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Two new species of *Inocybe* from Mediterranean Cistaceae heathlands

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ABSTRACT

This study explored a heathland region in Portugal, and through morphology, biogeography, and multilocus phylogeny, two new species of Inocybaceae are described. The first species, *Inocybe iberilepora*, belongs to "*I. flocculosa* group," whereas the second species, *Inocybe phaeosquamosa*, belongs to a relatively isolated and understudied clade, distantly related to *I. furfurea* and allies. Both species are tied to a west Mediterranean distribution and ecology, associating with the local Cistaceae ecosystems. By characterizing these new species, our research contributes to the understanding of European Funga and enriches the knowledge of the genus *Inocybe* on a global scale.

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Agaricales; biodiversity; cistus; ecology; European forests; fungi; inocybaceae; taxonomy; 2 new taxa

INTRODUCTION

Inocybe s. str. is thought to be a relatively recent genus of macrofungi, initiating a rapid and highly diversified evolution about 52–79 million years ago (Kosentka et al. 2013; Matheny et al. 2009; Ryberg and Matheny 2012; Sánchez-García et al. 2020; Varga et al. 2019). Such explosion in biodiversity is visible today as a rich genus, not only in terms of morphology or niche ecology (Ryberg et al. 2010), but also in number of taxa. It is estimated that the number of species could reach between 3000 and 5000 within *Inocybe* alone (Bhunjun et al. 2022). Taken together, this means that laborious taxonomic efforts necessarily await taxonomists to formally and adequately describe the new taxa. In fact, in the past 2 years, several dozens of taxa have been described in Europe alone (Bandini et al. 2021, 2022a, 2022b).

The western Mediterranean basin exhibits remarkably high biodiversity (Buira et al. 2021; Ramos Gutiérrez et al. 2021; Vila-Viçosa et al. 2023), especially with regard to Cistaceae (Civeyrel et al. 2011; Coello et al. 2021), a plant family known for its mycorrhizal associations (Daskalopoulos et al. 2021; Sanz-Benito et al. 2022). These shrubby plants not only form exclusive associations with several fungal species but also play a crucial role as surrogates, synthesizing ectomycorrhizae (EcMs) with fungi primarily associated with arboreal plants (Albuquerque-Martins et al. 2019; Comandini et al. 2006). In particular, *Inocybe*, among several macrofungal genera, is reported to be instrumental in facilitating the natural succession from old Cistaceae to young *Quercus* stands (Sanz-Benito et al. 2023). Another potential benefit of EcMs to hosts, especially Cistaceae, is the mitigation of parasitic overexploitation by endophytic plants (de Vega et al. 2010).

However, the same area is still largely unexplored from a mycological standpoint, with a steady stream of novelties emerging even from well-researched genera (Alvarado et al. 2022, 2010; Arraiano-Castilho et al. 2022; Garrido-Benavent et al. 2019). For a plant biodiversity hot spot, it is reasonable to expect that many rare and previously unknown species may exist in this territory, particularly within taxonomic groups that are both highly diverse and understudied, such as the genus *Inocybe* (Bandini et al. 2021, 2022a; Bhunjun et al. 2022; Matheny et al. 2006, 2020).

In this study, we employed alpha taxonomy and multilocus molecular phylogeny to unveil two new species belonging to the genus *Inocybe* in the Inocybaceae family. Supplementarily, we analyzed and discussed biogeographic annotations of *Inocybe iberilepora* and *Inocybe phaeosquamosa*, the two species described from the coastal heathlands of the western Mediterranean basin.

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MATERIALS AND METHODS

Field work.—The field expeditions took place between January of 2021 and December of 2022. For each species, we made two collections, each comprising several basidiomata in different stages of maturation, collected from different seasons. All macromorphological features were scale-recorded in every collection with a date-GPS-calibrated 5D Mark IV camera (Canon, Japan), equipped with a SP 1:2.5/90 mm objective (Tamron, Japan). Additionally, notes concerning ecology and organoleptics were taken for each collection. Processing of RAW files was performed in RawTherapee (Horváth accessed 29 Mar 2023), whereas morphometry was carried in Fiji (Schindelin et al. 2012). The color codes are taken from Munsell (Munsell 2009), and the terminology follows Kuyper (1986).

In order to preserve the taxonomically critical structures in *Inocybe*, basidiomata were carefully collected into a compartmented container. Within 1 hour after each field trip, specimens were processed on a ventilated dehydrator at 40 C (SilverCrest IAN 302447; Germany). Holotypes and paratypes were deposited in herbarium PO, at the Museum of Natural History of Porto University, Portugal.

Micromorphological studies.—The exsiccatae were rehydrated with water moisture and studied in 3% potassium hydroxide (KOH). All microscopic features were independently studied—and thus validated—by two different mycologists using (i) a BX50 BXFLA microscope (Olympus, Japan) coupled with a Axiocam 305 camera (Carl Zeiss, Germany), through Fluar 40×/0.7 and UPlanFL 100×/1.30 oil objectives (Olympus); and (ii) a DM750 microscope (Leica, Germany) coupled with a Axiocam ERc 5s camera (Carl Zeiss), through HI Plan 40×/0.65 and C Plan 100×/1.25 oil objectives (Leica). All illustrations were drawn from the latter setup.

For every collection, in order to observe cystidial distribution and ornamentation, carefully made sections were studied prior to conducting localized smears. In order to preserve microstructure reliability, all data were collected within 30 minutes of initial KOH incubation.

Cystidia and basidia were assessed excluding crystals and sterigmata, respectively. All spores were measured from the stipe preparations. The dimensions of all elements assessed are denoted as length × width. The Q value is equivalent to the proportion of spore length to spore width, which is computed for each individual spore. The abbreviations av. and SD stand for "average" and "standard deviation," respectively.

Micrograph processing was done in Fiji (Schindelin et al. 2012) and CombineZP (Hadleys accessed 29 Mar 2023), whereas morphometry was carried in Piximètre (Henriot and Cheype accessed 29 Mar 2023) and AxioVision 4.8 (Carl Zeiss).

DNA sequencing.—Total DNA was extracted from dry specimens employing a modified protocol based on Murray and Thompson (1980). The polymerase chain reactions (PCRs) (Mullis and Faloona 1987) included 35 cycles with an annealing temperature of 54 C. The primers ITS1F, ITS4, and ITS4B (Gardes and Bruns 1993; White et al. 1990) were employed to amplify the nuc rDNA internal transcribed spacer ITS1-5.8S-ITS2 region (ITS); LR0R, LR5 (Cubeta et al. 1991; Vilgalys and Hester 1990), and ITS4Brev (reverse of ITS4B) were used for the partial 28S nuc rDNA region (28S); and bRPB2-6F2 (reverse of bRPB2-6R2) and bRPB2-7R2 (Matheny et al. 2007) were used for the sites between domains 6 and 7 of the *rpb2* gene (encoding the second-largest subunit of nuclear RNA polymerase II; RPB2). The PCR products were checked in 1% agarose gels, and amplicons were sequenced with one or both PCR primers. Sequences were corrected to remove reading errors in chromatograms.

