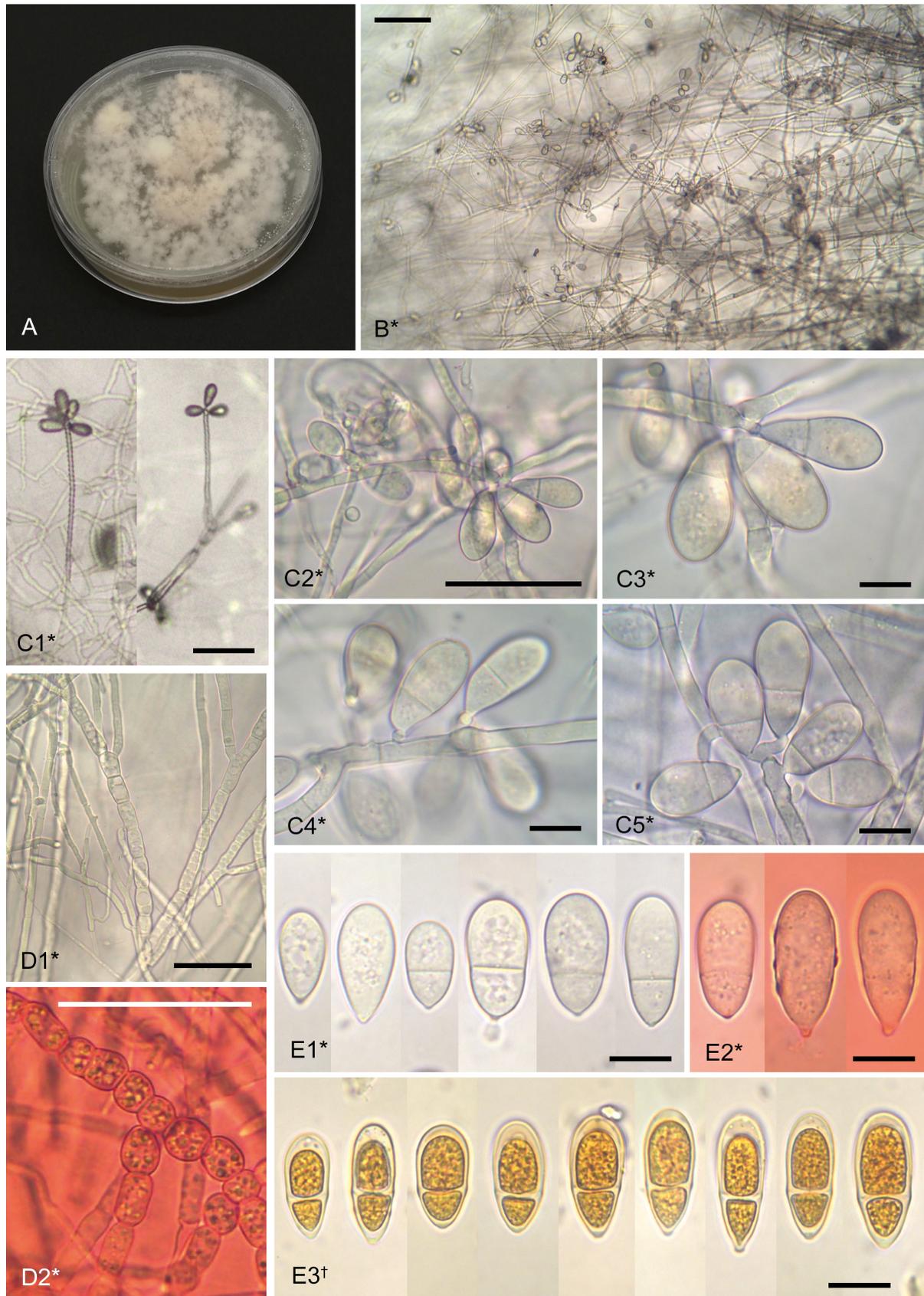


Fig. 4. Asexual morph of *Orbilia jesu-laurae* in culture – A: macromorphological aspect of colony in PDA; B: general view of vegetative hyphae, conidiophores and conidia; C1–5: conidiophores with attached conidia; D1: vegetative hyphae; D2: chlamydospores; E1–3: conidia. – Reagents: B, C1–5, D1, E1 = H<sub>2</sub>O; E2, D2 = CR; E3 = MLZ. – Scale bars: B = 100 µm; C1, D1, 2 = 50 µm; C2–5, E1–3 = 10 µm. – States: \* = living; † = dead.

