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G. aff. *albonigrum* (Geastraceae, Agaricomycetes): A new earthstar fungus from India

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Abstract

G. aff. *albonigrum* was found growing solitary or in cluster either fully or partially buried on lateritic soil in the dry deciduous *Shorea* forests. The fresh and dry fruiting bodies were collected and studied. The diagnostics characteristics for this new record is the hirsute ephemeral mycelial layer, non-delimited and fibrillose peristome, dark brownish endoperidium with single large robust rhizomorph attached at the base of the fruitbodies. Species description, images of basidiomes, SEM images of basidiospore are provided.

The gasteroid macro fungi *Geastrum* was first described in early 18th century by Persoon with *G. coronatum* Pers., (1801) as typified species[14]. *Geastrum* are widely distributed in tropical, sub-tropical and temperate regions of the world except Antarctica. These stipe-less mushrooms possess enclosed hymenophores within the endoperidium and discharge spore using bellow mechanism. Gasteroid fungi can easily be identified on the field due to star-like pattern of fruitbodies at maturity and are widely known as earthstar[10,27]. According to *Index Fungorum* database (http://www.indexfungorum.org/)[9], the genus comprises more than 330-350 species around the globe. Traditionally, taxonomy of the *Geastrum* mainly rely on morphological traits like basidiomata size and colour, exoperidium layers, endoperidium surface, hygroscopy behaviour of rays, rhizomorph, structure of hyphae from mycelial layers, type of peristome (fribrillos or, plicate), presence or absence of apophysis and stipe between exoperidium and endoperidium[22]. Although *Geastrum* superficially resemble *Astraeus* Phosri., or *Myriostoma* Desv., species but it is dissimilar in morphological, anatomical and phylogenetic traits. *Astraeus* has larger large pedicellate spores and lacks peristome and columella[15]. *Myriostoma* has multiple endoperidial stomata and wing-like

reticulate ornamentation of basidiospore. These should be should be sufficient traits to distinguished *Geastrum* from *Astraeus* and *Myriostoma*[21]. Zamora *et al.*,[27] reported the presence of Phenoloxidase and Calcium Oxalate deposits from rhizomorph of *Geastrum*. In India, about 25 distinct species of *Geastrum* are reported till date[25], but none of them is reported from Jharkhand, India. These indigenous edible agaricomycetes (mushrooms) locally known as "Rugra" inhabit the rhizosphere of *Shorea robusta* Gaertn. The tribal population collect and consume these wild edible mushroom, since a long time[18]. It is quite commonly found in moist deciduous forest, semi-evergreen forest, scared groves, Western ghats and West-coast, Karnataka, Kerala and Gujarat. Most of these species are terrestrial, lignicolous and coprophilous in habit, whereas some are reported form leaf litters, termite mounds, humus, decomposing twigs or barks (*Pongamia* and *Acacia* sp.) decaying twigs (*Sapium insigne*)[10,25]. The Indian subcontinent is blessed with high diversity of macrofungi due to diverse physiographic and agro-climatic conditions but there is a dearth of information on *Geastrum*. The vast gaps in our knowledge about macrofungal biodiversity and how these organisms are affected by trade, land management practices, deforestation, forest fire and upsurge global warming pose a serious threat and therefore demand special attention for restoration and conservation. This work is aimed to contribute to the knowledge of *Geastrum* diversity and distribution in Jharkhand mycoflora based on integrative taxonomic approach using morphological, molecular and phylogenetic studies.

Morphological studies:

Fresh and dry specimen were collected during the month of August 2020 from Bariatu, Ranchi, (23.39 N, 85.35 E) (Fig. 1) Jharkhand state of India and placed in paper bags prior to analysis. Micromorphological characters were examined under a light microscope at magnification(x) of X100, X400 & X1000. Colors based are coded on Kornerup & Wanscher[11]. Dried basidiospore were mounted in a mixture of 5% KOH in lactophenol and cotton blue, and stained with Congo red reagent[19]. About thirty spores were examined and measured. For SEM observation specimen was air-dried and observed in Carl Zeiss EVO18 scanning electron microscope. Basidiospore abbreviations followed by *Sousa et al.*[20] n= no. of randomly measured basidiospores; x = mean ± standard deviation of basidiospore diameter and height (including ornamentation), Qm= mean height/width ratio. The collected specimen (Geas-1a-JH) was deposited in the Department of Botany, Dr Shyama Prasad Mukherjee University, Ranchi, Jharkhand, India.

