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Morphology and molecular phylogeny uncover the first collection of *Paxillus orientalis* (*Paxillaceae*) from India

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ABSTRACT

During a 2016 foray to South District of Sikkim, *Paxillus orientalis*, a species belonging to *Paxillus* subg. *Alnopaxillus*, was collected for the first time in India. A detailed morphological description, *ITS*-based phylogenetic estimation, illustrations and comparisons with allied species are presented.

Keywords: Sikkim, *Basidiomycota*, *Boletales*, phylogeny, taxonomy

INTRODUCTION

The species *Paxillus involutus* (Batsch.) Fr. and *P. rubicundulus* P.D. Orton have long been known as the sole ectomycorrhizic representatives of the genus *Paxillus* Fr.: Fr. (Orton, 1969; Watling, 1970; Szczepka, 1987; Hahn, 2000) (here named as *Paxillus* s.s.). Lignicolous saprotrophic species were accepted in the genus *Tapinella* E.-J. Gilbert (Šutara 1992). Gradually, application of additional morphological markers (Hahn and Agerer, 1999) and phylogenetic studies (Vellinga *et al*., 2012; Gelardi *et al*., 2014; Jargeat *et al*., 2014; 2016) on the members of *Paxillus* s.s. led to the recognition of new species. So far two subgenera within *Paxillus* s.s. are recognized: *P.* subg. *Paxillus* and *P.* subg. *Alnopaxillus* Vizzini & Gelardi which encompasses the *Alnus*-associated species (Hedh *et al*., 2008; Vellinga *et al*., 2012; Gelardi *et al*., 2014;Jargeat *et al*., 2014; 2016). Four taxa, namely *P. rubicundulus* P.D. Orton, *P. olivellus* P.-A. Moreau, J.-P. Chaumeton, H. Gryta & P. Jargeat, *P. adelphus* J.-P. Chaumeton, H. Gryta, P. Jargeat & P.-A. Moreau and *P. orientalis* Gelardi, Vizzini, E. Horak & G. Wu are known under *P.* subg. *Alnopaxillus* whereas, the other subgenus, is also represented by four species, i.e. *P. involutus* (Batsch) Fr., *P. cuprinus* Jargeat, Gryta, Chaumeton & Vizzini, *P. obscurosporus* C. Hahn and *P. ammoniavirescens* Contu & Dessì (= *Paxillus validus* C. Hahn).

Sikkim, one of the small and Himalayan states in India, lies south of the Eastern Himalayas, one of the world's 18 biodiversity hotspots (**Fig. 1**). Sikkim's South district covers 750 km^2 and varies in elevation from 400 m to 2000 m. Within South District, Tendong (State) Biodiversity Park (**Fig. 1**) lies about 70 km west of Gangtok, the capital of Sikkim. The park is dominated by broadleaf tree species such as *Castanopsis*, *Alnus*, and *Juglans* and the coniferous *Cryptomeria japonica* (Thunb. ex L.f.) D. Don. During a routine macrofungal foray to this Biodiversity Park in 2016, one of us (KD) encountered an interesting specimen belonging to *Paxillus* growing under species of *Alnus* and *Castanopsis*. Morphological examination followed by phylogenetic analysis confirms the identification of this collection as *P. orientalis*, a species which was known so far only from China (Gelardi *et al*., 2014).

