NRC Research Press

ARTICLE

Two new species of *Inocybe* from Australia and North America that include novel secotioid forms

Christine C. Braaten, P. Brandon Matheny, Debra L. Viess, Michael G. Wood, Joseph H. Williams, and Neale L. Bougher

Abstract: The secotioid form of fruit bodies of mushroom-forming fungi may be an intermediate evolutionary modification of epigeous agaricoid or pileate–stipitate forms (i.e., with pileus, spore-bearing tissues, and stipe) and typically hypogeous, gasteroid- or truffle-forming species, in which the fruit bodies have been reduced to enclosed structures containing modified spore-producing tissues. To date, only a single secotioid species (*Auritella geoaustralis* Matheny & Bougher ex Matheny & Bougher) has been described in the ectomycorrhizal family Inocybaceae, a hyperdiverse clade of ca. 500–700 species with a cosmopolitan distribution. Fieldwork in Australia and western North America, however, has revealed two novel secotioid forms of *Inocybe* (Fr.) Fr., the first to be formally described in the genus. In this investigation, we analyze their phylogenetic relationships using molecular sequence data from multiple unlinked loci to test whether these are environmental variants of agaricoid forms or represent independent lineages. Results of phylogenetic analyses suggest these fungi have converged to the secotioid form independently. However, the California secotioid taxon (***Inocybe multifolia* f. *cryptophylla*** f. nov.) is a phenotypic variant of the newly described agaricoid taxon (***Inocybe multifolia*** sp. nov.). Similarly, the Australian secotioid form (***Inocybe bicornis* f. *secotioides*** f. nov.) is nested within a clade of otherwise agaricoid forms of a second novel species (***Inocybe bicornis*** sp. nov.) described from southwest Western Australia. Overall, four species with sequestrate forms within Inocybaceae can now be recognized, three of which are distributed in Australia and one in western North America, in the genera *Auritella* and *Inocybe*.

Key words: Agaricales, convergent evolution, Inocybaceae, sequestrate, systematics.

Résumé : La forme sécotioïde des fructifications des champignons formant des basidiomata peut constituer une modification évolutive intermédiaire de champignons agaricoïdes épigés ou formes piléostipitées (c.-à-d. avec un pileus, des tissus porteurs de spores, et un stipe), et des espèces de forme gastéroïde ou de truffes, chez lesquelles les fructifications ont été réduites à un enceinte contenant des tissus sporifères modifiés. Jusqu'à maintenant, une seule espèce sécotioïde (*Auritella geoaustralis* Matheny & Bougher ex Matheny & Bougher) a été décrite dans la famille ectomycorhizienne des Inocybaceae, un clade hyper diversifié d'environ 500–700 espèces, avec une distribution cosmopolite. Cependant, des travaux conduits sur le terrain, en Australie et dans l'Ouest Nord-Américain, ont révélé deux nouvelles espèces d'*Inocybe* (Fr.) Fr. de forme sécotioïde, les premières à être formellement décrites dans ce genre. Dans cette recherche, les auteurs analysent leurs relations phylogénétiques à l'aide de données de séquences moléculaires à partir de nombreux lieux non reliés, afin de vérifier s'il s'agit de variantes environnementales de forme agaricoïde ou des représentants de lignées indépendantes. Les résultats des analyses phylogénétiques suggèrent que ces champignons ont convergé vers la forme sécotioïde indépendamment. Cependant, le taxon sécotioïde californien (***Inocybe multifolia* f. *cryptophylla*** f. nov.) est une variante du taxon sécotioïde nouvellement décrit (***Inocybe multifolia*** sp. nov.). De la même façon, la forme sécotioïde australienne (***Inocybe bicornis* f. *secotioides*** f. nov.) se retrouve dans un clade comportant des formes plutôt agaricoïdes, constituant une deuxième nouvelle espèce (***Inocybe bicornis*** sp. nov.), décrite à partir de sud-ouest de l'Australie-Occidentale. Dans l'ensemble, on peut maintenant reconnaître quatre espèces aux formes séquestrées au sein des Inocybaceae, dont trois sont distribuées en Australie et une dans l'Ouest Nord Américain, appartenant aux genres *Auritella* et *Inocybe*. [Traduit par la Rédaction]

Mots-clés : Agaricales, évolution convergente, Inocybaceae, séquestré, systématique.

Introduction

Thiers (1984) hypothesized that selective pressure in areas, especially those with dry Mediterranean or arid climates, induces sequestration. This explanation was premised on the observation of high taxonomic diversity of secotioid and gasteroid taxa in such areas of western North America. Dry, warm environments may be driving the evolution of fruit body morphology, in which sexual spore-bearing tissues become enclosed to offset desiccation (Thiers 1984; Bruns et al. 1989; Justo et al. 2010). Selective pressures favoring retention of moisture and mycophagy by animals, particularly in regions with prolonged periods of drought such as western North America and areas of Australia, may account for why these two regions boast the greatest amount of sequestrate taxon diversity (Bougher and Lebel 2001; Mueller et al. 2007; Trappe et al. 2009). Indeed, sequestrate taxa have evolved independently among numerous distinct fungal lineages (e.g., Peintner

Received 5 August 2013. Accepted 19 October 2013.

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Published at www.nrcresearchpress.com/cjb on 18 December 2013.

et al. 2001; Hosaka et al. 2006; Matheny and Bougher 2006*a*; Læssøe and Hansen 2007; Henkel et al. 2010; Justo et al. 2010; Bonito et al. 2013).

However, phenotypic variants of otherwise normal, lamellate, or tubular forms, as opposed to evolution of independent phyletic lineages of sequestrate taxa, have also been demonstrated (Hibbett et al. 1994; Kretzer and Bruns 1997; Martín et al. 1999) or hypothesized (Hallen et al. 2003). Work by Hibbett and colleagues showed that the secotioid phenotype of *Lentinus tigrinus* (Bull.) Fr. is induced by a single mutation and possibly environmental factors. Hallen et al. (2003) suggest that the secotioid form of *Gastrocybe lateritia* Watling, a unique evolutionary lineage within the genus *Conocybe* Fayod, is environmentally induced by bacterial disease. Baura et al. (1992) suggest that a small number of mutations in development genes may influence multiple phenotypic traits exhibited by transient secotioid forms.

Reports of sequestrate forms in the Inocybaceae are extremely rare (Francis 2006). The first described sequestrate species was by Matheny and Bougher (2006*a*, 2006*b*) as *Auritella geoaustralis* Matheny & Bougher ex Matheny & Bougher). To date, this species remains taxonomically and phylogenetically autonomous from other agaricoid species of *Auritella* Matheny & Bougher ex Matheny & Bougher. DNA sequences of a second but undescribed taxon in *Inocybe* (Fr.) Fr from Victoria, Australia, were produced by Matheny et al. (2009), but without any taxonomic description of the fungus. "*Geoinocybe*" was an informal genus name referred to in works by Bougher and Lebel (2001) as "genus B" and by Francis and Bougher (2003) in reference to the secotioid form included in the molecular phylogenetic treatment of Matheny et al. (2009). However, "*Geoinocybe*" has never been formerly proposed and, if applied, would encompass a polyphyletic group. Here, we document two novel sequestrate forms of *Inocybe*, one from coastal northern California and the other from southwest Western Australia's wheatbelt region, and test the null hypothesis that these forms are the result of gasteromycetation (Singer 1986) and, thus, aberrant developmental phenotypes of agaricoid taxa. Alternatively, these sequestrate taxa of *Inocybe* could represent independent evolutionary lineages that warrant species-level recognition.

Materials and methods

Taxon sampling, gross morphology, and microscopy

Collections were made from southwest Western Australia (32°21′25.9″S, 117°44′17.1″E), Sierra County, California (39°37′53.1″N, 120°35′32.38″W), and Mendocino, California (39°19′5.48″N, 123°47′48.09″W). All are deposited at PERTH and (or) TENN. Herbarium abbreviations follow Thiers (continuously updated; http://sweetgum.nybg.org/ih/). Color notations of field specimens were noted with the aid of color guides produced by Ridgway (1912), Munsell Soil Color Charts (1954), and Kornerup and Wanscher (1967). In macroscopic descriptions, L indicates the number of lamellae that reach the stipe. After description, specimens were air-dried and preserved. Sections of dried material from each collection were rehydrated in 3% KOH and examined microscopically using a Nikon Eclipse 80i compound microscope. Measurements in parentheses fall outside two standard deviations and are considered statistical outliers. Total spores measured per collection are indicated as $n = x/y$. *Q* refers to the quotient of spore length divided by spore width. Terminology regarding basidiospores and cystidia follows Kuyper (1986). Line drawings of microscopic features were prepared using a camera lucida attached to the Nikon Eclipse microscope.

DAPI (4,6-diamidino-2-phenylindole) staining was performed to determine the number of nuclei in spores (Kapuscinski 1995). Microspectrofluorometry was undertaken using a Zeiss Axioplan 2 compound microscope equipped with an Axiocam digital camera. Material was hand sectioned and stained with DAPI for 1 h at room temperature in a light-restricted environment, then viewed using fluorescence optics.

DNA extraction, polymerase chain reaction (PCR), and sequencing

DNA extractions, PCR, and sequencing were performed following protocols outlined in detail by Judge et al. (2010). Amplification of the internal transcribed spacers 1 and 2 and the intervening 5.8S ribosomal RNA gene region (hereafter referred to as ITS) follows that of Judge et al. (2010), except for those of type specimens, which follows Ammirati et al. (2007) and Baroni and Matheny (2011). Amplification of portions of genes that encode the nuclear large-subunit ribosomal RNA (nLSU) and the second-largest subunit of RNA polymerase II (*rpb2*) follow that of Baroni and Matheny (2011).