Bioinformatics.—For each locus, chromatograms were bidirectionally assembled, trimmed, and edited for ambiguities in CodonCode Aligner (CodonCode, Massachusetts). The remaining sequences completing the alignment were selected by BLASTing each marker (ITS, 28S, and RPB2) against both GenBank and UNITE databases. We included closely related and confidently identified species, representative species from closely related clades, and species of significant morphological similarity. Additionally, we included all sequences (excluding duplicates) belonging to the same UNITE 1% species hypothesis (Kõljalg et al. 2013; Nilsson et al. 2019) as the sequences produced in this study. Finally, we have also included the sequences immediately adjacent to the aforementioned species hypotheses (excluding duplicates). The type sequences of *Inosperma saragum, Nothocybe distincta*, and *Inocybe flavoalbida* were included in the alignment as outgroups. All sequences used in this study can be referred to in TABLE 1.

The alignment was initiated with MUSCLE in MEGA X (Kumar et al. 2018) and manually adjusted for the three markers separately. Since the components within

Table 1. Sequences used in this study.

Classification	Country	Herbarium/*Source*	Voucher/*Sample*	GenBank sequence accession numbers			Study reference
				ITS	28S	RPB2	
I. aurantiobrunnea	Italy	STU	**F-0001816**	OP164016	OP164016	—	Bandini et al. (2022a)
I. botaurina	Germany	FR	**FR-0246008**	MK929259	—	—	Bandini et al. (2019a)
I. brijunica	Croatia	PUL	**F27673**	NR_172782.1	NG_075311.1	MT878449.1	Mešić et al. (2021)
I. deianae	France	STU	**F-0901538**	OK057117	—	—	Bandini et al. (2022b)
I. flavoalbida	Australia	TENN	**067000**	KJ729873	NG_057225	KJ729932	Matheny et al. unpubl.
I. flocculosa	Germany	STU	F-0901628	OK057165	OK057165	—	Bandini et al. (2022b)
I. flocculosa	Norway	WTU	PBM2392	—	AY380375	AY337375	Matheny (2005)
I. furfurea	France	G	**00053152**	MG012472	—	—	Bandini et al. (2019b)
I. gansuensis	China	HMJAU	**2012150**	KY402221	KY402217	KY402219	Fan and Bau (2020)
I. glabripes	Germany	STU	**F-0900979**	MW845881	MW845881	—	Bandini et al. (2021)
I. iberilepora	Portugal	PO	**PO-F2272**	OQ690007	OQ690007	OR360833	This study
I. iberilepora	Portugal	PO	PO-F2712	OQ690008	—	—	This study
I. minimispora	Austria	STU	**F-0901264**	MW845934	MW845934	—	Bandini et al. (2021)
I. mycenoides	Germany	STU	F-0901647	OK057156	OK057156	OK078899	Bandini et al. (2022b)
I. neorufula	Italy	STU	F-0901445	MT101876	MT101876	—	Bandini et al. (2020)
I. nitidiuscula	USA	TENN	062537	—	—	MH577476	Matheny et al. unpubl.
I. phaeoleuca	Hungary	GB	EL297-08	KJ399958	KJ399958	—	Larsson et al. (2014)
I. phaeosquamosa	Portugal	PO	**PO-F2346**	OQ690006	OQ690006	OR360834	This study
I. phaeosquamosa	Portugal	PO	PO-F2713	OQ690005	—	—	This study
I. psammobrunnea	France	LIP	**LIL-89226**	MW845926	—	—	Bandini et al. (2021)
I. queletii	USA	WTU	PBM 935	—	AY380390	AY337397	Matheny (2005)
I. rivierana	Austria	STU	**F-0901249**	NR_174866	MW845910	—	Bandini et al. (2021)
I. rufescens	Australia	PERTH	**08318468**	NR_152370	NG_057261	KM406231	Matheny et al. unpubl.
I. rufobrunnea	Netherlands	L	**0053539**	MZ667616	—	—	Bandini et al. (2022b)
I. rufuloides	Australia	PERTH	08305978	JN035291	—	MH577442	Matheny et al. unpubl.
I. rufuloides	Germany	STU	F-0901442	MT101878	—	—	Bandini et al. (2020)
I. saragum	India	CAL	**1360**	KY440103	KY549133	KY553249	Latha and Manimohan (2017)
I. somae	Germany	STU	**F-0901580**	OK057157	OK057157	OK078902	Bandini et al. (2022b)
Inocybe sp.	Canada	*EcM*	*OTU97*	JX630893	—	—	Timling et al. (2012)
Inocybe sp.	Canada	*Soil*	*OTU955*	KC965941	KC965941	—	Timling et al. (2014)
Inocybe sp.	Canada	*Soil*	*OTU2479*	KF297126	KF297126	—	Timling et al. (2014)
Inocybe sp.	Croatia	*Soil*	*TUE003033*	UDB02073561	—	—	Tedersoo et al. (2014)
Inocybe sp.	Italy	MCVE	21547	JF908222	—	—	Osmundson et al. (2013)
Inocybe sp.	Italy	MCVE	3665	JF908112	—	—	Osmundson et al. (2013)
Inocybe sp.	Italy	*Soil*	*TUE002696*	UDB02018119	—	—	Tedersoo et al. (2014)
Inocybe sp.	Italy	*Soil*	*TUE000394*	UDB03628993	—	—	Tedersoo et al. (2014)
Inocybe sp.	Italy	*EcM*	*G3489*	UDB026553	—	—	Tedersoo et al. (2014)
Inocybe sp.	Italy	*Soil*	*TUE002612*	UDB01996910	—	—	Tedersoo et al. (2014)
Inocybe sp.	Italy	*EcM*	*Inoc4*	GQ469523	—	—	Iotti et al. (2010)
Inocybe sp.	Morocco	*Soil*	*TUE000623*	UDB03650314	—	—	Tedersoo et al. (2014)
Inocybe sp.	Morocco	*Soil*	*TUE000620*	UDB03650179	—	—	Tedersoo et al. (2014)
Inocybe sp.	Morocco	*Soil*	*TUE000617*	UDB03650151	—	—	Tedersoo et al. (2014)
Inocybe sp.	USA	TENN	063941	—	—	MH577457	Matheny et al. unpubl.
I. tarda	Germany	STU	**F-0901730**	NR_185445	OP164094	—	Bandini et al. (2022a)
I. tigrina	Germany	STU	**F-0901532**	NR_174869	MW845933	—	Bandini et al. (2021)
I. tjallingiorum	Netherlands	L	**0053540**	MW845929	—	—	Bandini et al. (2021)
I. variispora	Spain	SMG-GME	**980504-01**	MT101872	—	—	Bandini et al. (2020)
I. venerabilis	Germany	STU	**F-0901605**	NR_176174	OK057198	—	Bandini et al. (2022b)
I. cf. *violaceoalbipes*	USA	TENN	062462	—	—	MH577486	Matheny et al. unpubl.
I. woglindeana	Germany	STU	**F-0901435**	NR_185414	MT101882	—	Bandini et al. (2020)
I. zethi	Netherlands	STU	**F-0901456**	NR_184511	ON003440	—	Bandini et al. (2022c)
N. distincta	India	CAL	**1310**	NR_173156	NG_057278	KX171345	Latha et al. (2016)

Note. Vouchers in bold indicate type material. Under column "Herbarium/*source*," the initials refer to herbaria according to Index Herbariorum, except when in italic (then referring to the isolation source).

