Taxonomic and phylogenetic affinities of *Hygrophorus chrysodon* from western Himalayan forests

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Abstract: *Hygrophorus chrysodon* plays a key role in the growth and stability of pine vegetation in two ways: mycorrhizal and antifungal activities against plant pathogens. This is the first taxonomic report from Pakistan of this species using morphological and molecular characters. Morphological characters of one collection were compared with literature for a reliable identification. The Internal Transcribed Spacers (ITS) of ribosomal DNA sequence was queried in GenBank database where it matches to 99% with sequences of the same species from different regions of the world. Phylogenetic analysis, description, photographs and line drawings of the Pakistanian collection are provided. *Hygrophorus chrysodon* has great potential to be used in the control of mycorrhization for afforestation and reforestation of *Pinus wallichiana.*

Zusammenfassung: *Hygrophorus chrysodon* hat eine zweifache Schlüsselrolle bezüglich Wachstum und Stabilität der Föhren-Vegetation: Mykorrhizawachstum und antifungale Aktivität gegen Schädlinge. Hier berichten wir erstmals taxonomisch über diese Art aus Pakistan unter Verwendung morphologischer und molekularer Merkmale. Morphologische Merkmale einer Aufsammlung werden mit den Literaturdaten verglichen. Der Abgleich der Internal Transcribed Spacer (ITS) Sequenz der ribosomalen DNA mit Sequenzen dieser Art aus verschiedenen Weltregionen brachte 99% Übereinstimmung. Eine phylogenetische Analyse, Beschreibung, Fotos und Zeichnungen der pakistanischen Aufsammlung werden gegeben. *Hygrophorus chrysodon* hat großes Potential für die Kontrolle der Mykorrhizierung bei Auf- und Wiederaufforstung von *Pinus wallichiana*.

Hygrophorus FR. (*Hygrophoraceae, Agaricales*) is a widley distributed genus across the globe and more than 200 species are known (KIRK & al. 2008). This genus is mainly characterized by tricholomatoid to omphalinoid basidiomata which have varying colours, subdecurrent lamellae, smooth, hyaline spores and hymenium without cystidia (BAS & al. 1990). All those species which have white to cream basidiomata are classified in sect. *Hygrophorus* while the colourful ones are in different sections and subsections. Those white mushrooms having a dry, floccose to almost cortina-like veil and a dry stipe are treated in subsect. *Chrysodonti* SING. (FRIES 1874, SINGER 1943, CANDUSSO 1997). LODGE & al. (2014) revised the classification of *Hygrophoraceae* and

classified the family with three new subfamilies, eight tribes (five new), eight subgenera (one new, one new combination and one stat. nov.), 26 sections (five new and three new combinations and two stat. nov.) and 14 subsections. They also introduced three subgenera in tribe *Hygrophoreae* P. HENN., and ranked up SINGER's (1986) subsect. *Chrysodonti* as *Hygrophorus* [subgen. *Camarophylli*] sect. *Chrysodontes* (SINGER) E. LARSS. with *Hygrophorus chrysodon* as type specimen and yellow granules on pileus and stipe apex as section characteristics.

Hygrophorus chrysodon is a very important fungus which is exclusively mycorrhizal with pine vegetation and antifungal against *Fusarium* spp. (GILARDONI & al. 2007). Pine roots depend upon its intercellular hyphal network, Hartig's net, extended into deep soil beneath the canopy of the tree for uptake of water and soils nutrients (SMITH & READ 1997). The roots have a covering sheath, called mantle, made up of a dense plectenchym of fungal hyphae which prevents pathogen attacks mechanically and biochemically. Chrysotriones, secondary metabolites, have been discovered in *H. chrysodon* which have strong antifungal potential against root pathogens like *Fusarium* spp. and help to prevent attack of root rot disease in pines (GILARDONI & al. 2007).