Alignment assembly and phylogenetic analyses

We constructed three different gene alignments to assess the phylogenetic position of the California sequestrate fungus. The first, an nLSU alignment, was assembled in MacClade version 4.08 (Maddison and Maddison 2005) based on initial BLAST results of the National Center for Biotechnology Information (NCBI) nucleotide sequence database that showed 98% similarity between nLSU sequences of the secotioid sample and a Swedish sequence of *Inocybe fuscidula* Velen. and the eastern North American species, *Inocybe semifulva* Grund & D.E. Stuntz. Additional nLSU sequences of relevant taxa were added to this data set from alignment group 2 in Ryberg et al. (2008), Ryberg (2009), and Matheny et al. (2009), as well as from an unpublished, in-house, curated nLSU alignment. *Inocybe pusio* P. Karst. (AY388643) was used for outgroup purposes based on Matheny (2005).

Results from the nLSU phylogenetic analysis, which suggest a sister relationship between the California secotioid fungus and *I. fuscidula*, were used to guide a finer scale ITS alignment using *Inocybe* sp. (JX316541) as the outgroup. To assemble this alignment, we performed a BLAST search using the following criteria to filter available GenBank sequences for taxon sampling: max identity >89%, query coverage >76%, and *E* value of 0.0. Sequences from GenBank were downloaded in FASTA format, uploaded into ClustalX version 2.0.9 (Larkin et al. 2007), and then aligned using default options. Two sequences with numerous autapomorphic changes were omitted after visual inspection in MacClade and preliminary phylogenetic analyses. Based on in-house BLAST searches, we identified an ITS sequence of MGW784 collected in Sierra County, California, (preliminary determination as *I.* cf. *fuscidula*) as nearly identical to the Mendocino sequestrate fungus.

We also assembled a third gene data set of *rpb2* sequences in MacClade by pruning an alignment from Matheny et al. (2009) to focus on clade "Ic" of *Inocybe* s. str. from Matheny (2005), within which we expected the California sequestrate fungus to be placed. This prediction was based on the relative phylogenetic proximity of nLSU sequences of the latter to *Inocybe flocculosa* Sacc. and its allies. This prediction is also borne out by the placement of both *I. flocculosa* and *I. fuscidula* in alignment group 2 in Ryberg et al. (2008) and Ryberg (2009). The alignment spans between conserved domains six and seven of the second-largest subunit of RNA polymerase II and includes a spliceosomal intron region, intron 4 (Matheny 2005). *Inocybe pusio* (AY337396) was used to root the resulting tree, as alluded to above.

All three data sets were analyzed phylogenetically using RAxML version 7.2.8 (Stamatakis 2006*a*, 2006*b*). Models of molecular evolution, including parameters for site rate heterogeneity, were applied following Matheny (2005). Single gene partitions were used for the nLSU and ITS data sets, whereas four gene partitions (i.e., four separate models) were applied to the *rpb2* data set, one for each codon position and one for the intron 4 region. Two hundred rapid bootstrap replicates were performed for each dataset (Stamatakis et al. 2008).

A secotioid collection of another species of *Inocybe* (BOU890/PERTH08320403) was collected in the wheatbelt region near Corrigin, in southwest Western Australia. This material was collected under compact moist sand, forced slightly upward, beneath numerous individuals of *Allocasuarina huegeliana* (Miq.) L.A.S. Johnson (family Casuarinaceae). In-house BLAST analyses of ITS and nLSU revealed high similarity to sequences of other Australian collections in the *Inocybe fibrillosibrunnea* O.K. Mill. & R.N. Hilton group. These sequences were combined for a phylogenetic analysis, hereafter referred to as the 'ITS+LSU *I. bicornis* data set'. Characters too ambiguous to align were excluded. Similarly, an *rpb2* alignment was performed of these taxa after a global phylogenetic inspection in RAxML. This data set was partitioned by each codon position and the intron 4 region. Three sequences from Western Australian (PBM3602/TENN 066573) and Queensland collections (PBM3774/TENN 067006, PBM3782/TENN 067014) from two undescribed species of *Inocybe* outside the ingroup were used for rooting purposes. Alignment files and phylogenetic trees were submitted to TreeBASE (submission number 14529).

Results

A total of 128 new sequences were produced for this study (38 ITS, 50 nLSU, 40 *rpb2*) and released to GenBank as accessions KC305452–KC305489, KC305362–KC305411, and KC305412–KC305451. The nLSU sequence of the secotioid form (DV12042011) from California was analyzed together with 48 additional nLSU sequences for an initial phylogenetic placement. Results from this analysis (Fig. 1; gray-shaded box) strongly suggest DV12042011 is (*i*) autonomous and (*ii*) most closely related to a European collection of *I. fuscidula*. Based on this result, a fine-scale phylogenetic approach was applied using ITS sequences to further determine the autonomy and phylogenetic relationships of the secotioid taxon. This exercise, based on a BLAST procedure, resulted in an alignment of 63 ITS sequences including 661 sites. Phylogenetic analysis of the ITS data set (Fig. 2) reaffirms that the secotioid taxon is most closely related to a Eurasian clade of *I. fuscidula* and an arctic North American clade composed of sequences labeled as "*Inocybe fuscidula*". We have labeled this latter North American clade as *I.* aff. *fuscidula* because they form a distinct clade apart from European–East Asian *I. fuscidula*. The California secotioid taxon is likewise autonomous in this analysis but clusters with a normal agaricoid form (MGW784) collected at higher elevations in Sierra County. The ITS sequences of the secotioid and agaricoid forms exhibit no variation; therefore, we designate both as a single new species, *Inocybe multifolia*, and describe the secotioid phenotype as *I. multifolia* f. *cryptophylla* (see Taxonomy section). The *rpb2* gene phylogeny indicates *I. multifolia* is most closely related to *Inocybe griseovelata* Kühner (sequences of New York material labeled as such) (Fig. 3). Sequences of *rpb2* from samples of *I. fuscidula* were not available for this analysis.

Because one species of *Inocybe* has been shown to be parasitized by a species of *Squamanita* Imbach (Vizzini and Girlanda 1997; Matheny and Griffith 2010), we wished to eliminate the possibility that the sequestrate forms were developmentally arrested by a mycoparasite. ITS sequences from each sequestrate form were homogeneous, suggesting the genomic DNA was indeed from a single individual. Because the secotioid material from California exhibited a particularly thick layer of fibrils enclosing the spore-bearing hymenium, we performed four multiple-DNA extractions from different areas of a single basidiocarp. All four resulted in homogeneous ITS sequences, each one identical to the other. However, these results do not preclude the possibility of deformation due to bacterial or viral infections.

The combined ITS+LSU *I. bicornis* data set included 2087 sites (1–655 ITS, 656–2087 nLSU). The *rpb2 I. bicornis* data set included 780 sites. We detected no strong conflict between the rRNA and *rpb2* gene trees aside from the rRNA placement of NB00905, which occupies a long branch distant from the *I. fibrillosibrunnea* group (Fig. 4). In the *rpb2* gene tree (Fig. 5), however, NB00905 shares the same sequences as *I.* aff. *fibrillosibrunnea*. At this time, we are investigating the source of this potential error. Thus, the rRNA sequences of NB00905 have not yet been released to GenBank.

DAPI staining of spores of *I. bicornis* f. *secotioides* (Figs. 6*a*, 6*d*), *I. bicornis* (Figs. 6*b*, 6*e*), and *I. multifolia* f. *cryptophylla* (Figs. 6*c*, 6*f*) (described below) reveal two nuclei per spore in both 4-sterigmate sequestrate taxa but four nuclei per spore in the 2-sterigmate species *I. bicornis*.

Taxonomy

Inocybe multifolia* f. *multifolia Braaten, M.G. Wood, & Matheny, sp. nov.
Figs. 7*a*, 8.

MYCOBANK NUMBER: 804817

DIAGNOSIS: Similar to *I. fuscidula*, *I. griseovelata*, and *Inocybe griseotarda* Poirier. *Inocybe multifolia* differs from these species by longer spores and geographic distribution in northern California and by forming a distinct monophyletic group apart from these other three species.

TYPUS: United States of America. California: near Sierra City and Bassets Station at Green Acres off Highway 49, on soil under high-elevation conifers, fruiting after snowmelt, elev. 1700 m, 39°37′5.31″N, 120°35′32.38″W, 8-Jun-2010, coll. *A.W. Wilson* & *M.G. Wood MGW784* (TENN 065795, holotype). GenBank DNA sequence accession No. KC335526.

ETYMOLOGY: (L.) *multi-* many, *-folia*, lamellae, in reference to the phenotypic plasticity of the lamellae.

DESCRIPTION: *Pileus:* 17–23 mm wide, broadly convex to nearly plane with a wide obtuse umbo, margin decurved but straight to weakly uplifted with age; surface with extensive velipellis, dry, entire, nowhere scaly; whitish mixed with warm brown or "Cinnamon Brown" ground color; context not changing color upon exposure, odor weakly spermatic. *Lamellae:* adnexed to sinuate, close to almost crowded, 45–55 L, "Wood Brown" to "Avellaneous" or pale brown to grayish-brown with a vinaceous or pinkish tint, not broad; edges not distinctly pallid or fimbriate. *Stipe:* 35–45 mm × 5–8 mm wide at the apex, terete, 10–12 mm wide at the enlarged to conjoined base; partial veil not directly observed but presumed fugacious, surface dry, indistinctly furfuraceous–pruinose at the apex, elsewhere finely fibrillose with scattered stringy fibrils above the stipe base; color dull whitish to dull olive-buff, white near the base where covered with soil.

