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Inocybe shawarensis sp. nov. in the Inosperma clade from Pakistan

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Abstract —A new species, *Inocybe shawarensis*, was collected during field research on ectomycorrhizal fungi associated with oak forests in Swat district, Pakistan. This species, supported by a combination of morphological and molecular phylogenetic analyses, is characterized by a brown, fibrillose campanulate pileus, grayish lamellae, slightly bulbous stipe base, slightly pruinose silvery white stipe, small phaseoliform spores, and clavate cheilocystidia. We present phylogenetic analyses based on DNA sequences from the internal transcribed spacer (ITS) region and large subunit (LSU) of the nuclear ribosomal RNA (rRNA). Phylogenies from both DNA regions cluster *I. shawarensis* within the Maculata subclade in the Inosperma clade.

Key words — *Agaricomycetes*, *Inocybaceae*, *Quercus*, Shawar Valley

Introduction

The *Inocybaceae* is one of the most taxonomically diverse families of *Agaricales*. Its representatives form ectomycorrhizal (EcM) associations with many angiosperms and gymnosperms in tropical and temperate areas. Between 70% and 80% of species in the family have been described in association primarily with ectomycorrhizal plant families *Fagaceae*, *Pinaceae* and *Salicaceae* (Kirk et al. 2008).

Inocybe (Fr.) Fr., one of three formally described genera in *Inocybaceae,* was first established by Fries as a "tribe" of *Agaricus* in 1821 and later elevated to genus rank in 1863 (Matheny et al. 2009). The genus has been divided into

subgenera and sections based on spore morphology, shape and distribution of cystidia on fruit body tissues, and stipe morphology. Multigene phylogenetic analysis by Matheny (2009) identified seven clades within *Inocybaceae*: Auritella, Inocybe, Inosperma, Mallocybe, Mallocybella, Nothocybe, and Pseudosperma.

The Inosperma clade is characterized phylogenetically as a robustly supported monophyletic group including species with radially fibrillose to rimose or squamulose pilei and ellipsoid to phaseoliform spores but lacking metuloid pleurocystidia. Many species of the Inosperma clade also feature unusual odors, and some may have rubescent or brunnescent flesh.

Among the more than 850 *Inocybe* species reported worldwide (Matheny et al. 2009, 2012; Kobayashi & Onishi 2010, Horak et al. 2015, Jabeen et al. 2016), at least 47 *Inocybe* species are resolved in the Inosperma clade (Matheny 2009, Matheny et al. 2009, Kropp et al. 2013, Pradeep et al. 2016, Latha & Manimohan 2016). From Pakistan, 26 species of *Inocybe* have been reported, but only a few studies have verified the taxonomic identity of specimens using molecular data (Ahmad et al. 1997, Ilyas et al. 2013, Saba et al. 2015, Jabeen et al. 2016). The only species in the Inosperma clade that has been reported from Pakistan is *Inocybe mimica* Massee (Saba et al. 2015).

Here we describe a new species, *Inocybe shawarensis*, from Shawar Valley, Swat district, Pakistan. The specimens were characterised by morphological characters as well as molecular datasets based on the internal transcribed spacer (ITS) and large subunit (LSU) of nrDNA. Based on traditional classifications (Kühner 1980, Kuyper 1986, Stangl 1989), our new species would be placed in *Inocybe* sect. *Rimosae* within *I.* subg. *Inosperma*.

Materials & methods

Sampling Site

The Swat district (34.34–35.55°N 72.08–72.50°E), situated in the Khyber Pakhtunkhwa Province of Pakistan, is well known for its unique biodiversity (Shinwari et al. 2003). Its Shawar Valley, which occupies an area of 48.77 km^2 within the Hindu Kush mountain range (Ahmad & Sirajuddin 1996), is topographically mountainous, varying in elevation from 1200 m to 3800 m (Anonymous 1999). Floristically the Valley is representative of the western Himalayan Province. The climate is moist temperate with temperatures averaging a winter minimum of 4.8°C and summer maximum of 33.5°C and an average annual rainfall of about 800 mm, with precipitation occurring in spring and summer seasons with snowfall at higher elevations (Ullah et al. 2014).