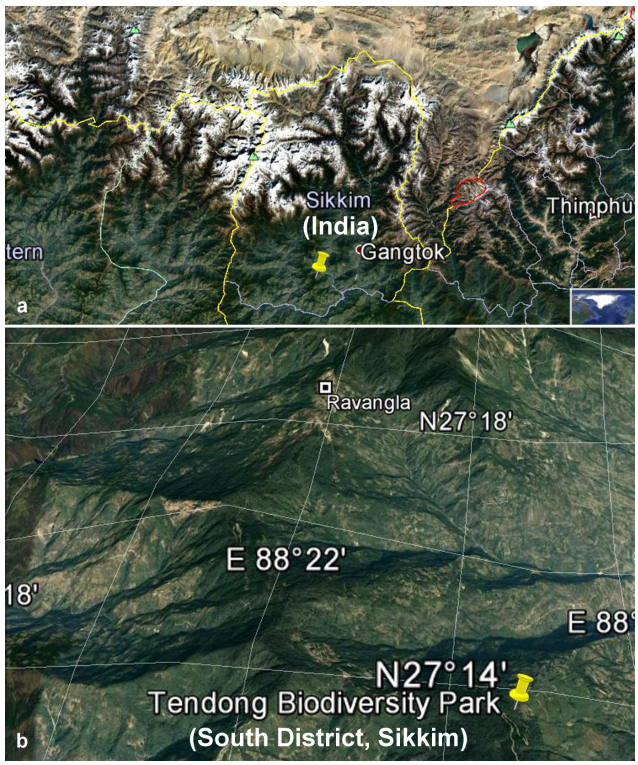

Fig. 1. a. Location of the collection site of *Paxillus orientalis*: Tendong (State) Biodiversity Park (yellow pointer) and the Sikkim state in India; **b.** Detail of map showing collection site (yellow pointer) in Tendong Biodiversity Park located in the South district of Sikkim. (Source: <www.google.com/earth>).

MATERIALS AND METHODS

Morphology: Macromorphological characters or field observations of the fresh and dissected young or mature basidiomata were noted in the forest or in the base camp. Photographs of the basidiomata and habitat were captured with a Canon Power Shot SX220 HS camera. Samples were dried in an aluminium field-drier. Freehand sections from dry materials mounted in a mixture of 3% KOH, 1% Phloxine and 1% Congo

red or in distilled water were examined microscopically using Nikon Eclipse Ni-U and Olympus CX 41 compound microscopes. Micromorphological characters were observed under 400× and 1000× and drawn at 1000× with the aid of a drawing tube attached to the Nikon microscope. Basidium length excludes that of sterigmata. Twenty basidiospores mounted from a spore print were measured in profile view. Spore measurements and length/width ratio (Q) are presented here as minimum mean maximum. Color codes and terms are mostly after the Methuen Handbook of Color (Kornerup and Wanscher, 1978). Herbarium codes follow Thiers (2017, continuously updated).

DNA isolation, PCR and sequencing: Genomic DNA was extracted from 100 mg of a dry basidiome using the InstaGeneTM Matrix Genomic DNA isolation kit (Biorad, USA) following the manufacturer's instructions. For PCR amplification of ITS region ITS1 and ITS4 primers were used (White *et al*., 1990). PCR amplification on "ABI Veriti" thermal cycler protocol was (for this ITS region) 2 min at 94°C, 35 cycles of 45 sec at 94°C, 1 min at 55°C, 1 min at 72°C, and a final stage of 10 min at 72°C. Amplified PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN, Germany). Both strands of the PCR fragments were sequenced on the 3730xl DNA Analyzer (Applied Biosystems, USA) using the amplifying primers and then assembled using Sequencher (Gene Codes Corporation, USA). Derived ITS (MF370339) sequence was deposited in GenBank (Benson *et al*., 2016).

Phylogenetic analysis: ITS dataset was assembled from previous studies on this genus (Gelardi *et al*., 2014; Jargeat *et al*., 2014; 2016) and from BLAST (Altschul *et al*., 1997) searches in GenBank (Clark *et al*., 2016) and UNITE (Kõljalg *et al*., 2013). Required ITS sequences were aligned in MAFFT 7.305 (Katoh and Standley, 2013). For this analysis 4 taxa of *P.* subg. *Paxillus* (*P. involutus, P. cuprinus, P. obscurosporus* and *P. ammoniavirescens*) were used as outgroup taxa (Gelardi *et al*., 2014; Jargeat *et al*., 2014; 2016). Maximum likelihood analysis was conducted using MEGA 6.0 (Tamura *et al*., 2013). One-thousand bootstrap replicates were analyzed to obtain nodal support values.

