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# Morphological and molecular evidence for the occurrence of *Itajahya galericulata* (*Basidiomycota*, *Phallales*) in India

Ravi S. Patel, Ajit M. Vasava & Kishore S. Rajput*

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**Abstract**. *Itajahya galericulata* (*Phallales*, *Phallaceae*) was previously reported from several countries in South America and Africa. Recently we found *I. galericulata* in the city of Vadodara, Gujarat State, India. To verify its identity we studied its morphology and performed molecular phylogenetic analyses using nuclear rDNA LSU and mitochondrial ATP6 loci. Here we also provide nuclear rDNA ITS sequences for the Indian collection, since up to now no sequences of this region have been available for *I. galericulata* in GenBank. This study furnishes the first evidence for the occurrence of *I. galericulata* in India and in Asia as a whole.

**Key words**: *Itajahya*, *Phallaceae*, ITS, molecular phylogeny, DNA barcoding, India, Asia

## Introduction

Members of the fungal family *Phallaceae*, classified within the order *Phallales* and subphylum *Basidiomycota*, are commonly known as stinkhorn mushrooms. The family contains 21 genera and 77 species (Kirk et al. 2008), including the genus *Itajahya*, which was first described by Möller (1895) from Brazil and named after the Itajahy River near Blumenau city in Santa Catarina State. The type of the genus, *Itajahya galericulata*, is rarely observed, so this genus remains one of the lesser-known members of the family *Phallaceae*. The main feature that distinguishes *Itajahya* from other taxa of this family is the presence of a structure termed the 'calyptra' located at the apex of the gleba (Möller 1895; Malençon 1953; Ottoni et al. 2010). Cabral et al. (2012) considered the taxonomic placement of *Phallus roseus*, a species assigned to the genera *Itajahya* or *Phallus* (e.g., Malençon 1984; Kreisel 1996). They carried out DNA sequencing and phylogenetic analyses of *Phallus roseus* and demonstrated that it does not cluster with other species of the genus *Phallus*. It was therefore separated from *Phallus* and accepted as a member of the genus *Itajahya* (Cabral et al. 2012).

Four species are currently included in *Itajahya*: *Itajahya galericulata* described from South America, *I. rosea* described from Egypt, *I. hornseyi* described from Australia, and *I. argentina* described from Argentina (e.g., Spegazzini 1898, 1927; Hansford 1954). Marincowitz et al. (2015) provided the first DNA sequence data for a poorly known yet taxonomically important member of the genus – *Itajahya galericulata* – and concluded that it is phylogenetically separated from species of *Phallus* and *Dictyophora*. Their study also confirmed that *Itajahya rosea* and *I. galericulata* (type of the genus) are phylogenetically related and indeed belong to the genus *Itajahya* (Cabral et al. 2012; Marincowitz et al. 2015).

During a field survey aimed at documenting the fungal diversity of Gujarat State in India, we collected an interesting fungus resembling *Itajahya galericulata* from the Community Science Centre and from the campus of the Maharaja Sayajirao University of Baroda, Vadodara. A literature survey revealed that there are no data on the occurrence of *I. galericulata* in India and Asia. The present study documents its occurrence in India and Asia, based on morphological and molecular analyses of the collected material.

## Materials and methods

### Collection

Fruiting bodies of *Itajahya galericulata* were collected from the Community Science Centre, Vadodara, Gujarat State, India (22°19′08.62″N, 73°09′11.53″E). These fruiting bodies were growing at the base of *Pithecellobium dulce*, a species native to northern South America, from where *Itajahya galericulata* was described for the first time. Fresh fruiting bodies were collected into a sterile polyethylene bag for further taxonomic study in the laboratory. These fruiting bodies were used for genomic

Department of Botany, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara 390002, India
* Corresponding author e-mail: ks.rajput15@yahoo.com

DNA isolation for molecular identification. Within a week, similar fruiting bodies were also observed growing on the campus of the Maharaja Sayajirao University of Baroda (an undisturbed area retained as natural forest), growing under *Prosopis juliflora*, another host species that is native to South America.

