

A NEW SPECIES OF *Clitopilus Shanglayensis* (ENTOLOMATACESAE, AGARICALES) REPORTED FROM KHYBER PAKHTUNKHWA, PAKISTAN

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Abstract

In current study a new species *Clitopilus shanglayensis* (Entolomatacesae, Agaricales) was reported based on morphological and molecular confirmation from northern region of Pakistan. Morphological parameters which differ the species from other species include white yellowish pileus (05-06 cm wide), light brown stipe and pale yellowish lamella. The fungal molecular markers nuclear ribosomal internal transcribed spacer (ITS) and larger subunits (LSU) were sequenced. These sequences were further used for evolutionary analysis through various bioinformatics tools. The phylogenetic analysis based on ITS and LSU sequences by using Maximum Likelihood method (ML) revealed that the novel species reported from current study had distinct characteristics features than other species. A complete detail description, color photographs and evolutionary relatedness of novel species *Clitopilus shanglayensis* were mentioned.

Keywords: Agaricales, *Clitopilus*, Entolomatacesae, Molecular, Phylogenetic analysis,

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INTRODUCTION

The current study was carried out at District Shangla, located in 72° 36'00" eastern longitudes and 34° 53' 12" northern latitude (Ahmad *et al.*, 2014). Till 1995 it remained a subdivision of district Swat. The district has small valleys and surrounded by big mountains. Annually 1778 millimetre of rain is recorded in the district which had impact on the rising of different flora including fungal species (Ajmal *et al.*, 2012; Gallois *et al.*, 1990). There are about 150,000 fungal species on the earth, in which 69000 are, documented (Ali *et al.*, 2012; Gafforov *et al.*, 2020). Many studies have reported the harmful effects of different species of fungi on crops because they compete for sunlight and nutrients resulting reduction in crop production (Hussain *et al.*, 2007; Rehman

et al., 2020). Therefore, the taxonomic identification of new species of fungi is very important.

One of the three largest euagaric families which contain 1500 known species is the Entolomataceae Kotl. & Pouzar (Agaricales, Basidiomycota) (David *et al.* 2009; Baroni *et al.*, 2011). The common characteristic feature of this family is the presence of basidiospores, which are angular, pinkish in color and consistent cyanophilic walls (David *et al.* 2009). This common microscopic appearance demonstrated that the family is a natural group and evolutionary molecular analysis showed its monophyly (David *et al.*, 2009, Baroni and Matheny 2011).

The genus *Clitopilus* P. Kumm (Entolomatacesae, Agaricales) was reported in 1821 by Fries. Presently *Clitopilus* is usually separated into three

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units namely *Clitopilus*, *Pleurotelloides* and *Scyphoides*. *Clitopilus* has average to fat size sporocarp, dissimilar and determined stipitipellis, (Singer 1986; Nooedeloos 1993). This genus is spread widely in northern temperate areas and include 30 species as suggested by an estimate at 2008 (David *et al.*, 2009; Kirk *et al.*, 2008). Some onwards studies suggested the redefinition of this genus as it include many members.

According to the best of our knowledge there is no proper study on *Clitopilus* from Pakistan related to the taxonomic classification and identification. In this study, a new species of *Clitopilus* was described from the Himalayan moist temperate region of District Shangla, Khyber Pakhtunkhwa, Pakistan with the help of morphological and molecular phylogenetic analysis.

MATERIALS AND METHOD

Sampling sites

During daytime the selected samples were excavated by using knife and photographs were taken and labelled. The type of vegetation in selected location of current study and collection date was noted for future reference. The samples were dried (having water content <15%) by using a gas heater at 50–60 °C and store properly. A slide was prepared in 3% of KOH and Congo red, spores' ornamentation was observed under (100X) objective in immersion oil and drawings were made under the light microscope with camera Lucida. Spore sizes were determined by measuring the diameter of minimum 20 mature spores including the ornamentation (Li *et al.*, 2012; Razaq *et al.*, 2012). The specimens were deposited in the Botany Department, University of the Punjab Herbarium, Lahore, Pakistan.

DNA extraction, amplification, and sequencing

A small piece of specimen was chopped and put in 2% CTAB buffer, followed by DNA extraction by using a previously reported protocol with some amendments (Porebski

et al., 1997). The universal primer pair (ITS4-F and ITS1-F) was used to amplify the ITS region of rDNA (White *et al.*, 1990). The total PCR reaction volume used was 25 µL (Gardes and Bruns 1993). The PCR product was then sequenced both in forward and reverse direction with the same primers (BGI, Shenzhen, China).