ITS have variable evolution rates (Hillis and Dixon 1991), ITS1, 5.8S, and ITS2 were split and treated as distinct partitions. A total of 5 partitions from 52 taxa and 3103 sites (43% missing data) were concatenated in Mesquite (Maddison and Maddison 2023) before maximum likelihood (ML) and Bayesian inference (BI) analyses (TABLE 2).

The substitution models for each partition were selected separately for ML and BI analyses based on the Bayesian information criterion score using ModelFinder (Chernomor et al. 2016; Kalyaanamoorthy et al. 2017), as seen in TABLE 2. The ML analysis was carried out in IQ-TREE 2.2.2.6 (Minh et al. 2020), by generating 10 000 ultrafast bootstrap (UFB) samples (Hoang et al. 2017) for two independent runs and allowing each partition to have its own evolution rate, resulting in a best log-likelihood of −14 763. The BI analysis was performed in MrBayes 3.2.7 (Ronquist et al. 2012) in two independent runs, each with four chains sampled every 1000 out of 10 million generations, resulting in an average standard deviation of split frequencies of <0.004. Posterior probabilities (PPs) for BI were then calculated after setting the burn-in to 25%.

Table 2. Partitions and respective models used for phylogenetic analysis.

Partition	No. of taxa	No. of sites	No. of informative sites	Selected models		Substitution rate (ML)*
				for IQ-TREE	for MrBayes	
ITS1	46	407	152	TPM2u+F+G4	HKY+F+G4	2.70
5.8S	47	154	1	TNe	K2P	0.05
ITS2	47	350	147	HKY+F+I+G4	HKY+F+I+G4	2.54
28S	26	1406	87	K2P+I+G4	K2P+I+G4	0.32
RPB2	16	786	147	K2P+G4	K2P+G4	0.83

*Normalized to the weighted average of 1, where the weights are the lengths of each partition divided by the final concatenation length.

The visualization and production of the final trees were carried out in FigTree 1.44 (Rambaut 2006–2018) and Inkscape 1.3 (http://www.inkscape.org). The aligned partitions and the respective trees produced in this study can be found in TreeBASE (study ID TB2:S30312) and as a nexus file in Supplementary Material (SUPPLEMENT 1).

Finally, the tool PlutoF (Abarenkov et al. 2010) was used to gather relevant environmental metadata from the selected sequences. Such metadata were then imported into dataframes with pandas (pandas development team 2020) and plotted with PyGMT (Uieda et al. 2023) in order to assess the newly described species' biogeography.

RESULTS

Phylogeny.—The tree in FIG. 1—derived from ML topology—outlines the multilocus phylogeny (ITS1+5.8S+ITS2+28S+RPB2) involving all taxa included in this study. The ML and BI analyses resulted in nearly identical tree topologies (see TB2:S30312 and SUPPLEMENT 1), with robust overall UFB and PP support for most nodes. The nodes denoting less support originate from distant taxa included for morphological comparison with *I. iberilepora* and *I. phaeosquamosa* (grayed out branches in FIGS. 1 and 2). The three genera of Inocybaceae used in our tree—*Inosperma, Nothocybe*, and *Inocybe*—received strong basal node support (UFB = 0.94). This allows us to infer that the new species described in this study diverged from each other at a relatively early node during *Inocybe* evolution (UFB = 0.95, PP = 0.99; annotated with a cyan-filled arrow in FIGS. 1 and 2).

Inocybe iberilepora shows affinity to the clade of *I. aurantiobrunnea* (UFB = 1, PP = 1; highlighted orange in FIG. 1). The latter is reflected by the ≈ 3.5% difference in the ITS+28S sequenced regions and ≈ 6% in ITS alone (see TB2:S30312). With robust support (UFB = 0.99, PP = 1), our results demonstrate that the holotype and paratype of *I. iberilepora* sit well within the UNITE's 1% species hypothesis (SH1959469.09FU), represented in FIG. 1 by *Inocybe* sp. TUE000620 and *Inocybe* sp. *Inoc4*, with ≥99.8% ITS similarity. However, falling just outside the 1% species hypothesis (≥1.6% differences in the ITS region) are sequences UDB02073561, UDB03650151, and GenBank JF908222 (FIG. 1). The latter set of sequences show at least 10 stable base pair (bp) differences toward all other *I. iberilepora* sequences, which, together with their well-supported nodes, restrains us from considering them part of the same species (FIG. 1). All of the above, including *I. iberilepora*, belong to the same broader clade as the relatively distant *I. flocculosa* and *I. tigrina* (UFB = 0.97, PP = 0.94; highlighted yellow in FIG. 1). The same results are supported by the individual analysis of the more conserved 28S and RPB2 genes, despite the lower node values and somewhat incoherent topology in the 28S tree (FIG. 2a, b).

Contrarily to *I. iberilepora, Inocybe phaeosquamosa* stands relatively far from any confidently known taxa (FIG. 1). It is well placed (UFB = 1, PP = 1), without stable bp differences (and always >99.7% ITS similarity), within UNITE's 1% species hypothesis (SH1368099.09FU), together with soil sequences UDB03650314, UDB02018119, UDB03628993, UDB01996910, and UDB026553 (FIG. 1). There are several closely related but well-separated sequences (UFB = 0.98, PP = 0.98; FIG. 1) belonging to two or three undescribed species from Italy and Canada, with at least 31 bp differences (≥4.4%) in the ITS region (highlighted dark pink in FIGS. 1 and 2). All these share an early node (UFB = 0.92, PP = 0.97; FIG. 1) with the clade of *I. furfurea, I. rufescens*, and *I. rivierana* (highlighted light pink in FIGS. 1 and 2). Although the node support is relatively low, the analysis of the RPB2 gene alone suggests a closer phylogenetic affinity between *I. phaeosquamosa* and an Australian sequence labeled *Inocybe* cf. *violaceoalbipes* than it does with *I. rufescens* (UFB = 0.71, PP = 0.53; FIG. 2b). Similarly, when analyzing the 28S gene alone, *I. rufescens* appears to be less closely related to *I. phaeosquamosa* compared with *I. rivierana* (FIG. 2a). However, due to the absence of common sequenced regions between *I.* cf. *violaceoalbipes* and the pair *I. furfurea*/*I. rivierana* (TABLE 1), their exact position relative to *I. phaeosquamosa* remains uncertain, resulting in low node confidence and a ML/BI discrepancy in tree topology (UFB = 0.58; FIG. 1).