Fruiting bodies of *Inocybe,* collected during a field investigation of ectomycorrhizal communities associated with the oaks of Swat during 2014-2016, were found in a pure *Quercus* forest in Shawar Valley. The type locality lies in a thick moist temperate forest

of *Quercus oblongata* D. Don [= *Q. incana* Roxb., nom. illeg.] along a tributary of the Swat River.

Morphological analyses

Fruiting bodies of *Inocybe* were collected and photographed in the field using a Nikon D70S camera. Morphological characters were recorded from fresh specimens. Color designations were based on the Munsell Color System (Munsell 1975). For preservation specimens were dried using an electric fan heater. Rehydrated material was examined microscopically in 5% KOH, phloxine, and Melzer's reagents.

Anatomical features of basidiospores, basidia, cystidia, stipe hyphae and pileus hyphae were measured at 1000× magnification and include: arithmetic mean of spore length and width for all spores measured, Q = spore length divided by spore width. Spore size range was determined by 30 basidiospore measurements from each fruiting body.

Specimens were deposited in the Herbarium, Department of Botany, University of the Punjab, Lahore, Pakistan (LAH) and the University of Florida Herbarium, Gainesville FL, USA (FLAS).

DNA extraction, amplification and sequencing

Genomic DNA was extracted from gill tissue using a modified CTAB method (Gardes & Bruns 1993). ITS and LSU regions were amplified by the primer pairs ITS1F/ITS4B and LR0R/LR5, respectively. All PCR products were evaluated for successful amplification using SYBR Green and 1.5% agarose gels with TAE buffer for gel electrophoresis. Amplicons were prepared for sequencing via enzymatic purification using Exonuclease I and Shrimp Alkaline Phosphatase enzymes (Werle et al. 1994). Purified products were sequenced by the University of Florida's Interdisciplinary Center for Biotechnology Research (http://www.biotech.ufl.edu/). Sequence chromatograms were trimmed, edited, and assembled using Sequencer 4.1 (GeneCodes, Ann Arbor, MI). DNA sequences generated for this study were deposited in GenBank.

Molecular phylogenetic analysis

For alignment and phylogenetic analysis, the top 100 BLAST search result sequences were selected from GenBank using NCBI BLAST (http://www.ncbi.nlm.niih.gov/). Other closely related species were also included based on the published literature in the final dataset. Sequences were manually edited and assembled using BioEdit (www.mbio.ncsu.edu/bioedit/bioedit.html). After sequence alignment by Muscle, all sequences were trimmed between the conserved motifs 5′–(…GAT) CATTA– and –GACCT(CAAA…)–3′ (Dentinger et al. 2011). *Auritella* species were selected as outgroup based on results reported by Larsson et al. (2009). Phylogenetic trees were constructed with the Maximum Likelihood (ML) algorithm using a general time-reversible model (Nei & Kumar 2000) and nearest-neighbour interchange as the ML heuristic search method using MEGA6 software. The topology was assessed by 1000 bootstrap replicates.

Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum

Composite Likelihood (MCL) approach. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013).

Taxonomy

Inocybe shawarensis Naseer & Khalid, **sp. nov.** Fig. 1

MycoBank MB 820130

Differs from *Inocybe quietiodor* by its brown pileus with a less prominent, campanulate umbo, its gray gills, its silvery lower stipe and dark gray upper stipe, and its smaller, more phaseoliform spores.

Type: Pakistan, Khyber Pakhtunkhwa province, Swat district, Shawar Valley, 2100 m a.s.l, solitary on ground under *Quercus oblongata* D. Don [= *Q. incana* Roxb., nom. illeg.], 14 July 2015, Arooj Naseer ASSW79 (Holotype, FLAS-F-S9456; GenBank KY616964, KY616966. Isotype, LAH35195; GenBank KY616965).

Etymology: The specific epithet *shawarensis* refers to the Shawar Valley, the location where the type was collected.

