



Ophiostoma ipsi-confusi sp. nov. Six, Marinc. & Duong, a consistent symbiotic fungus of the pinyon ips bark beetle, *Ips confusus* LeConte

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Abstract

Several tree-killing bark beetle species have nutritional mutualisms with specific fungi. However, few secondary bark beetles (those that colonize weak or dying trees) have been investigated for symbiotic fungi and most are thought to have only incidental fungal associates and no dependence on fungi for nutritional or other benefits. In contrast to this supposition, we consistently isolated (>97%) a fungus from adult *Ips confusus* (pinyon ips) collected from *Pinus edulis* (two-needle pinyon pine) from Arizona and New Mexico, USA. Using morphology and DNA sequences for three gene regions, we found the fungus is most closely related to an obligate mutualist fungus of *Dendroctonus ponderosae* (mountain pine beetle), *Ophiostoma montium* (Ascomycota: Ophiostomatales), but is morphologically and genetically distinct from it and other known species in *Ophiostoma*. It is also capable of growth at relatively high temperatures compared with other *Ophiostoma*, reflective of its southwestern USA distribution. The high frequency of its association with the beetle indicates it is symbiotic and suggests it may be a mutualist.

Keywords Mutualism · *Ophiostomatales* · *Pinus edulis* · Scolytinae · Symbiosis

1 Introduction

Many tree-killing bark beetles have associations with mutualist fungi that reliably provision them with nutrients (Ayres et al. 2000; Bleiker and Six 2007; Six and Elser 2019). Such mutualisms have been assumed to be confined to aggressive tree-killing beetles with complex mycangia (fungus-transport structures) and secondary bark beetles (those infesting weak or dying trees) have been mostly ignored. However, a recent study found two secondary beetles with consistent fungi that appear crucial for nutrient acquisition (Six and Elser 2020) suggesting nutritional mutualisms may be more widespread than previously thought. We investigated the fungi associated

with *Ips confusus* LeConte, the pinyon ips, a secondary bark beetle colonizing weak or dying pinyon pines in Utah, Colorado, California, Arizona, New Mexico, west Texas, USA, and portions of northern Mexico (Wood 1982; Cognato et al. 2003). The beetle specializes on *Pinus edulis* Engelm. and *P. monophylla* Torrey & Fremont (Lanier 1970) and is rarely found in other conifers (Cognato et al. 2003). While the beetle is not aggressive, recent increases in temperature and drought have increased the number of weakened and dying host trees leading to massive outbreaks in pinyon pines across the southwestern USA (Santos and Whitham 2010).

Little is known about the ecology of *I. confusus* including its association with fungi. In this study, we were interested in whether *I. confusus* possesses a consistent fungus (or fungi) which may indicate a nutrition-based mutualism. Fungi were isolated from adult *I. confusus* collected from multiple trees from two populations (one in New Mexico, USA, and one approximately 590 km away in Arizona, USA). We then used morphology, growth rates *in vitro* at various temperatures, and DNA sequences to identify and describe the most consistent fungus associated with the beetle.

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2 Methods

2.1 Isolates

Adult beetles were collected from emergence traps placed onto *I. confusus*-infested *Pinus edulis* near Strawberry Crater, Arizona (coord. 35.3926, -111.4301) and from under bark of *P. edulis* at Sevilleta National Wildlife Refuge in New Mexico (coord. 34.3860, -106.5289). Isolations were made from 15 adult beetles from the Arizona site and 62 adult beetles from the New Mexico site. Each live whole beetle was pushed into the surface of 2% malt extract agar (MEA: 2% malt extract and 2% agar, BD Difco) (New Mexico only) and CMEA (MEA amended with 0.05 g l⁻¹ cycloheximide, selective for *Ophiostomatales*) (New Mexico and Arizona) (Wingfield et al. 2022). After 7–10 d growth, individual colonies were sub-cultured onto MEA. Common environmental fungi (e.g., *Penicillium*, *Cladosporium*, etc.) were identified to genus using morphology and tallied. Yeasts were ubiquitous and not considered further. Subcultures of *Ophiostomatales* were sub-cultured two more times by removing hyphae from the actively growing edge to new MEA plates to obtain pure cultures.