Fusarium species are very important pathogens which attack badly the trees and agricultural crops in Pakistan (AKHTAR & al. 1999, IQBAL & al. 2006, MEHMOOD & al. 2013). In trees they infect the xylem and weaken the roots. Pakistani shesham (*Dalbergia sissoo*) and mango (*Mangifera indica*) are under pressure of *Fusarium* attacks (NENE & al. 1991, IQBAL & al. 2006). Similarly, the agricultural crops like wheat, rice, chickpea, maize etc, also suffer from yield loss due to *Fasurium* attacks (YASIN & al. 2003, PATHAN & al. 2003). Different fungicides have been used to control these diseases in agricultural crops and forest trees but their effects are marginal and not good enough to rehabilitate the plants (BAJWA & al. 2003, PATHAN & al. 2003). SUBHANI & al. (2013) used different microbes to determine their antifungal activity against *Fusarium* diseases in Pakistan and considered *Trichoderma harzianum* RIFAI. as most effective. *Hygrophorus chrysodon* is another effective possible biocontrol agent which can be used against wilt diseases in Pakistan. This macrofungus is naturally found in western Himalayas of Pakistan.

Western Himalayan forests, especially those between Great Himalaya and Siwalik mountains, are rich in diversity of plants, animals and macrofungi. More than 300 species of gilled fungi have been listed from Himalayan wet temperate forests belonging to different ecological groups like mycorrhizal, saprotrophic and wood decaying (AHMAD & al. 1997, RAZAQ & al. 2012 a, b, 2013 a, b; ILYAS & al. 2013 a, b). All these fungi are playing very important roles in the sustainability and dynamics of these forests (IRSHAD & KHAN 2012).

In this study, a new record of *Hygrophorus chrysodon* is described from Pakistan using morpho-anatomical details and molecular data. This species is associated with *Pinus wallichina* in Himalayan moist temperate forests of Pakistan. Morpho-anatomical and molecular description which is based on the internal transcribed spacer of ribosomal DNA (ITS-rDNA) will be useful for its re-collection and reliable identification which is required for culture raising in forestry and agriculture. The main objective of this study is to provide a reliable molecular taxonomic note on macrofungus which has fungicidal activity because of its secondary metabolites, chrysotriones.

Materials and methods

Morphological characterization. The basidiomata were carefully dug up using a knife and photographed in the field. For microscopic observation, sections were stained with Congo Red and Melzer's reagent. Dimensions were determined for 25 basidiospores, 20 basidia and 20 elements of pileus covering from each of the two basidiomata under the light microscope equipped with a camera lucida.

DNA isolation and amplification of ITS-rDNA region. A part of the fertile side of the dried basidoma (approx. 100 mg) was macerated by pestle and mortar using liquid nitrogen. The macerated tissue was gently shifted into a 1.5 ml centrifuge tube, and 1 ml of pre-warmed 2% CTAB buffer was added; it was mixed gently. All tubes were carefully incubated at 65 °C for one hour in a water bath with continuous shaking and swirling after every 20 min. At room temperature a 60% volume (to the CTAB used) of Isoamlyalcohol : Chloroform (1 : 24) was added in each tube and gently vortexed. The mixture was cleared by centrifugation for 10 min at maximum speed. The supernatant was taken in another tube by careful pipetting without touching the pellet between two layers. The DNA was precipitated by adding 2/3 volume of ice-cold Isopropanol or ½ volume 95% ethanol and placed at room temperature for a few minutes or in a freezer for overnight. DNA was collected by centrifugation at maximum speed and the pellet was washed with 70% ethanol or wash buffer. The final pellet was dried at room temperature or at 37 °C in an oven and dissolved in 50 µl TE buffer or distilled H_2O. ITS region of rDNA was amplified using universal primer pair ITS1F and ITS4 (WHITE & al. 1990). PCR was performed in 20-µl reaction volume following the protocol given by GARDES & BRUNS (1993). The size of PCR product was determined on 1.5% agrose gel in Gel documentation system (UVtec, Avebury House, Cambridge CB4 1QB UK) using default setting. PCR product was directly sequenced in both directions using the same pair of amplification primers by the company Macrogen (Korea).

