

## Studies on morphological and molecular identification of *Geastrum* from Gujarat, India

Ravi S. Patel, Ajit M. Vasava and Kishore S. Rajput\*

Department of Botany, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara 390002, India

\*Corresponding author Email: ks.rajput15@yahoo.com

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### ABSTRACT

The Genus *Geastrum* Pers. (*Geastraceae*), is a cosmopolitan gasteroid mushroom possessing enclosed hymenophore. Its species are commonly known as earthstars because of the splitting exoperidium giving the fungus a star like morphology. In the Western part of India, it is represented by three species, viz. *Geastrum saccatum* Fr., *G. rufescens* Pers. and *G. triplex* Jungh. These are being recorded for the first time from the Gujarat State. Specimens were collected from Vansda National Park and Jambughoda Wildlife Sanctuary during field survey undertaken from 2014-2019. Identification was carried out based on the morphological features and further confirmation was done by molecular phylogenetic analyses using nuclear rDNA ITS sequencing. Molecular data has been submitted to BOLD system for DNA barcoding.

**Key words:** Fungal diversity, *Geastraceae*, *Geastrum*, ITS, DNA barcoding.

### INTRODUCTION

The genus *Geastrum* was erected by Persoon (1801) with *G. coronatum* Pers., as the type species, which is commonly known as earthstar. It is one of the most widely distributed and complex genus of gasteroid fungi with enclosed hymenophore within the exoperidium. Different species of this genus can be easily recognised by their distinct star-like basidiomata at maturity. According to Zamora *et al.* (2014a, b), the genus comprises more than 100-120 species around the world. The taxonomy of the earthstars, as in many other macro-fungi, has been traditionally based on morphological features of the basidiomata (Sunhede, 1989). This genus is reported to harbor some of the medicinally important species including *G. saccatum*, the extract of which is reported to be anti-inflammatory, antioxidant and cytotoxic in actions (Dore *et al.*, 2007) while the extract of *G. triplex* is reported to be quite effective against several plant and human pathogenic bacteria (Chittaragi *et al.*, 2013).

In India, species of geaster are reported to be quite common in moist-deciduous forests, semi-evergreen forests, sacred groves, and coffee plantations of the Western Ghats of Karnataka and Kerala and Shola forests in the Western Ghats. Most of these are usually terrestrial, rarely lignicolous or even coprophilous in habit (Bhagwat *et al.*, 2005; Mohanan, 2011). However, some of its species are also reported from leaf litter, termite mounds, humus, decomposing twigs or even bark of *Pongamia pinnata*, *Acacia auriculiformis* and decaying twigs of *Sapium insigne* (Karun and Sridhar, 2014). In all about 24 different species of *Geastrum* are reported from India till date by the previous researchers (Verma *et al.*, 2018) and none of the documented species are from Gujarat.

In this manuscript summarized account of morphological and molecular studies undertaken during the present study on *G. saccatum* Fr., *G. rufescens* Pers., and *G. triplex* Jungh., has been presented.

### MATERIALS AND METHODS

**Collection of Fungi:** During extensive field visits in different forest regions of Gujarat in the years 2014-2019, specimens of *G. saccatum*., *G. rufescens* and *G. triplex* were collected from Vansda National Park, (20°45'31.86" N, 73°28'28.77" E), Jambughoda Wildlife Sanctuary (22°22'19.02" N,

73°39'57.85" E) and Mota Raska, Jambughoda Wildlife Sanctuary (22°21'26.06" N, 73°41'28.12" E) in Gujarat State, India and their morphological characters were noted in the field. The collected specimens were preserved for undertaking macroscopic and microscopic investigations. The additional specimens have been submitted under BARO in the Herbarium of Department of Botany, The Maharaja Sayajirao University of Baroda, Vadodara.

**Molecular analysis:** Genomic DNA was extracted from fresh fruiting bodies of collected specimens using a Plant/Fungi DNA isolation kit (Sigma Cat# E5038). ITS region was amplified using ITS1 and ITS4 primer pairs (White *et al.*, 1990). The PCR reactions were performed in a 20µL volume containing 1x final concentration of DreamTaq Green PCR Master mix (Cat# K1081), 50 ng of genomic DNA and 10 pmol of both primers i.e. ITS1 and ITS4. PCR reactions were carried out by using Veriti® thermal cycler (Applied BioSystems) under the following conditions: initial 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec and extension at 72 °C for 1:30 min., with a final extension at 72°C for 10 min. The PCR product was visualized on 2% agarose gel and amplified PCR products were purified using Purelink™ Quick PCR Purification kit (Cat# K310001). The purified PCR products were sequenced by Eurofins Genomics India Pvt. Ltd., Bangalore. The obtained sequences were compared with the database sequences available in the NCBI using the Basic Local Alignment Search Tool. The Barcode of Life Data System (BOLD) was used to generate DNA barcodes.