Spores: 10.0–12.0 μm × 5.0–6.5 μm, mean 11.2 μm × 5.7 μm (n = 23/1), *Q:* 1.77–2.21 (–2.30), mean *Q:* 1.97, smooth, amygdaliform with pointed apices, somewhat thick-walled, yellowish-brown with small but prominent apiculus. *Basidia:* 29–36 μm × 8–10 μm, clavate, hyaline, 4-sterigmate but 2- and 1-sterigmate forms also observed. *Pleurocystidia:* 62–96 μm × 12–17 μm, mostly slenderly fusiform, occasionally subcylindric; apices obtuse, less often swollen or indistinctly subcapitate, at times bearing crystals; thin-walled to slightly thick-walled, walls at most up to 2.0 μm thick, hyaline. *Cheilocystidia:* similar to pleurocystidia but scattered along the lamellar edges, mixed with paracystidia. *Caulocystidia:* restricted to extreme stipe apex, sparse, more or less similar to cheilocystidia; thin-walled to slightly thick-walled, hyaline. *Pileipellis:* a compact interwoven layer of hyaline and cylidnric hyphae, these smooth, thin-walled, mostly 5–12 μm wide, overlaying a light golden brown pigmented layer composed of hyphae similar to above, these not noticeably incrusted. *Clamp connections:* present.

ECOLOGY AND DISTRIBUTION: On soil after snowmelt in the Sierra Nevada, California, occurring in early June.

COMMENTS: *Inocybe multifolia* is most closely related to a small ensemble of species that includes *I. fuscidula*, *I.* aff. *fuscidula* (an arctic

Fig. 1. Best maximum likelihood (ML) tree produced by RAxML of nLSU sequences showing placement of the secotioid collection DV12042011 (as *Inocybe multifolia* f. *cryptophylla* f. nov.) as a sister lineage to *I. fuscidula* (gray-shaded box) and weakly supported as related to *I. griseovelata*. ML bootstrap values >50% are indicated above branches.

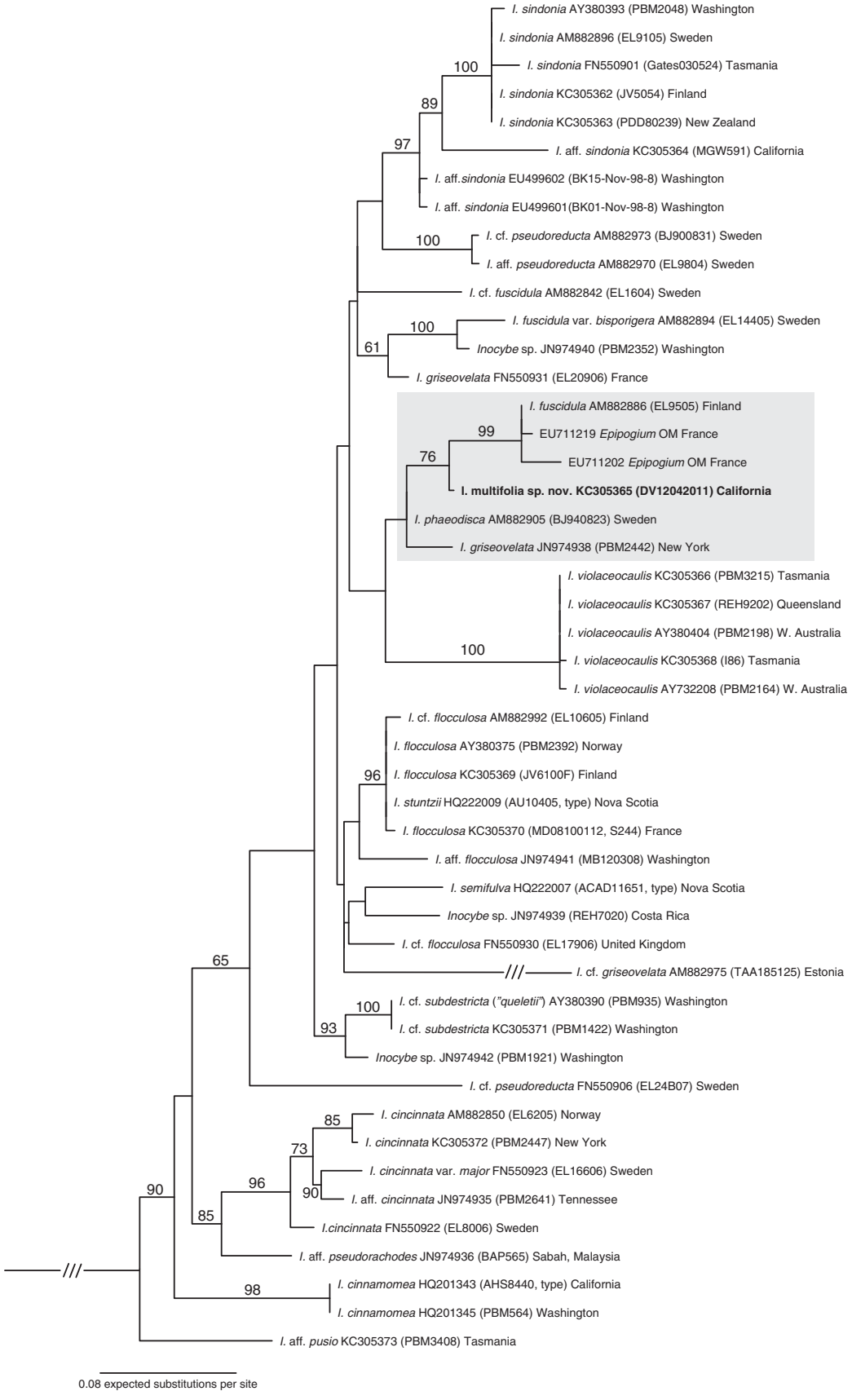
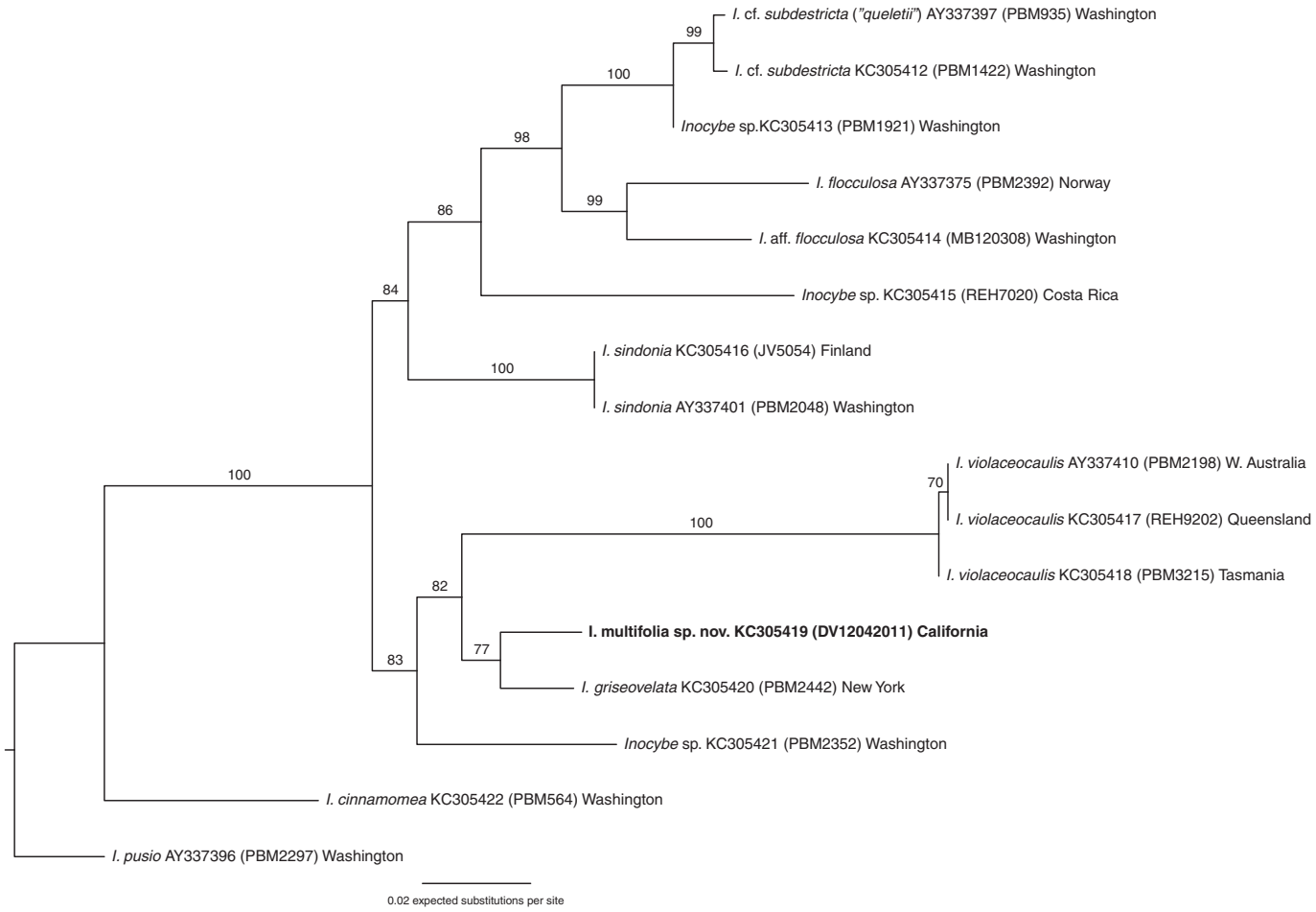


Fig. 2. Fine-scale phylogenetic maximum likelihood (ML) tree of internal transcribed spacers 1 and 2 and the intervening 5.8S ribosomal RNA gene region sequences from taxa closely related to *I. fuscidula*. The secotioid form (Mendocino, California) and an agaricoid form (Sierra County, California) cluster together (designated here as ***I. multifolia*** sp.nov.) as a sister lineage to an unclarified arctic species from North America known only from environmental sequences (*I.* aff. *fuscidula*) and the European–East Asian species *I. fuscidula*. Phylogenetic analysis performed in RAxML. ML bootstrap values >50% are indicated above branches.

