ISSN (print) 0093-4666 © 2013. Mycotaxon, Ltd. ISSN (online) 2154-8889

MYCOTAXON

http://dx.doi.org/10.5248/124.247

Volume 124, pp. 247–254 April–June 2013

Inocybe nitidiuscula and its ectomycorrhizae associated with *Alnus nitida* from Galyat, Pakistan

S. Ilyas*, A. Razaq & A.N. Khalid

Department of Botany, University of the Punjab, Lahore. 54590, Pakistan
*Correspondence to: *fungalcommunity@gmail.com**

Abstract — *Inocybe nitidiuscula* and its ectomycorrhizae are characterized from Pakistan morphologically and using rDNA internal transcribed spacer sequences. The sequences of above- and below-ground structures exhibit >97% similarity. This represents a new species record for Pakistan and the first description of ectomycorrhiza formed by *I. nitidiuscula*.
Keywords — alders, euagarics, Himalayan moist temperate forest, morphotype

Introduction

Inocybe (Fr.) Fr. (*Agaricales, Basidiomycota*) is a large, widely distributed genus of gilled fungi with more than 500 species worldwide (Kirk et al. 2008). This genus is characterized by a fibrillose pileus, equal or bulbous stipe, metuloids or thin walled cystidia, and yellowish brown smooth to angular-nodulose ellipsoid to amygdaliform spores. Many *Inocybe* species have thick walled pleuro-cheilocystidia (metuloids) that others lack (Heim 1931, Kühner & Romagnesi 1953, Kühner 1980, Kuyper 1986, Singer 1986, Stangl 1989, Kobayashi 2002).

Inocybe species play a very important role in the ecology of coniferous and deciduous trees by forming ectomycorrhizal associations (Larsson et al. 2009). The salient features of *Inocybe* ectomycorrhizae (EcM) are shiny ramifications, absence of rhizomorphs and cystidia, emanating hyphae with clamp connections, and a short exploration type (Agerer 2006). EcM have been reported for *Inocybe appendiculata* Kühner, *I. avellana* P. Kumm., *I. fuscomarginata* Kühner, *I. heimii* Bon, *I. lacera* (Fr.) P. Kumm., *I. lanuginella* (J. Schröt.) Konrad & Maubl., *I. obscurobadia* (J. Favre) Grund & D.E. Stuntz, *I. petiginosa* (Fr.) Gillet, and *I. terrigena* (Fr.) Kuyper. So far, EcM have not been described for *I. nitidiuscula* with any host plant.

Galyat, a narrow 250,000 acre strip of Himalayan moist temperate forest (HMTF) northeast of Islamabad extending on both sides of KPK-Punjab border, is considered the best representative of HMTF with a high species diversity (Jamal & Khadija 2009, Irshad & Khan 2012). The region is characterized by mixed vegetation of deciduous and coniferous trees. Niazi (2008) and Niazi et al. (2006, 2007, 2009, 2010) have reported on previous studies on ectomycorrhizal fungi from this area.

During fieldwork in Koza Gali and Jhika Gali in Galyat, *Inocybe nitidiuscula* was collected along with belowground ectomycorrhizae associated with *Alnus nitida*. Anatomical and molecular characterization of both above- and below-ground parts determined its distribution with coniferous and deciduous vegetation.

Materials & methods

Basidiomata were collected, photographed and dried in the field. Collected material was characterized anatomically and using DNA sequence data. For microscopic observation, free hand sections were stained with Congo Red and Melzer's reagent. Drawings were made using a camera lucida attached to a compound microscope.

Soil blocks of 10 × 12 cm under selected trees were dug out and washed to isolate, characterize, and identify EcM. Morphotypes were characterized morphologically following Agerer (1987–2006) and have been deposited in the Herbarium of the Botany Department, University of the Punjab, Lahore (LAH).