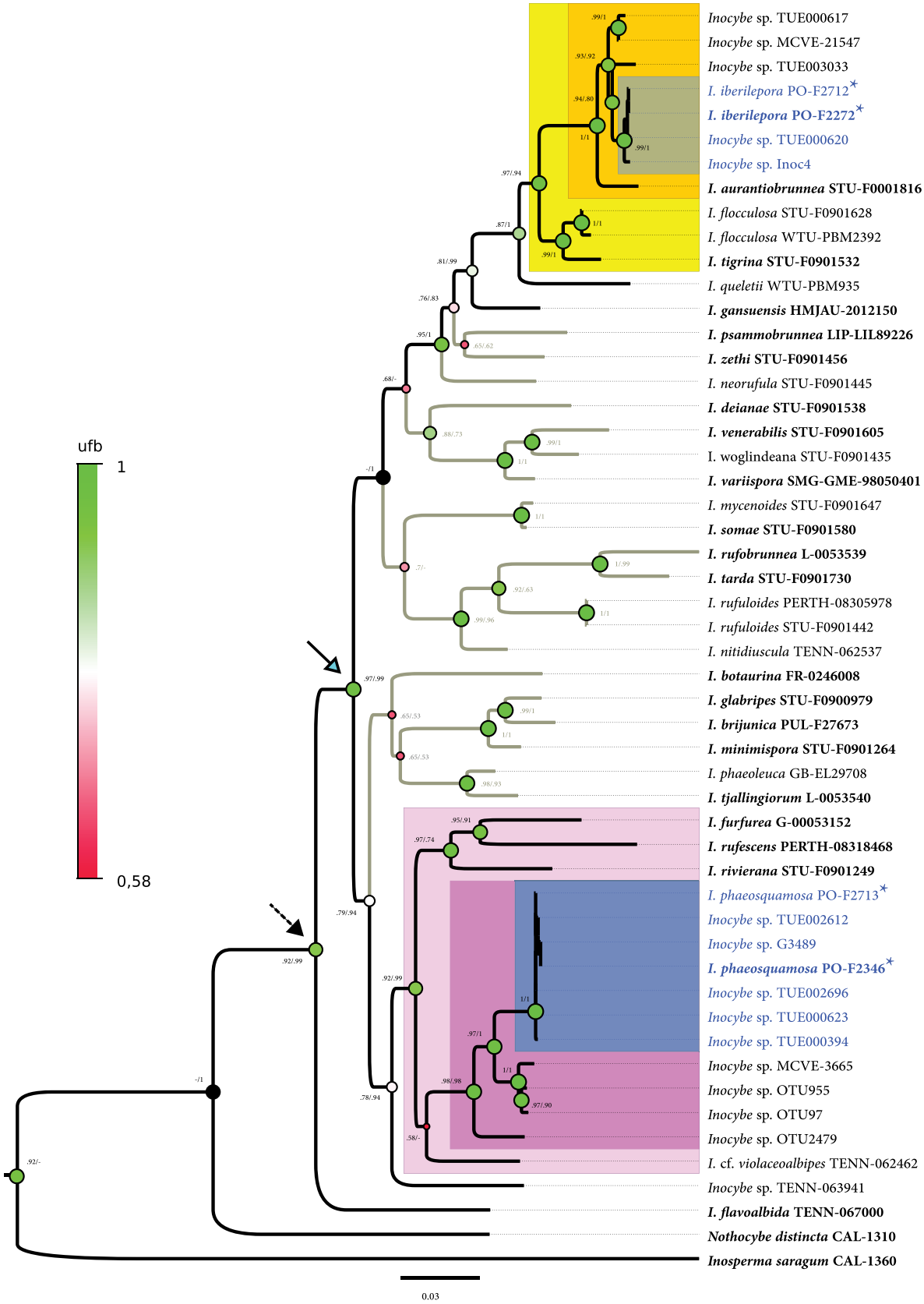

Figure 1. Phylogeny from concatenated data (ITS1+5.8S+ITS2+28S+RPB2) with topology derived from ML. Node support is represented by circle size and color, and by UFB (ML) and PP (BI) values, respectively. Dashed black-filled arrow indicates *Inocybe* s. str. lineage; cyan-filled arrow indicates split between *I. iberilepora* and *I. phaeosquamosa* lineages. Grayed-out branches indicate distant clades with morphologically similar species to *I. iberilepora* and *I. phaeosquamosa*. Every leaf in bold signify a type record. Asterisks (*) mark the vouchers sequenced in this study. Orange and yellow indicate *I. iberilepora*'s inner and outer clades, respectively. Dark and light pink indicate *I. phaeosquamosa*'s inner and outer clades, respectively. Blue highlights species newly described in this study.