DNA extraction, PCR amplification and sequencing :

The genomic DNA was extracted from approximately 10 mg of gleba of mature dry basidiome using EXpure Microbial DNA isolation kit (Bogar Bio Bee stores Pvt Ltd, Coimbatore, India). PCR amplification and sequencing of 5.8S ribosomal RNA gene cluster of ITS region were carried out according to Sousa *et al.*[20] Consensus sequence were assembled and edited with BioEdit 5.0.6 software[8]. Prior to the alignment, query

sequence were compared with homologous sequence in GenBank/DDBJ using the MEGABLAST algorithm of National Centre for Biotechnology Information (NCBI)[12]. The newly generated sequence Geas-1a-JH has been deposited on NCBI GenBank with the accession number MW676787 for *G.* aff. *albonigrum.*

Phylogenetic Analysis :

The alignment was analysed under a heuristic search with Tree Bisection and Re-connection (TBR) as branch swapping algorithm using PAUP 4.0a[23] and a default setting to stop the analyses when reaching 100 trees to obtained maximum parsimonious tree. The Bayesian analysis was done assuming HKY+ G best fit-model, using Mr Bayes v. 3.2.6[16] with default parameters (Nst=6 with 2 runs, 4 chains per run, each run searching for 1M generations sampling every 1K generation), as described in Cabral *et al*[3]. The 50% maturity-rule consensus tree and the posterior-probability (PP) of the nodes were calculated. Phylogenetic tree was viewed with Fig Tree v1.31.1 (http://tree.bio.ed.ac.uk/software/figtree/).

Taxonomy :

G. albonigrum. Calonge& M. Mata. (2004). Bol. Soc. Micol. Madrid 28: 332. Fig. 2 A-J; Fig. 4 A-C

Unexpanded Basidiomata semi-hypogeous, globose to sub-globose, whitish 12-17mm in diam., hirsute surface, brownish, single large robust rhizomorph attached at the base, persistent rhizomorph, 25-28mm long, encrusted with debris. **Expanded Basidiomata** saccate, 34-40mm in diam., 18-20mm tall. **Exoperidium** non-hygroscopic, split into 5-7 revolute rays, mycelial layer greyish brown (6D3) to dark brown (6F4), external hirsute layer of aggregate hairs up to 120-130µm long, encrusted, peel off at maturity exposing the fibrous layer. Fibrous layer brown to greyish brown (6D3), persistent, coriaceous, glabrous, thick with attached rhizomorph. Pseudoparenchymatous layer dark brown (6F4, 6F3), with transverse crack. **Endoperidium** globose to subglobose, 12-16mm in diam., 14-18mm tall, sessile, dark brown, glabrous without apophysis, peristome fibrillose, not-delimited, mammiform, circular, slightly darker than endoperidium. **Gleba** greyish brown (7F2) and dusty at maturity. **Basidiospore** globose to subglobose, 3.2-5.1 × 3.1-5.1µm [x = 4.3 ± 0.5 × 4.5 ±0.4µm, Qm = 1.05, n=30], brownish, verrucose, short cylindrical warts 0.3-0.5 µm long with rounded tips; apiculus conspicuous. Capillitial hyphae 2.9-5.9µm diam., straight, thick-walled, not encrusted, surface verrucose, brown, lumen present. Mycelial layer brownish in KOH consists of sinuous-walled hyphae, 2-4µm diam., with narrow lumen. Fibrous layer consists of sinuous -walled hyphae, 2-4µm in diam., with narrow lumen, greenish to hyaline in KOH. Pseudoparenchymatous layer consist of thin-walled hyphal cells (<1 µm), subglobose to pyriform, hyaline to greenish in KOH. Crystalline matter: Rhizomorph white, thick-walled, 1.015 X 1.086µm diam., surface encrusted, branched, clamped present and rhizomorph with distinct calcium oxalate pyramidal crystal.

Substrate: Sandy lateritic soil covered with litter, fruiting frequently in monsoon season (June-October). It is found as solitary or scattered in small groups under the Dipterocarpaceae sal dry deciduous forest.

Specimen Examined: India, Jharkhand, Bariatu, Ranchi, alt. 287m, Aug 01, 2020, 23.39 N, 85.35 E, on the ground under near root of *Shorea robusta*, V. Vishal and S. S. Munda, S. Lal and G. Singh Gea-1a (JH).