DNA was extracted using the Extract-N-Amp™ Plant kit (Sigma, St Louis, MO, USA). The rDNA internal transcribed spacer (ITS) region was amplified using the primer pair ITS1F (Gardes et al. 1991) and ITS 4 (White et al. 1990) with denaturation at 94°C for 4 min, followed by 35 cycles of 45 sec at 94°C, 45 sec at 54°C, and 1 min 30 sec at 72°C, and a final extension at 72°C for 2 min. PCR products from basidiomata and EcM morphotypes were sent for bidirectional sequencing (Macrogen, Korea). Sequences were queried to GenBank using the NCBI BLAST interface for sequence comparison and identification of both basidiomata and fungi on EcM root tips. Sequence alignment and phylogenetic analysis were performed using Molecular Evolutionary Genetics Analysis (MEGA) software (Tamura et al. 2011). Maximum Likelihood (ML) optimization was conducted with a Jukes-Cantor substitution model and Nearest-Neighbor-Interchange (NNI) as the heuristic search method. Branch support was assessed using 1000 nonparametric bootstrap replicates. Selected sequences were aligned using Clustal W and corrected manually. The specimens are deposited in the Herbarium of the Department of Botany, University of the Punjab, Lahore (LAH) and the Herbarium of Hokkaido University Museum, Sapporo, Japan (SAPA). Nucleotide sequences are deposited in GenBank as accession numbers HE862959 (basidiome), and HF565509 (ectomycorrhizal root).

Results

Inocybe nitidiuscula and its ectomycorrhizae were identified morpho-anatomically and molecularly. The ITS sequences from basidiomata and EcM morphotypes obtained in the present study exhibited 97–99.8% sequence identity, with identification confirmed by phylogenetic analysis. In both maximum likelihood and neighbor-joining methods, all sequences of

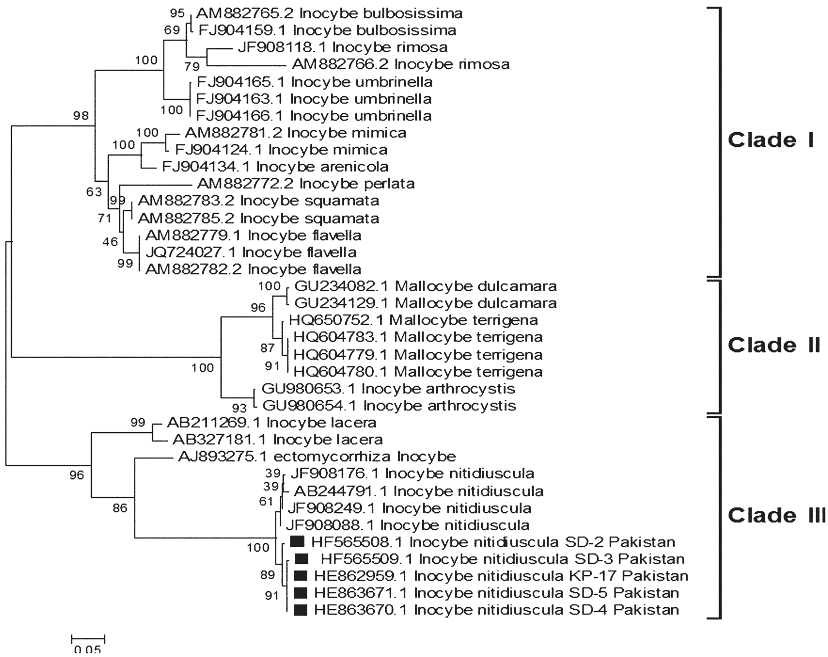

Fig. 1. Phylogenetic relationship of *I. nitidiuscula* (■) with other members of *Inocybe* based on Maximum Likelihood analysis of nrITS sequences. Bootstrap values based on 1000 replicates are shown above the branches (values <50 are not shown). The analysis included 35 sequences. All positions containing gaps and missing data were eliminated, yielding a total of 616 nucleotide positions in the final dataset. Clade I contains members of *Inocybe* sect. *Rimosae*, Clade II members of *Inocybe* subg. *Mallocybe*, and Clade III members of *Inocybe* having metuloid pleuro-cheilocystidia.

