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Morphological, ultrastructural and phylogenetic study of *Calvatia candida* and *Calvatia craniiformis* reported from Northern western Ghat of India

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Abstract

Two species of *Calvatia* viz., *Calvatia candida* and *Calvatia craniiformis* were collected from Savitribai Phule Pune University campus, Pune, Maharashtra, India. They were identified based on morphological features of Basidiomata and basidiospore. The Scanning Electron Microscopy of spore shape and ornamentation of two species of *Calvatia* were observed. The basidiospores of *Calvatia candida* are pedicellate subglobose and spiny whereas pedicellate, globose and spiny ornamentation observed in *Calvatia craniiformis*. Both species were identified by using Internal Transcribed Spacer and the Large Subunit regions of the nuclear rDNA gene sequence analysis.

Keywords – ITS – LSU – Lycoperdaceae – SEM

Introduction

Calvatia is a cosmopolitan genus mostly medium to large-sized epigeous puffball. Preliminary surveys have revealed rich representation of these genera in the Himalayan forests (Ahmad 1941, Gupta et al. 1974, Khare 1976, Thind & Thind 1982). The species of *Calvatia* have pharmacological importance. Calvacin a new anti-tumour agent was reported (Roland et al. 1960). All *Calvatia* species are edible, but before spore maturation (Morris 1987, Gray 1973, Rinaldi & Tyndalo 1974, Purkayastha & Chandra 1985). After maturity the gasterocarp dehisces by irregular rupturing the peridium and regularly septate capillitium and spores, which comprises of 36 species worldwide (Kreisel 1994). Bisht et al. (2006) reported *Lycoperdon ovalicaudatum* and *Calvatia longicauda* reported for the first time from India. Based on genetic and molecular studies, Kirk et al. (2008) treated them as a polyphyletic assemblage representing different orders within Agaricomycetes of subdivision Agaricomycotina and mentioned the existence of 55 (*Bovista*) and 40 (*Calvatia*) species worldwide while as Index Fungorum showed list of 195 and 138 records of these genera respectively. Syed et al. (2008) reported first time *Bovista aestivalis* and *Calvatia craniiformis* from Karnataka, India. Verma et al. (2018) reported 16 species from central India especially from Himachal Pradesh, Assam, Madhya Pradesh and Uttarakhand. Traditionally some species of *Calvatia* namely *C. cyathiformis, C. craniiformis, C. excipuliformis, C. gigantean and C.*

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utriformis have been used as a source of food and medicine (Coetzee & van Wyk 2009). *Calvatia rubroflava* and *Calvatia cyathiformis* reported from Hollongapar Gibbon Wildlife Sanctuary (HGWLS), Jorhat, Assam, India by Gogoi & Vipin (2015).

Pune District is situated in the North-western Ghat of Maharashtra, India, having the forest fall under tropical to semi evergreen forest. In the present study, two species of *Calvatia* are described and illustrated for the first time in north-western Ghat of Maharashtra, India. SEM analysis and ITS-LSU based molecular analysis were performed for the accurate identification of *Calvatia* species.

Materials & methods

Collection site

The gasterocarps were collected from Savitribai Phule Pune University Campus, Maharashtra, India. Five fresh specimens of each species were uprooted from soil. *Calvatia candida* was collected near Alice garden and *Calvatia craniiformis* were collected from Department of Botany, Garden. Images of fresh Basidiomata were captured with help of Canon EOS 700 D camera. The fungal specimens were dried in oven at 55ºC for 3 hours.

The morphological and micro-morphological characters

The microscopic characters were recorded from fresh and dissected gasterocarp with help of compound microscope (Leica Company). The basidiospore and capillitium were observed by mounting them in lactophenol. Alphanumeric nomenclature of colors is based on Kornerup & Wanscher (1978). For Scanning Electron Microscopy (SEM) of spore and ornamentation, the dry sample of spores were mounted on aluminum stub with the help of carbon tape and coated with gold (Quorum Technologies; model-Q150R), coated samples were loaded and observed under FESEM (Field Emission Scanning Electron Microscope) at high vaccume. FEMES (FEI Nova NanoSEM 450) were operated at 5-10 KV with 3-3.5 spot size, ETD and TLD detector was used to detect the signals and images were captured by using CCD camera. The average of thirty basidiospore were taken from each species. Dried gasterocarp of *Calvatia candida* and *Calvatia craniiformis* and cultures of *Calvatia candida* were deposited in the National Fungal Culture Collection, India, Agharkar Research Institute, Pune, Maharashtra, India (NFCCI-ARI).