2.2 Morphological examinations

Isolates of *Ophiostomatales* were then examined microscopically, and using measurements and characteristics of the hyphae, conidiophores, and conidia, were deemed to be one morphospecies. Two cultures of this morphotype from each site were further purified using single spore isolations. These four isolates were used in growth studies while two isolates, CMW 62,099 (New Mexico) and CMW 62,100 (Arizona), were used for morphological and cultural descriptions, and DNA sequencing.

Mycelial plugs of the two isolates were placed on pieces of pine wood embedded in water agar (WA). The cultures were kept in the dark at room temperature until fungal fruiting structures were produced. Fungal structures grown on the pine wood were removed with the tip of a hypodermic needle and placed on a water drop on a microscope slide. The water drop was replaced with 85% lactic acid in which all observations and measurements were made. Nikon Eclipse Ni mounted with a Nikon DS-Ri2 camera was used with the Imaging Software NIS-Elements Ver 4.30.

Cultures for the growth study were prepared by placing a 7-d old mycelial plug (5 mm diam) of the four strains at the center of 90 mm Petri dishes containing 2% MEA. Four replicate cultures of each strain were incubated at 5–35 °C with a 5 °C interval for 10 d. The radial growth of mycelia was recorded when the cultures reached the edge of the

Petri dishes when grown at the optimum growth temperature. Two perpendicular diameters were measured for each replicate.

2.3 PCR amplification, sequencing and phylogenetic analyses

The two cultures (CMW 62099 and CMW 62100) used for morphological studies were also used for PCR amplification and sequencing of the nuclear ribosomal internal transcribed spacer (ITS), β -tubulin (β T), and translation elongation factor 1- α (TEF) gene regions. DNA extraction was carried out as described in Duong et al. (2012). The primer pairs ITS1-F (Gardes and Bruns 1993) and ITS4 (White et al. 1990), Bt2a and Bt2b (Glass and Donaldson 1995), and EF2F (Marincowitz et al. 2015) and EF2R (Jacobs et al. 2004) were used for PCR amplification of the ITS, β T, and TEF regions, respectively. PCR amplification and sequencing were carried out as described in Duong et al. (2012).

Consensus sequences were generated from forward and reverse-complement sequences using Geneious Prime (Geneious, MA, USA). The resulting sequences were used to carry out BLASTn analyses against the NCBI nt database. Based on the result of BLAST analyses, a dataset of β T gene regions from representative members of the *O. ips* complex were assembled and used for phylogenetic analyses.

The assembled dataset was aligned using MAFFT v7.0 (Katoh and Standley 2013) and trimmed using trimAl (Capella-Gutiérrez et al. 2009) with -automated1 option selected. The aligned and trimmed dataset was subjected to Maximum Likelihood (ML) analyses in IQ-TREE v2 (Minh et al. 2020) with nucleotide substitution automatically determined. Bayesian analysis was carried out using MrBayes (Ronquist et al. 2012) using the same model parameters as determined in IQ-TREE. Ten independent runs, each with four chains, were conducted and trees were sampled after every 100th generation. Tree sampling was stopped when average standard deviation of split frequencies reaches 0.01 or smaller. Burn-in was set at 25% of tree sampled and Bayesian posterior probability values were calculated from the remaining trees.

3 Results

One fungus was consistently isolated from *I. confusus* from both populations (New Mexico 98%, Arizona 100%). In the phylogenetic analysis conducted using the ITS sequence dataset, two isolates (CBS153553 and CBS153554) of this fungus, together with an additional two sequences from GenBank (KT264296 and KT264301) of the fungi isolated

from *Pinus edulis* in New Mexico (A347P12T4s2z and A362P6T5s4z), formed a distinct lineage closely related to *Ophiostoma montium*, an obligate nutritional mutualist of the mountain pine beetle, *Dendroctonus ponderosae* Hopkins (Online Resource 1). A similar result was obtained from phylogenetic analysis of the β T sequence dataset where two isolates (CBS153553 and CBS153554) formed a lineage distinct but closely related to *O. montium* (Online Resource 1). In the phylogenetic analyses of the concatenated sequence dataset (ITS+BT+TEF), isolates of the newly isolated fungus (CBS153553 and CBS153554) and those from *Pinus edulis* in New Mexico (A347P12T4s2z and A362P6T5s4z) consistently formed a well-supported lineage distinct from *O. montium* (Fig. 1). All sequences used in these analyses are listed in Online Resource 2.