I. nitidiuscula from Pakistan clustered with sequences of *I. nitidiuscula* (Fig. 1, clade III) with significant bootstrap support.

Taxonomy

Inocybe nitidiuscula (Britzelm.) Lapl., Dict. Iconogr. Champ. Sup.: 523, 1894. Fig. 2

Pileus 2.2–3.5 cm broad, light brown to dark brown from margins to centre, conical when young and plano-convex at maturity, sharply umbonate, shiny, smooth or minutely woolly at very center, umbo dark brown to blackish, outwards radially fibrous, uniformly aligned brown fibrils move toward margins which lighten in colour, margins not smooth, somewhat dentate. Lamellae cream to light brown, sinuate in attachment to stipe, margins dentate, lamellulae two-tiered, alternating with lamellae. Stipe 3.4–5.4 × 0.35–0.42 cm, central,

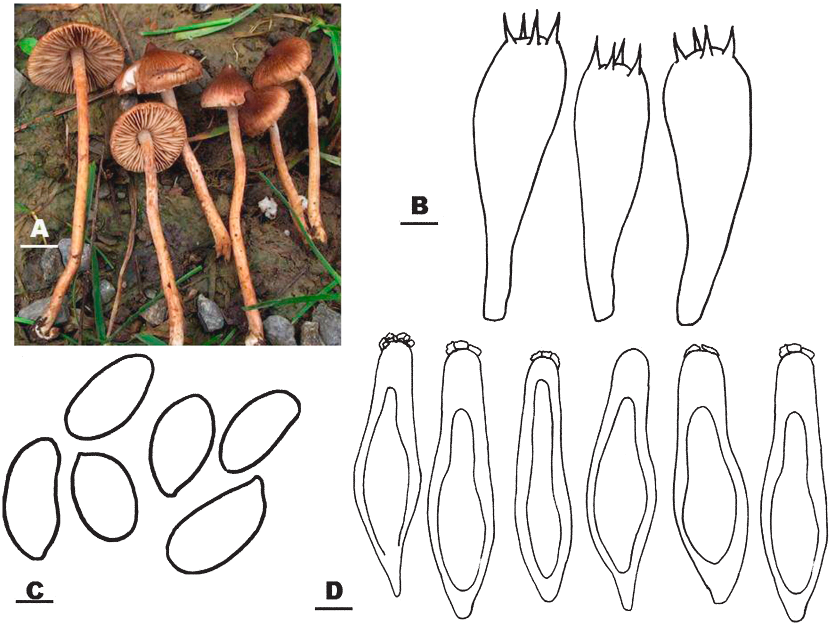

FIG. 2: *Inocybe nitidiuscula*. A. Basidiomata; B. Basidia; C. Basidiospores; D. Metuloids (cheilocystidia and pleurocystidia). Scale bars: A = 1 cm, B–C = 3.45 µm, D = 6.25 µm.

light brown, smooth, cylindrical, slightly swollen at base. ODOR not distinctive. TASTE not recorded.

Basidiospores 10–11 × 6.5–8µm, smooth, amygdaliform in side view, elliptical to ellipsoid in front view, yellowish brown, thick walled, with a prominent apiculus. BASIDIA 29–31 × 9.0–10.5 µm, tetra sterigmate, clavate, with olive green droplets in 5% KOH. PLEUROCYSTIDIA metuloids, 47.0–71.5 × 12.0–17.5 µm, abundant, cylindrical to fusiform, contents hyaline to pale yellow in 5% KOH with thick brown walls, apices heavily encrusted with colourless crystals. CHEILOCYSTIDIA similar to pleurocystidia, abundant, 38–41 × 11.8–13 µm, hyaline, clustered, thick walled, with encrusted apices. PILEIPELLIS a cutis of loosely arranged hyphae, light yellowish-brown, 7–9 µm wide. CLAMP CONNECTIONS abundant in all tissues.