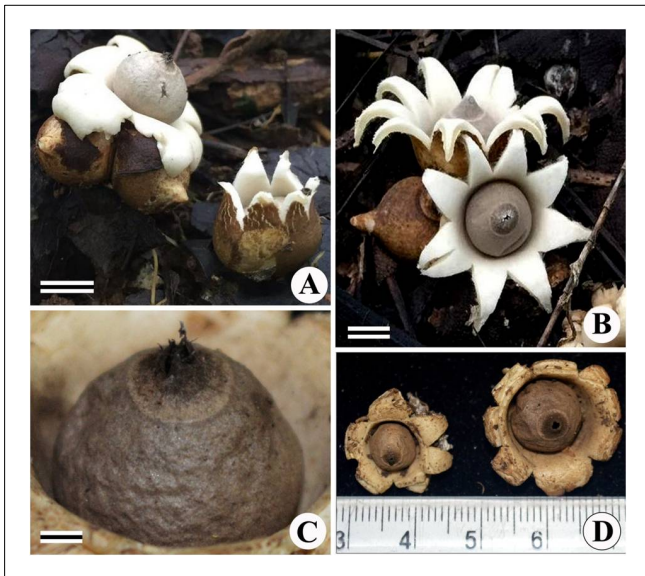
**Phylogenetic analysis:** The phylogenetic tree is generated by using nucleotide sequences of ITS gene. Nucleotide sequences of *Geastrum* species are downloaded from GeneBank in FASTA format. Downloaded sequences were supplemented with the newly generated sequences of *G. saccatum*, *G. rufescens* and *G. triplex*, whereas *Myriostoma coliforme* was used as outgroup taxa. The nucleotide sequences were aligned with ClustalW (Thompson *et al.*, 2002) embedded in MEGA X (Kumar *et al.*, 2018). Aligned data was analysed with Partition Finder (Lanfear *et al.*, 2012) for optimal partitioning strategy and evolutionary substitution model. Maximum Likelihood (ML) analyses were employed to infer phylogenetic relationships in RAxML

(Silvestro and Michalak, 2012). An ML analysis was run for 1000 bootstrap replicates under the GTR + I model to assess clade support.

## TAXONOMIC DESCRIPTIONS

### 1. *Geastrum saccatum* Fr., *Syst. Mycol.* 3:16, 1829 **Fig.1 (A-D)**

Commonly known as the rounded earthstar, bulblight brown, surmounting a star-shaped base, which raises the spore sac above the surrounding substrate (**Fig.1A**). Immature basidiomata epigeous, light brown, sub-globose to oval, fibrous to squamulose. On maturity, exoperidium splits at the apex into 5-8-pointed non-hygroscopic starfish-like rays, involute and saccate (**Fig.1B**). Mature sporophores measured from 0.6–2.5 cm in diameter, 1–1.2 cm in height (**Fig.1C**); peristome slightly darker than endoperidium, delimited, pale, fimbriate, conically protruding, surrounded by a finely depressed circular halo region (**Fig.1D**). Mycelial layer felty but does not encrust litter and there is a basal attachment point to the below ground mycelium; endoperidium pale brown, sessile, thin and papery



**Fig. 1:** **Morphology of *Geastrum saccatum*.** A: Immature brown bulb and growing fruiting bodies. B: Mature fruiting bodies with 5-8-pointed non-hygroscopic saccate rays. C: Exposed endoperidium. D: Mature fruit body measuring 0.6–2.5 cm in diameter. **Scale bars:** A=5 mm, B=5 mm, C=1 mm, D=scale indicating cm marking.

**World distribution:** This species is well distributed in Africa, Asia, Australia, Europe, North America, South America and West Indies (Jeppson *et al.*, 2013).

**Collection Examined:** Gujarat: Navasari, Vandsa National park. Growing on soil along with mixed leaf litter of Bamboo, Dr. K.S. Rajput, Ravi S. Patel and Ajit M. Vasava. BARO 123450030716, August 14, 2016.

