MOLECULAR AND MICROGRAPHIC STUDY OF CHLOROPHYLLUM HORTENSE, USING ITS-RDNA MARKER AND SCANNING ELECTRON MICROSCOPY (SEM) TECHNIQUE

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Abstract

Chlorophyllum hortense (Agaricales, Agaricaceae) has been collected during a survey of macrofungi from Rana resort, a Safari park, Kasur district, Punjab, Pakistan. The fruiting bodies were characterized morphoanatomically as well as by molecular analysis. The shape, size and colour of pileus, gills and stipe were noted and compared with the available data in literature. Further, the fine shape and size of basidiospores were confirmed by scanning electron microscopy (SEM) technique. Molecularly, the identification of macrofungus was confirmed by Internal Transcribed Spacer of ribosomal DNA (ITS-rDNA) sequence using Polymerase Chain Reaction (PCR) and sequencing techniques. Sequence BLAST analysis and phylogenetic description showed that Pakistani species is an isotype of European and North American collections and *Chlorophyllum hortense* has been reported previously from hilly areas of Pakistan. This study will help to understand the genetic variations and morphological differences of two collections from two different extreme climatic zones. Further, recent collection has been analyzed not only molecular basis but also on refined anatomical footings.

Keywords: Agaricaceae, *Chlorophyllum*, BLAST, SEM

Introduction

Chlorophyllum Massee is a genus in the family Agaricaceae, typified by *Chlorophyllum molybdites* (G. Mey.) Massee. This genus is characterized by the presence of big fleshy mushrooms having cap with large, flat scales, smooth stipe comprising annulus, hyaline or green spores often truncated with un-covered germ pore or with no germ pore (Vellinga, 2003; Vizzini *et al.*, 2014; Ge *et al*., 2018). *Chlorophyllum* species are cosmopolitan. A total nineteen (19) species of *Chlorophyllum* have been reported worldwide while thirty three (33) records of this genus are available on Index Fungorum (http://www.indexfungorum.org/Names/NAMES.ASP) (Ge *et al*., 2018). From Pakistan, a good diversity data of macrofungi is being reported in recent years using molecular marker, ITS-rDNA but this genus is under represented (Razaq, 2013; Razaq *et al.*, 2012, 2013, 2014, 2016, 2018; Nawaz *et al.*, 2013).

It has always been of prime importance to discover the diversity of organisms in a given area to study their impact on environment and other organisms. Molecular markers give a modern approach

to identify a genus and its relation with other genera.

Materials and Methods

In present study, fruiting bodies of a mushroom were collected from Rana resort Safari park in Nankana Sahib, Kasur district of Punjab province, which is second largest province of Pakistan. Nankana Sahib is situated at 31.45 latitude and 74.45 longitudes and it is sited 194 meter above the sea level. Sampling site has a little annual rainfall so, climatic state of this site is considered as semi-arid, exposed with thick cultivated vegetation of different plants. The fruiting bodies were collected and photographed in their natural habitat. After proper labeling samples were packed and brought to laboratory for microscopic examination. Specimens were dried to 5% total moisture content. Free hand section of lamellae were prepared in 5% KOH and observed using compound microscope and Scanning Electron Microscopy (SEM) was conducted at Central Resource Laboratory (CLR), Department of Physics, University of Peshawar, Pakistan.

For molecular characterization, the genomic DNA was isolated using CTAB buffer method following Gardes and Bruns (1993) and the same protocol was followed for PCR amplification and profiling using universal primer set ITS1F and ITS4 (White *et al.* 1999). The PCR products of about 750 bp were directly sent to Macrogen, Korea for sequencing with forward and reverse primers. Consensus sequences of ITS regions of all collections were submitted to Basic Local Alignment Search Tool (BLAST) analysis using National Center for Biotechnology information (NCBI), USA for initial sequence comparison and closely related sequences were retrieved from GenBank for alignment and phylogeny analysis. Sequence alignments and phylogenetic analysis were performed using molecular evolutionary genetics analysis (MEGA X) software (Tamura *et al.,* 2011), Maximum likelihood (ML) method was based on the jukes- Cantor model of nrITS sequences using nearest- Neighbor-Interchange (NNI) as ML heuristic search method. Phylogeny was tested by bootstrap value of 1000 replicates. All the newly generated sequences were submitted to GenBank and their accession numbers are mentioned in phylogenetic tree.