DNA extraction, Polymerase Chain Reaction (PCR) and sequencing

DNA extraction from powder of basidiomata was done as described by Aamir et al. (2015). The two regions from rDNA gene were amplified by using ITS and LSU regions (White et al. 1990, Vilgalys et al. 1990). The PCRs were performed in a 25µl reaction volume containing 20–50ng of template DNA, 16µl Milli-Q water (Sigma), 2.5 µl PCR buffer (10×), 2.5 µl of 10mM dNTPs mix (Sigma-Aldrich), 1µl of each primer (20 pmol/µl), 1 µl (5 U/µl) of Taq polymerase (Sigma-Aldrich). PCR was performed in a Himedia Master Cycler (Himedia Prima-16).

The amplification program for ITS1/ITS4 consisted of an initial denaturation step at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 1 min, annealing for 30 seconds at 58°C and extension for 1 min at 72°C. A final extension step at 72 °C for 7 min was included at the end of the amplification. The amplification program for LR0R/ LR7 consisted of same programmed as in ITS amplification programme except annealing i.e. 52°C for 30 seconds. The PCR products were sequenced by Sanger sequencing from 1st Base laboratory, Malaysia. The sequences were analyzed by BLAST in the NCBI nucleotide database. The sequences were submitted to NCBI for getting accession numbers.

Deposition of Gasterocarp, Culture and sequences:

The Gasterocarp deposition Number from NFCCI-ARI namely *Calvatia candida*: AMH10029 *Calvatia craniiformis*: AMH10025

Culture deposition number given by NFCCI-ARI: *Calvatia candia*: NFCCI4462 (Fig. 1E-F)

NCBI deposition numbers for ITS sequences: *Calvatia candida*: MH916575; *Calvatia craniiformis*: MH916598

LSU sequences: *Calvatia candida*: MH916599; *Calvatia craniiformis*: MH916600

Phylogenetic analysis

Phylogenetic analyses based on ITS data were carried out to establish the phylogenetic placement of our collected isolates. Forty-five sequences were selected from Genebank including our two isolates and one *Chlorophyllum pseudoglobosum* were used as an outgroup for phylogenetic analysis (Table 1). Alignment of all the sequences were performed by using CLUSTALW and the phylogenetic tree was constructed with Neighbor Joining and Maximum Likelihood algorithms with the bootstrap value of 1000 replicates for each tree. The Maximum Likelihood tree was inferred by Kimura 2-parameter model with Subtree-Nearest-Neighbor-Interchange (NNI) Method from MEGA X software (Saitou & Nei 1987, Felsenstein 1985, Tamura & Nei 1993, Kumar et al. 2016). Initial tree was Maximum Parsimony with very weak branch swap filters and four search threads. The gaps were treated as missing data that were eliminated.

Table 1 List of sequences of *Calvatia* used for Phylogenetic analysis of *Calvatia* sp.