TAXONOMY AND PHYLOGENY

Paxillus orientalis Gelardi, Vizzini, E. Horak & G. Wu; *Mycol. Progress* **13**: 336 (2014) **Figs. 2 & 3**

Pileus 30-85 mm diam., broadly convex when young, becoming plano-convex to plane with a shallow to deep depression at centre to infundibuliform at maturity, rarely with a small central umbo in young basidiomata; margin strongly inrolled to incurved, with a very narrow flap of tissue, often irregularly lobed, entire when young gradually ribbed-striate with maturity; surface, dry, with loosely covered and adpressed more or less radially arranged

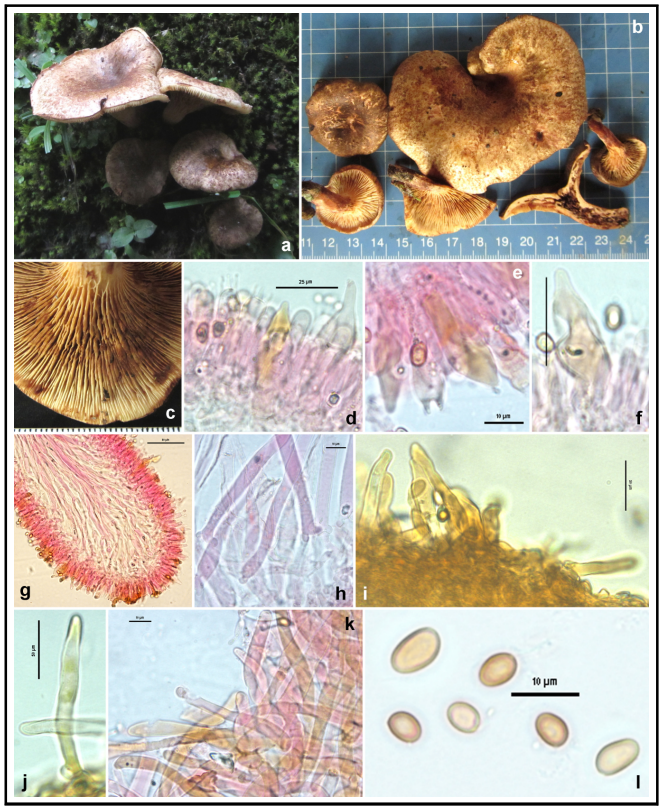

Fig. 2. *Paxillus orientalis* (KD 16-16): a & b. Fresh or dissected basidiomata c. Lamellae and lamellulae d & f. Pleurocystidia e. Lamellae edge showing cheilocystidia and basidia g. Divergent pattern of hymenophoral trama h. Hyphae with clamp connections in hymenophoral trama i & j. Transverse section (TS) through stipitipellis showing the elements k. TS through pileipellis showing the suberect elements k. Basidiospores. Scale Bars: d & f = 25 µm; e, h, k, l = 10 µm; g, j, i = 50 µm.