Results

Chlorophyllum hortense (Murrill) Vellinga, Mycotaxon 83: 416, 2002**.** Figures 1, 2

Macroscopic description

The pileus is 3- 4 cm, at first sub-cylindrical ovoid, then conical to flattened finally, regularly shaped, with a definite obtuse umbo at maturity, soft-textured and a bit fragile in all developmental stages; margin initially involute with the partial veil but finally completely expanded at maturity,

regular to faintly waving, not exceeding beyond the lamellae, slightly striated at maturity; surface dry, ornamented by appressed and radially arranged scales, with whitish remnants of the universal veil upon the discal zone, rimose-fibrillose at margin; scales yellow-ochreous to pale brownish on a whitish ground, ochraceous to brown in the umbonate zone; unchangeable on handling; sub-cuticular layer whitish. Hymenophore lamellate, lamellae straight to slightly ventricose, free, thin, longer than pileus context thickness (up to 0.6 cm high), intermixed by one lamellula, not furcate nor interveined, concolorous edges entire; pale cream, not changing in color on bloom or pressure.

The stipe is 5.6 cm- 6.7 cm, commonly as long as the pileus diameter at maturity, central, hollow, firm, dry, straight to curved, cylindrical but always considerably enlarged downwards, frequently knobby and rounded at the very base, not rooting; young specimens exhibit a partial veil enclosing the fertile tissues which soon interrupts leaving the upper half of the stipe ornamented by a showy and long lasting, ascending, simple, membranaceous, cream-whitish to pale ochraceous ring; surface very finely fibrillose length-wise, whitish to brownish depending on the developmental stage but always with silvery streaks, brownish at the base, constant on handling but strongly flushing on rubbing or when injured; basal mycelium whitish. **Context** soft and brittle in the pileus (up to 0.4 cm thick in the central zone), tougher and more fibrous in the stipe, whitish in the pileus, whitish-gray in the stipe, turning vinaceous red on disclosure, starting from the apical umbo and all along the stipe, more penetratingly downwards and towards the peripheral cortical layers, eventually fading brownish, nearly unchangeable to slightly staining pale pinkish-vinaceous in the pileus. **Smell** negligible.

The basidiospores is 4.68-8.11 µm - x 4.05-5.38 µm , Q = 1.15-1.50, , roughly ellipsoid to ovoid both in face and side view, rarely larmiform, smooth, thick-walled, with a well pronounced apiculus and no suprahilar depression, having a single large oil drop, without germ pore, hyaline in water, dextrinoid, cyanophilic and with metachromatic reaction. **Basidia** rising from an irregularly-shaped sub-hymenium, 30-42 × 9-11 µm, clavate, temperately thick-walled, primarily 2-spored but also 1-spored, bearing relatively long sterigmata (up to 8 µm), typically containing a granular pigment. **Cheilocystidia** 22-67 × 6-8 µm, frequent, scarcely clavate to subcylindrical or more rarely sub-fusiform, smooth, moderately thick-walled, sometimes containing a pale yellowish granular pigment, without epiparietal coverings.

Pleurocystidia not observed. **Pileipellis** at disc consisting of two layers; an inner layer made up of parallel to moderately interwoven, septate, colourless, thick-walled, cylindrical hyphae, 36.5- 52.2 × 3.5-6 µm and an upper layer consisting of clavate terminal elements arranged as a tricho-hymeniderm which are very adjustable in size, 28-180 × 5.2-20 µm with an apex rounded-obtuse; sometimes over the tricho-hymeniderm are present cylindrical, tightly interlaced, hyaline, hyphae, offcuts of the universal veil. **Pileipellis** towards the margin of the pileus with the very same arrangement of the inner layer found at disc. **Stipitipellis** a texture of parallel and longitudinally running hyphae, 5.2-7.8 µm across; caulocystidia not observed. **Annulus trama** comprising of interwoven, diverticulate, septate, more or less cylindrical hyphae with a brownish plasmatic pigment; terminal elements arranged as a palisade of parallel cells, cylindrical to clavate, more rarely slightly capitate, 29.6-78.3 × 4.3-10.4 µm, temperately thick-walled. **Hymenophoral trama** made up of interwoven, thick-walled, smooth, subcylindrical and sometimes diverticulate hyphae; terminal elements 70-180 × 4.3-11.3 µm broad, with rounded apex. **Oleipherous hyphae** observed in the hymenium and pileipellis. **Clamp connections** with an incomplete morphology (rudimentary clamps with incomplete junction) only perceived in the stipe tissues (Figure 1 and 2).