Distribution: Central America: Costa Rica[4]; North America:Mexico[4]; South America: Brazil, MatoGrosso State (Treirveiler Pereira et al. 2011b), Rio Grande do Norte State[20]. India, Jharkhand.

Phylogenetic Analysis

The molecular phylogenetic analysis carried out on nineteen ITS datasets including *Myriostoma* sp. as an outgroup placed the reported species from India within the genus *Geastrum*. The alignment of ITS dataset resulted into 895 unambiguously aligned nucleotide position consists of 477 constant, 155 parsimony-uninformative and 263 parsimony-informative. The 100 most parsimonious tree (not shown) give a length of 813 steps, CI =0.7022, HI = 0.2928, RC =0.4783. The 50% Bayesian majority rule combined consensus tree exhibited similar topologies. The *Geastrum* isolates were resolved as monophyletic in highly supported clade (MPbs = 100%, PP = 1.00), Fig. 3. The Bayesian tree generated three main clades (I-III) of which Clade I consists *G. ovalisporum, G. hirsutum* from South America while *G. coronatum* from United Kingdom. The clade II is represented by section Exaerolata comprises of *G. aculeatum, G. echinulatumand G. rufescens*, from South America and Europe. However, clade III is represented *G. albonigrum* and *G. inpaense* earlier reported from South America, North America and now from India.

Based on morphological data and molecular phylogenetic analysis *G.* aff. *albonigrum* is closely related to species grouped in section Exareolata[26], like: *Geastrum albonigrum* Calonge & M. Mata, *G. inpaense* Cabral *et al*., *G. rufescens* Pers., *G. echinulatum* Cabral *et al*. and *G. aculeatum* da Silva *et al*. All the species have distinct hyphal outgrowth over outer peridium like *G.* aff. *albonigrum*, (Fig. 2B). *G.* aff. *albonigrum* has characters such as hirsute exoperidium, large robust rhizomorph, ephemeral mycelial layer, a white fibrous layer, a black pseudoparenchymatous layer, sessile black endoperidium and fibrillose non-delimited, mammiform peristome without apophysis [4, 20, 24].

Though the collected sample shows affinities with *G. albonigrum*, in morphological and phylogenetic traits (Clade III on tree, Fig. 3), *G. albonigrum*, however, has reports of occurrence in distinct and distant ecoregion (Neotropics: Brazil, Costa Rica, Mexico and Panama) compared to the location of our sample (IndoMalayan - India)[13]. The collection from Neotropical region were found in humid forests like Amazon forest and Atlantic Rainforest in Brazil[20], where as the collections from India were found on dry deciduous forests. Besides, there are some inconspicuous traits that can distinguish *Geastrum albonigrum* from the collected sample. Based on protologue description [19] *Geastrum albonigrum* has longer cylindrical warts (0.6 - 1µm long), where as the samples collected from Jharkhand has short cylindrical 0.3-0.5µm long warts. On comparison with *Geastrum albonigrum's* collection from Brazil, samples from Jharkhand, India has distinct semi-hypogeous growth of unexpanded basidiomata, while in Brazil the

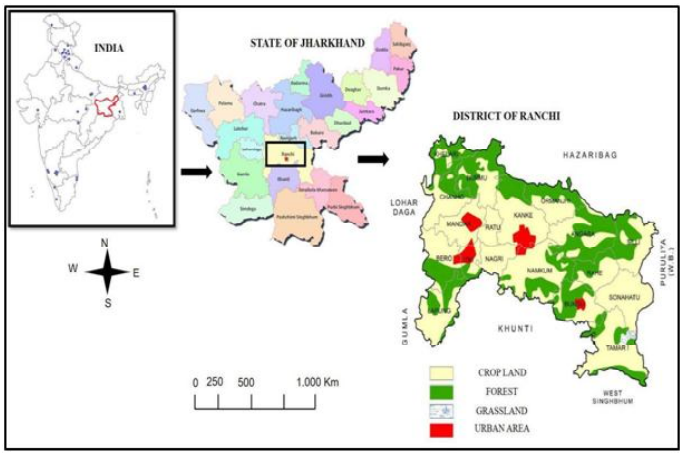

Fig. 1- Map of India showing study and collection sites (23.39 N, 85.35 E) and distribution of Gasteroid fungus- *Geastrum*.