Sequence analysis and phylogeny. The generated sequence was edited manually using BioEdit sequence alignment editor version 7.0.9.0 (TOM HALL, Ibis Biosciences, Carlsbad, California) and a consensus sequence was generated from forward and reverse sequences. For initial sequence analysis, the sequence was queried by BLAST (Basic Local Alignment Search Tool) against the National Center for Biotechnology Information (NCBI), USA, database. The newly generated sequence and the closely related ones retrieved from the GenBank were aligned by using Clustal W program of Molecular Evolutionary Genetics Analysis (MEGA) software (TAMURA & al. 2011). Phylogenetic analysis was determined following RAZAQ & al. (2013b). The sequences included in this analysis had 2293 characters of 37 sequences, from which 598 characters were used in final analysis after trimming the alignment from both 5' and 3' sides. In the phylogenetic analysis gaps are treated as missing data. Consensus nucleotide sequence of *H. chrysodon* was submitted to European Molecular Biology Laboratory (EMBL) database.

Results

Morpho-anatomical characterization:

Hygrophorus chrysodon (FR.) FR. Epicr. Myc. 1838: 320. (Fig.1)

P i l e u s : 3.5–4.0 cm wide, tricholomoid to somewhat omphalioid, white with yellowish ochre central disc, overall spread with yellow granules, convex to planoconvex, viscid and dry, deep umbonate to umbilicate; context moderately thick, firm, white to pale yellow, unchanging when bruised or cut; margin striate, with yellowish ochre or golden yellow patches and granules.

L a m e l l a e : subdecurrent, spaced to moderately close, white to yellowish white, denticulate, fleshy, thick; lamellulae truncate, of two types lengthwise, distributed between each pair of lamellae.