Material examined: Pakistan: Punjab, Kasur, Pattoki. Department of Botany, University of Veterinary and Animal Sciences, Lahore. Ayesha Nazir, 04-07-2018, collected by Razaq A, LAH0407181, LAH0407182.

Molecular characterization:

Blast match of *ITS-rDNA* sequences both from fruiting bodies (AN4 and S2) showed maximum similarity with *Chlorophyllum sp.* (KR155063, KR154963) with 98% match collected from India, *C .hortense* (MF773636 and AY243612) with 98.93% and 99% similarity respectively with collections of USA. In phylogenetic tree, studied taxon was found clustering with *C. hortense* (MG741968, MG741967 and MG741971). *Coniolepiota spongodes* (HM488756) was selected as an out group (Figure.3)

Discussion

In current research a mushroom species belonging to genus *Cholorophyllum* was collected from Rana resort, Kasur district of Punjab province in Pakistan. Mushroom was identified using morpho-anatomical as well as molecular methods. Spores were found ovoid, with rounded apex in shape print white to cream in color and spore size is

(4.68-8.11 µm - x 4.05-5.38 µm, Q = 1.15-1.50).

Chlorophyllum africanum, a species from Yunnan (China) is characterized by yellow grey to grey orange basidiocarps. Cheilocystidia 28- 50 x 6.0-10.0 µm, cylindrical to slightly fusiform, spore colour is print white to cream yellow, spores are 8.2 ± 0.4 × 6.2 ± 0.3 µm, Q (1.2)1.3–1.4 (1.5), Qav = 1.3 ± 0.05 in size *C. hortense* a species from Knoxvile, USA is characterized by pileus white with yellow scales yellows-brown umbo hymenidermal pileipellis. Spores often truncate and with an un- covered germ pore (Vellinga *et al*., 2003). Broadly ellipsoid to ovoid in shape. Spore (7)9.2±1.3 (13.1) × (5.2)6.4±0.8 (8.7) µm, Q = 1.2-1.7, Qm = 1.4 in size. Phylogenetic analysis is also done by forming distinct clades. Clade III has group of *Chlorophyllum* species, *C. hortense*, *C. africanum* and *Chlorophyllum demangei*. *Chlorophyllum sp.* AN4 is closely related to Knoxville (USA) species *C. hortense*, because it shares 99.32% of its characteristics to Pakistani species. Thus, molecular analysis showed a close relationship between Knoxville (USA) and Pakistani collections of this species.

It is a widely diffused species, being known from Rana resort, Kasur district of Punjab province in Pakistan as *Leucoagaricus hortensis* or *Lepiota humei* (Smith 1966; Akers & Sundberg, 1997), Central and South America (Dennis 1952; Pegler 1983; Franco 1994; Sobestiansky 2005; Gimenes 2007), Africa as *Leucoagaricus bisporus* (Heinemann 1973), Japan as *Lepiota alborubescens* or *Macrolepiota alborubescens*, China (Ge & Yang 2006), India (Vrinda *et. al.,* 1999; Farook *et al.,* 2013), Thailand (Vellinga *et al.,* 2011) and Australia (Aberdeen 1962); as *Lepiota naucina* (Fr.) P. Kumm. Aberdeen (1992); as *Leucoagaricus fimetarius* (Vellinga 2003). It befalls on dung of cattle and horse pastures, sandy soils, meadows and garden loamy soil.

C. hortense has also being described from Brazil (Vizzini *et. al* 2014). The sequences in Blast analysis of the species sequence belong to the same genus or different genus of the same family. The percentage base similarity is noted maximum with submitted from Pakistan. Other closely related sequences in the Blast below the closest sequence showed 99.32%, 99.15%, 98.74%, 96.32% and 93.66% with *C. hortense* (MF773636, USA), *Chlorophyllum alborubescens* (AY243610, USA), *Chlorophyllum sp. voucher*. (KY636372, Pakistan) and *Chlorophyllum demangei* (MG741966, China) respectively.