Sr no.	Species	Voucher/strain, isolate	Geographic region	Genebank accessions
1	*Calvatia candida*	PB101	India	GU939632
2	*Calvatia candida*	MJ3514	Sweden	DQ112624
3	*Calvatia pachydermica*	276161	USA	MF073285
4	*Calvatia pachydermica*	AN014692 (ARIZ)	USA	EU833653
5	*Calvatia holothurioides*	CMU-CLA-STPNK2013	Thailand	KX064242
6	*C. holothurioides*	*Voucher LE 287408*	*Vietnam*	JQ734547
7	*C. holothurioides*	*Voucher KA11-0287*	*South Korea*	KJ909662
8	*Calvatia* aff. *Rugosa*	MEL:2382849	Australia	KP012724
9	*Calvatia craniiformis*	TNS Kasuya B734	Japan	KF551248
10	*Calvatia candida*	ASIS22727	Republic of Korea	KF668328
11	*Calvatia* cf. *leiospora*	AN014671 (ARIZ)	USA	EU833652
12	*Calvatia bicolor*	LMG756-58 (ARIZ)	USA	EU833651
13	*Calvatia cyathiformis* var. *cyathiformis*		USA	AJ486867
14	*Calvatia cyathiformis*	TENN:070786	USA	MF686508
15	*Calvatia cyathiformis* var. *cyathiformis*	AH 25225	USA	AJ486866
16	*Calvatia cyathiformis*	Isolate MP12	Ontario, Canada	KY706183
17	*Calvatia cyathiformis var. crucibulum*	Voucher HAJB 2811	La Habana, Cuba	AJ486869
18	*Calvatia chilensis*	AH 19509	Chile	AJ486965
19	*Calvatia fenzlii*	*Strain Jz01 Unknown,*	*could be from China*	FJ772413
20	*Calvatia fragilis*	AAH 25226	Ghana	AJ486964
21	*Calvatia fragilis*	GFW (Kreisel) leg. Lopez Nov. 1990	France	AJ617493
22	*Calvatia fragilis*	AH 25227 (duplo Herb. Kreisel)	Pakistan	AJ486958
23	*Calvatia fragilis*	*Voucher AH 24114*	Corrientes, Argentina	AJ486959
24	*Calvatia fragilis*	Voucher K 56043	Swan River, Australia	AJ486960
25	*Calvatia fragilis*	*Voucher AH 18553*	*Baja California, Mexico*	AJ486870

Table 1 Continued.

Sr no.	Species	Voucher/strain, isolate	Geographic region	Genebank accessions
26	*Calvatia fragilis*	*Voucher Sydow 941 (M)*	*South Dakota, USA*	AJ486871
27	*Calvatia fragilis*	*Voucher Cragin 523 (NY)*	*Kansas, USA*	AJ486957
28	*Calvatia fragilis*	*Voucher AH 21915*	*Madrid, Spain*	AJ486961
29	*Calvatia fragilis*	*Voucher PAD 3309*	*Italy*	AJ486962
30	*Calvatia fragilis*	*Voucher AH 25228*	*Charaa-Tal, Mangolia*	AJ486963
31	*Calvatia aff. Rugosa*	MEL:2382849	Australia	KP012724
32	*Calvatia holothurioides*	LE 287408	Viet Nam	JQ734547
33	*Calvatia holothurioides*	KA11-0287	South Korea	KJ909662
34	*Calvatia rubroflava*	TENN59078	USA	AF485064
35	*Calvatia rubroflava*	*Strain TFB11269*	*Urugua Provincial Park, Argentina*	KY559335
36	*Calvatia sp.*	*Voucher KA08-0253*	*South Korea*	KJ909664
37	*Calvatia sp.*	*Voucher KA12-1061*	*South Korea*	*KF995352*
38	*Calvatia cretacea*	MJ4302	Sweden	DQ112598
39	*Calvatia turneri*	ML295	Sweden	DQ112596
40	*Calvatia gigantea*	CG13	Czech Republic	EF190318
41	*Calvatia gigantea*	*Strain 9-7A*	*Sichuan, China*	*HM237179*
42	*Chlorophyllum pseudoglobosum*	CUH:AM155	India	NR137967
43	*Calvatia fenzlii*	Jz01	China	FJ772413
44	*Calvatia* cf. *leiospora*	AN014671 (ARIZ)	USA	EU833652
45	*Calvatia candida*	AMH10029	India	MH916575
46	*Calvatia craniformis*	AMH10025	India	MH916598

Culture of *Calvatia* species:

Culture of *Calvatia candida*: The basidiospores of gasterocarp were inoculated on PDA medium, growing for 7-10 days at 27^{o}C. The culture was identified by ITS1/ITS4 gene sequencing analysis.

Results

Taxonomy

Calvatia candida (Rostk.) Hollós Fig. 1 A-D

Material examined – INDIA, collected from Alice garden Savitribai Phule Pune University Campus, Maharashtra, India. AMH10029, (living culture, NFCCI4462).