Morphological observations revealed that the fungus also has distinct morphological (conidia dimensions, see notes section below) and physiological (optimal growing

temperature, see notes section below) features compared to that of *O. montium*. Thus, this fungus represents a new species in *Ophiostoma* (*Ophiostomataceae*) and is described herein as *Ophiostoma ipsi-confusi*.

3.1 Taxonomy

Ophiostoma ipsi-confusi sp. nov. Six, Marinc. & Duong, sp. nov. Figure 2.

MycoBank: MB 857,689.

Etymology Named after the epithet of the host beetle from which the strain was isolated.

Diagnosis The ITS, β T, and TEF sequences distinguish *O. ipsi-confusi* from its closest relative, *O. montium*. Additionally, unlike *O. montium* (Moore and Six 2015), *O. ipsi-confusi* grows on 2% MEA at 35 °C (Fig. 3), and *O. ipsi-confusi*

Fig. 1 Phylogenetic tree derived from maximum likelihood analyses of concatenate sequence dataset (ITS+BT+TEF) of species in the *Ophiostoma ips* complex. Tree is rooted to *O. piceae*. Isolates of *Ophiostoma ipsi-confusi* isolated and described from this study are indicated in bold type. Maximum likelihood bootstrap values (ML $\geq$ 75%) and Bayesian posterior probability values (BI $\geq$ 95, calculated from 11820 trees sampled across 10 runs, each with 157500 generations) are presented at nodes as ML/BI. T=type