lineage), and *I. griseotarda* (Fig. 2). *Inocybe fuscidula* appears to be a widespread species in Europe and east Asia based on our phylogenetic analysis of ITS sequences. Sequences of this species have yet to be confirmed from North America. *Inocybe multifolia* does indeed resemble forms of *I. fuscidula*, which are marked by a distinct velipellis imparting a dirty whitish to yellowish brown pileus color as described by Kuyper (1986) and depicted by Stangl (1989). Kuyper and Stangl, however, both describe and illustrate spores

Fig. 3. Best maximum likelihood (ML) tree produced by RAxML of *rpb2* sequences partitioned by codon position showing placement of ***Inocybe multifolia*** sp. nov. as closely related to *I. griseovelata* with high support. ML bootstrap values >50% are indicated above branches. All taxa shown belong to clade Ic of *Inocybe s.str.* in Matheny (2005).



that are shorter (7.5–10.5 μm × 4.5–6.0 μm) and with less pronounced pointed apices. *Inocybe* aff. *fuscidula* represents a distinct evolutionary lineage of an arctic North American taxon known only from environmental ITS sequences of *Dryas* Hübner and *Salix* L. root systems (as *I. fuscidula*; Timling et al. 2012). Sequences produced by Timling et al. (2012) form a distinct clade apart from European–Asian *I. fuscidula*. The sole and unpublished sequence labeled *I. griseotarda* in Fig. 2 stems from a barcoding initiative of the Venice Museum fungal collection (Osmundson et al. 2013). Based on the protologue (Poirier 2002), this species is morphologically very similar to *I. multifolia*. It appears to differ from *I. multifolia* by the rose-tinted stipe, occurrence on calcareous ground under *Pinus* L. in central and southern Europe, and ITS genetic differences. A sequence of *I. fuscidula* var. *bisporigera* Kuyper is indicated as distantly related to *I. multifolia* (Fig. 1). Thus, *I. fuscidula* does not appear to represent a monophyletic species. *Inocybe fuscidula* var. *bisporigera* is reported to occur under hardwoods and is known only from England and Denmark (Kuyper 1986), though it may also occur in Spain as suggested by Esteve-Raventós (1998).

Inocybe multifolia is a new species of the mountain spring flora in California as the type was collected after snowmelt in early June. However, the secotioid form of this species (f. *cryptophylla*, described below) was collected in a residential area less than a mile from the Pacific Ocean, buried in damp, grassy ground adjacent to native, mixed-conifer forest, with only the cap exposed, in Mendocino, California, in early December.

Inocybe multifolia* f. *cryptophylla Braaten, Viess, & Matheny, f. nov.

Figs. 7*b*, 9.

MYCOBANK NUMBER: 804823

DIAGNOSIS: Genetically same as *I. multifolia* but differs phenotypically by the secotioid habit.

TYPUS: United States of America. California: Mendocino, in grass lawn adjacent to strip of native, mixed-conifer forest, Larkin Road above Highway 1, 39°19′5.48″N, 123°47′48.09″W, 12 Apr. 2011, coll. *D. Viess 12042011* (TENN 066926, holotype). GenBank DNA accession Nos. KC305365, KC305452, KC305419.

ETYMOLOGY: (Gk.) *crypto-* hidden, *-phylla*, lamellae, in reference to the secotioid form of *I. multifolia*.

DESCRIPTION: *Pileus:* 15–25 mm wide; pulvinate, obtusely conical, to deeply convex, disc bluntly pointed or with a low broad umbo, margin conspicuously incurved and enclosed or in places undulate and extruding small portions of the hymenophore, surface grayish to café au lait (7B3 to 7C3) in places due to hoary covering of grayish fibrils; ground color henna brown (7E8), umbrinous, or yellowish-brown where exposed or beneath the superficial fibrillose layer; context up to 6 mm thick, primarily pale yellowish-white (1A1 to 1A2) but with some pinkish buff areas (7B3 to 7C3), odor and taste not remarkable. *Lamellae:* enclosed within irregular hymenial chamber, at times crushed and folded into pileal context,

Fig. 4. Best maximum likelihood (ML) tree produced by RAxML of combined internal transcribed spacers 1 and 2 and the intervening 5.8S ribosomal RNA gene region and nLSU sequences of taxa in the *Inocybe fibrillosibrunnea* group. ***Inocybe bicornis*** sp. nov. and its sequestrate relative, *I. bicornis* f. *secotioides* f. nov. form a strongly supported cluster separated from at least ten other Australasian lineages. ML bootstrap values >50% are indicated above branches.

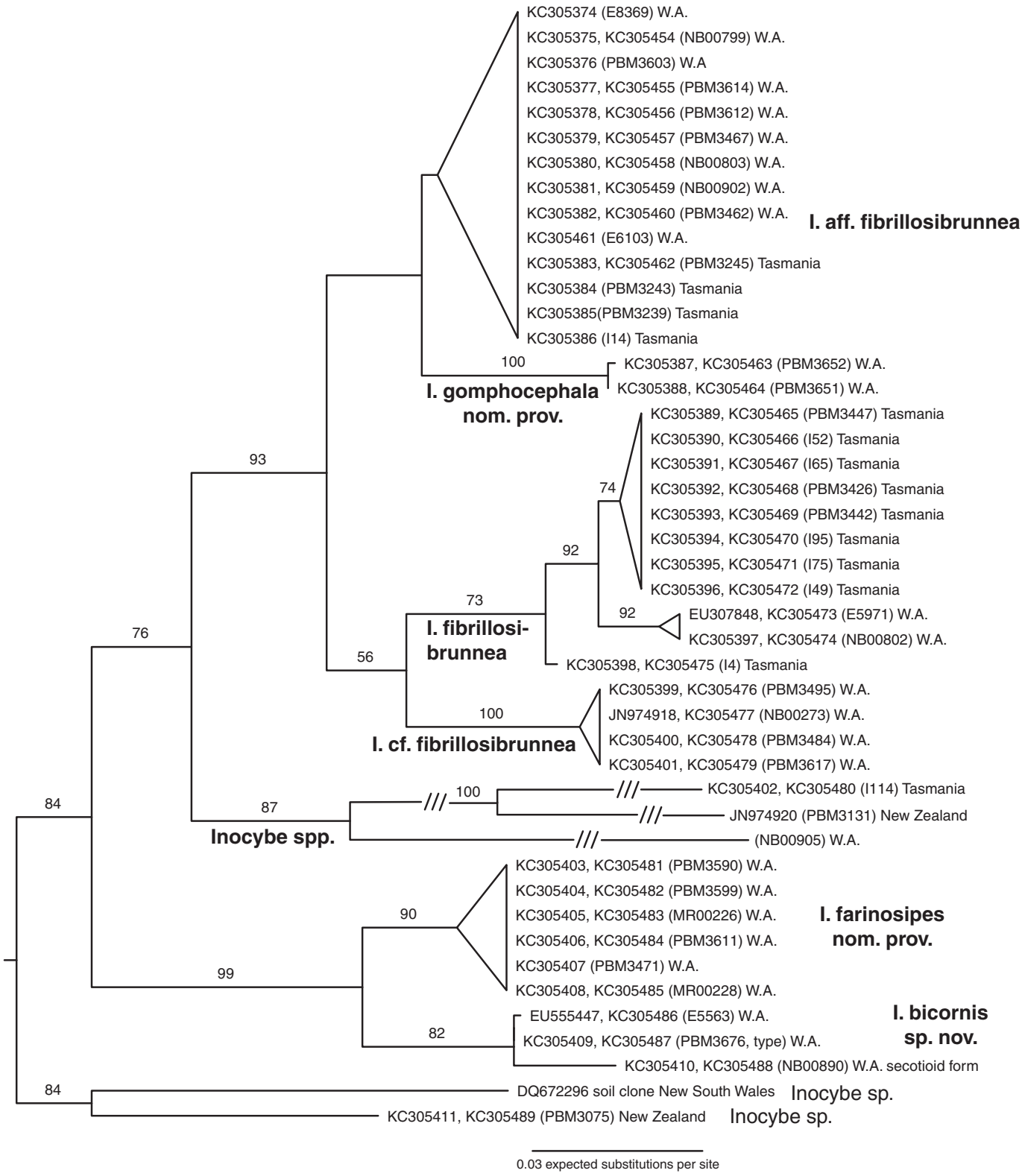
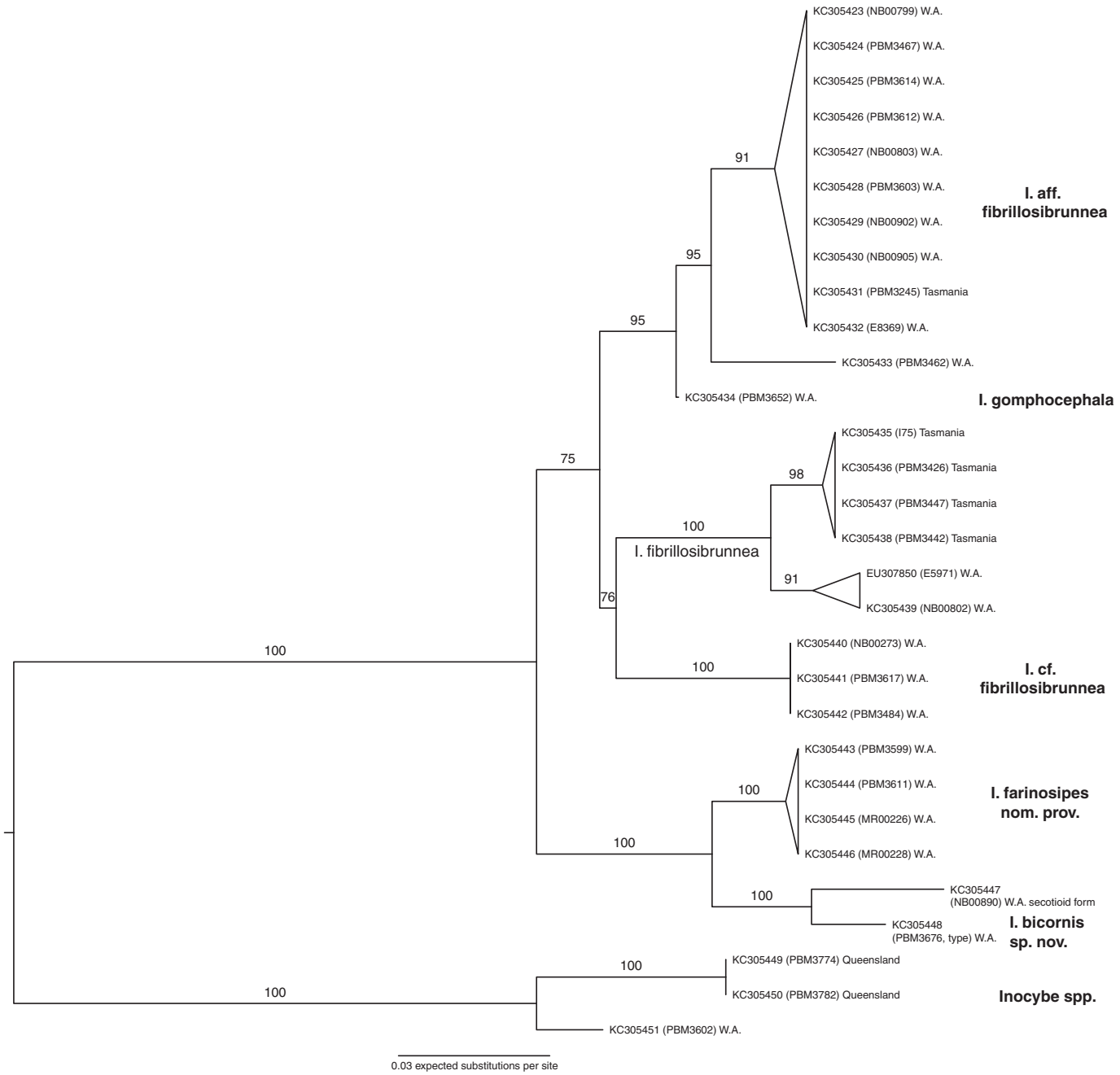