Gasterocarp – Growing on soil and bed of litter, Pseudostipe rounded at base, ostiole lacking, Exoperidium Chinese yellow (4B7). Gasterocarp up to 110 mm high, 80 mm diam., subglobose, with presence of pseudostipe which is 30-40mm in diameter and 60mm height. During maturation, exoperidium becomes dull yellow (3B3), smooth, thin (<2 mm thick) and membranous, forming sub-pyramidal patches which gets dehisces. Endoperidium adhered to the exoperidium. Gleba more or less persistent and blonde color (4C4) at maturity. Subgleba larger than gleba and occupying more than 2/3 of the size of the gasterocarp, cellular.

Basidiospores 2.5 x 3.5 µm diam., subglobose, echinate, with short spines (average 0.554µm) and pedicellate (Average 0.605µm). The SEM image showed the ornamentation is formed on the spore surface by short pyramidal spines which are connected by thin filaments. The photograph showed the culture of *C. Candida.*

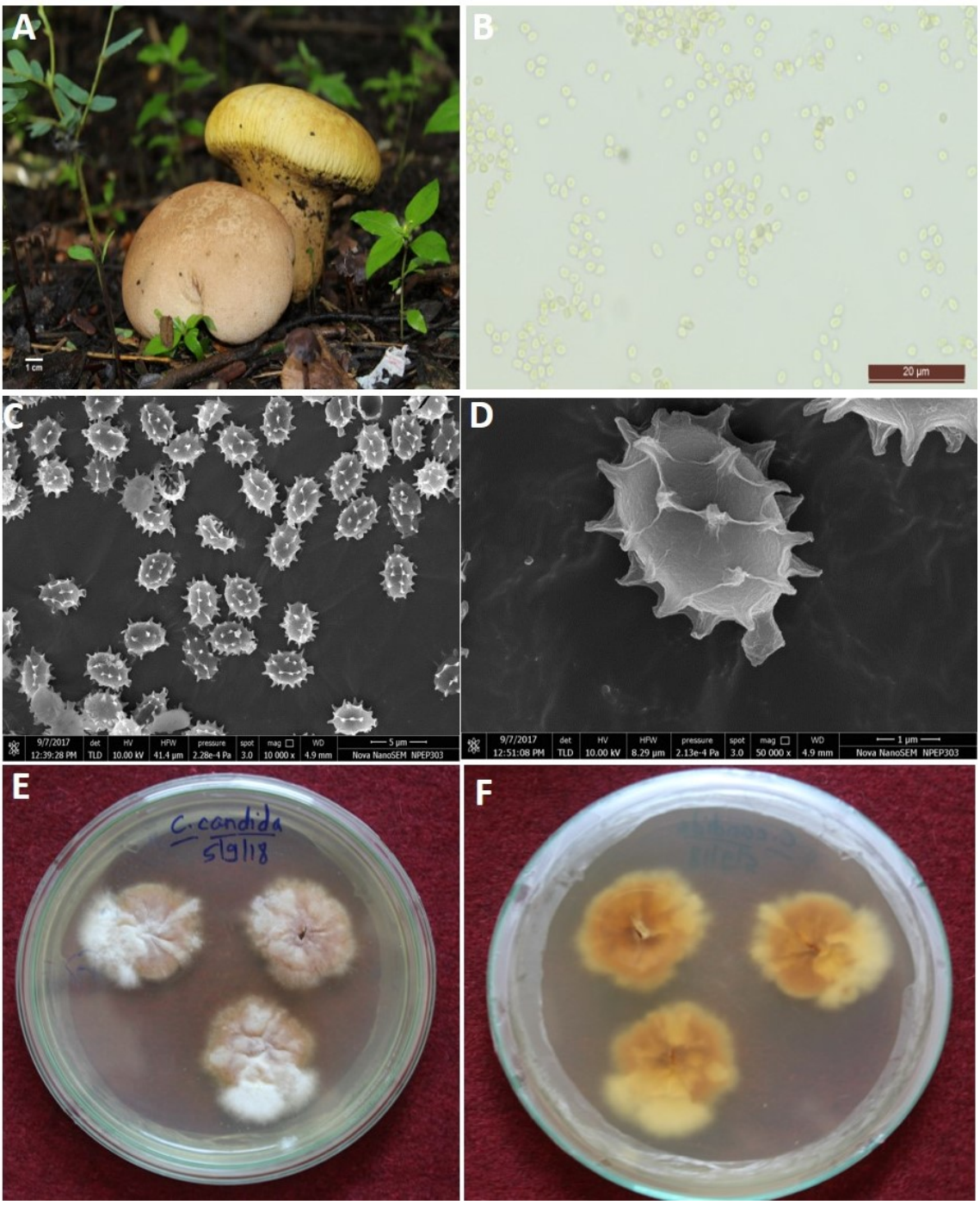