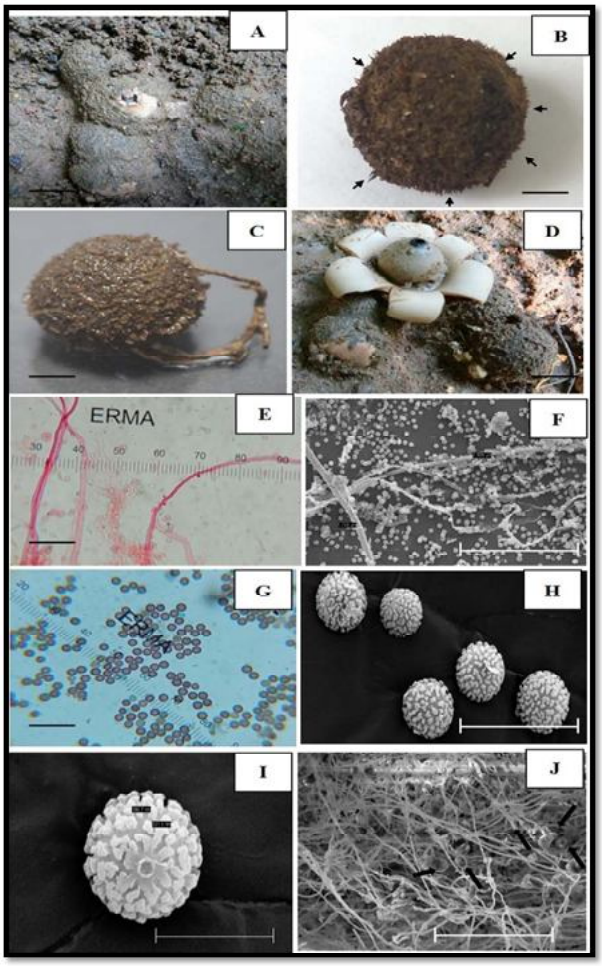

Fig. 2- *G.* aff. *albonigrum*. A. Basidiomata partially buried in soil; B. Basidiomata showing external hirsute layer of aggregate hairs; C. Basidiomata with robust rhizomorph. D. Star-like pattern of basidiomata with not-delimited fibrillose peristome; D. Capillitium hyphae; E. SEM of Capillitium hyphae; G. Basidiospore; H-I. SEM of Basidiospore showing verrucose ornamentation with short cylindrical warts; J. SEM of rhizomorph showing pyramidal shaped crystals. Scale bar A-D = 10mm; E = 10µm; F= 1µm; G =10µm, H = 30 nm; and J = 4µm.

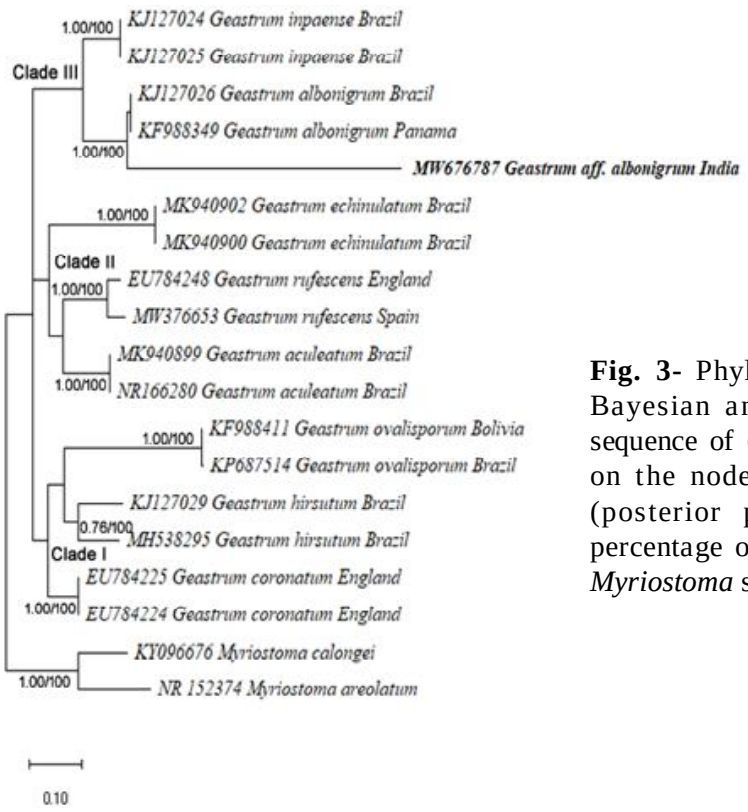

Fig. 3- Phylogenetic tree obtained by Bayesian analysis derived from ITS sequence of *Geastrum* Isolates. Number on the node indicate support values (posterior probabilities values and percentage of parsimonious bootstrap). *Myriostoma* sp. were used as an outgroup.