MATERIAL EXAMINED: **PAKISTAN: KHYBER PAKHTUNKHWA,** Galyat, Ayubia, Koza Gali, 2250 m a.s.l., gregarious, on moist ground under *Alnus nitida*, 23 August 2010, Abdul Razaq, KP-17 (LAH 230817, SAPA 100000; GenBank, HE862959).

Morpho-anatomical description of ectomycorrhizae FIG. 3

ECTOMYCORRHIZAL SYSTEM dichotomous, axis 3–6 × 1.0–1.5 mm., in close contact with surrounding soil due to gelatinous mantle. UNRAMIFIED ENDS straight to beaded, 1–2 mm long and 0.5–1 mm in diam.; younger tips creamy

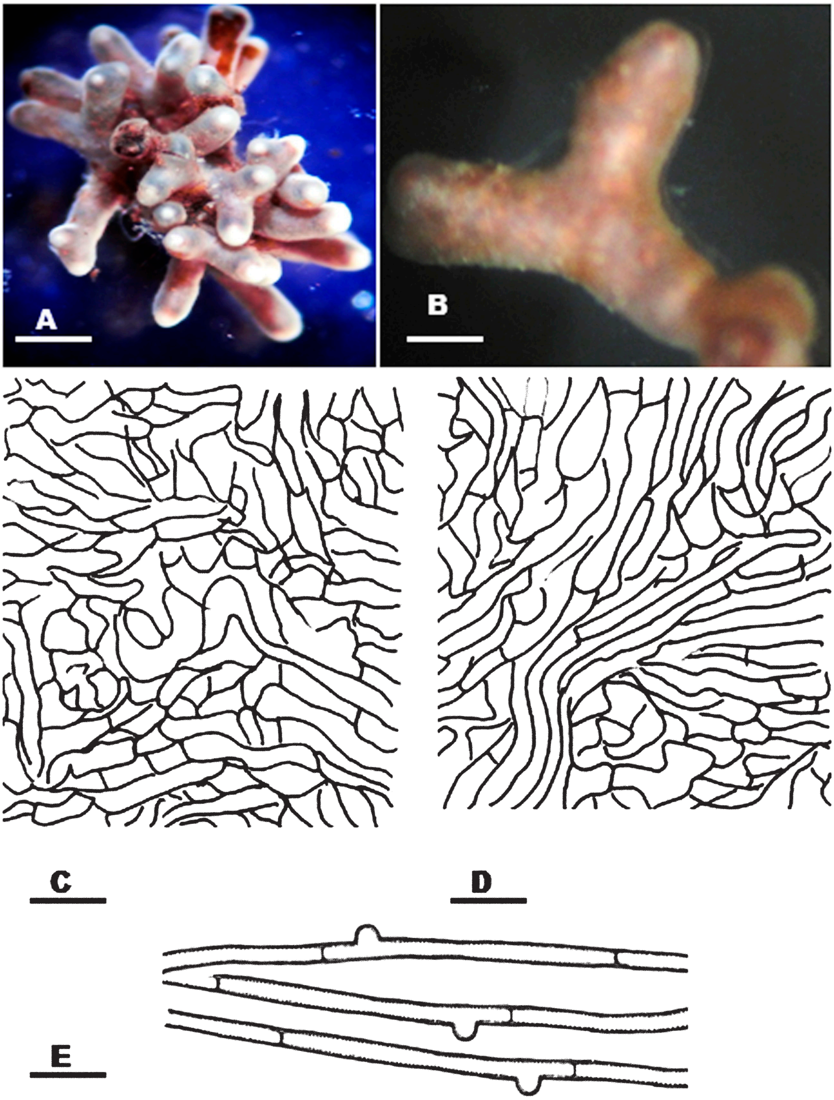

Fig. 3: *Inocybe nitidiuscula* A,B. Ectomycorrhizae. C. Outer mantle layer D. Inner mantle layer. E. Emanating hyphae. Scale bars: A = 5.4 mm, B = 3 mm, C–E = 9 µm

white, older tips light brown. Texture of system smooth with shiny luster; host tissue visible under the sheath. Rhizomorphs absent. Emanating hyphae frequent, straight, frequently clamped; clamps large, half or more than a half semicircle; anastomoses H-shaped. Cystidia absent, Color Reaction absent with lactic acid & KOH.