Conclusion

The Pakistani collection of *Chlorophyllum hortense*, is compared with the isotypic descriptions of European and

North American collections and it was found that all the collections belong to cold climatic conditions and our collection is first time collected from extremely hot climate zone with minor variations. On the morpho-anatomical descriptions and phylogenetic similarities, its cosmopolitan distribution is evident.

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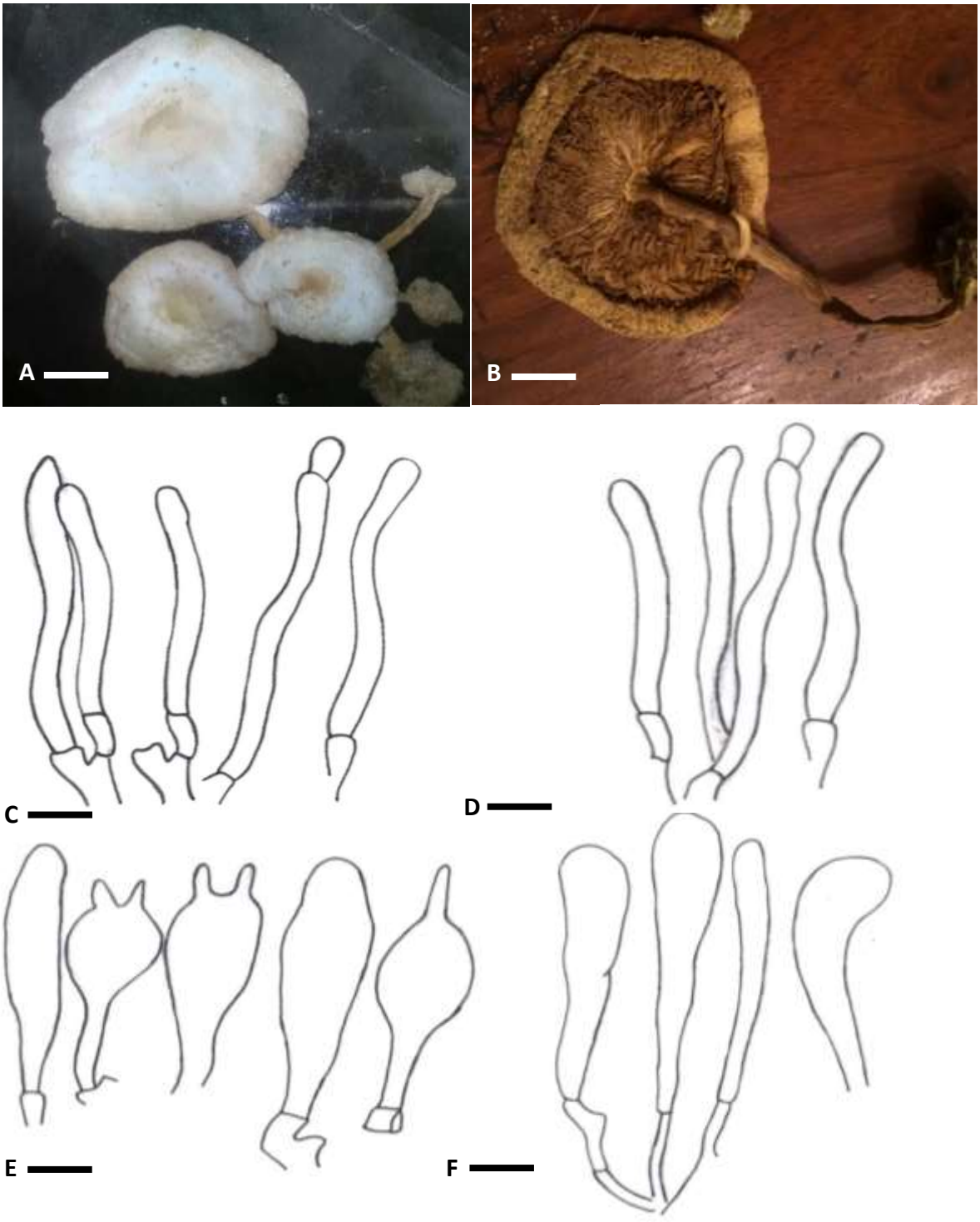