Fig. 5. Best maximum likelihood (ML) tree produced by RAxML of *rpb2* sequences partition by codon of taxa in the *Inocybe fibrillosibrunnea* group. ***Inocybe bicornis*** sp. nov. and its sequestrate relative, ***I. bicornis* f. *secotioides*** f. nov. form a strongly supported cluster separated from at least seven other Australasian lineages. ML bootstrap values >50% are indicated above branches.



thick, distorted, uneven; at maturity yellow-gray (4B2) or grayish olive-buff. *Stipe:* 25–35 mm × 8–10 mm, squat, thick, even to slightly bulbous at the blunt base; surface at times with shallow longitudinal folds, pale yellowish-white to pinkish-buff, long white fibrils extending from pileus to base of stipe enclosing the hymenium; context mostly solid, compressed and folded into pileal tissue leaving gaps and folds, pale yellowish-white but pinkish-buff in areas where tissue folds.

Spores: 8.5–12.5 μm × 5.0–6.0 μm, mean 10.0 μm × 5.5 μm, *Q*: 1.57–2.27, *Q* mean: 1.83 (*n* = 31/1), smooth, elliptic to subamygdaliform in profile, apices rounded, slightly thick-walled, brown to yellowish-brown, apiculus small but distinctive. *Hymenial cystidia:* present as metuloids but not frequent, occurring on faces of the convoluted lamellae and thus homologous to pleurocystidia. *Basidia:* 27–31 μm × 9–10 μm, clavate, hyaline, 4- or 2-sterigmate. *Caulocystidia:* absent, stipe surface with abundant superficial, hyaline, cylindrical hyphae. *Pileipellis:* composed of layer of loosely interwoven hyphae, these cylindric, slightly pigmented, cylindric, mostly 5–10 μm wide, subpellis with more densely packed, uniformly slender hyphae. *Clamp connections:* present.

ECOLOGY AND DISTRIBUTION: In grass lawn under mixed-conifer forest, Mendocino, northern California, occurs in December.

COMMENTS: *Inocybe multifolia* f. *cryptophylla* is the first documented secotioid or truffle-like (sequestrate) species of *Inocybe* from North America. Two fruiting bodies were found and examined here. Morphologically, they form an obvious alliance to *Inocybe* due the presence of the smooth and yellowish-brown basidiospores and

Fig. 6. Differential interference contrast microscopy (*a*–*c*) and DAPI fluorescence microcopy (*d*–*f*) of basidiospores of *I. bicornis* f. *secotioides* (*a*, *d*; BOU890, PERTH08320403), *I. bicornis* f. *bicornis* (*b*, *e*; PBM3676, PERTH 08319103), and *I. multifolia* f. *cryptophylla* (*c*, *f*; DV12042011, TENN 066926). Two nuclei per spore can be visualized in *I. bicornis* f. *secotioides* (*d*) and *I. multifolia* f. *cryptophylla* (*f*). Four nuclei are observed in spores of *I. bicornis* f. *bicornis* (*e*). Scale bar equals 10 μm.

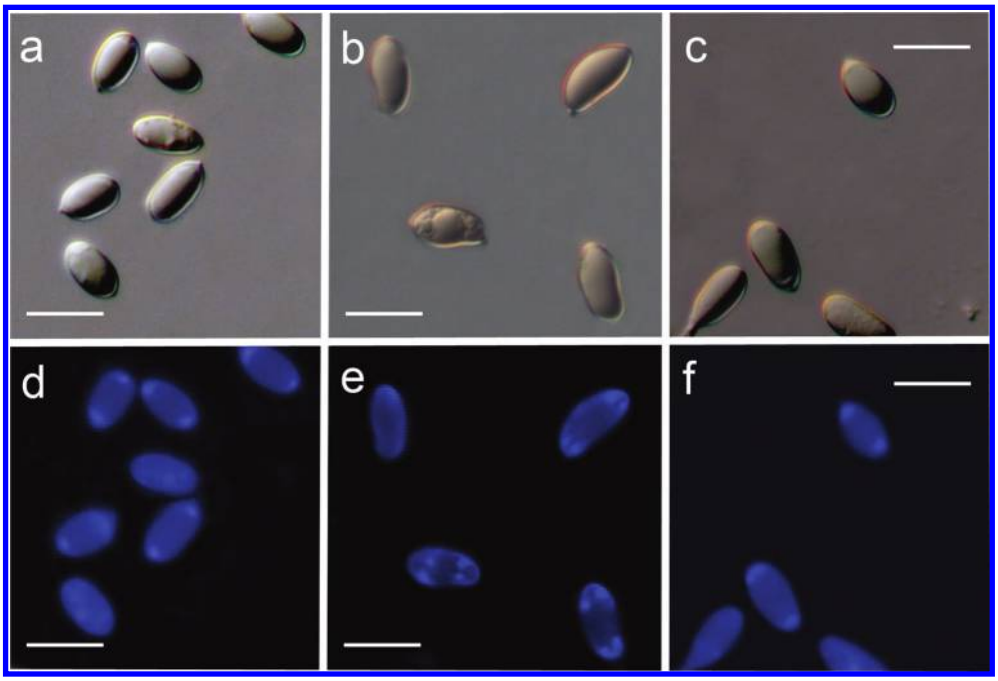
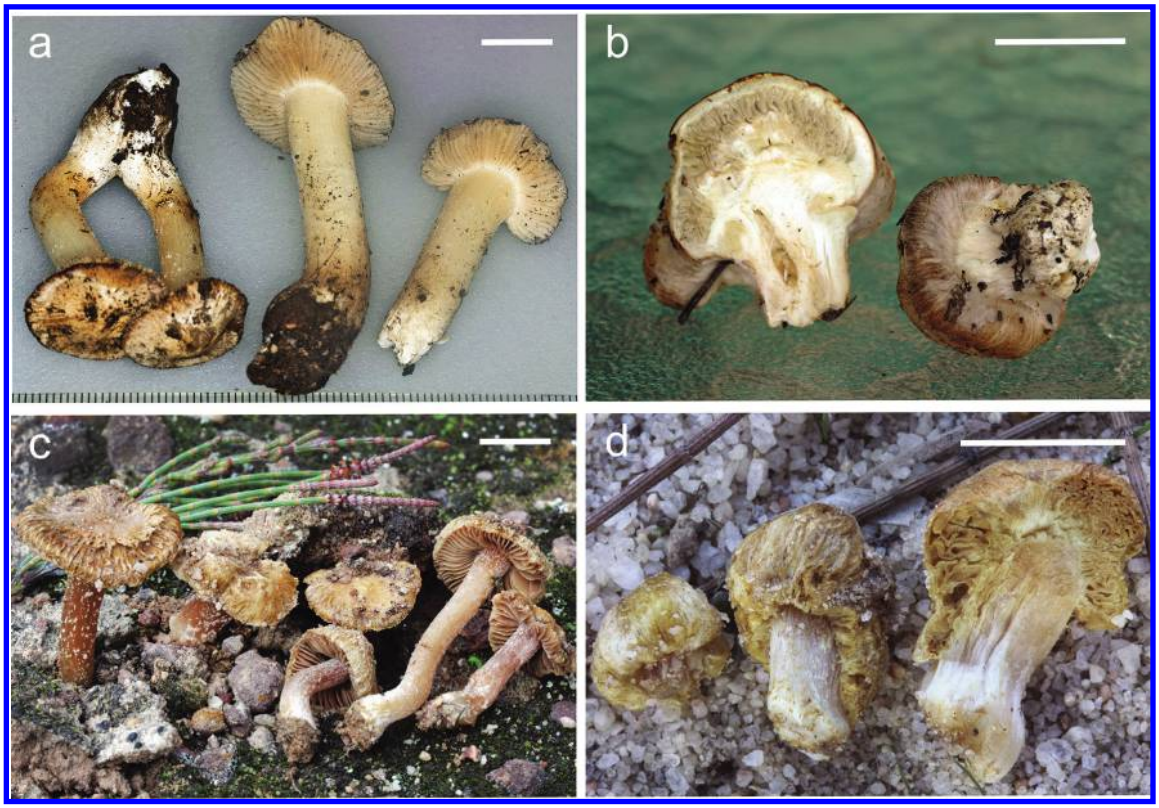