### DNA isolation, PCR and sequencing

Genomic DNA was extracted from a fresh fruiting body of *Itajahya galericulata* using a Plant/Fungi DNA isolation kit (Sigma-Aldrich, USA). DNA sequences were obtained for three different regions: internal transcribed spacer (ITS), nuclear ribosomal large subunit (LSU), and mitochondrial ATPase subunit 6 (ATP6). For these regions we used, respectively, the primers ITS1 and ITS4 (White et al. 1990), LROR and LR5/LR10 (Vilgalys & Hester 1990), and ATP6-1 and ATP6-2 (Kretzer & Bruns 1999). Subsequently, PCR reactions were carried out for the ITS and LSU regions in a final volume of 20 µl containing 1× final concentration of DreamTaq Green PCR Master mix (ThermoFisher Scientific, USA). For amplification, 50 ng genomic DNA and 10 pmol of each primer were used under the following PCR conditions: 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 min 30 sec, with final extension at 72°C for 10 min. For the ATP6 region the initial PCR cycling conditions were 2 min at 95°C for initial denaturation, 5 cycles of 94°C for 35 sec, annealing at 37°C for 55 sec and extension at 72°C for 1 min. This was followed by 30 cycles of denaturation at 94°C for 35 sec, annealing at 45°C for 55 sec and extension at 72°C for 1 min, with final extension at 72°C for 10 min and hold of 4°C. The PCR product was visualized on 2% agarose gel and the amplified PCR product was purified using a Purelink® Quick PCR Purification kit (ThermoFisher Scientific, USA), following the manufacturer's instructions.

The purified PCR products were sequenced by Eurrofins Genomics India Pvt. Ltd., Bangalore. The obtained sequences were compared with the database sequences available in the NCBI database, using the Basic Local Alignment Search Tool. The Barcode of Life Data System (BOLD) was used to generate DNA barcodes for nucleotide sequences.

### Phylogenetic analyses of sequence data

The phylogenetic tree was generated using a concatenated rDNA LSU and ATP6 dataset that included *Itajahya galericulata* from India and other species of the order *Phallales* from GenBank, which were selected according to Marincowitz et al. (2015) (Table 1). Multiple sequence alignment was done using Clustal-W embedded in MEGA7.0 (Kumar et al. 2016). The nucleotide substitution models that best fit our phylogenetic analyses were found using jModelTest 2 (Darriba et al. 2012), with models selected based on the Akaike information criterion (AIC). The TrN+I+G model was identified as optimal for LSU, and TPM2uf+I+G for the ATP6 region. A phylogenetic tree for two genes was constructed based on maximum likelihood (ML) analysis using PAUP* ver. 4.0 (Swofford 2002), with GTR+I+G as nucleotide substitution model. A heuristic search was generated with random taxon addition of sequences (10 replicates) and tree-bisection-reconnection branch swapping (TBR), as well as 1000 replicates to reach the ML bootstrap values. All positions containing gaps and missing data were eliminated during construction of the phylogenetic tree.