Pileus: 30 mm diam., campanulate, umbonate, margin slightly incurved or deflexed; surface fibrillose, rimose or cracked towards the margin; dark brown (7.5YR4/8) at the centre, becoming lighter brown in patches towards the margin that is creamy white (10Y8/2). Lamellae subdistant, fimbriate, eroded, light gray (2.5GY8/2) when young; Lamellulae of varying lengths, alternating with lamellae. Stipe: 4.8 × 0.4 cm, cylindrical, central; surface fibrillose, apex slightly pruinose, slightly narrower towards apex, base slightly swollen to bulbous; base light brown (7.5YR4/8) with lower two third creamy or silvery white and upper one third dark gray (2.5YG5/2)

Basidiospores [60/2/1], (4.5–)4.7–6.5(–6.8) × (2.7–)2.8 × 3.7(–3.8) µm, avl × avw = 5.2 × 3.2 µm, Q = (1.2–)1.3–1.9(–2.5), avQ = 1.63, yellowish brown in KOH, smooth, thick walled, phaseoliform. Basidia 22–31 × 6–10 µm, light brown, clavate, blunt ended, four–spored. Cheilocystidia 21–55 × 9–11 µm, clavate, thin walled, in groups. Pleurocystidia none. Pileipellis a radially orientated cutis of thin-walled hyphae, 5–7 µm diam., cylindrical, walls smooth or finely encrusted, light brown in KOH, blunt ends, septate, clamped. Caulocystidia only at apex, more or less similar to cheilocystidia. Stipitipellis hyphae 4–7 µm diam., septate, filamentous, unbranched. Clamp connections present. Odour not recorded.

Molecular phylogenetic analysis

Inocybe shawarensis sequences of the amplified products of ITS (KY616964, KY616965) and LSU (KY616966) were BLAST searched at NCBI. Both the ITS and

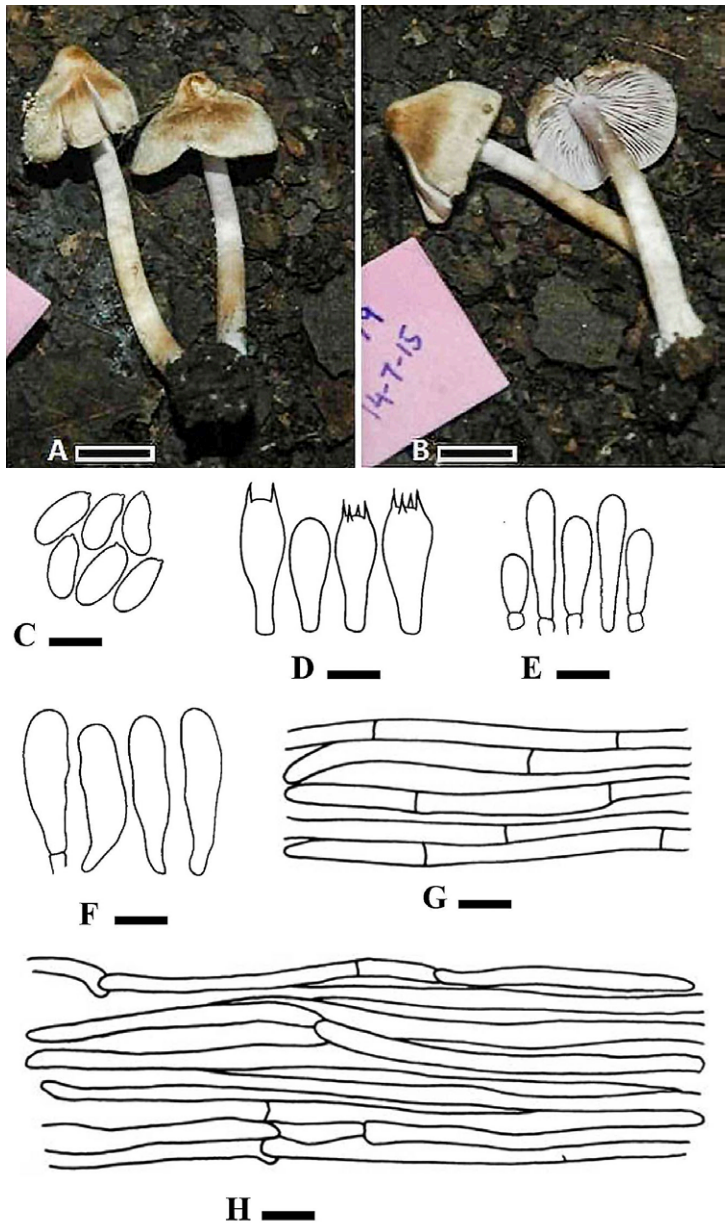

Fig. 1. *Inocybe shawarensis* (isotype, LAH35195). A, B. basidiomata; C. basidiospores; D. basidia; E. cheilocystidia; F. caulocystidia; G. pileipellis; H. stipitipellis. Scale bars: A, B = 1.5 µm; C = 5 µm; D = 12 µm; E, F = 16 µm; G, H = 13 µm.