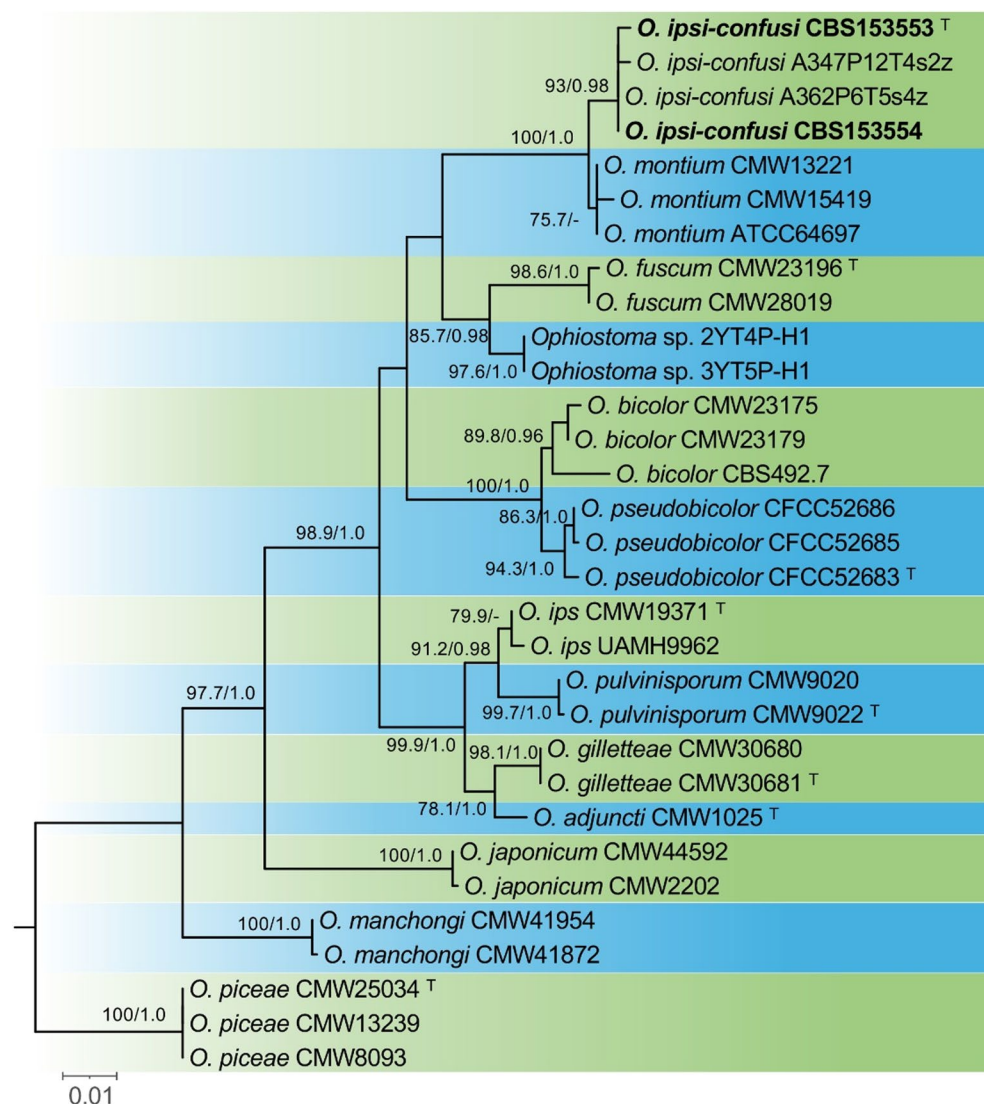




Fig. 2 Micrographs of *Ophiostoma ipsi-confusi* sp. nov. (ex-holotype, CMW-IA 6010, CMW 62099). A–C. Ascoma with a curved neck. D, E. Tip of the neck. F. Neck. G, H. Ascospores covered with gelatinous sheath (H. Off-focus image showing spore and sheath), I–M. Close-up

of ascospore (I, J. Top view; K, L. Ascospore without sheath; M. Ascospore covered with sheath). N, O. *Hyalorhinochloidiella*-like conidiophore. P. Conidiogenesis showing annellide (arrows). Q. Conidia. Scale bars: A–C = 100 µm; D–H, N–Q = 10 µm; I–M = 2.5 µm