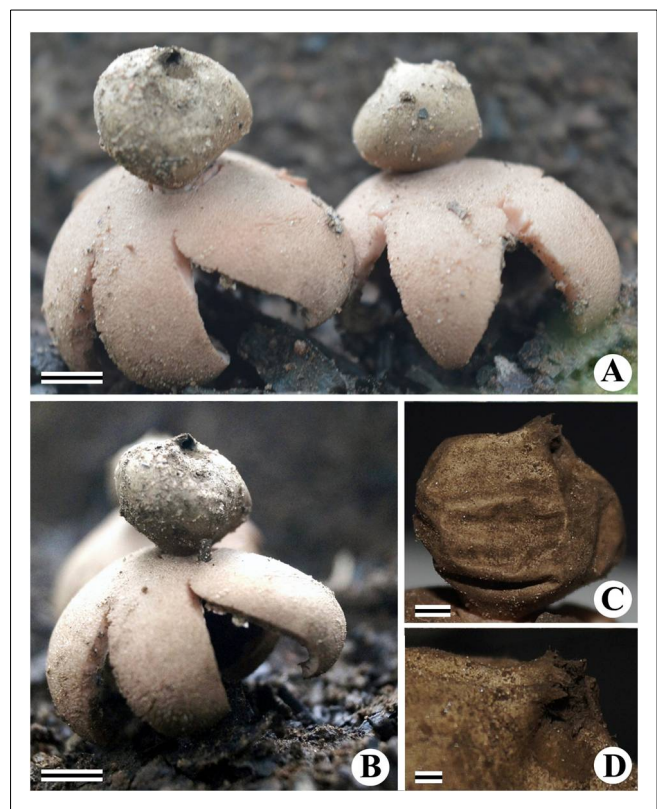
**Remarks:** *Geastrum saccatum* was described based on Brazilian specimens. According to Baseia *et al.*, (2003), *G. saccatum* can be distinguished by its saccate exoperidium,

sessile endoperidium, fibrillose and delimited peristome. According to Trierveiler-Pereira *et al.*, (2011), this species is very similar to *G. lageniforme*. Both species have saccate basidiomata, sessile endoperidium, fibrillose and delimited peristome. *G. lageniforme* usually has longer and more slender rays and longitudinal ridges in the external layer. However, *G. saccatum* may also have such ridges. According to Sunhede (1989) these two species can be distinguished by the presence of clamped hyphae in the external mycelial layer, which only occur in *G. lageniforme*. In India *G. saccatum* was previously reported from Kerala (Karun and Sridhar, 2014), Uttar Pradesh (Khare, 1976) and Assam (Gogoi and Vipin, 2015).

### 2. *Geastrum rufescens* Pers., *Syn. Meth. Fung. (Goettingen)* 1:134, 1801. **Fig. 2 (A-D)**

Popularly known as the rosy earthstar, bulb up to 2 cm in size, light greyish, coarsely scaly, sessile and brittle. At maturity, exoperidium splitting into 5-8 pointed starfish like rays, which reflect back to reveal the light flesh colour layer (**Fig. 2A, B**). Fully grown fruiting body 3-5 cm in diameter, endoperidium (spore sac) sub spherical, papery, opening by a slight elevated apical pore (**Fig. 2 C, D**). Gleba (spore mass) at first pallid and firm, becoming brown.

**World distribution:** *G. rufescens* is well distributed in different parts of the world including Asia, Europe, Central America and North America (Jeppson *et al.*, 2013).



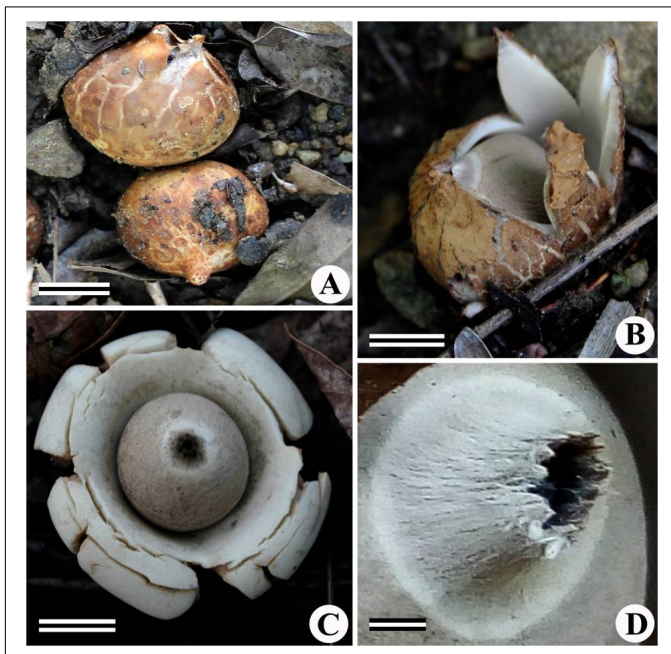
**Fig. 2:** **Morphology of *Geastrum rufescens*.** A&B: Mature fruiting bodies with 5-8-pointed starfish like rays. C&D: Endoperidium with Peristome surrounding the slight elevated apical pore. **Scale Bars:** A=4 mm, B=4 mm, C=2 mm, D=1 mm