Fig. 1 – A Basidiomata of *Calvatia candida*. B *Calvatia candida* spores. C SEM of *Calvatia candida* spores. D SEM of single *Calvatia candida* spore. E Culture of *C. candida* front view. F Culture of *C. candida* ventral view. Scale bars: A = 1 cm, B = 20 µm, C = 5 µm, D = 1 µm.

Calvatia craniiformis (Schwein.) Fr. ex De Toni Fig. 2 A-D

Material examined – INDIA, collected from Botanical Garden, Department of Botany, Savitribai Phule Pune University Campus, Maharashtra, India. AMH10025

Gasterocarp – Growing on soil and bed of litter, scattered or gregarious, pyriform to turbinate, 20-30mm broad, 30–35 mm high. Pseudostipe present. Exoperidium dark dull yellow (3B3), spinose, spines up to 0.5 mm, dense, prominent on the upper part, scattered, blunt on pseudostipe. Endoperidium rough, opens by apical aperture. Gleba greyish brown, pulverulent at maturity, cottony, Pseudocolumella indistinct**.** Subgleba chambered, one- quarter of basidiomata in length, tapering towards base.

Basidiospores Globose, Average of Thirty basidiospore is 2.9 µm, spiny, spines are 0.95µm guttulate, pedicellate, pedicels hyaline, long, curved, up to 0.809 µm in length.