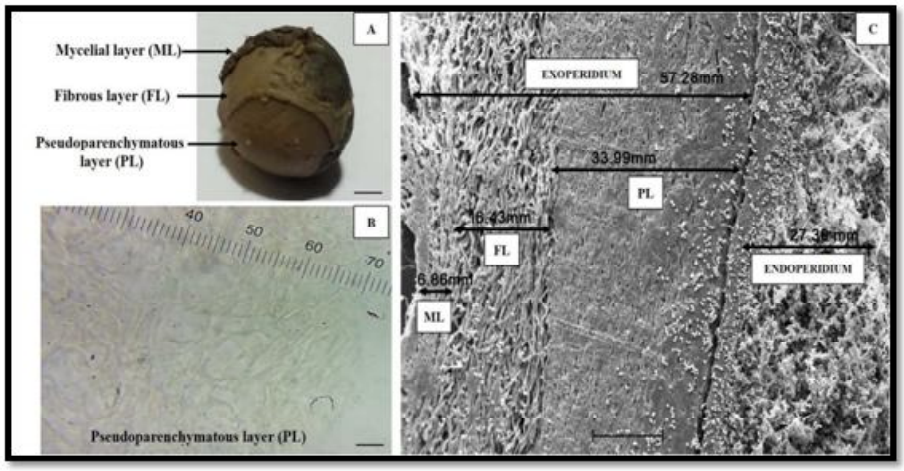

Fig. 4-*G.* aff. *albonigrum*- Exoperidium**.** A. Different layers of exoperidium; B. Cross-section through exoperidium showing pseudoparenchymatous layer with subglobose to pyriform cells; C. SEM of different layers of exoperidium- Mycelial layer (ML), Fibrous layer (FL), Pseudoparenchymatous layer (PL) and endoperidium. Scale bar A = 10mm; B = 10µm and C= 20µm.

basiomata on epigeous habit (Sousa *et al.*[20], Trierveiler-Pereira *et al.*[24]). SEM analysis demonstrated presence of calcium oxalate crystals on rhizomorph surface (Fig. 2J), but there is no information about crystalline structure in the Neotropical reports on *Geastrum*.

Another species related with the collected samples is *Geastrum inpaense*. However, G. inpaense has smaller hair on mycelial layer (up to 0.5 mm long), delimited peristome and smaller basidiospore (2.6-3.8µm)[2]. Other species from sect. Exareolata are: *Geastrum rufesnces, G. echinulatum, G. aculeatum, G. carirense* and *G. lanuginosum*. *Geastrum rufescens* is distinguished from *G.* aff. *albonigrum* by having larger basidiospores (5-6µm) and strongly encrusted with soil debris[22]; *G. echinulatum* has well developed subiculum and reddish pseudoparenchymatous layer when fresh, on the other hand, *G. aculeatum* has larger basidiospore (5-6µm) and aculeate hyphal tuft over outer peridium[17]; *G. carirense* is differentiated by cracks on mycelial layer without hairs and larger basidiospores (4.5-6.5 µm); *G. lanuginosum* is distinct by cottony and persistent mycelial layer, and whitish pseudoparenchymatous layer[5,7]. Other species with hair on exoperidium layer are *G. brunneocapillatum, G. hirsutum, G. piquiriunense, G. pusillipilosum* and *G. rubellum*, although those species have silimarity on external pedirium layer, they are grouped in a distinct section (*Myceliostroma*) and are distinguished by delimted peristome and presence of subiculum[1,6].

In India, *G. fimbriatum, G. lageniforme, G. Pseudostriatum, G. schweintzii*, and *G. triplex* were described by various workers[10,25] and distinguished from present collection by external hirsute of aggregate hairs on mycelial layer, basidiospore size and rhizomorph with distinct calcium oxalate pyramidal crystals that are absent in the basidiomes of described species. Taking in account the analysis and bibliographic review for the identification of the present *Geastrum* samples, it can be considered as the first report of a species of sect. Exareolata from India.

Declaration on conflict of Interest :

The authors declare no conflict of interest.

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