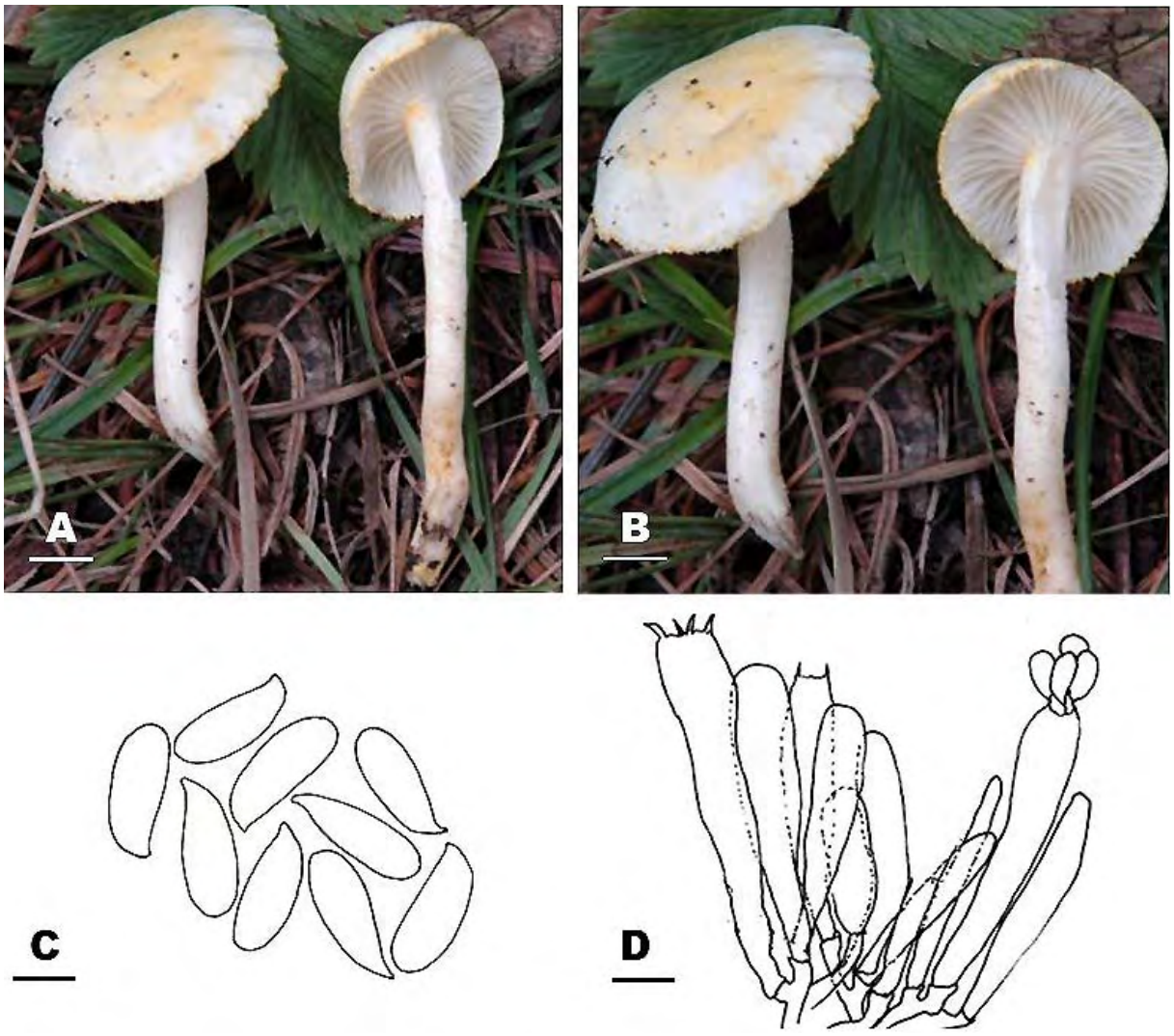

Fig. 1. *Hygrophorus chrysodon. A, B* Basidiomata, *C* basidiospores, *D* hymenium with basidia. Bars: A, B 0.85 cm, C 5.6 µm, D 7 µm.

Stipe: 4.5–5.6 × 0.56–0.65 cm, white, smooth, fragile, cylindrical; woody texture, context thick, white slightly swollen base; golden yellow granules in the form of a veil which is dry and almost cortina like, on the apical region of the stipe.

Basidiospores: 6.5–9.5 × 4.0–5.5 µm, avl × avw = 10.5 × 4.75 µm, Q= 2.13–2.27, avQ= 2.2; apiculate, thin-walled, ellipsoid to oblong, proximal poles less wide than distal poles, hyaline to light green in 5% KOH, inamyloid, yellow in Melzer's reagent.

Basidia: 34.5–48.5 × 7.5–8.5 µm, 4-sterigmate, hyaline to pale yellow in 5% KOH, thin-walled, subclavate to rod-like with oil-like contents.

Lamellar trama: hyphal. Cheilocystidia: absent. Pileus covering: a cutis of wider elements 71.0–140.5 × 24.0–35.5 µm, hyaline, thin-walled. Clamp connections: present.