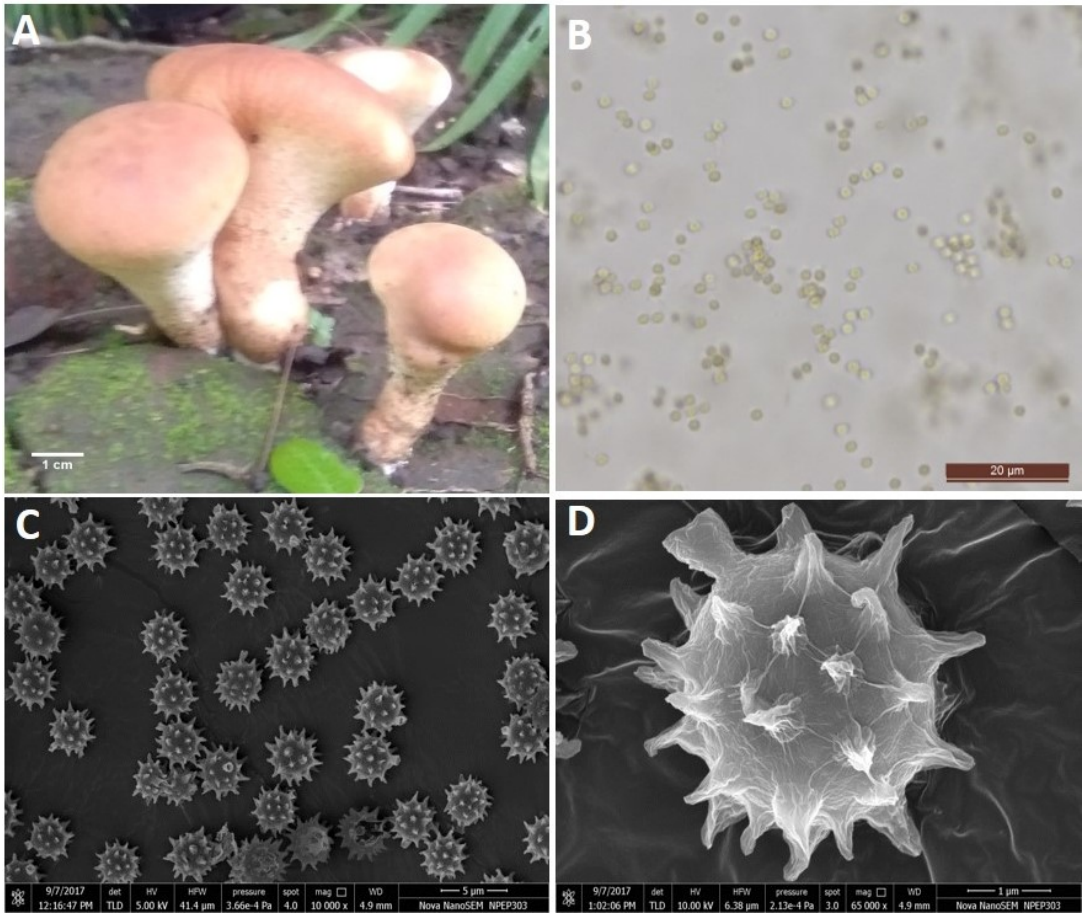

Fig. 2 – A Basidiomata of *Calvatia craniformis*. B *Calvatia craniformis* spores. C SEM of *Calvatia craniformis* spores. D SEM of single *Calvatia craniformis* spore. Scale bars: A = 1 cm, B = 20 µm, C = 5 µm, D = 1 µm.

ITS sequences and Phylogenetic study

The multiple ITS sequences of 45 different species of *Calvatia* togethering our Indian isolate of *Calvatia candida* and *Calvatia craniiformis* were analyzed. *Chlorophyllum pseudoglobosum* were chosen as out-group taxa (Table 1). The phylogenetic tree based on ITS sequences was constructed by using Neighbor Joining and Maximum Likelihood method by MEGA X (Figs 3, 4). In Neighbor Joining tree the sequences of the *Calvatia* species fall out into two distinct clades. Clade I comprising one species of *C. rubroflava*. and rest of other forty-three specimens including our isolates were formed in second clade. The results showed by ITS gene sequences are shown here. Our Indian isolates *C. candida* (MH916575) was found be closed clustered amongst the sequences of *Calvatia candida* (GU939632) from India with 99% query cover. Our isolate *Calvatia craniiformis* formed clade with sequence of *C. craniiformis* (KF551248) from Japan with 99% query cover and with *Calvatia* sp. from South Korea with 100% query cover. The phylogenetic tree by Maximum Likelihood method, the *Calvatia* species fall in out in to two distinct clade, Clade I comprised two species viz., *C. cretacea* and *C. turneri*. Clade II comprises forty-three specimens with fifteen different species of *Calvatia*. Our Indian isolates *C. candida* (MH916575) was found be closed clustered amongst the sequences of *Calvatia candida* (GU939632) from India with 97% query cover. Our isolate *Calvatia craniiformis* formed clade with sequence of *C. craniiformis* (KF551248) from Japan with query cover 97%.