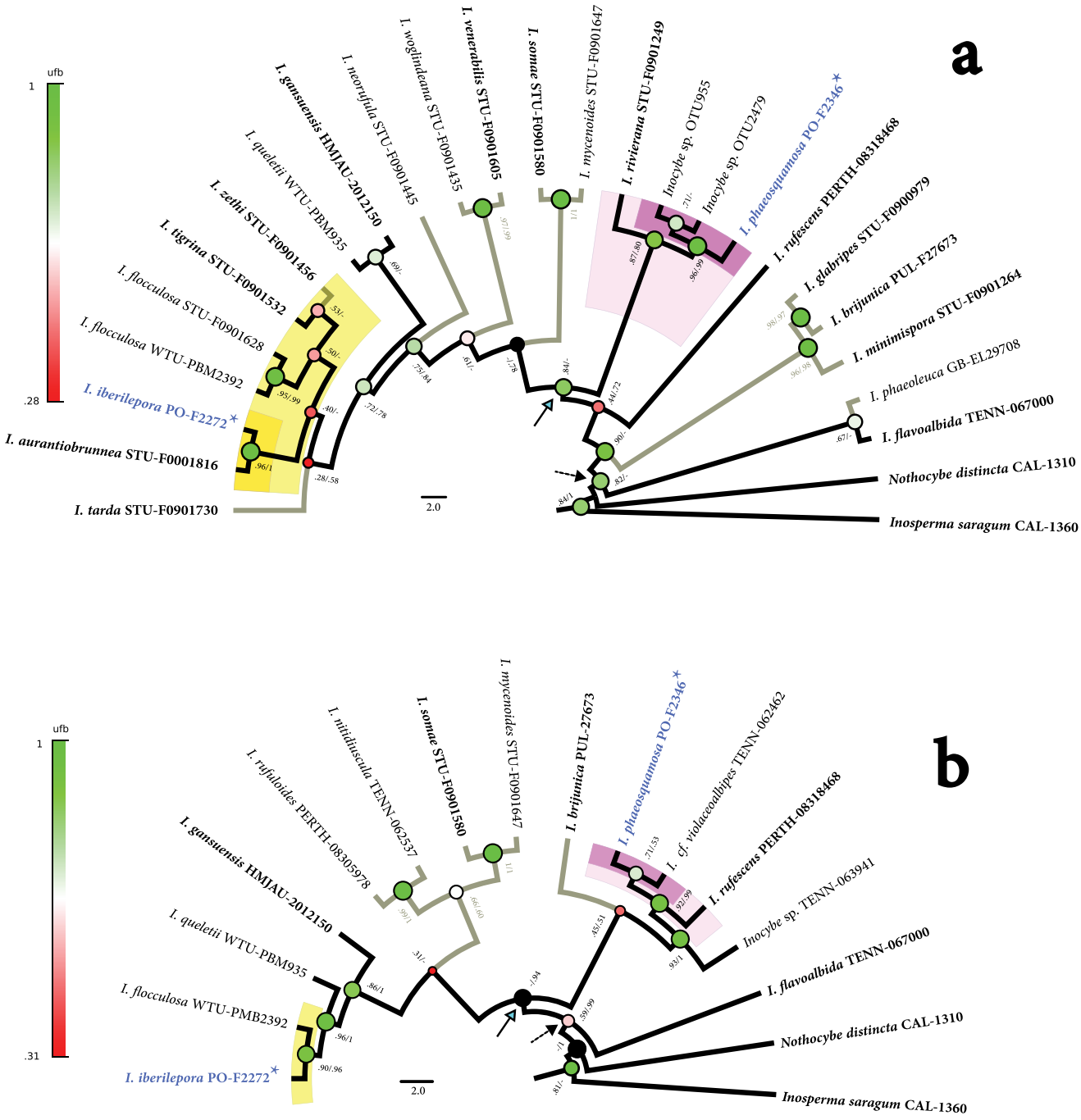

Figure 2. Phylogeny from isolated genes 28S (a) and RPB2 (b), represented by ML topology and polar layout with proportionally transformed branches. Node support is represented by circle size and color, and by (ML) and PP (BI) values, respectively. Dashed black-filled arrow indicates *Inocybe* s. str. lineage; cyan-filled arrow indicates split between *I. iberilepora* and *I. phaeosquamosa* lineages. Grayed-out branches indicate distant clades with morphologically similar species to *I. iberilepora* and *I. phaeosquamosa*. Every leaf in bold signify a type record. Asterisks (*) mark the vouchers sequenced in this study. Orange and yellow indicate *I. iberilepora*'s inner and outer clades, respectively. Dark and light pink indicate *I. phaeosquamosa*'s inner and outer clades, respectively. Blue highlights species newly described in this study.

TAXONOMY

Inocybe iberilepora Fachada & Bandini, sp. nov.

FIG. 3

MycoBank MB848093

Etymology: Referring to the striking resemblance to the local Iberian hare's fur.

Diagnosis: Inocybe iberilepora is characterized by its gray-mottled, fibrillose-(sub)lanose pileus, plumpish-fusiform, often (sub)capitate hymenial cystidia, and poorly metuloid and catenate caulocystidia. It prefers acidic soils, associating with heathland *Cistus* and *Pinus* species.

Figure 3. *Inocybe iberilepora*. a, b, and c. Basidiomata (holotype). d. Basidiospores (paratype). e. Pleurocystidia (paratype). f. Collection locality (paratype). g. Microscopical characters (holotype). Ca = caulocystidia; Cpa = cauloparacystidia; Ch = cheilocystidia; Pa = paracystidia; Pl = pleurocystidia; Sp = spores. h. Caulocystidia and cauloparacystidia (paratype).

Typification: PORTUGAL. Sesimbra, Faúlha (WGS84 coordinates: 38.478707, −9.085062, elevation 121 m), with *Cistus salviifolius, Pinus pinaster* 20 m away, 1 Jan 2021, leg. *Vasco Fachada* (**holotype** PO-F2272, isotype private herbarium [priv. herb.] V.F. VF010121IR1). GenBank: ITS+28S = OQ690007; RPB2 = OR360833.

Description: Pileus 12–30 mm wide, at first (sub) campanulate or hemisperical, with age hemispherical-convex, without or with low large umbo, margin at first incurved, later decurved, young and sometimes also older basidiomata with sometimes fugacious remnants of a whitish velipellis; color usually mottled and therefore sometimes contrasting light grayish-brown to dark brown (Mu 10 YR 4/2–4/4, 3/3–3/6; 7.5 YR 3/2), sometimes somewhat paler at the center; surface at first finely tomentose, later coarsely tomentose or fibrillose, sometimes sublanose, at the margin sometimes effaced; young basidiomata with remnants of a whitish cortina. Lamellae somewhat crowded (ca. 45–65, l = 1), adnexed to almost broadly adnate, straight to (sub) arched, hardly with ventricose portion, edge minutely fimbriate, at first whitish later brownish to brown with or without faint grayish tinge. Stipe 11–25 × 5–8 mm; stocky, cylindrical straight, with gently enlarged base; densely and entirely covered with whitish tomentum but only very faintly pruinose at apex, giving whitish appearance in early stages, later longitudinally striate, red-brown beneath the tomentum, often retaining whitish base in maturation. Context white in stipe, grayish in pileus, smell subspermatic, taste indifferent. Color of exsiccata brownish gray.

Spores 8.5–12 µm (av. 10.0 µm, SD 0.7 µm) × 4.8–6.7 µm (av. 5.6 µm, SD 0.3 µm); Q = 1.4–2.1 (av. 1.8, SD 0.1) (n = 178 of 2 collections [coll.]); smooth, ellipsoid to sometimes (sub)phaseoliform or (sub)amgydaloid with gentle suprahilar depression, apex often (sub) conical, sometimes clearly obtuse. Basidia 19–38 × 6–10 µm; generally tetrasporic, rarely bisporic. Pleurocystidia 48–82 µm (av. 63 µm, SD 9 µm) × 11–17 µm (av. 14 µm, SD 2 µm); Q = 3.4–5.7 (av. 4.5, SD 0.6) (n = 33 of 2 coll.); mostly plump-fusiform to almost "sac-shaped," sometimes (sub)clavate, rarely (sub)cylindrical, frequently (sub)capitate with faint gelatinous "cap"; without or with only short neck, with short pedicel or without pedicel, and then sometimes with rounded base, often without crystals, but occasionally quite crystalliferous; occasionally filled with pale amorphous content; walls rather thin up to 1.0 (≤1.7) µm thick at the apex, pale yellowish-greenish with 3% KOH. Cheilocystidia very much like pleurocystidia, only somewhat shorter in average, intermixed with numerous colorless, (sub)clavate, thin-walled paracystidia; 36–66 µm (av. 52 µm, SD 7 µm) × 12–18 µm (av. 15 µm, SD 1 µm); Q = 2.4–5.0 (av. 3.6, SD 0.6) (n = 29 of 2 coll.). Caulocystidia 24–84 µm (av. 44 µm, SD 13 µm) × 7–19 µm (av. 10 µm, SD 2 µm); Q = 2.1–7.9 (av. 4.4, SD 1.2) (n = 48 of 2 coll.); poorly metuloid without crystals and hard to define, only present at the apex as thick-walled (sub)clavate or (sub)cylindrical terminal cells, arising from catenate hyphoid structures, which in turn are present on the entire stipe length, walls up to 0.5 µm thick, pale yellowish-greenish with 3% KOH; intermixed with some colorless, shorter (sub)clavate, thin-walled cauloparacystidia. Pileipellis a clearly layered cutis, with a subpellis of dark brown incrustating pigment (cells ≤20 µm wide), and a more hialine suprapellis but with very faint zebra-like incrustations (cells ≤12 µm wide). Clamp connections present, abundant in pileipellis and conspicuous in caulocystidia.