Figure 1: *Chlorophyllum hortense* (MG515a- GDGM57301) A. Basidiocarp with pileus and stipe B. Lamellar side of basidiocarp. **C-F**. Microscopic features: **C, D**. Annulus trama **E**. Basidia and cheilocystidia, **F**. Pileipellis

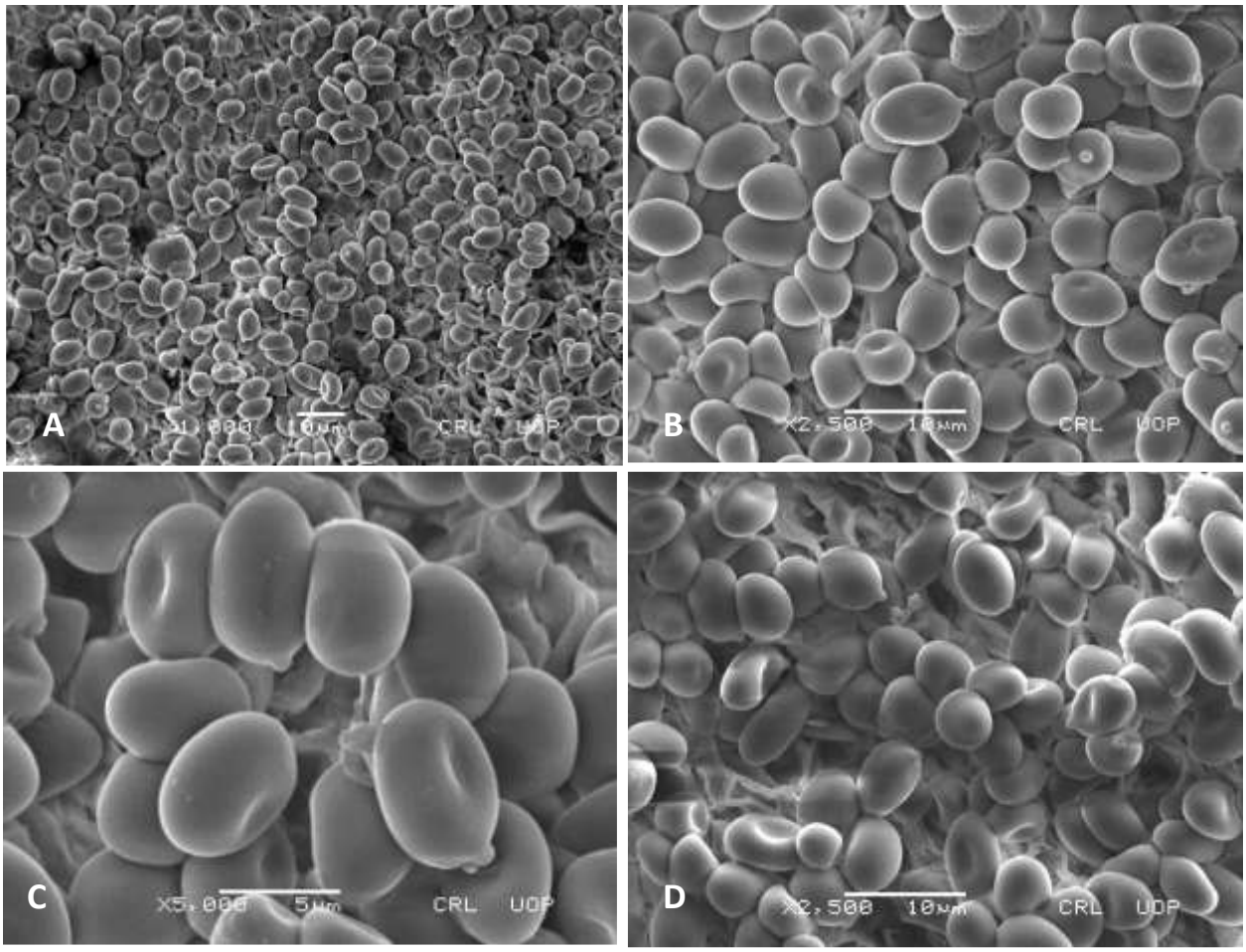

Figure 2: SEM photographs of basidiospores of *Chlorophyllum hortense*

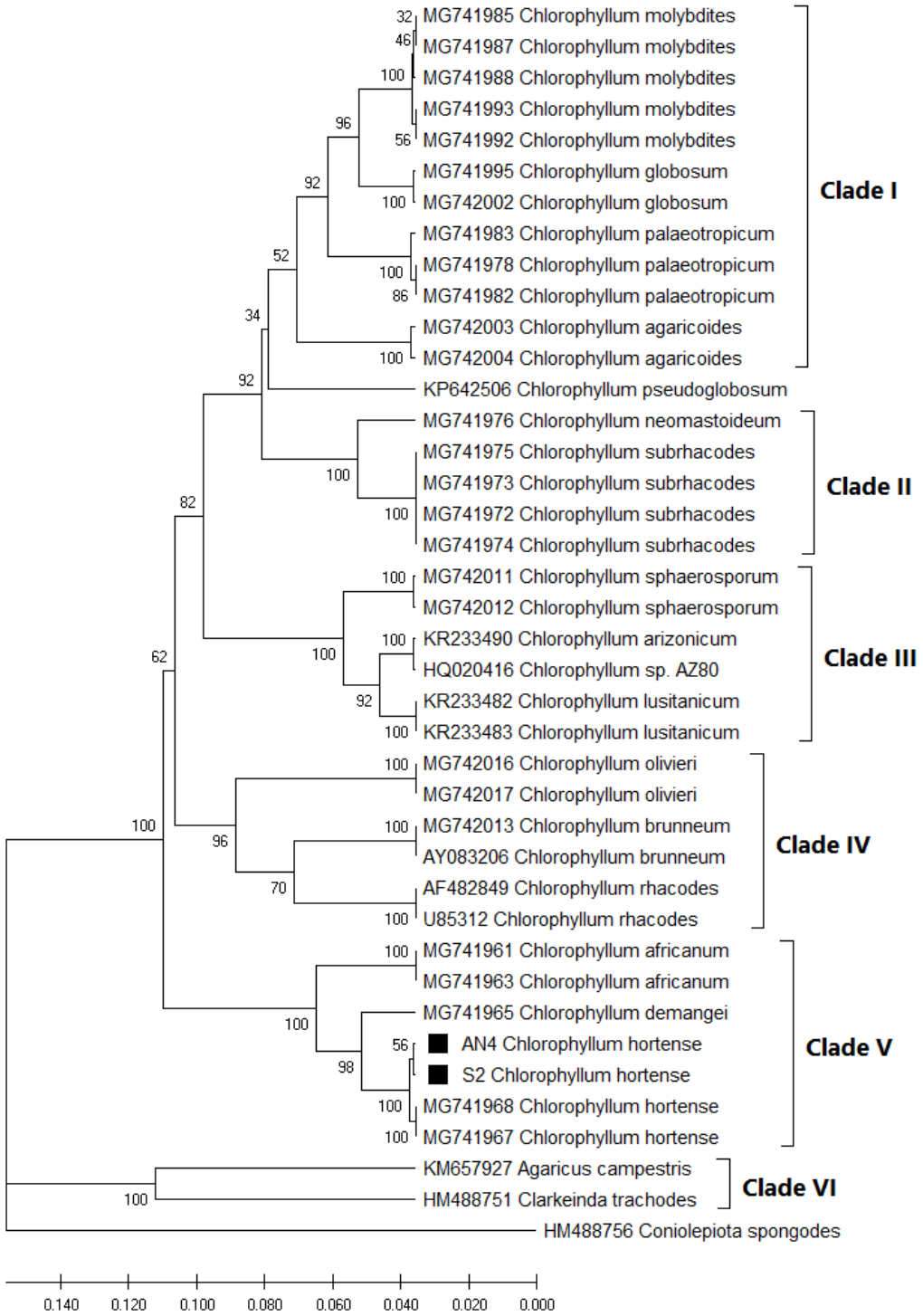

Figure 3: Phylogenetic anaylysis of *Chlorophyllum hortense* based on ITS-rDNA using maximum likelihood method following Juke-Contor model. A total 39 sequences of ITS region of *Chlorophyllum*,the new species *Chlorophyllum sp.* AN4. are included which form different cluster under significance bootstrap value. Pakistani collection is labeled with rectangle in the phyloclade.