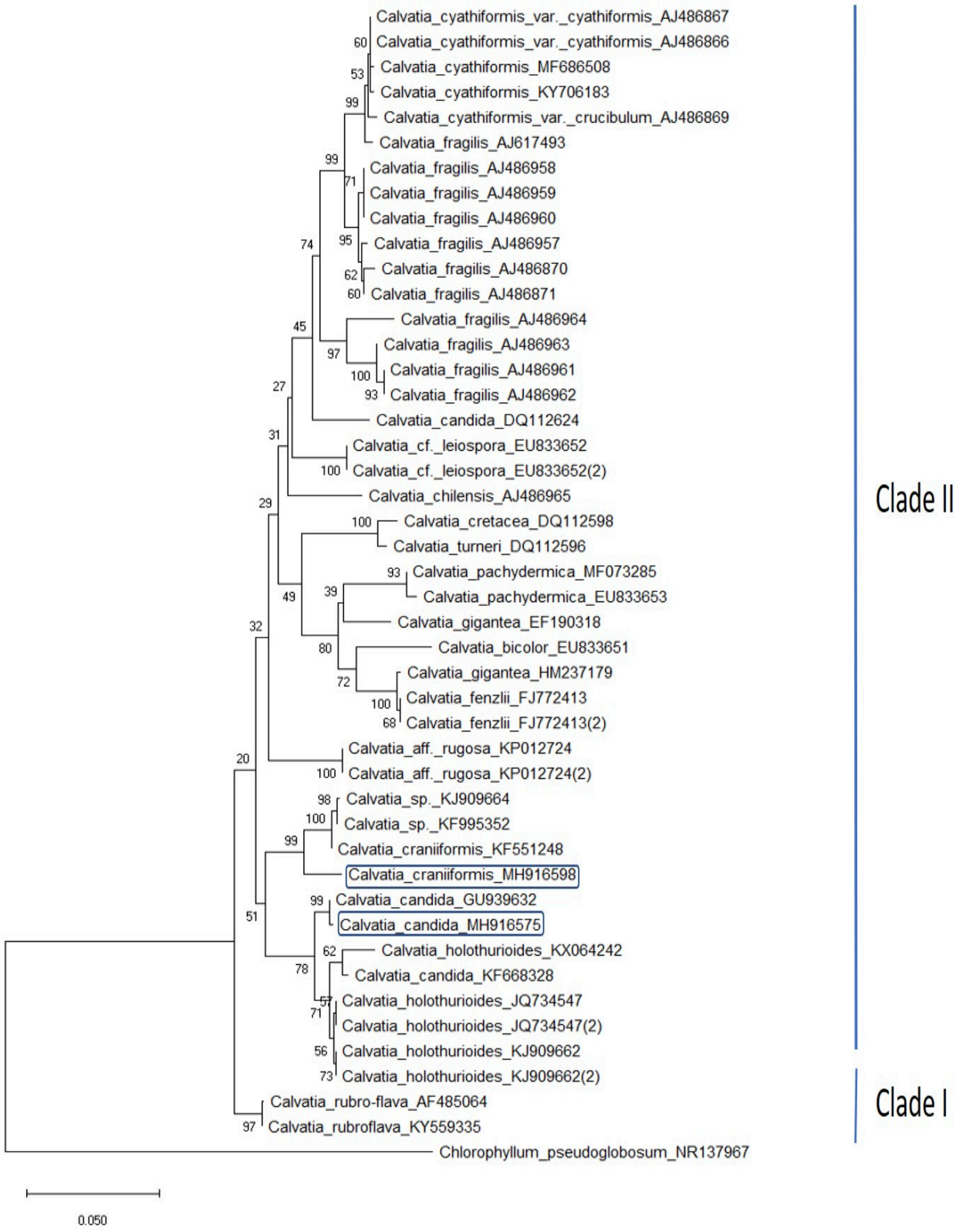

Fig. 3 – The Phylogenetic tree was generated using the Neighbor-Joining method based on ITS sequences (Saitou & Nei 1987). The optimal tree with the sum of branch length = 0.74094278 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown above the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method [3] and are in the units of the number of base substitutions per site. The differences in the composition bias among sequences were considered in evolutionary comparisons. This analysis involved 46 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option).