Mantle plectenchymatous in all layers. Outer Mantle loosely plectenchymatous with netlike hyphal arrangement (Agerer 1987–2006, type A), hyphae 2–3 µm diameter, lacking clamps and cystidia, transparent, no cell contents visible, anastomoses present. Inner Mantle densely plectenchymatous (Agerer 1987–2006, type A), hyphae 2–3 µm diameter, colorless to light yellowish, cell contents clear, no clamps or cystidia observed (Fig. 3).

Material examined: **PAKISTAN: Khyber Pakhtunkhwa**, Galyat (Koza Gali & Jhika Gali), roots of *Alnus nitida*, 23 August 2010, Sobia Ilyas, SD-3 (LAH; GenBank, HF565509).

Discussion

Inocybe is an integral part of almost all ectomycorrhizal communities in association with coniferous and deciduous trees from different areas of the world (Cullings et al. 2001, Yamada & Katsuya 2001, Obase 2006, Tedersoo et al. 2009, Walbert et al. 2010, Kennedy et al. 2011, Ma et al. 2012). Pakistani forests are well represented by different ectomycorrhizal fungi associated with wide host range (Niazi 2008, Niazi et al. 2006, 2007, 2009, 2010, Jabeen et al. 2012), but no published data on *Inocybe* is available even though it seems to be the most abundant group in this area among all euagarics. Basidiomata of *I. nitidiuscula* are also reported from different forests associated with *Picea*, *Pinus*, *Larix*, *Quercus*, *Corylus*, *Carpinus*, *Fagus*, *Salix*, and *Dryas* but no description of its mycorrhizae has yet been published (Kuyper 1986).

Inocybe nitidiuscula and *I. lacera* are closely related as both clustered together in phylogenetic analyses (Fig. 1, Clade III). Both species produce metuloid cystidia on lamellar face and edges and form ectomycorrhizal symbioses. The sequence (HE862959.1) from the *I. nitidiuscula* Pakistani basidiome clustered with *I. nitidiuscula* isolates from other regions of the world. The Pakistani sequence shows a 94–95% match with *I. nitidiuscula* (FN550920.1, HQ604085.1), although its morphological details are same as given by Obase et al. (2006). The genetic variation may be due to geographic distance.

The *I. nitidiuscula* ectomycorrhiza is characterized by smooth ramification, white to light brown color, lack of rhizomorphs and cystidia, prominent clamped emanating hyphae, and a plectenchymatous mantle. These resemble the ectomycorrhizae of *I. appendiculata* associated with *Picea* and *Abies* and *I. fuscomarginata* associated with *Salix* spp., except that the ramification type more closely resembles *I. terrigena,* reported with *Pinus* (Agerer 1987–2006).

Phylogenetic analyses are a useful tool for identifying unknown *Inocybe* sequences to the species level (Ryberg et al. 2008). Both above- and below-ground parts of the Pakistani collections cluster in the *I. nitidiuscula* clade (Fig.1, Clade III). Although *Inocybe* has been reported to associate with alders (Tedersoo et al. 2009), to our knowledge this is the first report of EcM of *I. nitidiuscula* with that host. In one study, Tedersoo et al. (2006) reported ectomycorrhiza of *Inocybe* sp. 5 (AJ893275.1) that showed maximum sequence identity with *I. nitidiuscula* (AJ534934), although with only 77% sequence similarity. In our phylogenetic analysis, this sequence clustered in the same clade as *I. nitidiuscula* but significantly distant from sequences from Pakistan, Japan, and Italy. Previous studies have shown that alder ectomycorrhizal communities tend to be host specific (Rochet et al. 2011). Tedersoo et al. (2009) indicated that ascomycete EcM symbionts of *Alnus* might not be host-limited, although their results suggested high *Alnus* specificity in basidiomycete symbionts. A detailed study on *Alnus* ECM communities from Pakistan will determine the level of host specificity.