**Table 1**. GenBank accession numbers of taxa used for phylogenetic analyses. The newly obtained sequences are in bold.

| Species | GenBank accession numbers | |
|---|---|---|
| | LSU | ATP6 |
| *Anthurus archeri* | DQ218624 | DQ218913 |
| *Abrachium floriforme* | JF968440 | JF968438 |
| *Aseroe rubra* | DQ218625 | DQ218914 |
| *Clathrus chrysomycelinus* | DQ218626 | DQ218915 |
| *Dictyophora duplicata* | DQ218481 | DQ218765 |
| *Dictyophora indusiata* | DQ218627 | DQ218917 |
| *Dictyophora multicolor* | DQ218628 | DQ218918 |
| *Gelopellis* sp. 1 | DQ218630 | DQ218919 |
| *Gelopellis* sp. 2 | DQ218631 | DQ218920 |
| *Ileodictyon cibarium* | DQ218633 | DQ218922 |
| *Ileodictyon gracile* | DQ218636 | DQ218925 |
| *Itajahya rosea* | JF968441 | JF968439 |
| *Itajahya galericulata* | KR071851 | KR071848 |
| *Itajahya galericulata* | **MH168327** | **MH175196** |
| *Kobayasia nipponica* | DQ218638 | DQ218926 |
| *Laternea triscapa* | DQ218640 | DQ218928 |
| *Lysurus borealis* | DQ218641 | DQ218929 |
| *Lysurus mokusin* | DQ218507 | DQ218791 |
| *Mutinus elegans* | AY574643 | AY574785 |
| *Phallobata alba* | DQ218642 | DQ218930 |
| *Phallus costatus* | DQ218513 | DQ218797 |
| *Phallus hadriani* | DQ218514 | DQ218798 |
| *Phallus ravenelii* | DQ218515 | DQ218799 |
| *Protubera borealis* | DQ218516 | DQ218800 |
| *Protubera canescens* | DQ218645 | DQ218932 |
| *Protubera jamaicensis* | DQ218647 | DQ218933 |
| *Protubera maracuja* | DQ218518 | DQ218802 |
| *Protubera parvispora* | DQ218648 | DQ218934 |
| *Protubera sabulonensis* | DQ218649 | DQ218935 |
| *Simblum sphaerocephalum* | DQ218521 | DQ218806 |
| *Trappea darkeri* | DQ218651 | DQ218938 |

## Results

### Molecular identification and phylogenetic analyses

The newly generated nucleotide sequences are deposited in GenBank (www.ncbi.nlm.nih.gov) under the following accession numbers: MF506819 (ITS), MH168327 (LSU) and MH175196 (ATP6). A BLAST search in the GenBank database of LSU and ATP6 sequences revealed 99% base pair similarity to sequences of *Itajahya galericulata* from South Africa (Marincowitz et al. 2015). The ITS sequences were not available in GenBank; our generated ITS sequences of *I. galericulata* from India are the first for this species. The newly generated nucleotide sequences