Fig. 7. Fruit body images of (*a*) *Inocybe multifolia* f. *multifolia* (MGW784, TENN 065795), (*b*) *I. multifolia* f. *cryptophylla* (DV12042011, TENN 066926), (*c*) *I. bicornis* f. *bicornis* (BOU890, PERTH 08320403), and (*d*) *I. bicornis* f. *secotiodes* (PBM3676, PERTH 08319103).



the presence of metuloid cystidia in the hymenium. The nLSU data from the holotype indicates a close relationship (98% similarity) to *I. fuscidula*, *I. griseovelata*, and members of the *I. flocculosa* group such as *I. semifulva* (Fig. 1). In outward respects, *I. multifolia* f. *secotioides* resembles published treatments of *I. griseovelata* (Kuyper 1986; Stangl 1989) in overall coloration and by the lack of caulocystidia on the stipe. ITS data from the type material indicates it is genetically the same as an agaricoid form, *I. multifolia*,

Fig. 8. Micromorphological features of *I. multifolia* f. *multifolia* (MGW784, TENN 065795) (*a*) pleurocystidia, (*b*) cheilocystidia, (*c*) basidiospores, and (*d*) basidia. Scale bars equal 10 μm.

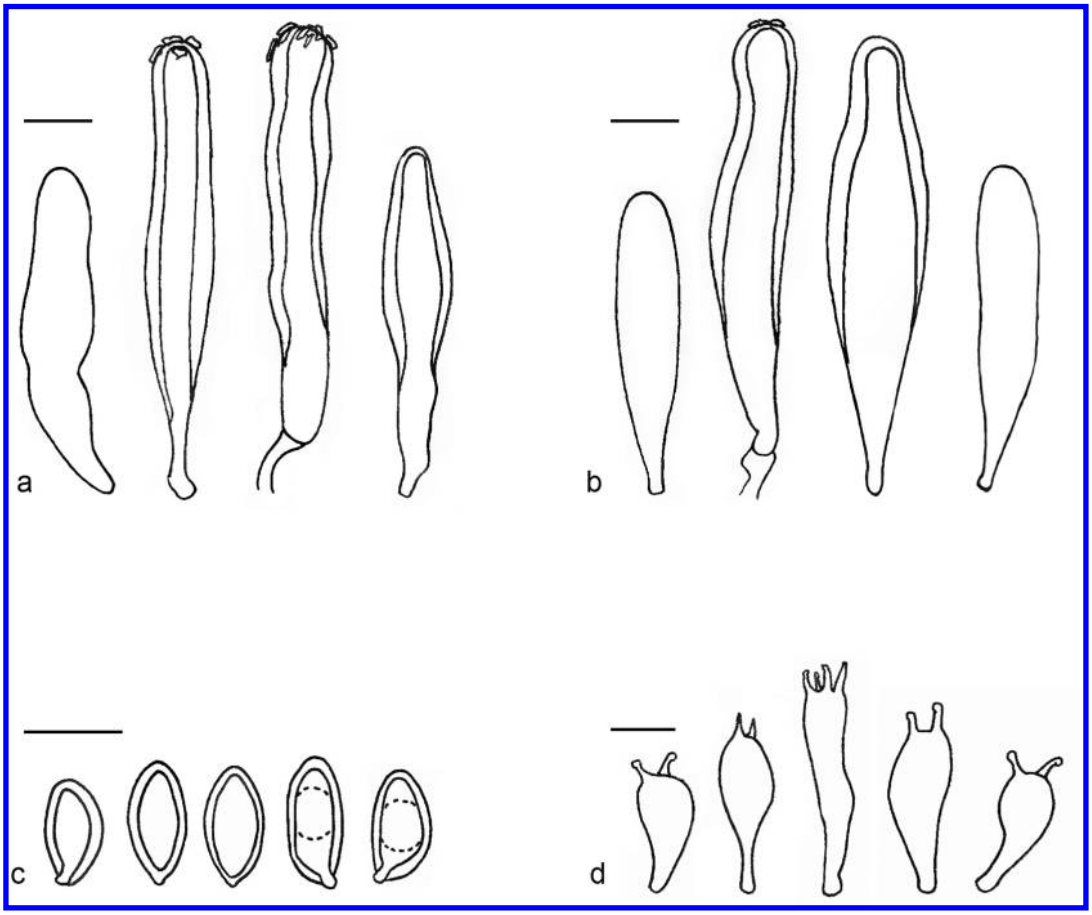


Fig. 9. Micromorphological features of *I. multifolia* f. *cryptophylla* (DV12042011, TENN 066926) (*a*) hymenial cystidia, (*b*) basidiospores, and (*c*) basidia. Scale bars equal 10 μm.

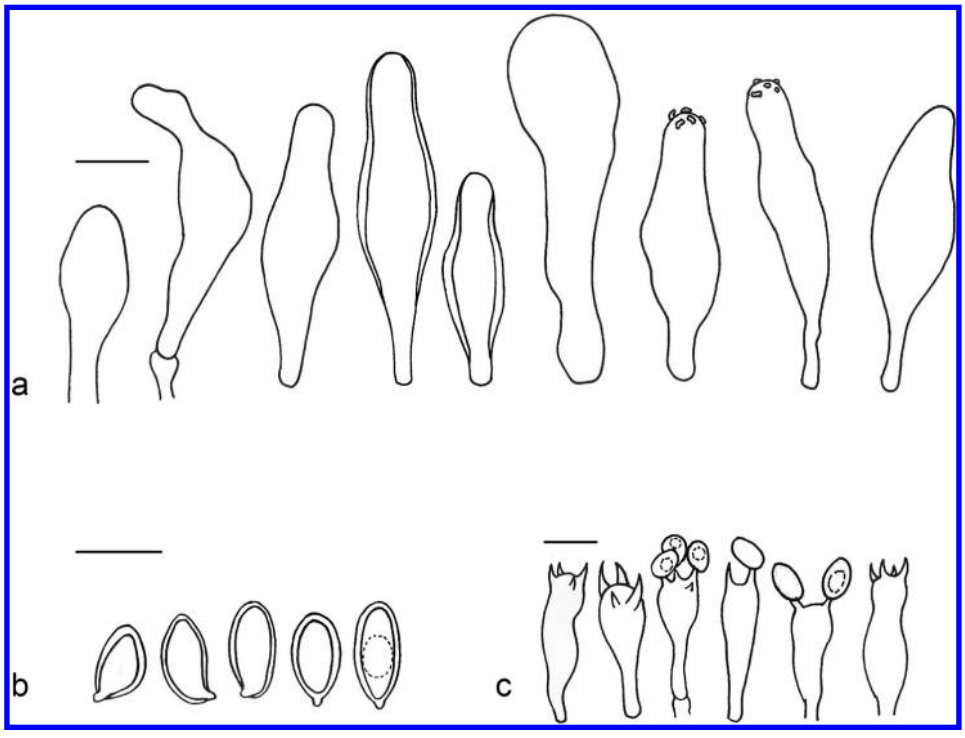
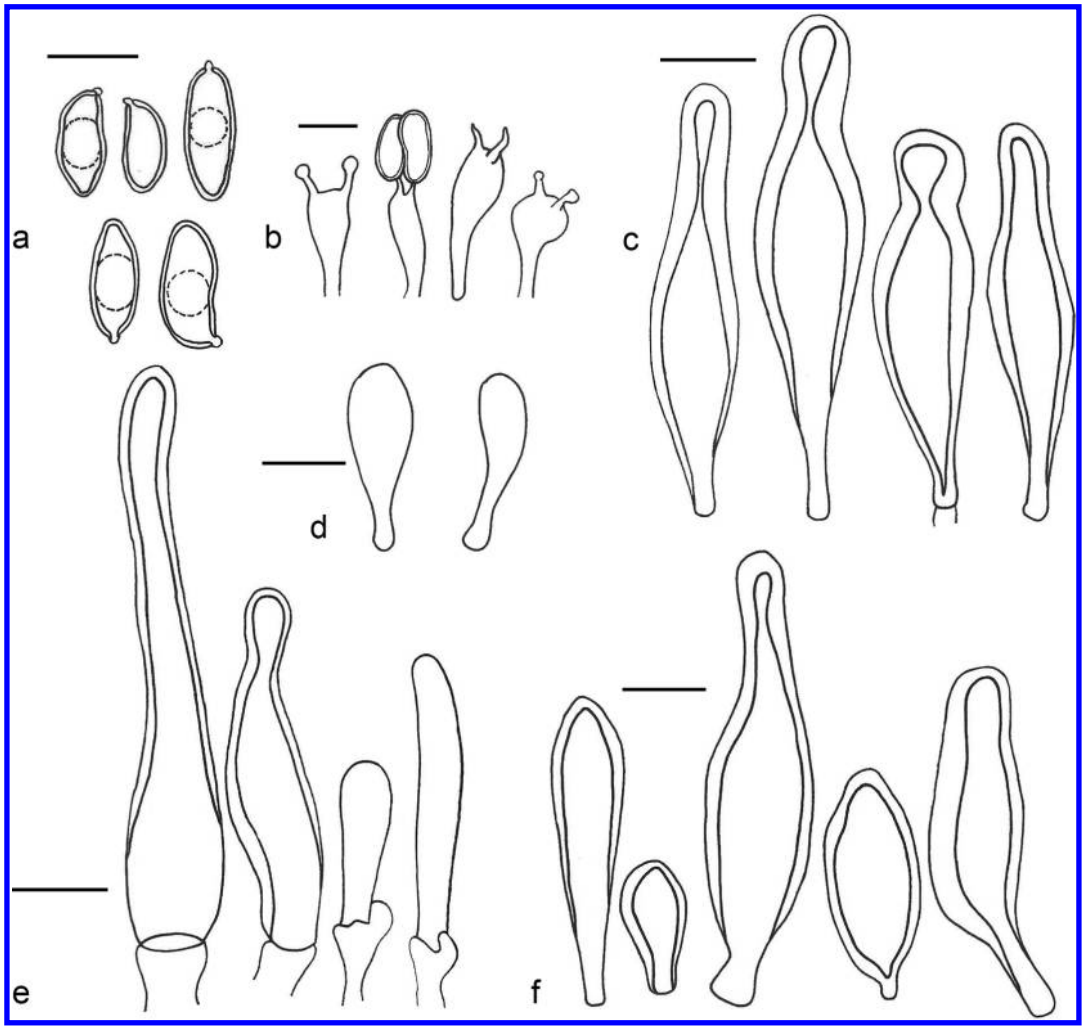