**Collection Examined:** Gujarat, Panchmahal, Jambughoda wildlife Sanctuary. Growing on soil along with mixed leaf litter of *Madhuca longifolia*, Dr. K.S. Rajput, Ravi S. Patel and Ajit M. Vasava. BARO 123450030717, August 21, 2016.

**Remarks:** *G. rufescens* has a pale pinkish-buff to pinkish exoperidium and starfish like backwardly reflecting rays. It was traditionally considered similar to *G. fimbriatum*. However, according to Zamora *et al.* (2014a) *G. rufescens* is distinguished from *G. fimbriatum* in having larger basidiospores. Moreover, *G. rufescens* has reddish tone which is absent in *G. fimbriatum* (McKnight and McKnight, 1987). In India, *G. rufescens* was previously reported from Gorakhpur, Uttar Pradesh by Vishwakarma *et al.*, (2014).

**3. *Geastrum triplex*** Jungh., *Tijdschr. Nat. Gesch. Physiol.* 7: 287, 1840. **Fig. 3 (A-C)**

Universally known as collared earthstar. *G. triplex* is differentiated from closely related species by the presence of collar-like structure of the inner layer of the exoperidium. *G. triplex* has the largest fruiting body-amongst the earthstar mushrooms. Immature basidiomata 2-3 cm in diameter, hypogeous, dull orange brown, onion shaped, coarsely fibrous to squamulose (Fig. 3A) while fully grown fruiting bodies 6-8 cm in diameter. At maturity, exoperidium splits into 5-6 non-hygroscopic starfish-like rays and form a prominent collar around the endoperidium (Fig. 3C). Endoperidium greyish brown, thin, papery and smooth. Peristome delimited, light brown, conically elevated and surrounded by a distinct pallid to brownish black halo (Fig.



**Fig. 3:** Morphology of *Geastrum triplex* A: Immature basidiomata with coarsely fibrous to squamulose surface. B: Growing basidiomata. C: Mature fruiting bodies with 5-6 non-hygroscopic starfish-like rays forming a prominent collar around the endoperidium. D: Conically elevated Peristome surrounding the ostiole. **Scale Bars:** A-10 mm, B-10 mm, C-15 mm, D-3 mm.

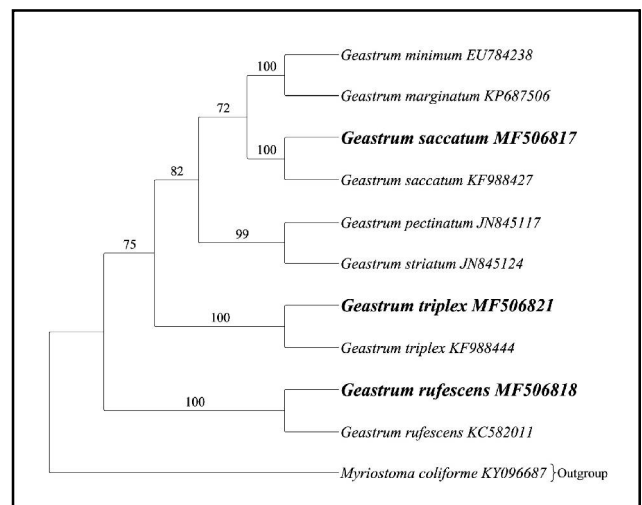
3D). At first, gleba pallid and firm, with age it becomes brown and powdery.

**World distribution:** *G. triplex* is distributed in Asia, Africa, Europe, Central America, New Zealand, North America and South America (Jeppson *et al.*, 2013).