Phylogenetic analysis

Sequences editing was performed by using Bio edit (v7.0) and alignment sequences were BLAST in (NCBI) USA databank. For the phylogenetic study the closely related sequences were retrieved from the Gen Bank. The alignment of sequence and phylogenetic tree construction was done with MEGA 7 (Tamura *et al.*, 2011). Phylogeny was tested with bootstrap value of 1000 replicates. The current study sequences were submitted to NCBI Gen Bank for accession number.

RESULTS

Molecular and phylogenetic characterization

The ITS segment of the nrDNA of local specie was amplified by ITS, F1 & F4 primers. The agreement sequences of the local species were explored in NCBI and BLAST. It shows 95% character with *Clitopilus prunulus* (KC885965), 95% query covers 0.0 E. values. The comparable sequences were retained from the Gen Bank. The sequences were trim and aligned by using online tools (MAFFT-version-7 and UPGMA phylogeny CBRC (<https://www.mafft.cbrc.jp/alignment/server/>)). The primary aligned file contained 1981 primary characters, of which 930 characters were used. This data set contained 602 conserved sites, 311 variable sites, 97 parsimony-info sites and 212 singleton sites. Phylogenetic analysis was inferred using maximum likelihood method in MEGA 7.0. the model used for this analysis was Jukes cantor model. The analysis has two major clades viz: clade I and clade II, Fig (01). Our species sequences clustered with *Clitopilus*

prunulus (AY228348) and (JQ281487) in clade II with a high bootstrap value. Other species i.e. *Clitopilus prunulus* (LN714533) *C.* cf. *scyphoides* (KC176279), (KC176280), (KC176290), (KC176289) *C. cystidiatus* (HM623130) *C. hobsonii* (FJ770385), (FJ770398), (FJ770395)

C. scyphoides (FJ770401), (KC176282) *C. passeckerianus* (FJ770388), (FJ770406), (MG282460), (FJ770386), (KY962494) *C. giovanellae* (EF413030) *C. austroprunulus* (KC139085) were clustered in same clade with higher bootstrap value

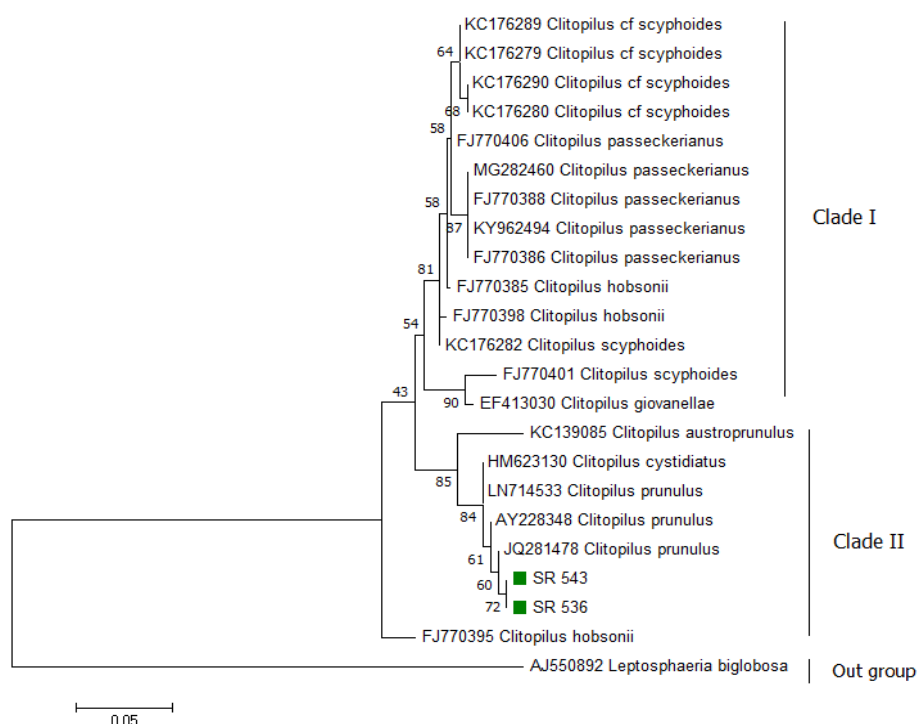


Figure 1: ITS sequence molecular phylogenetic analysis by Maximum Likelihood method. Tamura 3-parameter model was used for evolutionary analysis. The sequences generated in present study were marked with ■.