LSU sequences suggest a close affinity with *I. quietiodor* Bon. The LSU sequence was 97% identical to *I. quietiodor* (FJ904174) with 100% query coverage, whereas the ITS sequence showed 93% sequence similarity with *I. quietiodor* (FJ936168) with 100% query coverage. The ITS analysis comprised 52 nucleotide sequences with 1405 positions total in the final dataset, while the LSU analysis comprised 40 nucleotide sequences with 852 positions in the final dataset.

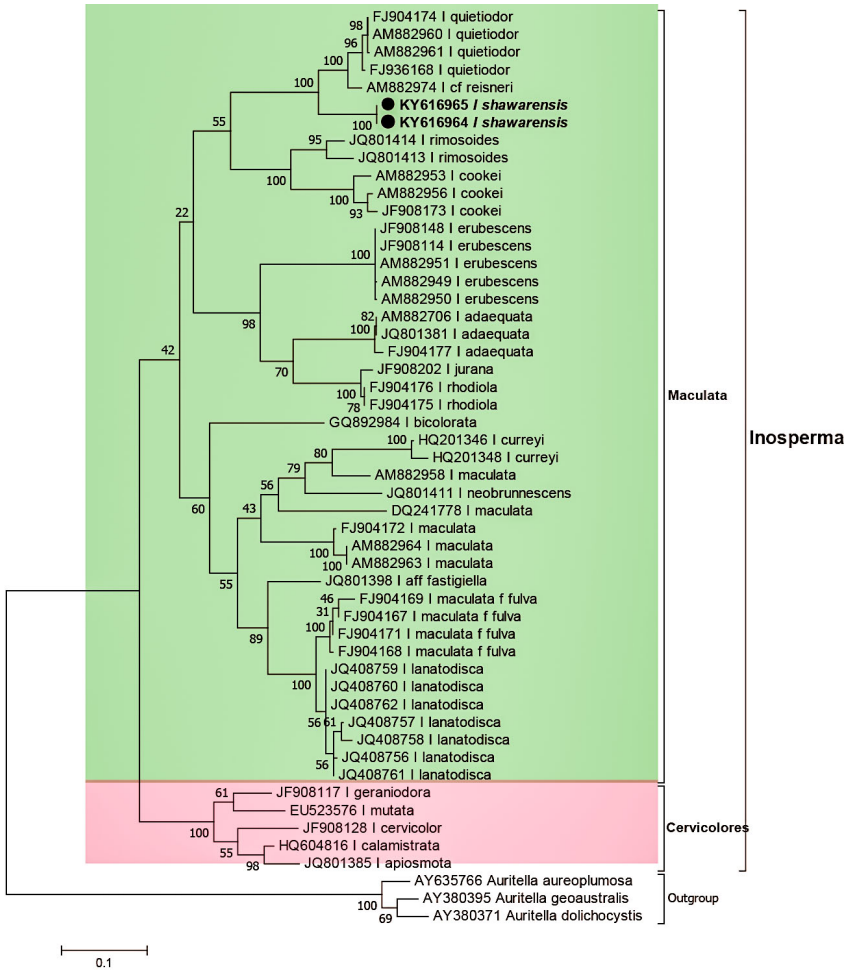

Fig. 2. Molecular phylogenetic analysis of ITS sequences from *Inocybe* spp. in the Inosperma clade inferred by using the Maximum Likelihood method. The analysis involved 52 nucleotide sequences. The tree with the highest log likelihood (–9223.6738) is shown. Sequences generated during this study are represented by dots.