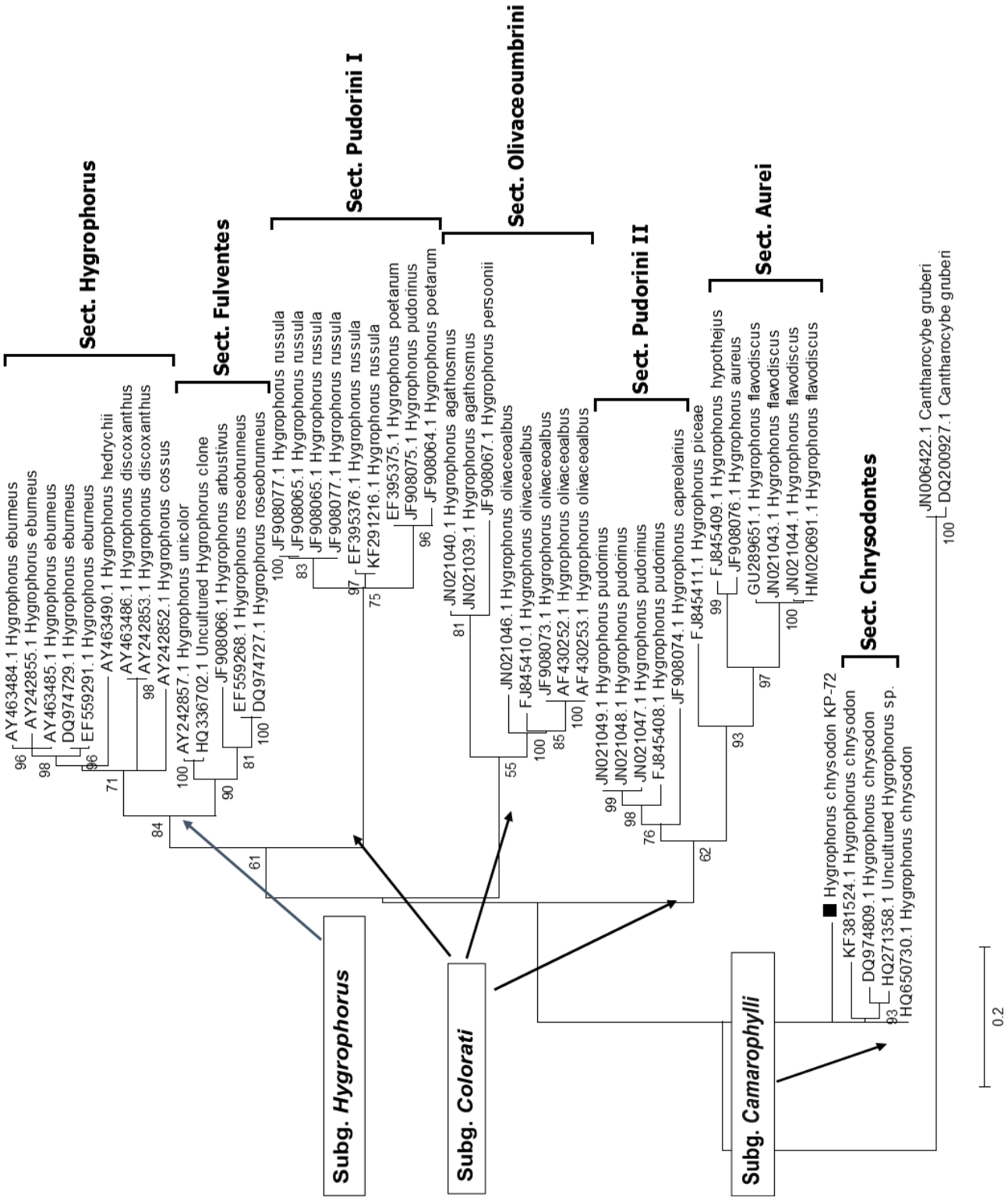

Fig. 2. Phylogenetic analysis of Pakistanian *Hygrophorus chrysodon* (■) based on nrITS-rDNA marker using maximum likelihood method. The analysis involved 50 nucleotide sequences. There were a total of 639 positions in the final dataset. Bootstrapping values more than 50% are shown.

M a t e r i a l e x a m i n e d : Pakistan, Khyber Pakhtunkhwa, Abbottabad, Ayubia-Khanspur (Halley pad side), 2400-2580 m s. m., on humified soil under *Pinus wallichina* vegetation, solitary, leg. ABDUL RAZAQ, KP-72, 23. August 2010; deposited in the fungarium LAH (no. 13081072).

Molecular characterization and phylogenetic analysis:

The target region comprising the internal transcribed spacers (ITS1, 5.8S, ITS2) of rDNA gave fragments of approximately 600 bp on amplification in PCR using the primers pair ITS1–ITS4. Initial BLAST analysis of nucleotide sequences revealed that the Pakistanian collection matched 99% with *Hygrophorus chrysodon* (GenBank accession no. DQ974809.1, USA).