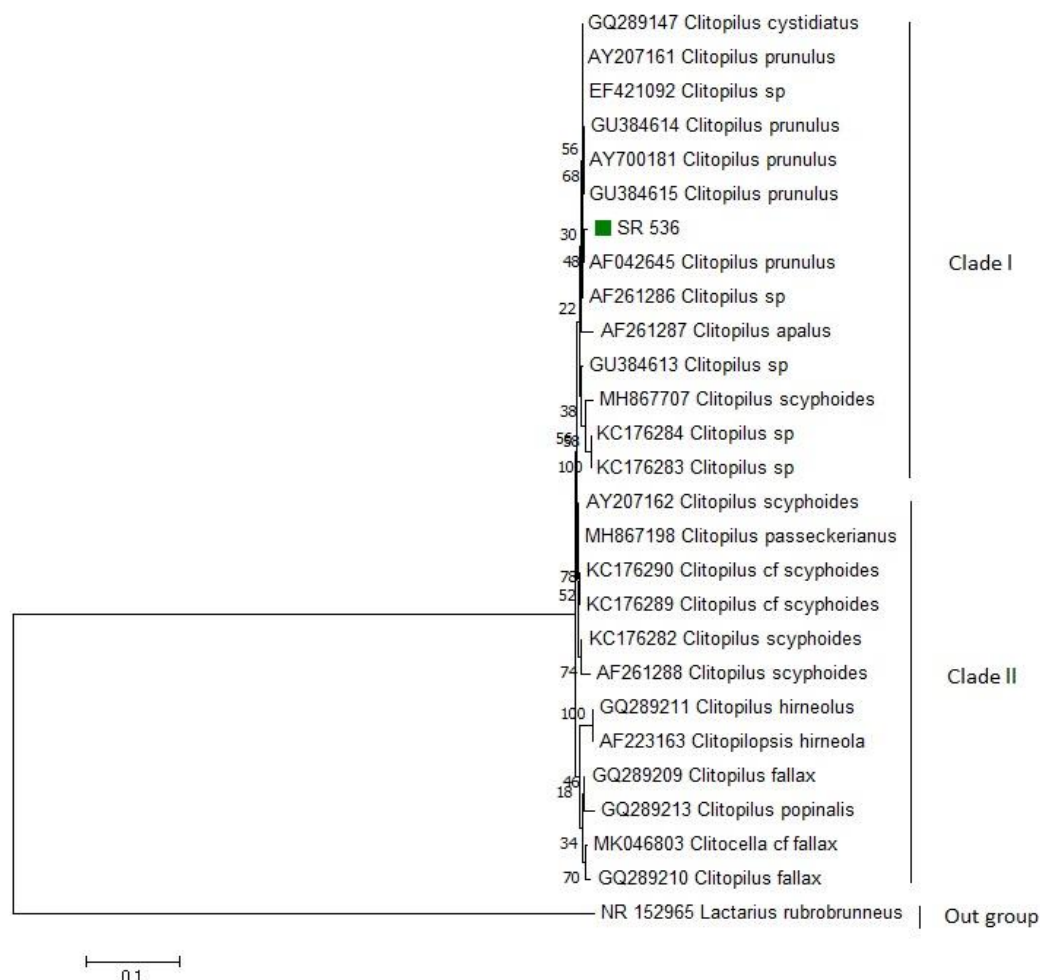


Figure 2: LSU sequence analysis for Molecular Phylogenetics by Maximum Likelihood method. Tamura 3-parameter model in MEGA7 was used. The sequences generated in present study were marked with ■.

Taxonomic

***Clitopilus shanglayensis*. Sp. nov.**

Etymology: — The term *Shanglayensis* refers to the region district Shangla.

Mycobank: — No.MK344431.

Holotype: — It is collected from the moist dark rich humours soil of Ajmer Forests, District Shangla, Khyber Pakhtunkhwa, Pakistan, 2500 masl, under the *Juglans regia* on September 06, 2017, Saif ur rehman SR-536. (Holotype, LAH35495. Genbank ITS no, MK348559 and LSU no MB829253).

Comments: — Morphological and molecular characterization exposed that *Clitopilus shanglayensis* previously undefined species.

Description__Pileus 05-06 cm wide, white light yellowish (7.5Y9/4) color, disk white yellowish red (10YR 7/6), umbonate with incised, smooth surface with rolled margins; **Lamellae** 04-05 cm wide, white pale yellowish (5Y 9/4) color, adnate, even short gills, forked near margin; **Stipe** 01-1.4 cm broad, 5-7 cm long, white light brown,(5Y 9/2) color, central, hollow bulbous base, glandular clotted surface with caespitose base; **Basidiospore** 7-8 x 4-5 µm, hyaline, ellipsoid to amygdaliform with acute apex in side view, ovoid in frontal view, thin walled with angular

surface, 2-spored sharp sterigmata; **Basidia** 19-22 x 5-6 μm , narrowly clavate to clavate shape, sharp and small sterigmata with thin wall, hyaline; **Pileipellis** 2-4 μm , irregular trichoderm, unbranched, hypha non septate with thin walled, **Stipitipellis** 30-38 x 3-6 μm , divergent, trichymeniderm, branched with septate hypha.