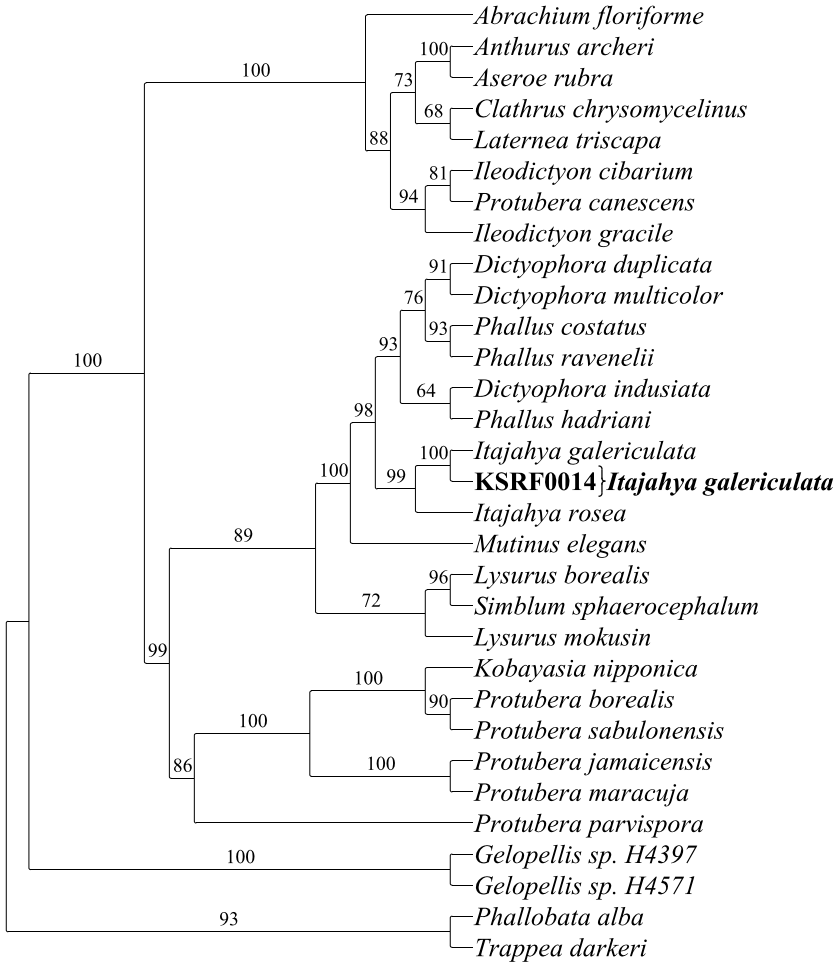


**Figure 1**. Maximum likelihood cladogram generated from concatenated LSU and ATP6 dataset for *Itajahya galericulata* and related taxa. *Phallobata alba* and *Trappea darkeri* were taken as outgroup. Bootstrap values are indicated on the tree branches.

of *I. galericulata* were also submitted to the Barcode of Life Data System (BOLD) to generate DNA barcodes (Sample ID: KSRF-0014). Phylogenetic analyses based on the two-gene (LSU, ATP6) dataset placed the sequences from sample KSRF-0014 (collected in the present study) in a strongly supported clade with *Itajahya galericulata* from South Africa (Marincowitz et al. 2015) (Fig. 1).

### Taxonomy

***Itajahya galericulata*** Möller, Bot. Mitt. Trop. 7: 79, 148. 1895 (Figs 2 & 3)

Synonym: *Phallus galericulatus* (Möller) Kreisel, Czech Mycol. 48: 275. 1996.

**Description.** Rod-shaped fungus popularly known as stinkhorn, with fruiting body 8.5–20 cm tall, shaped like a phallus emerging from an egg and possessing a white stipe with a cottony cap. Egg medium to large, oval, greyish white. Fruiting body develops from the egg during the night; fully developed fruiting body emerges from peridium 10–15 h later. Stipe white to light pink, smooth, sponge-like appearance due to the presence of several small compartments on it; stipe hollow, cylindrical in shape, tapering at both ends (i.e., top and base) and developing from volva with rhizomorphs at its base. Cap wiglike, turning black once the gleba has fallen; sometimes remnants of volva seen attached to cap; top of cap shows cottony white calyptra consisting of fine white lamellate plates. Gleba greenish-brown, with very strong and foul odour, making the fungus noticeable from a long distance. Basidia not observed. Spores smooth, slimy or sticky, hyaline, elliptical or slightly curved.

**Edibility.** Not known.

**Habitat.** Typically this fungus appears in sandy soils after rainfall between August and October, and is found associated with the roots of *Pithecellobium dulce* and *Prosopis juliflora* (*Fabaceae*) in India.

**Distribution.** Brazil, India, Paraguay and South Africa.

**Material examined.** INDIA. Gujarat State. Vadodara, Community Science Centre (voucher material) and the campus of the Maharaja Sayajirao University of Baroda (observation), 12 Aug. 2016, K. S. Rajput, R. Patel & A. Vasava (BARO 00357).

## Discussion

The morphology and molecular identification based on DNA sequences (ITS, LSU, ATP6) confirmed that the stinkhorn fungus collected in India belongs to *Itajahya galericulata.* This fungus was previously reported from South America (Brazil, Paraguay) (Möller 1895;


**Figure 2**. Morphology of *Itajahya galericulata*: A – Greyish white immature eggs ready for opening; B – Developing fruiting body emerging from the egg; C – Mature fruiting body; D – Habit and morphological features in natural habitat. Scale bars: A, B, C = 3 cm, D = 15 cm.

Campi Gaona et al. 2017) and from Africa (Republic of South Africa) (Marincowitz et al. 2015). This is the first report of the species for India and for the whole of Asia. According to Marincowitz et al. (2015), in South Africa *I. galericulata* commonly occurs in very close association with the root system of *Jacaranda mimosifolia*, a tree of the *Fabaceae* family. This tree is native to South America, where the fungus was originally described. Therefore they suggested that *I. galericulata* probably was introduced to South Africa together with *J. mimosifolia*. Interestingly, in India we also found *I. galericulata* to be associated with trees of the *Fabaceae* family: *Pithecellobium dulce* and *Prosopis juliflora*, which are native to South America. This suggests that the occurrence of *I. galericulata* in India and Asia is the result of introduction from South America. The newly generated molecular