Fifty sequences of *Hygrophorus* species were included in the phylogenetic analysis to clarify the phylogenetic position of the Pakistanian collection. The sequences of three subgenera (subg. *Hygrophorus* E. LARSS., subg. *Colorati* (BATAILLE) E. LARSS. and subg. *Camarophylli* (FR.) E. LARSS.) were retrieved from GenBank and their analysis showed different clades according to their sections. There are six major clades formed in the phylogenetic tree (Fig. 2) which have been labeled as sect. *Hygrophorus*, sect. *Fulventes*, sect. *Pudorini* (two clades), sect. *Olivaceoumbrini*, sect. *Aurei* and sect. *Chrysodontes*. The light-coloured mushrooms of subgenus *Hygrophorus*, clustered monophyletically in sect. *Hygrophorus* (BULL.) FR. and sect. *Fulventes* (FR.) E. LARSS. under a significant bootstrap value. The members of the latter section showed a closer resemblance with sect. *Hygrophorus* than SINGER (1986)'s sect. *Discoides* and therefore *H. arbustivus* FR. has been accommodated in sect. *Fulventes*. All the colourful species of subg. *Colorati* recovered in different sections (Fig. 2) and as this subgenus is polyphyletic, especially the species of sect. *Pudorini,* this needs revision. *Hygrophorus russula* and *H. poetarum* are a sister clade to subg. *Hygrophorus* while *H. pudorinus* and *H. capreolarius*, morphologically closely resembling *H. russula*, classified in subg. *Colorati* showed closer affinities with sect. *Aurei*. *Hygrophorus russula* and *H. poetarum* also recovered in sister clade to each other to be grouped either into different subsections like SINGER (1986) did. The other important clade recovered in *Colorati*, sect. *Aurei*, derived from sect. *Discoidei,* is monophyletic. *Hygrophorus piceae* is a sister clade to sect. *Aurei*, therefore it was treated in a separate section, sect. *Picearum* E. LARSS. by LODGE & al. (2014). The third subgenus, *Camarophylli*, contains the clade of sect. *Chrysodontes* characterized by light coloured basidiomata with decurrent to subdecurrent lamellae and yellow granules on pileus and stipe apex. In this clade, only the sequences of the type species of this section, *H. chrysodon*, clustered as no other sequence data were available in GenBank. The Pakistanian collection of *H. chrysodon* from western Himalaya also recovered in this clade together with European and North American ones. All the sequences showed some intraspecific variation and the Pakistanian collection received low bootstrap value but the morphological characters are conspecific with *H. chrysodon*. Morphologically, this species was treated in sect. *Hygrophorus* subsect. *Chrysodontini* by SINGER (1986) and other taxonomists, until LODGE & al. (2014) raised its rank to section level. Both, sect. *Hygrophorus* and sect. *Chrysodontes* on molecular basis (ITS-rDNA) are distinct (Fig. 2).

Discussion

Hygrophorus chrysodon is characterized by white to cream pileus with yellow granules spread over the whole pileal surface, conclorous subdecurrent lamellae, a yellow ring of granules on the stipe apex, hyaline basidiospores, and its specific mycorrhizal association with pine vegetation for uptake of water and nutrients.

Hygrophorus species are found near woody, arboresent trees either pine or deciduous or mixed vegetation. The basidiomata are viscid or glutinous especially when

young (BAS & al. 1990). The presence of clamp connections and subdecurrent lamellae are the differences from *Tricholoma* species otherwise both genera are mycorrhizal and their basidiomata are viscid and the colour of the lamellae is the same when young, namely white (SINGER 1986). Among all the species of *Hygrophorus*, *H. chrysodon* is easily identifiable due to the yellow granules on pileus and stipe apex. The Pakistanian collection is morphologically similar to the description given by BAS & al. (1990) and BREITENBACH & KRÄNZLIN (1991) for European materials and LARGENT (1985) for North American specimens. North American material has a more yellowish colouration of the pileus due to excessive granulation than the Pakistanian one. Microscopically, our collection is in accordance with American and European collections (LARGENT 1985, BAS & al. 1990). BREITENBACH & KRÄNZLIN (1991) also mentioned that this species is non-specific for its phytobionts either it may form mycorrhiza with hard wood or coniferous trees but the Pakistanian specimen is found only with coniferous trees.