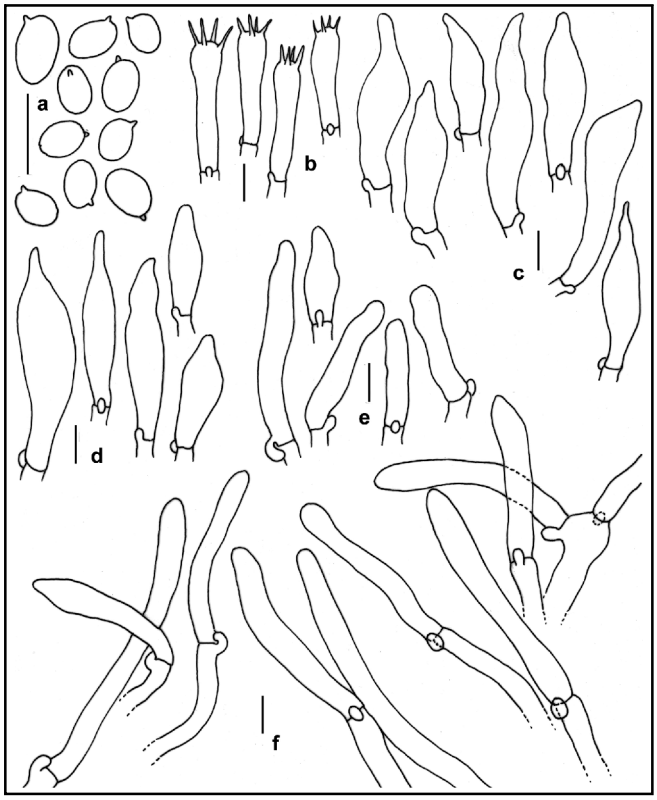

Fig. 3. *Paxillus orientalis* (KD 16-16): a. Basidiospores b. Basidia c. Pleurocystidia d. Cheilocystidia e. Caulocystidia f. TS through pileipellis showing erect to suberect hyphal elements. Scale bars: af = 10 µm.

squamules, becoming denser at center gradually more distant towards margin, scales dark brown (7-8F4-5), on yellowish white to pale yellow (3-4A2-3) to pinkish background but gradually paler with age, becoming rusty brown when bruised. Lamellae (including lamellulae) crowded (24/cm), decurrent, mostly forked and anastomosing near stipe, easily detachable from pileus, yellowish white (4A2) to pale yellow, becoming dark rusty brown when bruised; lamellulae at least in 5 series, concolorous, edges entire. Stipe 28-40 × 8-15 mm, central to eccentric, solid, straight or curved, cylindrical or tapering towards the base; surface smooth, glabrous, concolorous to lamellae at apex, slightly pinkish towards base but becomes reddish brown (9D7) or dark rusty brown; basal mycelium olivaceous. Context in stipe cream yellow initially, becoming pinkish on exposure. Odour strong and pleasant. Taste not recorded. Spore print light brown (6D45) or darker.

Basidiospores 5.0-5.9-8.5 × 3.7-4.2-5.3 µm, (n=20, Q=1.18-1.39-1.68), broadly ellipsoid to ellipsoid, more rarely subcylindrical, smooth, with a short apiculus, moderately thick-walled (0.5-0.7 µm), straw coloured in water and 5 % KOH, with one large oil drop at maturity, inamyloid, germ pore absent. Basidia 25-40 × 5-8 µm, cylindrical to narrowly clavate, moderately thick-walled (0.6-0.8 µm), 2- to 4-spored; sterigmata 3-6 µm long, basal septa clamped. Pleurocystidia 32-55 × 8-14 µm, abundant, fusiform to ventricose to ventricose-rostrate or appendiculate, moderately thick-walled, with brownish yellow pigmentation in water and 5% KOH. Lamellae edge fertile, composed of basidia, basidioles and cystidia. Cheilocystidia 30-67 ×7-15 µm, abundant, similar to pleurocystidia. Hymenophoral trama bilateral-divergent, loosely arranged; hyphae 6-12 µm wide, septate, branched. Pileipellis 200-250 µm thick, a trichoderm when young, becoming somewhat a cutis at maturity, consisting of erect to suberect, branched, septate and strongly interwoven hyphae; terminal element 40-110 × 6-9 µm, long, cylindrical with rounded to subfusoid apex, moderately thick-walled (0.6-1 µm), with intracellular brownish yellow pigmentation in water and 5% KOH, basal septa always clamped. Stipitipellis (from stipe apex) with longitudinally arranged parallel hyphae and cystidia. Caulocystidia 27-53 × 5-10 µm, abundant, subcylindrical to cylindrical or subfusoid, moderately thick-walled (0.6-0.8 µm), with brownish-yellow intracellular pigmentation in water and 5% KOH. Clamp connections present in all tissues.

Specimen examined: INDIA. Sikkim: South-district, Tendong (State) Biodiversity Park, N27°13′40.5″ E88°24′38.7″, under *Alnus* sp. and *Castanopsis* sp., 17 August 2016, K. Das, KD 16-16 (CAL).