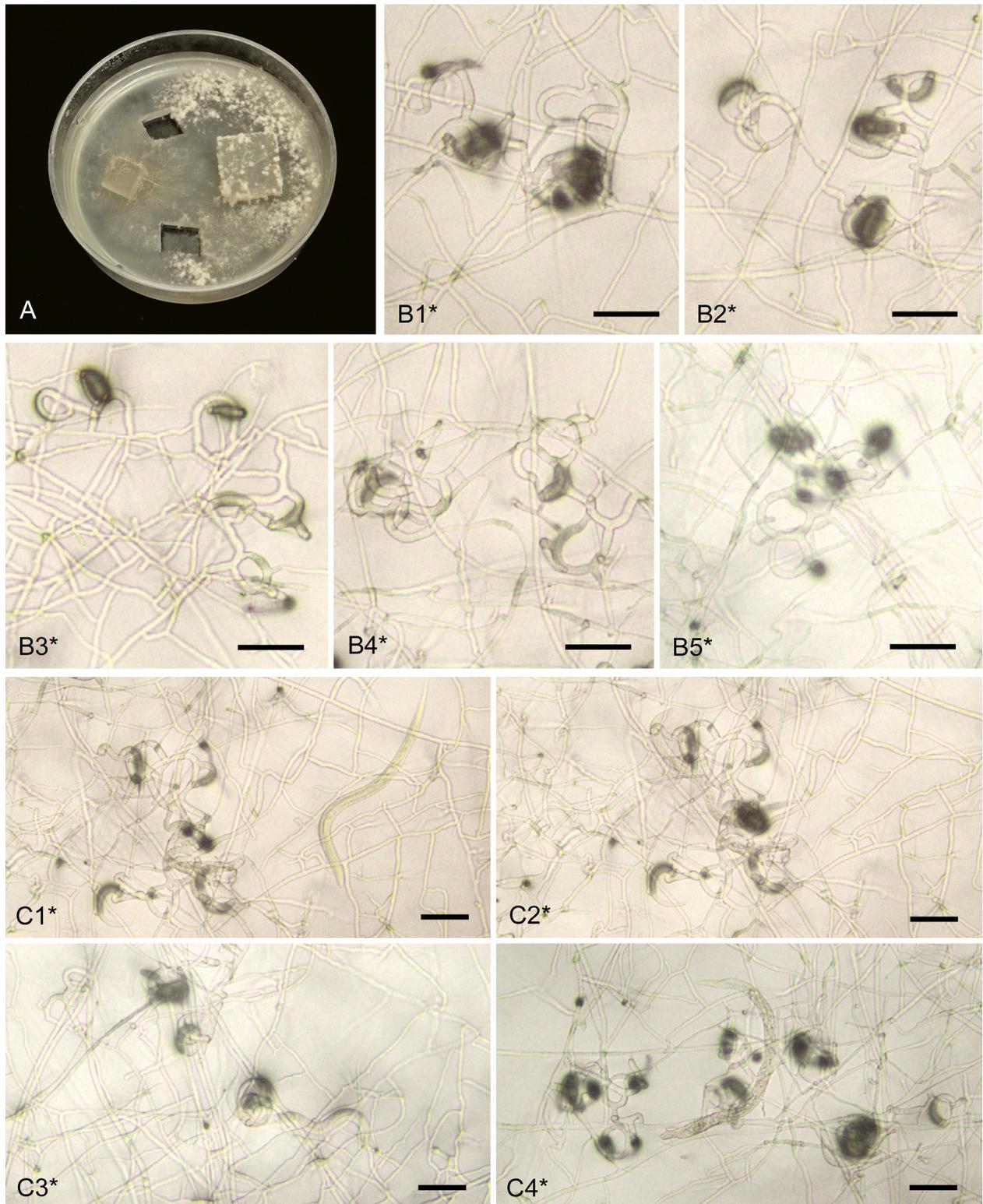


Fig. 5. A: asexual morph of *Orbilina jesu-laurae* in culture formed in contact with cultured nematodes; B1–5: three-dimensional adhesive networks formed after one day in contact with nematodes; C1: living nematodes close to traps; C2, 3: living nematodes trapped; C4: dead nematode with hyphae inside nematode. – Scale bars: B1–5, C1–4 = 50  $\mu\text{m}$ . – State: \* = living.

cells from lower to upper flank, cells \*(7–)8.5–10(–11.5)  $\times$  (4.5–)7–8.5(–9)  $\mu\text{m}$ , at margin with only 1 or 2 rows of cells, \*(5–)6.5–8(–9)  $\times$  (2.5–)3.5–4.5(–5)  $\mu\text{m}$ . Outermost cells from lower flank to margin covered by strong yellow

low (84.s.Y) to deep yellow (85.deepY) refractive glassy exudate, \*†1–2(–2.5)  $\mu\text{m}$ . LBs sparse and small, only observed in lower cells of paraphyses and ectal excipular cells, SCBs or VBs absent. *Asexual morph*: *Arthro-*

*botrys*-like. Vegetative hyphae  $*(1-3.5-5(-10)) \mu\text{m}$  wide, chlamydospores present, hyaline, smooth, multi-guttulate,  $*(8.5-9.5-11.5(-13)) \times (7.5-8-9.5(-10.5)) \mu\text{m}$ . Conidiophores erect,  $*90-200 \mu\text{m}$  long,  $4-6 \mu\text{m}$  wide at base,  $3-4 \mu\text{m}$  at apex, branched only at apex,  $(1-3)$  or  $4(-6)$  conidia formed on inconspicuous nodules or distinct denticles ( $2.5-5 \times 2-3.5 \mu\text{m}$ ) at hardly swollen apex. Conidia obovoid to obpyriform,  $*(14-20-22(-26)) \times (7.5-10-11(-13)) \mu\text{m}$ ,  $\dagger(16.5-18-19.5(-22)) \times (6-7.5-8(-9)) \mu\text{m}$ , consistently 1-septate (rarely aseptate), often slightly constricted at septum, upper cell wider and longer than lower cell, containing groups of non-refractive vacuoles and a few small LBs. Trapping nematodes by 3-dimensional adhesive networks with loops with an inner diameter of  $*(12.5-23-30(-47.5)) \mu\text{m}$ , loop cells  $*(15.5-20-30(-36)) \times 6.5-8 \mu\text{m}$ , formed after adding nematodes.