Material Examined: It is collected from the moist dark rich humours soil of Ajmer Forests, District Shangla, Khyber Pakhtunkhwa, Pakistan, 2500 masl, under the *Juglans regia* on September 06, 2017, Saif ur rehman SR-536. (Holotype, LAH35495. GenBank ITS no, MK348559 and LSU no, MB829253)



Figure 3: Basidiomata of *Clitopilus shanglayensis*. A. Morphology of *Clitopilus shanglayensis* sp. nov. A: View of Pileus, lamellae and stipe of Basidiomata.

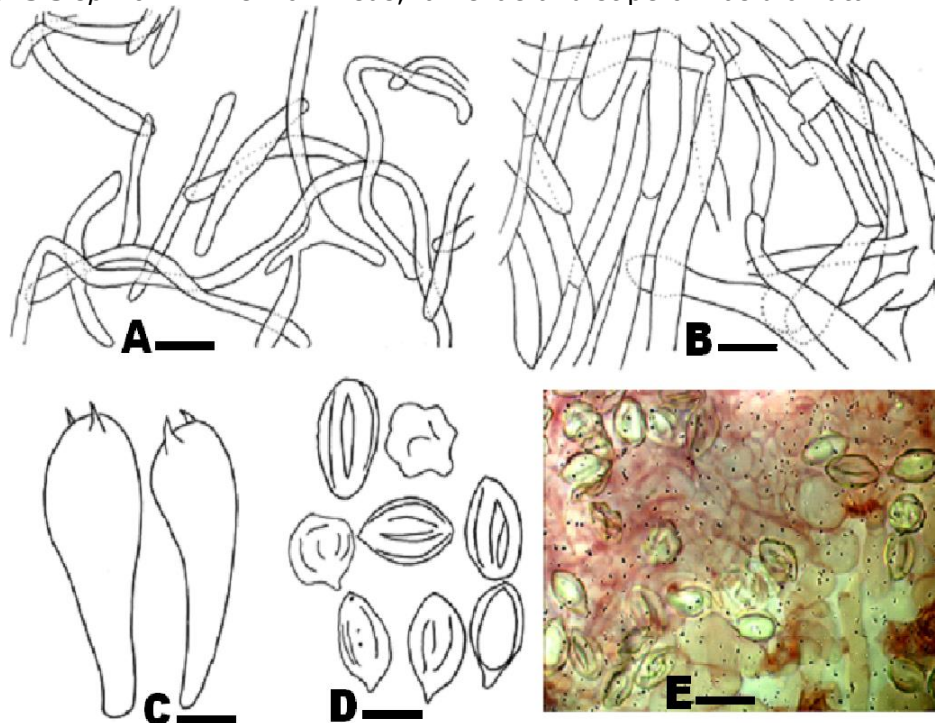


Figure 4: A-F. Microscopic characteristics of *Clitopilus shanglayensis*: A) Pileipellis, B) Stipitipellis, C) Basidia, D-E) Basidiospores.

Scale bars: A = 4.3 μm , B = 4.1 μm , C = 29.7 μm , D = 22 μm

DISCUSSION

We entitled a different type of *Clitopilus shanglayensis*, that was spread in high altitude area of Shangla district in KP, Pakistan, and was further confirmed by ITS and LSU sequence analysis. The phylogenetic analyses of the ITS and LSU sequences were shown individually, many of them were sequenced from different voucher specimens. *C. shanglayensis* fits to genus *Clitopilus*, total nine species in the clade II in which two species with different voucher number *C. fusiformis* one species *C. cf prunulus* two species *C. cystidiatus* and three species *C. prunulus* were included in the analysis. *C. shanglayensis* was sited in a distinct clade that is different from these species on the bases of ITS sequences. While morphologically they were comparing with *C. fusiformis* the pileus 2.5-4.5 cm, lamella 0.3 cm tall, Stipe 2.2-4.0 cm in length, 2.5- 3.5 mm in width, Basidiospores $10.5-14 \times 5.0-7.0 \mu\text{m}$ in size with thick walled and 4-spored sterigmata (Wang *et al.*, 2017) which show that *C. shanglayensis* is described a new species.

CONCLUSIONS

It is concluded from the current study that morphological characterization in combination with molecular phylogenetic analysis clearly provide evidence for the identification of novel species of fungus "*Clitopilus shanglayensis*" from the northern region (Shangla) Pakistan. This research support further deep molecular studies of the fungi in Pakistan to clearly understand the ecology and distribution pattern of genus *Clitopilus*. Furthermore, the genus *Clitopilus* will be further elucidated for medicinal purposes due to the presence of many important secondary metabolites.

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