Fig. 10. Micromorphological features of *I. bicornis* (PBM3676, PERTH 08319103) (*a*) basidiospores, (*b*) basidia, (*c*) pleurocystidia, (*d*) paracystidia, (*e*) caulocystidia, and (*f*) cheilocystidia. Scale bars equal 10 μm.



described above from the Sierra Nevada in northern California. These same data reveal homogeneous sequences from variable parts of a single basidiocarp, a result that rejects the hypothesis of sequestrate inducement by a mycoparasitic fungus.

Inocybe bicornis* f. *bicornis Braaten, Bougher, & Matheny, sp. nov.
Figs. 7*c*, 10.

MYCOBANK NUMBER: 804824

DIAGNOSIS: Forms a monophyletic group with *I. bicornis* f. *secotioides* but differs by the agaricoid habit and exclusively 2-sterigmate basidia.

TYPUS: Australia, Western Australia: On ground under *Eucalyptus wandoo* Blakely, revegetation site in Maurice Barnes' paddock, near Letchford, north of Kellerberin, 20-Jul-1995, *W. Dunstan E5563* (PERTH 07605684, holotype); GenBank DNA accession Nos. EU555447, KC305486, KC305448.

ETYMOLOGY: (L.) *bi-* two, *-cornis,* horns, in reference to the exclusively 2-sterigmate basidia.

DESCRIPTION: *Pileus:* 14–25 mm, convex to broadly so in youth, becoming plane with age, umbo absent, velipellis occasionally present over the disc; margin decurved, undulating often on older specimens; surface shaggy to coarsely fibrillose, at times with appressed fibrillose squamules, fibrillose towards the margin, here fibrils diverging by maturity; brownish-yellow or fulvous to yellowish-brown or brown (near 6D6 or "Buckthorn Brown" to "Russet"); context stark white to whitish, up to 4 mm thick under the disc, not changing color where exposed, odor absent. *Lamellae:* adnexed to uncinate, moderately close to close with 32–40 L and several tiers of lamellulae, yellowish-brown or brown (10YR 5/3 to 6/3) in youth or "Avellaneous" (near 6C4) to "Wood Brown", dark yellowish-brown with age; edges pallid-fimbriate but not distinctly so or edges appearing concolorous with faces; ventricose, medium, up to 3 mm deep. *Stipe:* 15–35 mm × 3–6 mm, terete, squat at times in youth when under soil but elongating by maturity, base swollen or weakly bulbous but not distinctly marginate; partial veil not observed but presumably present due to presence of floccose fibrils above the stipe base; surface pruinose at the apex or at least visibly so down towards the center, at times furfuraceous in appearance, lower part with scattered dense clusters of whitish floccules or fibrils, these more densely arranged above the base; light brown or Cinnamon Buff to Tawny-Olive; context dull colored or brownish to reddish-brown, stark white in the slightly bulbous base.

Spores: 10.0–15.5 (–16.0) μm × 5.5–7.5 μm, mean 12.6 μm × 6.3 μm, *Q:* 1.67–2.41 (–2.58), mean *Q:* 2.01 (*n* = 42/3), smooth, oblong-amygdaliform to cylindric with bluntly pointed to obtuse apices, at times reniform in appearance due to kinked or depressed ventral side, less often subangular or sinuous in outline, apiculus small and not very distinctive, yellowish-brown. *Basidia:* 31–41 μm × 9–11 μm, clavate, hyaline, 2-sterigmate. *Pleurocystidia:* 62–92 μm ×

11–15 (–19) μm, variable in shape — slenderly fusiform to slenderly lageniform or more ventricose, at times nearly cylindric, arising from a narrow pedicellate or truncate base; apices obtuse, swollen, or indistinctly subcapitate, crystalliferous or bare; thick-walled to slightly so, 1.0–3.0 μm thick, walls hyaline. *Cheilocystidia:* similar to pleurocystidia or shorter and more variable in shape (clavate, ventricose, cylindric), mixed with hyaline paracystidia, these multiseptate and arising from narrow subtending hyphae (unlike more typical basidia-like paracystidia). *Caulocystidia:* 45–141 × 12–18 μm, shape highly variable, at apex similar to pleurocystidia but also flexuous or sublageniform to cylindric, thin- or thick-walled; at apex and center of stipe mixed with shorter, thin-walled, hyaline (sub)cylindric to clavate cystidioid cells of various size, these dissimilar to paracystidia of the lamellar edge; end cells undifferentiated or occasionally cystidioid (viz., fusiform) above the base. *Pileipellis:* a cutis of lightly incrusted hyphae, these mostly cylindric, up to 11 μm wide, yellowish-brown in mass. *Clamp connections:* present.

ECOLOGY AND DISTRIBUTION: On sandy soil or completely hidden under sandy soil under planted or revegetated sites with *Eucalyptus wandoo*, *Eucalyptus* sp., and (or) *Allocasuarina*, southwest Western Australia, occurring in July.

COMMENTS: *Inocybe bicornis* is the only species of *Inocybe* in Australia observed to date that bears exclusively 2-sterigmate basidia. In outward appearance, the species looks similar to *Inocybe sinuospora* Matheny & Bougher, *Inocybe emergens* (Cleland) Grgur., and *Inocybe farinosipes* nom. prov., the first two of which co-occur in the wheatbelt region of southwest Western Australia. However, all three of these species differ by their 4-sterigmate basidia. *Inocybe emergens* features nodulose spores and is closely related to *I. sinuospora*, both of which are phylogenetically quite distant to *I. bicornis*. *Inocybe farinosipes* may be the sister taxon to *I. bicornis* but differs by its 4-sterigmate basidia and smaller spores. This appears to be widespread in Jarrah forests in southwest Western Australia. *Inocybe aberrans* (E. Horak) Garrido, described from *Nothofagus* Blume forests in Papua New Guinea, features 2-sterigmate basidia but differs readily by its large stellate spores and slender scaly appearance.

A secotioid form (BOU890) with 2- and 4-sterigmate basidia has been identified in the *bicornis* lineage sharing very similar LSU and ITS sequences with normal lamellate forms of *I. bicornis*. Both secotioid and agaricoid forms have been collected from the same general area to the west of Corrigin, in the wheatbelt region under *Allocasuarina*.

OTHER SPECIMENS EXAMINED: Australia. Western Australia: 14 km west of Corrigin, 32°21′25.9″S, 117°44′17.1″E, Old Kunjin Railway, emerging from compact coarse sandy soil or pushing up plaques of wet sand under *Allocasuarina* (Black Tamma), 26-Jul-2011, *P.B. Matheny PBM3676* (PERTH 08319103, TENN 066454) (KC305409-nLSU, KC305487-ITS, KC305448-*rpb2*); same locality as previous, under *Allocasuarina*, 26-Jul-2011, *P.B. Matheny PBM3680* (PERTH 08319057, TENN 066488); same locality as previous, scattered completely under sandy soil under planted *Eucalyptus* and *Allocasuarina*, 26-Jul-2011, *N.L. Bougher BOU00873* (PERTH 08320586, TENN 066711); 5 km west of Corrigin, Corrigin Wildflower Drive, 32°20′19.1″S, 117°40′20.0″E, scattered singly on yellow sand under *Allocasuarina*, 27-Jul-2012, *P.B. Matheny PBM3691* (PERTH 08318948, TENN 066640).

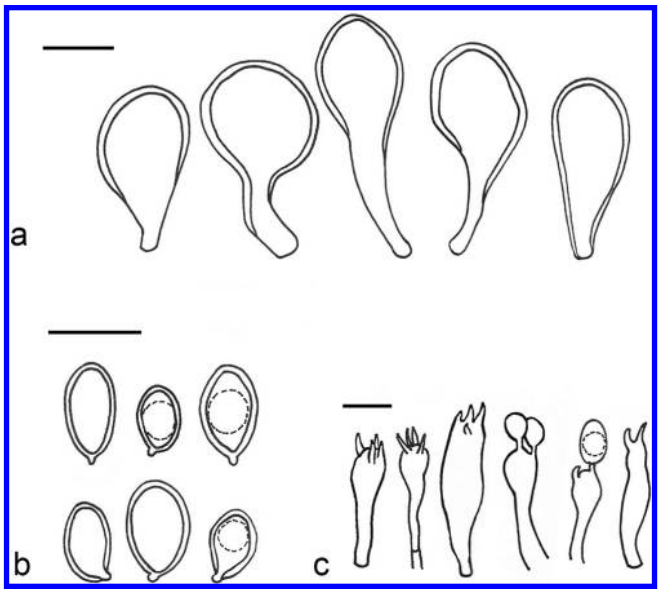
Inocybe bicornis* f. *secotioides Braaten, Bougher, & Matheny, f. nov.
Figs. 7*d*, 11.