**Phenology** — Fruit bodies collected in the wet season (July); living asci and ascospores observed when rehydrated after 2–3 weeks (desiccation-tolerant).

**Distribution and ecology** — So far known from a single collection in the Neotropics, in a lowland tropical rain forest of northeastern Puerto Rico on unidentified angiosperm wood on the ground.

**Etymology** — The specific epithet refers to Jesús Laura Rodríguez Armas in recognition of her many contributions to mycology, of her work in education and of our friendship.

## Discussion

The *Arthrobotrys* clade was proposed in Baral & al. (2018) for a subgroup of *Orbilina* with predominantly nematode-trapping capabilities using diverse types of trapping organs. Species in this clade (Baral & al. 2018) are characterized by narrowly sickle-shaped (Fig. 3, E1–3), rod-shaped or ellipsoid ascospores and apothecia being either tolerant of or sensitive to desiccation (Baral & al. in press; Quijada & Baral 2017). The connection between an *Orbilina* teleomorph and an *Arthrobotrys* state was reported for the first time by Pfister (1994). Only three years later, ITS phylogenetic analysis showed that trapping organs reflect evolutionary relationships in this group of fungi (Liou & Tzean 1997). Here, we use cultures obtained from ascospores to link an anamorph (Fig. 4) to the Puerto Rican apothecial collection and demonstrate the type of trapping organs formed (Fig. 5). Morphology of both teleomorph and anamorph was studied, and sequence comparisons supported the connection (Fig. 1). None of the species reported from Puerto Rico in the *Arthrobotrys* clade (Pfister 1997; Pfister pers. comm.; Cantrell & Lodge 2008) agrees with the one described here. Informative features such as asci,

ascospores and spore body shape and biometry indicate a relationship of *O. jesu-laurae* with *O. auricolor* and related species of the *Arthrobotrys* clade, but morphological, phylogenetic and biogeographic differences have been noted.