**Collection Examined:** Gujarat, Panchmahal, Jambughoda wildlife Sanctuary. Growing on soil along with mixed leaf litter of *Madhuca longifolia*, Dr. K.S. Rajput, Ravi S. Patel and Ajit M. Vasava BARO123450030718, August 28, 2016

**Remarks:** This species can be distinguished by its involute rays, prominent collar around the endoperidium, sessile endoperidium, fibrillose and delimited peristome (Trierveiler-Pereira *et al.*, 2011). Other species (*G. fimbriatum*, *G. saccatum*, *G. lageniforme*, *G. rufescens*) may also show a small pseudoparenchymatous collar, but it is never as conspicuous as in *G. triplex* (Sunhede, 1989). Basidiomata of *G. triplex* is usually large (up to 6.4 cm diam. in the examined material) and the European material is reported to reach 15 cm diam. (Calonge, 1998). In India *G. triplex* was previously reported from Kerala by Karun and Sridhar (2014), Mohanan (2011) and from Madhya Pradesh by Verma *et al.* (2018).

**Molecular identification:** The generated nucleotide sequences were used for BLAST search in the GenBank database ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) for identification at species level. Identification was done by 99% basepair match of the sequence obtained to the closest available reference sequences (Leung *et al.*, 1991). Nucleotide sequences of *G. saccatum*, *G. rufescens* and *G. triplex* were submitted to NCBI database using Bankit nucleotide sequence submission tool with accession numbers MF506817, MF506818 and MF506821, respectively. The nucleotide sequences are also submitted to BOLD data system (MIFDG Project) to generate DNA barcodes with BOLD Id's are KSRF-0011, KSRF-0013 and KSRF-0010, respectively. Phylogenetic relationship of all the three species of *Geastrum* is represented in Fig. 4.



**Fig. 4:** Phylogenetic tree based on Maximum likelihood analyses of the ITS sequence dataset.

## DISCUSSION

The earthstars have a sub-cosmopolitan distribution and have been recorded from all continents except Antarctica and are more abundant in the temperate zones and in the tropics (Ponce de Léon, 1968). The present study documents new distributional record for three species of *Geastrum* from Gujarat State (India). The morphology and molecular identification based on ITS sequences data confirmed that the collected earthstar fungi belong to *G. triplex*, *G. saccatum* and *G. rufescens*. Amongst the earthstar fungi recorded in the present study, *G. triplex* is reported to have antibacterial activity against several plant and human pathogenic bacteria (Chittaragi *et al.* 2013). Dore *et al.* (2007) reported anti-inflammatory, antioxidant and cytotoxic actions of extract from *G. saccatum* and *G. rufescens*. Several geasters occurring in Europe have been considered as threatened, critically endangered and are red listed. Nitare (2000) and Benkert (2003) opined that geasters are under threat due to anthropogenic pressures and thus those locations endowed with geasters needs special attention for restoration and conservation. Number of molecular studies are appearing now as that of Hibbett *et al.* (1997) and Douanla *et al.* (2005) wherein phylogenetic relationship between *Gasteromycete* and *Geastrum* spp. has been investigated by sequencing large subunit of rDNA. Kasuya *et al.* (2012) also carried out molecular studies for the phylogenetic placement of *G. melanocephalum* and *G. triplex*. Zamora *et al.* (2014c) re-described a forgotten species of *G. argentinum* based on molecular traits. Jeppson *et al.*, (2013) established phylogenetic relationships of European earthstars based on molecular sequence data (nuclear rDNA, ITS1 and ITS2; LSU; Tef- $\alpha$ ), morphological data and ecological characteristics. The rDNA, ITS region is a useful molecular tool for fungal taxonomic and phylogenetic studies. As has been observed in the present study, gross morphology supplemented with microscopic and molecular studies are important in deciding the taxonomic vagaries amongst the closely related taxa.

## CONCLUSION

Present study report *G. triplex*, *G. saccatum* and *G. rufescens* from Gujarat state for the first time using molecular studies for the species level identity. Different species of the *Geastrum* have various medicinal properties and several of them have also been considered as threatened, critically endangered and red listed throughout the world. Therefore it is very important to study the diversity of *Geastrum* species for restoration and conservation. On the other hand, the rDNA, ITS sequencing data is useful to distinguish the morphologically closely related species such as *G. saccatum* and *G. lageniforme*. The newly generated molecular data in the present study should stimulate further study to understand the diversity and systematics of *Geastrum* species.

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