In the phylogenetic analysis, species of all three subgenera (*Hygrophorus*, *Colorati*, *Camarophylli*) clustered in different clades and subclades. The species of subg. *Hygrophorus* were monophyletic (Fig. 2) and even two sections of this subgenus were recovered. LODGE & al. (2014) also described the same relationship among light coloured species of two clades of subg. *Hygrophorus* on molecular basis. The species of sect. *Hygrophorus* clade (Fig. 2) were also treated in the sect. *Hygrophorus* by SINGER (1986) and BAS & al. (1990) but sect. *Fulventes* clade is purely derived on molecular data from morphologically erected sect. *Discoidei* (SINGER 1986). The sequences of *H. roseobrunneus* also lie in this section. The coloured mushrooms of subg. *Colorati* were not monophyletic; the species of different sections clustered in the same clade while the species of the same section clustered in different clades (Fig. 2). In this subgenus the species of sect. *Olivaceoumbrini* and of the newly formed sect. *Aurei* clustered monophyletic while the sect. *Pudorini* bifurcated into two parts. Section *Pudorini* I showed very close relationship with subg. *Hygrophorus* while sect. *Pudorini* II is more inclined to sect. *Aurei*. LODGE & al. (2014), on molecular basis, also recovered the same relationship between the different sections of subg. *Colorati* and the positions of *H. pudorinus* and *H. capreolarius* were not clearly defined though the latter has close relation with *H. russula* (BREITENBACH & KRÄNZLIN 1991). According to SINGER (1986) the species of the sect. *Pudorini* I clade, morphologically, are very close to sect. *Hygrophorus* clade and its subclades are also according to SINGER (1986) two subsections (subsect. *Erubescentes* SM. & HESL. and subsect. *Fulvoincarnati* SM. & HESL.) with exception of *H. arbustivus* which was treated in subsect. *Fulvoincarnati* by SINGER (1986), in sect. *Discoidei* by BAS & al. (1990) and in sect. *Fulventes* by LODGE & al. (2014). The position of sect. *Pudorini* I clade is justified both on morphological and molecular clustering while the members of sect. *Pudorini* II clade are either misidentified or sequences of closely related members of subsection of sect. *Aurei*. The morphological relationship of different sections of coloured fungi was not recovered on molecular basis, for instance, SINGER (1986) and BAS & al. (1990) treated *H. leucophaeus* (*H. unicolor*) in sect. *Discoidei* on morphological basis while it was recovered in the newly formed sect. *Fulventes* on molecular basis. Morphologically, the previous taxonomists (SINGER 1986, BAS & al. 1990) unanimously agreed to place the light coloured *H. chrysodon* in sect. *Hygrophorus* despite the yellow granules on its pileus and stipe apex, a complex character. Placement was solved by LODGE & al. (2014) by

shifting it to a new section, *Chrysodontes*, of new subgenus, *Camarophylli,* based on molecular data. In the phylogenetic tree, the geographically distinct sequences of *H. chrysodon* clustered in the same clade. Section *Chrysodontes* showed intraspecific variation that is evident by the clustering pattern within the clade but the yellow granules on pileus and stipe are an easy character to recognize this species. This easily recognizable character may lead to misidentification of very closely related species and subsequently, the availability of their sequences under the name of *H. chrysodon* can provide the basis for a maybe cryptic species complex.

The occurrence of a high level of natural genetic variability in this species may be the other possibility. The chrysotriones, secondary metabolites, from *H. chrysodon* can also be used against plant pathogens. GILARDONI & al. (2007) discovered a group of secondary metabolites from cultures of *H. chrysodon* which are very efficient against fungal plant pathogens like *Fusarium*. The association of this species with *Pinus wallichiana* in Pakistan is believed to be responsible for its good vigor and growth as it provides water and nutrients to the plants and protects its roots against pathogen attacks.

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