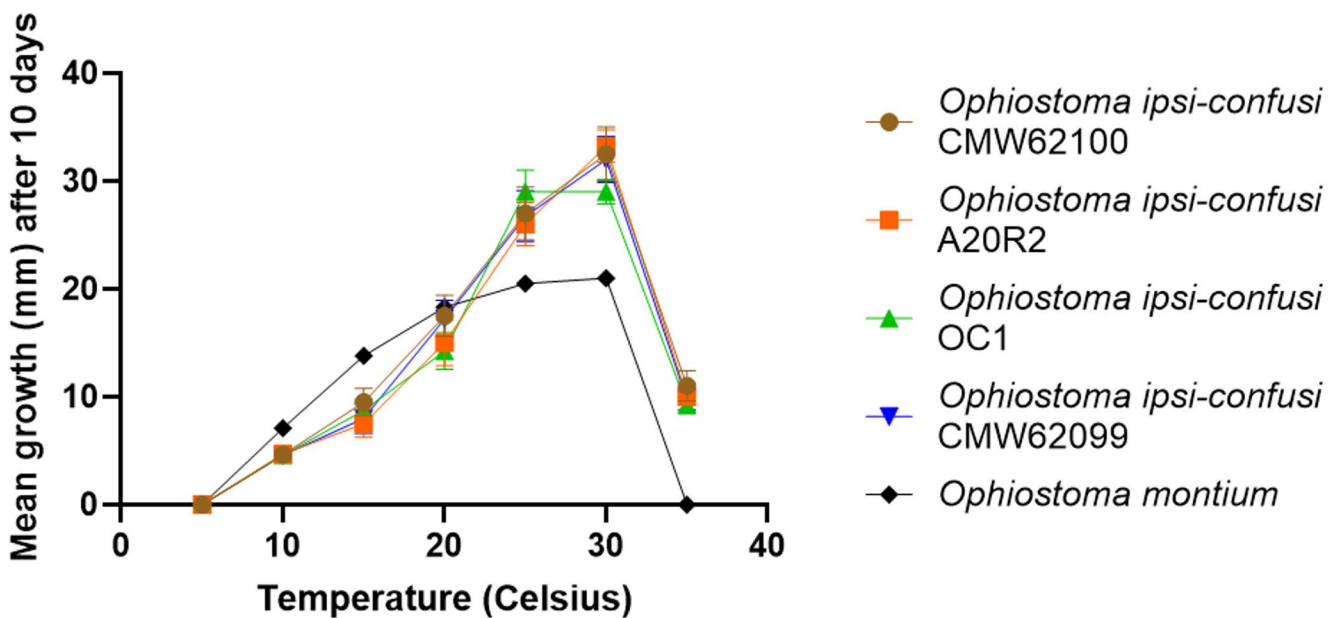


Fig. 3 Mean growth of two isolates of *Ophiostoma ipsi-confusi* from Arizona (CMW62100, A20R2) and two isolates from New Mexico (CMW62099, OC1) compared with mean growth (38 isolates) of *O. montium* (from Moore and Six 2015)

(8–15 × 3–4 μm) has larger conidial dimensions than *O. montium* (6.5–8 × 4–5 μm).

Type USA: New Mexico, Sevilleta National Wildlife Refuge (coord. 34.3860, -106.5289). Isolated from *Ips confusus* adult collected from *Pinus edulis*. July 2023. *Diana Six* IC2. (**Holotype** PRU(M) 4630, stored in a metabolically inactive state; ex-holotype culture CMW-IA 6010, CMW 62099, CBS 153553). GenBank: PX994915 (ITS); PZ012684 (βT), PZ012680 (TEF).

Description *Ascomata* on pine twigs in WA, singular or gregarious, superficial or semi-immersed, upright or spreading; base globose to sub-globose, dark brown to black, 154–317 × 165–317 (230.5 ± 153.8 × 228.8 ± 165.2) μm, *n* = 25; ostiolar necks curved irregularly, 508–1708 (960.7 ± 284.9) μm long, 21–56 (40.9 ± 7.08) μm wide at base, 12–31 (17.6 ± 4.46) μm wide near apex, ostiolar hyphae absent. *Asci* evanescent. *Ascospores* hyaline, oblong in side view, globose in top view, covered with gelatinous sheath giving impression of pillow, 5–6 × 3–4 (5.6 ± 0.31 × 3.1 ± 0.25) μm, *n* = 50. *Conidiophores* *Hyalorhinocladiella*-like, branched. *Conidiogenous cells* holoblastic, cylindrical, annellidic, hyaline, 8–28 (17.4 ± 4.70) μm long, 2–3 (2.2 ± 0.20) μm wide at base, 2–3 (2.4 ± 1.77) μm wide near apex, *n* = 25. *Conidia* hyaline, cylindrical with round apex or slightly inflated middle, base truncate, 8–15 × 3–4 (11.7 ± 1.77 × 3.6 ± 0.34) μm, *n* = 50.

Culture characteristics on MEA: The optimum growth temperature was 30°C, reaching 32.0 mm in 10 d, followed

by 25°C (26.8 mm), 20°C (52.2mm), 35°C (9.8 mm), 15°C (8.0 mm), and 10°C (4.6 mm). There was no growth at 5°C (Fig. 3). After 28 d, cultures at 5°C were light yellow with flat mycelia, at 10–15°C had sparse white aerial hyphae and were light yellow and slightly darker in the centre or edges, at 20°C were olivaceous grey to brown with white aerial hyphae, at 25–30°C were evenly fuscous black with sparse white aerial hyphae, and at 35°C were honey-coloured with white aerial hyphae in patches.

Ecology Found in symbiosis with the scolytine (Curculionidae Scolytinae) bark beetle, *I. confusus* from *P. edulis*.

Distribution USA (Arizona, New Mexico).

Material examined USA: Arizona, Strawberry Crater, Arizona (coord. 35.3926, -111.4301). Isolated from *Ips confusus* adult collected from *Pinus edulis*. July 2023. *Diana Six* A20R1. (**Herbarium** PRU(M) 4631, stored in a metabolically inactive state; culture CMW-IA 6011, CMW 62100, CBS 153554). GenBank: PX994914 (ITS); PZ012683 (BT), PZ012679 (TEF).