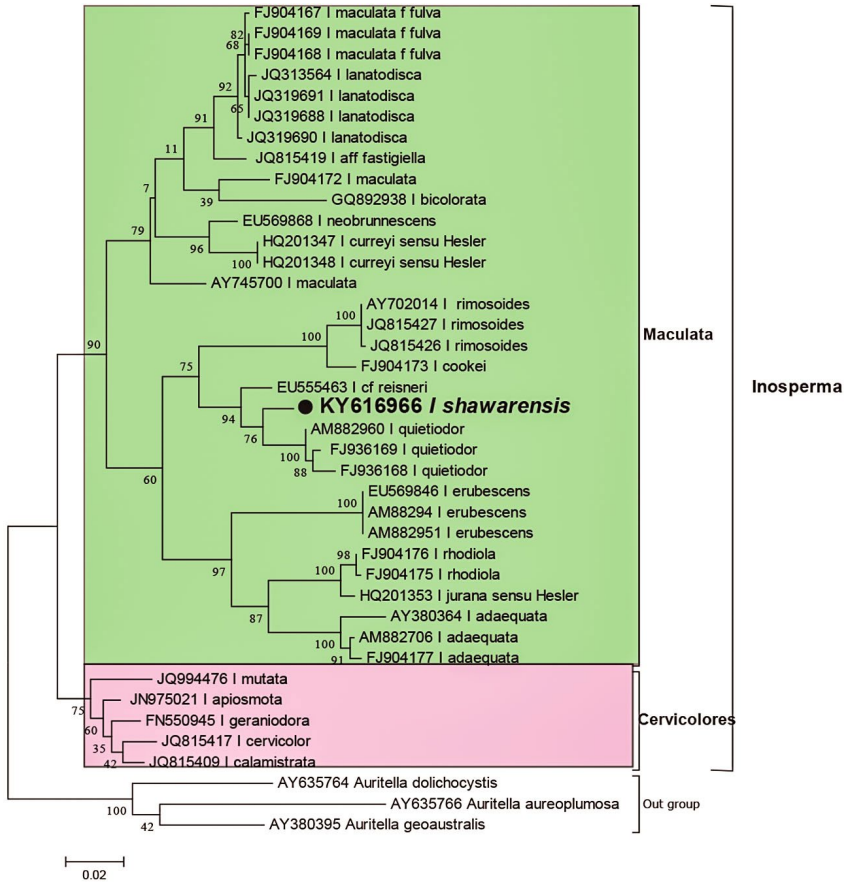

Fig. 3. Molecular phylogenetic analysis of LSU sequences from *Inocybe* spp. in Inosperma clade inferred by using the Maximum Likelihood method. The analysis involved 40 nucleotide sequences. The tree with the highest log likelihood is shown. The sequence generated during this study is represented by a dot.

Our analysis revealed two major lineages within the Inosperma clade. The new species clustered with *I. quietiodor* in the Maculata subclade, whereas the Cervicolores subclade was resolved as a distinctly monophyletic group.

The percentage of trees in which the associated taxa clustered together is shown next to the branches.

Discussion

Species in the Maculata subclade are characterized in part by the presence of thin-walled often clavate to pyriform cheilocystidia, phaseoliform spores, and specific odours, and the stipe base tends to be distinctly bulbous, as found in *I. cookei* Bres., *I. maculata* Boud., and *I. quietiodor* (Larsson et al. 2009).

Inocybe shawarensis is characterized by its brown fibrillose campanulate pileus, grayish hymenium, a stipe surface that is silvery white on the lower two-thirds and dark gray on the upper third, a cylindrical stipe that is slightly pruinose at apex, slightly bulbous stipe base, an absence of pleurocystidia, and small phaseoliform yellowish brown spores. Our new species is morphologically similar to *Inocybe quietiodor* but separated with 70% bootstrap value from *I. quietiodor* (FJ93618, AM882950, AM882961), with which it forms a sister clade that includes *Inocybe* cf. *reisneri* (sampled from Japan).

Compared to *I. shawarensis,* characterized with a brown campanulate pileus and gray gills, *I. quietiodor* has a more prominently umbonate yellowish (5Y8/10) pileus and yellowish lamellae. *Inocybe quietiodor* is further distinguished by its yellow cylindrical stipe with the whitish bulbous base and larger and less phaseoliform spores. *Inocybe cookei* is distinguished morphologically by its conspicuously marginate bulbous base (bulb ≤16 mm) and pyriform cheilocystidia.

The Indian species *I. gregaria* K.P.D. Latha & Manim., *I. virosa* C.K. Pradeep et al., and *I. carnosibulbosa* C.K. Pradeep & Matheny falling in the Inosperma clade "Old World tropical clade 2" may be comparable to our taxon. *Inocybe shawarensis* differs from these, however, by its placement in the Maculata subclade and its smaller basidiomata (Pradeep et al. 2016, Latha & Manimohan 2016).

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