MYCOBANK NUMBER: 804825

DIAGNOSIS: Forms a monophyletic group with *I. bicornis* but differs by the secotioid habit and presence of some 4-sterigmate basidia.

TYPUS: Australia. Western Australia: Vinegar Well, off Barber Road, Kunjin, west of Corrigin, 32°23′31.7″S, 117°40′6.5″E, 27-Jul-2011, coll. *P.B. Matheny & N.L. Bougher BOU890* (PERTH8320403, holotype; TENN066804, isotype). GenBank DNA accession Nos. KC305410, KC305488, KC305447.

Fig. 11. Micromorphological features of *I. bicornis* f. *secotiodes* (BOU890, PERTH 08320403) (*a*) hymenial cystidia, (*b*) basidiospores, and (*c*) basidia. Scale bars equal 10 μm.



ETYMOLOGY: (L.) *secotioides*, in reference to the secotioid morphology.

DESCRIPTION: *Pileus:* 7–12 mm diam, margin appressed and clasping the stipe, convoluted button-shaped with strongly incurved margins, no evidence of a veil, surface with some appressed radial fibrils; color dull yellowish (near 5C5) to yellowish-brown and mixed with paler areas; context isabelline, no odor. *Lamellae:* convoluted or a lamellate-like gleba, pale avellaneous to yellowish-brown. *Stipe:* 12–15 mm× 5 mm, even but slightly swollen at the base, with some pruinosity below insertion of pileal margin, otherwise finely fibrillose, pallid or almost white; context solid.

Spores: 7.5–13.5 μm × 5.0–7.5 (–8.4) μm, mean 10.5 μm × 5.9 μm, *Q*: 1.46–2.43, *Q* mean: 1.80 (*n* = 33/1), smooth, yellowish-brown with an oblique apiculus, highly variable in size, shape, wall thickness, and appearance of the cytoplasm, mostly ellipsoid–subcylindric, or oblong, subcylindric, lacrymoid, or broadly elliptic. *Basidia:* 17–26 μm × 6–8 μm, variable in shape, often squat-cylindric with a broad truncate base and narrow apex but some more slender, hyaline, 2- or 4- sterigmate, ballistosporic. *Hymenial cystidia:* 31–33 μm × 18–24 μm, scattered singly or at most with several in close proximity, not abundant, broadly clavate to sphaeropedunculate; thick-walled, walls mostly 2.0–3.0 μm thick, yellowish-brown, not infrequent on faces of the convoluted lamellae. *Caulocystidia:* infrequent, stipe apex with patches of fertile hymenium including an occasional cystidium. *Pileipellis:* a cutis composed of loosely interwoven septate hyphae mostly 8–11 μm wide, slightly pigmented pale yellow. *Clamp connections:* present.

ECOLOGY AND DISTRIBUTION: Underneath cracks in sand on track (hypogeous), hidden underneath or barely emerging above surface of sand, near *Allocasuarina huegeliana* with no eucalypts nearby.

COMMENTS: The individuals described here are best interpreted as secotioid forms of a usually lamellate species common in Western Australia's wheatbelt region, ***I. bicornis*** sp. nov. As there is molecular evidence suggesting genetic divergence, it possible that the secotioid morphology may be the result of small genetic changes, however, environmental factors may also have played some role in altering the shape of the fruit body. We suspect the individuals are likely to have struggled to emerge from compacted sand along

a track and thus resulted in a compressed secotioid form. Molecular data from two loci support a close taxonomic affinity to normal lamellate forms of *I. bicornis*. Of 769 nucleotide positions between the highly variable conserved domains 6 and 7 of *rpb2*, BOU890 (f. *secotioides*) differs at twelve sites (2.6% pairwise sequence divergence) from f. *bicornis* (PBM3676). BOU890 differs at seven positions in a pair-wise comparison at the nLSU-rRNA locus with f. *bicornis* (E5653) but two of these differences entail polymorphic sites in E5653 and three insertions in BOU890. The same three inserted sites in BOU890 were observed in comparison with another collection of f. *bicornis* (PBM3676) but with only one nucleotide site difference (a single transition). While we have variable nucleotide data from the ITS region sampled from f. *bicornis* (E5653 and BOU890), direct ITS sequence results from the secotioid form were highly heterogeneous and not useable. The *rpb2* and LSU sequences of BOU890 are nested within monophyletic groupings of sequences from normal lamellate forms of *I. bicornis*. We therefore propose a novel intraspecific taxon within *I. bicornis* to document the unusual secotioid features of BOU890.

Several differences were also observed microscopically between *I. bicornis* f. *secotioides* and lamellate forms: (*i*) the asymmetric elongate–amygdaliform to cylindric spore shape of f. *bicornis* is absent in the secotioid form, which has predominantly ellipsoid–subcylindric spores; (*ii*) fusiform–ventricose hymenial cystidia of f. *bicornis* contrast with the clavate–sphaeropedunculate cystidia of the secotioid form; and (*iii*) the abundant and variously shaped paracystidia that accompany the cheilocystidia on the gill edge in f. *bicornis* are absent in the hymenium of the secotioid form.

Morphologically, the condition of exclusively 2-sterigmate basidia in species of Inocybaceae is extremely rare (Kuyper 1986). This condition is observed for the normal lamellate forms of *I. bicornis* while f. *secotioides* possesses both 2- and 4-sterigmate basidia. Also, in f. *secotioides*, we observe much greater variance in spore size and shape, perhaps attributable to the deformed formation of the lamellae. The spores are unusually variable in shape, size, and wall development. In addition, there are a high proportion of likely nonviable and (or) aborted spores, as is also indicated by the variable appearance of the cytoplasm. This variability may be partly explained by the spores being produced from either 2- or 4-spored basidia; however, as the cystidia are also anomalous and variable, it may also be suggestive of some stress during development of the fruit bodies. Ecologically, *I. bicornis* f. *secotioides* may be an ectomycorrhizal associate of *Allocasuarina*.

Discussion

Secotioid morphology may represent a preliminary step in an evolutionary direction towards a gasteroid and hypogeous habit (Thiers 1984). As has been observed within Agaricales, Boletales, and Russulales (Hibbett et al. 1994; Kretzer and Bruns 1997; Martín et al. 1999; Peintner et al. 2001), the evolution of secotioid and gasteroid forms has occurred in multiple unrelated lineages. In some cases, the secotioid phenotype may result from relatively modest genomic deviations as in *L. tigrinus* (Hibbett et al. 1994). In some environments, particularly those with xeric climates, sequestration may actually convey a selective advantage (Thiers 1984).

Observations of sequestration in the Inocybaceae, however, are extremely rare. This may be because they lack suitable vectors for presumably toxic fruit bodies as many species of Inocybaceae contain muscarine, a quaternary ammonium compound that binds and stimulates some receptors of the parasympathetic nervous system (Benjamin 1995; Kuyper 1986; Kosentka et al. 2013). Within these taxa, sequestration may represent a poorly adapted phenotype with low fitness.

Phylogenetic analysis and molecular sequence data from *I. bicornis* f. *secotioides* and *I. multifolia* f. *cryptophylla* suggest these two forms are phylogenetically unrelated and have converged to the secotioid form independently. Our results suggest that *I. multifolia* f. *cryptophylla* is a phenotypically induced variant given that ITS sequences of it and a typical lamellate form in the Sierra Nevada are identical. We suspected this might be the case as well in the Australian *I. bicornis* f. *secotiodes*, but this form is somewhat genetically distinctive. Morphologically, it features several traits that separate it from lamellate forms of *I. bicornis*, namely, a mixture of 2- and 4-sterigmate basidia, highly variable spores, and clavate to sphaeropedunculate hymenial cystidia. The latter two attributes could be altered by lamellae conformation, but the presence of 4-sterigmate basidia, in otherwise an exclusive 2-sterigmate basidial clade, may suggest further taxonomic separation than admitted here.

Only two other sequestrate forms of Inocybaceae, both secotioid, have been documented, and only one of these has been formally described. DNA sequences of the former (T18260, OSC) were used in a global biogeographical analysis (Matheny et al. 2009). Current unpublished data support a very close phylogenetic relationship of this secotioid fungus (collected in Victoria, Australia) with unpublished agaricoid forms collected in Queensland and Western Australia. Formal documentation of this third from is planned for future publication. The fourth sequestrate form was described as *A. geoaustralis* in Matheny and Bougher 2006*a* and validated as such in Matheny and Bougher 2006*b*. To date, this species remains phylogenetically distinct from seven other described species of *Auritella* (Matheny et al. 2012) and the only sequestrate species in this genus. Thus, three of the four known sequestrate forms of Inocybaceae are known only from Australia.

Acknowledgements

We thank Pierre-Arthur Moreau (Université Lille Nord de France) for assistance with the literature and Brian O'Meara (University of Tennessee) for assistance with phylogenetic analyses. This work was supported by a National Science Foundation (NSF) Research Experience for Undergraduates supplement for Braaten and NSF award DEB-0949517 to Matheny and Bougher. Also Bougher and Matheny acknowledge a research grant from the Australian Government's Australian Biological Resources Study National Taxonomy Research Grant Program (RFL211-31) and associated financial support from the Western Australian Naturalists' Club Inc. We thank Joshua Birkebak (University of Tennessee) for assistance with MycoBank submissions.

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