Habitat and known distribution: The two occasions this species was observed by us were in rather isolated and exposed sandy clearings, having *Cistus salviifolius* as the apparent mycorrhizal partner (FIG. 3f). Other farther and less likely associated plant species were *Pinus pinaster* and *Halimium* sp. (Cistaceae). The available records suggest that *I. iberilepora* favors the months of December and January.

The holotype and paratype sequences of *I. iberilepora* are nearly identical (<0.2%, or 1 bp difference) to an Italian sequence obtained from a *Pinus pinea* root (GenBank GQ469523) and a Moroccan soil sequence taken from amongst *Cistus* spp. and *Pinus pinaster* (UDB03650179). The molecular results, supported by the biogeographic data, prompt us to believe that *I. iberilepora* is a Mediterranean species associated with pine and rockroses from acidic heathlands (FIG. 4).

Other specimen examined: PORTUGAL. Sesimbra, Faúlha (WGS84 coordinates: 38.479083, −9.085333, elevation 123 m), with *Cistus salviifolius, Halimium* sp., and *Thymus vulgaris*, nearest *Pinus* 25 m away, 18 Dec 2022, leg. *Vasco Fachada* (paratype PO-F2712, isoparatype priv. herb. V.F. VF181222IS2). GenBank: ITS = OQ690008.

Taxonomic notes: Inocybe iberilepora is characterized by its mottled gray-brown and dark brown, coarsely fibrillose to (sub)lanose pileus. The reddish-brown stipe is entirely covered by pale tomentum made of catenate cells, which seldom produce thick-walled cystidia near the apex. Importantly, it is a species with frequently subcapitate hymenial cystidia (FIG. 3e, g). The latter feature can help to separate *I. iberilepora* from all the similar species discussed below.

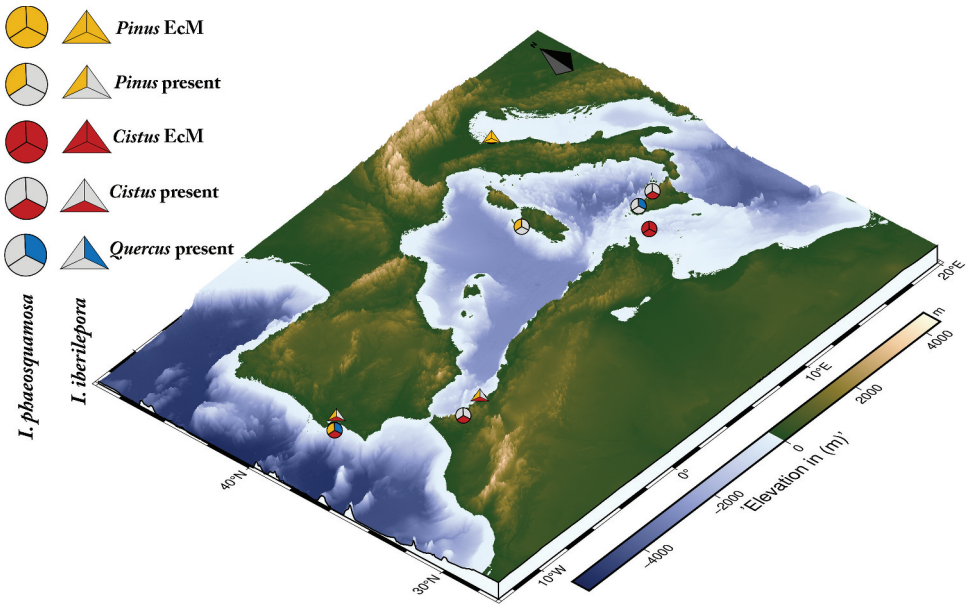

Figure 4. Biogeographic annotation of *I. iberilepora* and *I. phaeosquamosa* within the western Mediterranean basin. Plotted coordinates of known occurrences, together with the ecological data. Markers filled with a single color represent sequences from ectomycorrhizae.

Phylogenetically, *I. aurantiobrunnea* is the closest known species to *I. iberilepora* (FIG. 1). However, the former is easily separated by its intensely yellow-orange lamellae, larger spores, and mostly (sub)cylindrical hymenial cystidia (Bandini et al. 2022a; Esteve-Raventós et al. 2003).

Although *I. iberilepora*'s stipe is entirely covered by catenate cellular structures, these did not seem to us like true caulocystidia for the most part, with only a few longer and thicker-walled cystidia-like cells near the apex (FIG. 3h). Therefore, this differentiates it from species belonging to section *Splendentes* Singer, such as *I. phaeoleuca* (Bandini et al. 2019c; Kuyper 1986).

In addition, *I. iberilepora* could be mistaken for *I. venerabilis* (Bandini et al. 2022b) and *I. woglindeana* (Bandini et al. 2020); however, both these species possess an abundant velipellis, larger spores, and prefer calcareous soils. A comparison with *I. woglindeana* could not be complete without mentioning its sister species, *I. variispora*, which can be separated by its lack of velipellis, its longer spores, and its cystidia with a well-developed pedicel, and being not so plumpish as in *I. iberilepora* (Bandini et al. 2020; Fernández Sasia 2002).

Furthermore, the (sub)lanose pileus of *I. iberilepora* can bear similarity toward species such as *I. rufuloides* (Bandini et al. 2020; Bon 1984; Lantieri 2004) and *I. deianae* (Bandini et al. 2022b; Brugaletta et al. 2019; Eyssartier 2007). Even so, these species tend to present much coarser lanosity or even squamulosity on their pileus surface; besides, both species have larger spores than *I. iberilepora* (Bandini et al. 2020, 2022b; Bon 1984; Brugaletta et al. 2019; Eyssartier 2007; Lantieri 2004).

Conversely, the studied collections of *I. iberilepora* have always shown a somewhat fibrillose pileus surface, which separates it from the usually much smoother pileus of *I. tarda*, a common and frequently misidentified species. Moreover, the latter also has larger spores and prefers calcareous soils (Bandini et al. 2022a; Kühner 1955; Marchetti and Franchi 2008; Poirier 2012).