Phylogeny: ITS-based phylogenetic analysis (**Fig. 4**) with 40 ITS sequences (including the present species) resolved the genus *Paxillus* (including its two subgenera) with full support. Four species of *P.* subg. *Alnopaxillus* are clearly separated in the ingroup. Present Indian ITS sequence derived from KD 16-16 (represented by GenBank accession no. MF370339) is nested in the clade including all the known sequences of *P. orientalis* (in *P.* subg. *Alnopaxillus*) derived from Chinese collections with strong support (bootstrap value = 95%) showing the conspecificity between the collections

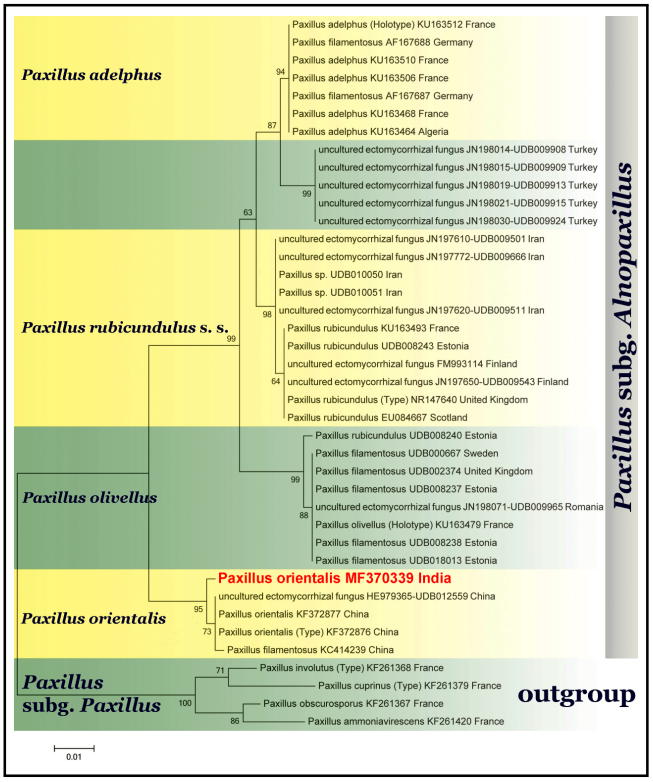

Fig. 4. The phylogenetic estimation inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 40 sequences. The sequence derived from the Indian collection of *Paxillus orientalis* is shown in red and bold font.

from two Asian countries. Like the analyses conducted by other authors (Gelardi *et al*., 2014; Jargeat *et al*., 2016) our phylogeny shows the species of the *P. rubicundulus* complex (or *P. rubicundulus* s.l., including *P. rubicundulus* s.s., *P. adelphus* and *P. olivellus*) as sister to *P. orientalis*.

DISCUSSION

Different species of *Paxillus* can hardly be separated distinctively only with the help of macromorphology in the field. It has been realized that most of the macromorphological features overlap each other amongst different species, hence making difficult to segregate them in the field (Hahn and Agerer, 1999). Therefore, combination of micromorphology and molecular phylogeny (even ITS-sequence data alone) coupled with macromorphological features are required for the species delimitation in *Paxillus*.

Paxillus orientalis is characterized by small to medium-sized pileus, presence of dark coloured squamules on pinkish exposed background colour of pileus surface, small basidiospores, stipe that is yellow at apex and pinkish towards base but becoming dark brown when bruised, yellow context that becomes pinkish to reddish where bruised (Gelardi *et al*., 2014). The combination of morphological features like, squamulose pileus surface, yellow context, comparatively small basidiospores and occurrence under *Alnus*, undoubtedly place this Asian species under *P.* subg. *Alnopaxillus*. Moreover, macro- and micromorphological

features of the Indian collection of *P. orientalis* is in conformity with those of the Chinese collections except the pleurocystidia which are slightly longer [(34-) 43-72 (-80) × 8-19 (-22) µm] in Chinese materials (Gelardi *et al*., 2014). The morphologically and phylogenetically closest taxa, the species of the *P. rubicundulus* complex differ from *P. orientalis* by the distinguishingly "ribbed-costate" pileus margin throughout the entire life of basidiomata, the brown-yellowish to olive-brown pileus always lacking pinkish tinges in the background of the scales, the faintly pruinose apex of stipe without any pinkish tinges and deeper lamellae (Gelardi *et al.,* 2014 and Jargeat *et al*., 2016).

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