Acknowledgements

We are thankful to Dr. Abdul Rehman Khan Niazi and Dr. Todd Osmundson for acting as expert reviewers to improve the manuscript and Dr. Takahito Kobayashi, Curator of SAPA Herbarium, Japan, for the identification of specimens. Higher Education Commission, Pakistan, provided financial support for this research work.

Literature cited

Agerer R. 1987–2006. Colour atlas of ectomycorrhizae. 1–13th delivery. Einhorn-Verlag Edward, Dientenurger Schwabisch Gmund, Germany.

Agerer R. 2006. Fungal relationships and structural identity of their ectomycorrhizae. Mycol. Prog. 5: 67–107.

Cullings KW, Vogler DR, Parker VT, Makhija S. 2001. Defoliation effects on the ectomycorrhizal community of a mixed *Pinus contorta Picea engelmannii* stand in Yellowstone Park. Oecologia 127: 533–539.

Gardes M, White TJ, Fortin JA, Bruns TD, Taylor JW. 1991. Identification of indigenous and introduced symbiotic fungi in ectomycorrhizae by amplification of nuclear and mitochondrial ribosomal DNA. Can. J. Bot. 69: 180–190. http://dx.doi.org/10.1139/b91-026

Heim R. 1931. Le genre *Inocybe*. Encyclopédie mycologique 1. Lechevalier & Fils, Paris.

Irshad S, Khan S. 2012. Impacts of protection on floral diversity of Himalayan moist temperate forests of Galyat. Pakistan Journal of Environment 1(4): 119–125.

Jabeen S, Ilyas S, Niazi AR, Khalid AN. 2012. Diversity of ectomycorrhizae associated with *Populus* spp. growing in two different ecological zones of Pakistan. Int. J. Agric. Biol. 14: 681–688.

Jamal S, Khadija. 2009. Watershed issues assessment in Galyat. M.Sc. Thesis, Hazara University, Pakistan.

Kennedy PG, Garibay-Orijel R, Higgins LM, Angeles-Arguiz R. 2011. Ectomycorrhizal fungi in Mexican *Alnus* forests support the host co-migration hypothesis and continental-scale patterns in phylogeography. Mycorrhiza 21: 559–568. http://dx.doi.org/10.1007/s00572-011-0366-2

Kirk PM, Cannon PF, Minter DW, Stalpers JA. 2008. Ainsworth & Bisby's dictionary of the fungi. 10th edn. CAB International, Wallingford, UK.

Kobayashi T. 2002. The taxonomic studies of the genus *Inocybe*. Nova Hedwigia 124: 1–246.

Kühner R. 1980. Les Hyménomycetès agaricoïdes. Bulletin Mensuel de la Société Linnéenne de Lyon 49: 1–1027.

Kühner R, Romagnesi H. 1953. Flore analytique des champignons supérieurs. (Agarics, Bolets, Chanterelles). Masson, Paris.

Kuyper TW. 1986. A revision of the genus *Inocybe* in Europe. I. Subgenus *Inosperma* and the smooth-spored species of subgenus *Inocybe*. Persoonia Suppl. 3: 1–247.