Although there are a number of other species that may resemble *I. iberilepora*, these can usually be separated macroscopically by their reddish pilei and ecologically by their calciphilous tendency, such are the cases of *I. neorufula* (Bandini et al. 2020; Esteve-Raventos et al. 2011), *I. rufobrunnea* (Bandini et al. 2022b; Favre 1955; Kuyper 1986), and *I. psammobrunnea* (Bandini et al. 2021; Bizio et al. 2017; Bon 1990; Ludwig 2017; Poirier 2002). In collections of unclear morphology and ecology, *I. iberilepora* can be distinguished from *I. rufobrunnea* by its smaller spores (Bandini et al. 2022b; Favre 1955; Kuyper 1986) and from *I. neorufula* by its somewhat longer spores (Bandini et al. 2020; Esteve-Raventos et al. 2011).

Inocybe phaeosquamosa Fachada & Bandini, sp. nov. FIG. 5.

MycoBank MB848090

Diagnosis: Inocybe phaeosquamosa produces small but stocky basidiomata, with quite dark and somewhat

Figure 5. *Inocybe phaeosquamosa*. a and b. Basidiomata (holotype). c. Basidiomata (paratype) d. Basidiospores (paratype). e. Cheilocystidia (paratype). f. Caulocystidia (paratype). g. Microscopical characters (holotype). Ca = caulocystidia; Cpa = caulopar-acystidia; Ch = cheilocystidia; Pa = paracystidia; Pl = pleurocystidia; Sp = spores. h. Pleurocystidia (paratype).

scaly pileus, pruinosity only on the upper third of the stipe, large pleurocystidia, and very small spores. Such combination of characters sets it apart from other known *Inocybe* species.

Etymology: Referring to the dark scaly look.

Typification: PORTUGAL. Sesimbra, Faúlha (WGS84 coordinates: 38.481500, −9.086444, elevation 115 m), among *Cistus crispus* and *Halimium* cf. *umbellatum* on sandy soil, few *Quercus suber* and *Pinus pinaster* nearby, 6 Jan 2022, leg. *Vasco Fachada* (**holotype** PO-F2346, isotype priv. herb. V.F. VF060122IS2). GenBank: ITS+28S = OQ690006; RPB2 = OR360834.

Description: Pileus 15–28 mm wide, soon expanded straight, often with a wavy margin in age; usually without or with low large umbo, margin soon uplifted; young basidiomata with faint remnants of a pale grayish velipellis; color dark chestnut brown, up to blackish brown, darker at the center (Mu 7.5 YR 3/2–3/4, 10 YR 3/3–3/6), sometimes paler at the umbo because of the velipellis; surface entirely minutely subsquamulose to squamulose with very small squamules, at the center sometimes somewhat warty; cortina remnants faint or not detected. Lamellae moderately crowded (ca. 35–60, l = 1–3), somewhat thickish, adnate, (sub) ventricose, edge fimbriate, first beige, later light caramel-brown. Stipe 15–30 × 3–6 mm; cylindrical, robust, only slightly swollen at base; faintly pruinose in upper third, longitudinally striate white on a reddish brown background, often whitish at the base due to the mycelium. Context whitish with bluish tinge, especially in the pileus and the cortex; smell and taste indistinct. Color of exsiccata dark brown.

Spores 6–9.5 µm (av. 7.6 µm, SD 0.6 µm) × 3.8–5.9 µm (av. 4.9 µm, SD 0.4 µm); Q = 1.2–1.9 (av. 1.6, SD 0.1) (n = 120 of 2 coll.); smooth, (sub)ellipsoid, (sub)amygdaloid, mostly without suprahilar depression but sometimes faint in side view, apex (sub)obtuse to (sub)acute. Basidia 18–30 × 6.0–10.0 µm, generally tetrasporic. Pleurocystidia 42–83 µm (av. 60 µm, SD 11 µm) × 10–17 µm (av. 13 µm, SD 2 µm); Q = 2.9–6.8 (av. 4.7, SD 0.9) (n = 56 of 2 coll.); common but not abundant; mostly (sub)utriform, also subcylindrical, rarely sublageniform, often with rather long neck, with short pedicel, apex crystalliferous, walls usually thin at ventral part (≈ 1 µm) and up to 2.5 µm thick at the apex, occasionally strongly thickened or coalescing toward the apex (≤4 µm), pale yellowish-greenish with 3% KOH. Cheilocystidia 30–72 µm (av. 46 µm, SD 10 µm) × 11–18 µm (av. 14 µm, SD 2 µm); Q = 2.2–4.5 (av. 3.4, SD 0.7) (n = 22 of 2 coll.); more variously shaped than pleurocystidia and only occasionally similar, usually shorter (neck), slightly wider and with thinner wall at neck; often with yellowish intracellular content in KOH, intermixed with numerous colorless, (sub)clavate, thin-walled paracystidia. Caulocystidia 34–84 µm (av. 57 µm, SD 11 µm) × 7–19 µm (av. 13 µm, SD 3 µm); Q = 2.4–7.1 (av. 4.4, SD 1.0) (n = 43 of 2 coll.); common only at the extreme stipe apex, uncommon and scattered along the rest of the upper third, not found in middle and lower portions of the stipe; usually (sub)lageniform or (sub)utriform, with short, sometimes slightly constricted apex, sometimes somewhat contorted, apex without or with only few small crystals, walls up to 1.5 (–2.5) µm thick at the apex, pale yellowish-greenish with 3% KOH; intermixed with thin- to slightly thick-walled, oblong, (sub)clavate cauloparacystidia. Pileipellis composed of an epicutis of occasional uplifted tufts (scales), with strongly dark-brown-pigmented incrustations in 3% KOH; terminal cells creatively and weirdly shaped, often bifurcated. Clamp connections present in all tissues, abundant in pileipellis.

Habitat and known distribution: The two occasions this species was observed by us were in the months of December and January, in sandy soil among *Cistus crispus* and *Halimium* cf. *umbellatum*, appearing to be associated with Cistaceae and sharing habitat with *Entoloma cistophilum*. Nonetheless, a few individuals of *Pinus pinaster* and *Quercus suber* in the vicinity could not be fully excluded as potential partners in situ. Supporting the *Cistus* partnership hypothesis, one of the environmental sequences (UDB026553) belonging to *I. phaeosquamosa*, from Pantelleria (Strait of Sicily), was produced from an EcM sample belonging to *Cistus salviifolius*. Other environmental samples similarly indicate soil acidity and *Cistus* preference. *Inocybe phaeosquamosa* is only known from the coastal heathlands of the western Mediterranean basin (FIG. 4).

Other specimen examined: PORTUGAL. Sesimbra, Faúlha (WGS84 coordinates: 38.481556, −9.086389, elevation 114 m), among *Cistus crispus* and *Halimium* cf. *umbellatum*, few *Quercus suber* and *Pinus pinaster* nearby, 18 Dec 2022, leg. *Vasco Fachada* (paratype PO-F2713, isoparatype priv. herb. V.F. VF181222IP1). GenBank: ITS = OQ690005.