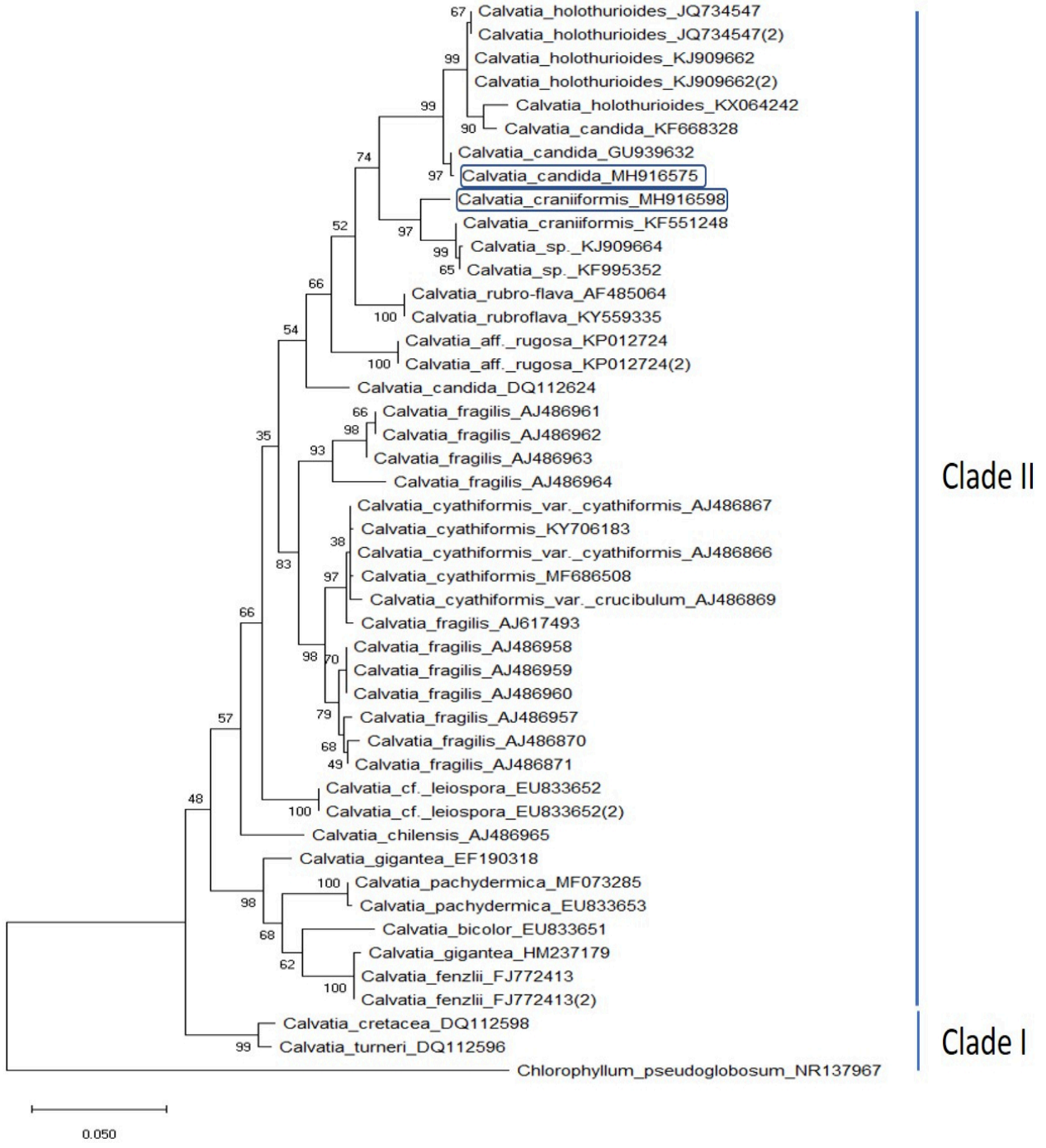

Fig. 4 – The Phylogenetic tree was generated using the Maximum Likelihood method and Kimura 2-parameter model based on ITS sequences. The tree with the highest log likelihood (-3639.92) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. This analysis involved 46 nucleotide sequences. There was a total of 733 positions in the final dataset. Evolutionary analyses were conducted in MEGA X

Discussion

The *C. craniiformis* formed clade with *C. craniiformis* isolate from Japan and not formed a clade with *C. cyathiformis.* Thus the morphological and molecular characters in clear identification of *C. craniiformis*. The *C. craniiformis* resembles morphologically with *C. Cyathiformis* but not to lilacineous gleba, smaller pedicillate spores about 2.9 µm in diameter, small and regularly distributed verrucae-spines as observed with SEM, similar observation reported by Moreno et al. (2007). Similarly, from phylogenetic study, *C. craniiformis* formed a clade with *C. craniiformis*

isolate from Japan not formed a clade with *C. cyathiformis.* Earlier only two species of *Calvatia* viz., *C. cyathiformis* and *C. gardneri* were reported from S. P. Pune University campus by Nair et al. 1978. The *C. craniiformis* distributed in North America, China and Japan, United States, North Carolina, Michigan, Oklahoma and Texas (Kreisel 1994, Zeller & Smith 1964). The *C. candida* showed close proximity with C. *holothurioides*. Calonge et al. (2003) assigned C. *holothurioides* to "Calvatia sect. Sporocristata" based on its echinulate and ellipsoidal spores. This character clearly observed under SEM analysis.

The combination of morphological and molecular feature supports the *C. candida* and *C. craniiformis* are new record of Northern Western Ghat of India. The 16 species of *Calvatia* were recorded from 8 different states of India except Northern Western Ghat of Maharashtra out of which *C. candida* were reported from Uttar Pradesh, India (Khare 1976) and *C. craniiformis* were reported from Karnataka but only based on morphological features (Syed Abrar et al.(2008).

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