Larsson E, Ryberg M, Moreau PA, Mathiesen ÅD, Jacobsson S. 2009. Taxonomy and evolutionary relationships within species of section Rimosae (Inocybe) based on ITS, LSU and mtSSU sequence data. Persoonia 23:86–98. http://dx.doi.org/10.3767/003158509X475913

Ma D, Zang S, Wan L, Zhang D. 2012. Ectomycorrhizal community structure in chronosequences of *Pinus densiflora* in eastern China. African Journal of Microbiology Research 6(32): 6204–6209. http://dx.doi.org/10.5897/AJMR12.902

Niazi AR. 2008. Biodiversity of ectomycorrhizas in conifers from Himalayan moist temperate forests of Pakistan. Ph.D. Thesis, Department of Botany, University of Punjab, Lahore, Pakistan.

Niazi AR, Iqbal SH, Khalid AN. 2006. Biodiversity of mushrooms and ectomycorrhiza. 1. *Russula brevipes* Peck and its ectomycorrhiza, a new record from Himalayan moist temperate forests of Pakistan. Pakistan J. Bot. 38(4): 1271–1277.

Niazi AR, Khalid AN, Iqbal SH. 2007. *Descolea flavoannulata* and its ectomycorrhiza from Pakistan's Himalayan moist temperate forests. Mycotaxon 101: 375–383.

Niazi AR, Iqbal SH, Khalid AN. 2009. Ectomycorrhizae between *Amanita rubescens* and Himalayan spruce (*Picea smithiana)* from Pakistan. Mycotaxon 107:73–80. http://dx.doi.org/10.5248/107.73

Niazi AR, Khalid AN, Iqbal SH. 2010. New records of ectomycorrhizae from Pakistan. Pakistan J. Bot. 42(6): 4335–4343.

Obase K, Kobayashi T, Miyamoto T, Tamai Y, Yajima T. 2006. *Inocybe nitidiuscula*, new to Japan. Mycoscience 47: 293–297. http://dx.doi.org/10.1007/s10267-006-0304-x

Rochet J, Moreau PA, Manzi S, Gardes M. 2011. Comparative phylogenies and host specialization in the alder ectomycorrhizal fungi *Alnicola*, *Alpova* and *Lactarius* (Basidiomycota) in Europe. BMC Evolutionary Biology 11:40. http://dx.doi.org/10.1186/1471-2148-11-40

Ryberg M, Nilsson RH, Kristiansson E, Töpel M, Jacobsson S, Larsson E. 2008. Mining metadata from unidentified ITS sequences in GenBank: A case study in *Inocybe* (*Basidiomycota*). BMC Evol. Bio. 8: 50. http://dx.doi.org/10.1186/1471-2148-8-50

Singer R. 1986. The *Agaricales* in modern taxonomy. 4th ed. Koeltz Scientific Books, Koenigstein.

Stangl J. 1989. Die Gattung *Inocybe* in Bayern. Hoppea 46: 5–388.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol. Bio. Evol. 28: 2731–2739. http://dx.doi.org/10.1093/molbev/msr121

Tedersoo L, Suvi T, Larsson E, Kõljalg U. 2006. Diversity and community structure of ectomycorrhizal fungi in a wooded meadow. Mycol. Res. 110: 734–748. http://dx.doi.org/10.1016/j.mycres.2006.04.007

Tedersoo L, Suvi T, Jairus T, Ostonen I, Põlme S. 2009. Revisiting ectomycorrhizal fungi of the genus Alnus: differential host specificity, diversity and determinants of the fungal community. New Phytologist 182: 727–735. http://dx.doi.org/10.1111/j.1469-8137.2009.02792.x

Walbert K, Ramsfield TD, Ridgway HJ, Jones EE. 2010. Ectomycorrhiza of *Pinus radiata* (D. Don 1836) in New Zealand — an above- and below-ground assessment. Australasian Mycologist 29: 7–16.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. 315–322, in: MA Innis et al. (eds). PCR Protocols. London: Academic Press.

Yamada A, Katsuya K. 2001. The disparity between the number of ectomycorrhizal fungi and those producing fruit bodies in a *Pinus densiflora* stand. Mycol. Res. 105: 957–965.