Notes *Ophiostoma ipsi-confusi* is a sister taxon to *O. montium* in the well-defined clade. *Ophiostoma montium* was first reported as *Ceratostomella montium* Rumbold, isolated from *D. monticolae* and *D. ponderosae* (Rumbold 1941). It was renamed later as *Ophiostoma* (Von Arx 1952). Their ascospore dimensions are similar (*O. ipsi-confusi* 5–6 × 3–4 μm, *O. montium* 3.7–5.8 × 2–3.4 μm), but conidia dimensions are different with *O. ipsi-confusi* being larger than *O. montium*.

(*O. ipsi-confusi* 8–15 × 3–4 µm, *O. montium* 6.5–8 × 4–5 µm). It grows at 35 °C whereas *O. montium* does not. Indeed, to the best of our knowledge, this is the only known *Ophiostoma* to grow at such a high temperature.

4 Discussion

Ips confusus occupies a relatively large geographic range and we were only able to collect beetles to isolate fungi from two populations. However, given the strong fidelity between the beetle and the fungus (we were able to isolate the fungus from greater than 97% of the beetles in these collections) and the considerable distance between collection sites (almost 600 km), we predict that this fungus is a consistent associate with the beetle across much if not all of its range, particularly if the symbiosis is ancient. Cognato et al. (2003) conducted a nested clade analysis of MtDNA COI from 10 broadly distributed populations of the beetle from across the western USA and from both host trees. Host tree species did not influence genetic structure, but three main haplotype lineages were detected: eastern, southwestern, and western. The analysis suggested that these maternal beetle lineages originated in the southwestern US (the region where we collected beetles and fungi) and that subsequent populations expanded outward from that region. If the association of *I. confusus* with *O. ipsi-confusi* originated prior to this expansion, the fungus may have moved west and north along with its host insect. Such an expansion would be especially supported if the fungus is an obligate mutualist as is suggested by its high incidence with the host beetle. Such co-expansions of beetles and mutualist fungi have been noted for *D. brevicornis* and *D. ponderosae* (Rice and Langor 2009; Bracewell et al. 2018).

Interestingly, *O. ipsi-confusi* is a very close relative to *O. montium*, an obligate nutritional mutualist associated with *Dendroctonus ponderosae*. *Ophiostoma montium* is tolerant of relatively warm temperatures and is common and often dominant with its host beetle in warmer locations (Six and Bentz 2007). *Ophiostoma ipsi-confusi* is also heat tolerant and has among the highest known temperature optima (~30 °C) and tolerances (+35 °C) reported for an *Ophiostoma* species. Such a high tolerance might be expected for a fungus that occurs in the hot southwest of the USA.

A consistent association between fungus and beetle implies a mechanism to maintain contact from generation to generation. *Ips confusus*, like most bark beetles, remains uninvestigated for mycangia (complex or pit) or for mutualisms with fungi. With *I. confusus*, its consistent association with *O. ipsi-confusi* suggests the presence of a selective force maintaining the symbiosis which, in turn, suggests this association is beneficial and a mutualism. Future work

on this beetle should include efforts to conduct range-wide sampling including from *P. monophyla*, and investigations into whether this beetle possesses mycangia and if the fungus provisions nutrients to its host beetle. *Ips* species, in general, are poorly studied regarding fungal associates and work on this species and other *Ips* could yield a wealth of information to aid in our understanding of the function and evolution of symbioses among bark beetles and fungi.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s13199-026-01135-9>.

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Author contributions DLS conceived of the study, conducted isolations and growth trial, and wrote first draft of the manuscript. SM did morphological measurements, photography, and wrote species description, TAD conducted sequencing and genetic and phylogenetic analyses. All authors contributed to the final draft of the manuscript.

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Data availability Sequence data is available in GenBank.

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

Ethics approval No approval of research ethics committees was required to accomplish the goals of this study because work was conducted with an unregulated invertebrate species.

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