Taxonomic notes: Inocybe phaeosquamosa can be found on the acidic heathlands of the Mediterranean basin. It is characterized by its dark, scaly pileus often covered with a light gray velipellis, and a reddish ochre stipe that is pruinose only near the apex. Microscopically, it comprises long pleurocystidia and very small spores, setting it apart from most species (FIG. 5).

Nevertheless, small spores are a trait equally shared by *I. glabripes* and *I. minimispora*. These two taxa can be macroscopically separated from *I. phaeosquamosa* by their lighter and smoother pilei, together with their

ecological preference for rich-basic soils. In the case that habit and habitat information is not available, the slightly smaller spores in *I. minimispora* and narrower in *I. glabripes* may aid in diagnosis. However, the decisive diagnosing microscopic feature is the much longer pleurocystidia of *I. phaeosquamosa* (Bandini et al. 2021; Ferrari 2006; Kuyper 1986; Lange 1917; Ricken 1915 (1980); Stangl 1989).

The dark and squamulose pileus of *I. phaeosquamosa* can make it mistakable for a number of species. One of them is *I. botaurina*, a hygrophilous species fruiting with *Salix* spp. that has much larger spores (Bandini et al. 2019a). Likewise, the pileus of *I. furfurea* can be squamulose, but it is usually paler in color and the spores are larger on average; moreover, this species prefers basic soils (Bandini et al. 2019b; Gminder 2010; Kühner 1955; Kuyper 1986; Schwobel and Stangl 1982; Zitzmann 2002).

With slightly less pronounced pileus squamulosity, there are *I. neorufula, I. rivierana*, and the polymorphic *I. tigrina*. These can be distinguished from *I. phaeosquamosa* by never developing such dark tones on the cap, and by their larger spores and more alkaline habitats (Bandini et al. 2020, 2021; Esteve-Raventos et al. 2011). Additionally, *I. neorufula* tends to present reddish colors (Bandini et al. 2020; Esteve-Raventos et al. 2011), whereas *I. rivierana* possesses peculiar cystidia with undate walls (Bandini et al. 2021).

There a few other species that may be confused with *I. phaeosquamosa*, but they all have considerably smoother pilei and larger spores. These species include *I. tarda* (Bandini et al. 2022a; Kühner 1955; Marchetti and Franchi 2008; Poirier 2012), *I. tjallingiorum* (Bandini et al. 2021; Kuyper 1986; Stangl 1989), and *I. zethi* (Bandini et al. 2022c).

Lastly, several dark-colored species are found within section *Splendentes* Singer, for instance, *I. phaeoleuca*. These species, however, are characterized by a pruinose stipe in their entire length and are thus easily distinguished from *I. phaeosquamosa* (Bandini et al. 2019c; Kuyper 1986).

DISCUSSION

In this study, we describe two new species within the genus *Inocybe*. The studied specimens of both species were found 5 km inland from the North Atlantic Ocean, on semixeric heathlands with sandy soil—generally acidic with occasional pockets of calcareous bedrock—dominated by *Erica* spp., *Ulex* spp., and various Cistaceae, with *Quercus suber* and *Pinus pinaster* common as well. The same acidic and plant ecology was found from other western Mediterranean sequences deposited in UNITE and PlutoF. The combination of the morphological, molecular, and biogeographic characters make these species recognizable and distinct from any other known *Inocybe* taxa.

With regard to phylogeny, the partition analysis of ITS1+5.8S+ITS2+28S+RPB2 reveals that the two species belong to different early split branches of *Inocybe* evolution (FIG. 1). Whereas *I. iberilepora* clearly falls to the *I. flocculosa* clade, *I. phaeosquamosa*, on the other hand, appears to belong to a relatively unknown and isolated clade, with its probable closest known relatives found in the distant group of *I. furfurea*/*I. rivierana*. Both in terms of early branching and in terms of near-leaf resolution, the RPB2 gene proved to be more informative than 28S (TABLE 2 and FIG. 2).

Within the Mediterranean context, the western basin is exceptionally diverse when it comes to the Cistaceae present in northern Morocco and the Iberian Peninsula (Civeyrel et al. 2011; Coello et al. 2021; Guzmán and Vargas 2005). Just last year, *I. mecoana* from Portugal and Malta (Bandini et al. 2022a) and *I. velatipusio* from Spain (Muñoz et al. 2022) were described from the western Mediterranean coasts. More new species are expected from these understudied and rich biomes.

In the case of *I. phaeosquamosa*, there is evidence that it can form EcMs with *Cistus* (FIG. 4). Although our own *I. iberilepora* observations are even more suggestive of a partnership with *Cistus* (FIG. 3f), there are data supporting its association with *Pinus* (FIG. 4). Whether these specific mutualisms are exclusive or preferential is something we cannot answer with this study, but it is not uncommon for macrofungi to exhibit symbiotic flexibility toward different plant species, including Cistaceae (Águeda et al. 2008; Comandini et al. 2006; Molina et al. 1992). Regardless, the ability for Inocybaceae to associate with Cistaceae is well established (Comandini et al. 2006; Daskalopoulos et al. 2021; Martín-Pinto et al. 2006). Further investigations, including both field and in vitro experiments, are necessary in order to conclusively identify *Inocybe* species exclusively associated with Cistaceae stands and those playing transitional roles in ecosystem succession (Sanz-Benito et al. 2023).

In the UNITE database, *Inocybe* alone boasts an extensive collection of over 275 000 soil DNA sequences, spanning various continents and latitudes (Kõljalg et al. 2013; Nilsson et al. 2019). However, we found *I. iberilepora* and *I. phaeosquamosa* sequences only from the western Mediterranean region (FIG. 4), which suggests a potential case of

endemism in this area. Judging from the number of sequences, *I. phaeosquamosa* seems to be more prevalent than *I. iberilepora*, which in turn appears to belong to a complex of yet undescribed species (FIG. 1). The closest sequences to these species were predominantly Mediterranean, with an intriguing exception of three Canadian records related to *I. phaeosquamosa* (GenBank JX630893, KC965941, KF297126). This finding piques our curiosity about the interactions *Inocybe* species may have toward endemic Cistaceae in North America, such as in the genus *Hudsonia* (Malloch and Thorn 1985; Massicotte et al. 2010), and their potential relationship with Mediterranean counterparts.

The taxonomic characterization of these species, as well as their broader biogeographic relationships, remains puzzling and warrants further studies by *Inocybe* systematists. Continued research will be crucial in unraveling the complexities of these species and their distribution patterns.

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DISCLOSURE STATEMENT

No potential conflict of interest was reported by the author(s).

DATA AVAILABILITY STATEMENT

The alignment and phylogeny data produced in this study are accessible in TreeBASE (study ID TB2:S30312). The assembled sequences are deposited in GenBank under the accession number provided. The vouchers are deposited in herbarium PO in Porto, Portugal. All remaining data, such as micrographs, measurement data, model computation data, etc., can be made available upon request.

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