The species most similar to *Orbilina jesu-laurae* in apothecial and conidial characters are *O. blumenaviensis*, *O. terrestris* Raitv. & Faizova, *Arthrobotrys javanicus* (Rifai & R. C. Cooke) Jarrow. and *A. musiformis*. All these species have similar falcate ascospores (Baral & al. in press). *Orbilina blumenaviensis* has a pantropical distribution (Qiao & al. 2012) which includes neotropical (Caribbean belt, subtropical humid eastern South America), afrotropical (central Africa, Comoros), Indo-Malayan (subtropical humid SE Asia) and European (thermo-temperate NW Spain) areas (Baral & al. in press). The isolates of *O. blumenaviensis* included in our phylogeny (Fig. 1) reflect this distribution. The three collections clustered unsupported and are distant with respect to the placement of *O. jesu-laurae*. Morphologically, *O. blumenaviensis* differs in its lanceolate paraphyses and 1–3-septate conidia (Qiao & al. 2012). *Orbilina terrestris* lacks sequence and anamorph data. It is only known from the type collection in mountainous central Asia. Its ascospores are wider than in *O. jesu-laurae* ( $\dagger 1.4-1.7 \mu\text{m}$  vs.  $\dagger 0.9-1.1 \mu\text{m}$ ) and have a distinct tail-like base (Baral & al. in press; Raitviir & Faizova 1983). *Arthrobotrys javanicus* and *A. musiformis* are the morphologically most similar species to *O. jesu-laurae*. *Arthrobotrys javanicus* has an Indo-Malay distribution (Fig. 1), appearing in tropical or subtropical forests of Java, Yunnan and Taiwan (Baral & al. in press). *Arthrobotrys musiformis* is widespread with a Palearctic and Indo-Malayan distribution (Fig. 1); specimens have been reported in temperate (Europe, North America) and tropical (China) ecosystems (Baral & al. in press; Drechsler 1937; van Oorschot 1985). The new species is phylogenetically distant from these two species. The clade that includes the two collections from Mexico and the new species from Puerto Rico have a neotropical distribution (Fig. 1). In addition, we found biometric differences among them (measurements for *A. javanicus* and *A. musiformis* taken from Baral & al. in press). First, the ascospore length of *O. jesu-laurae* ( $*10-11.8 \mu\text{m}$ ) is larger than in the other two species (*A. javanicus* =  $*7-9 \mu\text{m}$ ; *A. musiformis* =  $*7.7-10 \mu\text{m}$ ). Second, the conidial length of *O. jesu-laurae* is shorter than in the other two species and the width is in between (*O. jesu-laurae* =  $*14-26 \times 7.5-13 \mu\text{m}$ ; *A. javanicus* =  $*25-42.5 \times 9.5-15.5 \mu\text{m}$ ; *A. musiformis* =  $*18.5-32.5 \times 6-8.5 \mu\text{m}$ ).

Our phylogenetic and morphological analysis showed that *Orbilina jesu-laurae* is not related to any of the taxa described so far in the *Arthrobotrys* clade (Baral & al. 2018). The new species has a neotropical distribution, at least so far as can be said based on a single collection. The closest phylogenetically related strains are two

Mexican isolates identified in GenBank as *A. musiformis* (EVLL02, EVLL-2), here called “*Arthrobotrys* sp.” because the ex-type culture of *A. musiformis* is in a different clade (Fig. 1). Our work contributes to the knowledge of this group of nematode-trapping fungi in a little-explored geographic area of the world.

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