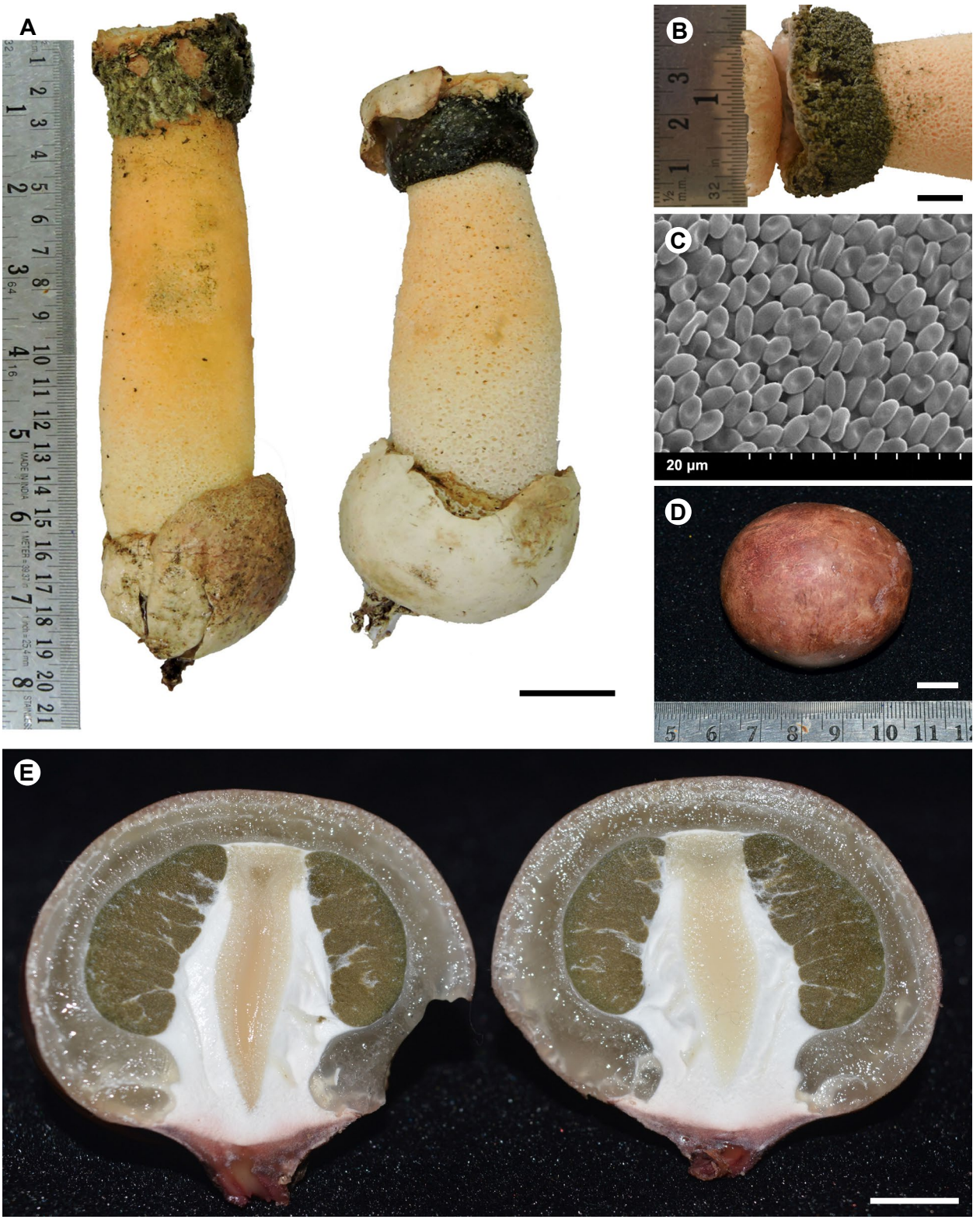


**Figure 3**. Dimensional details of *Itajahya galericulata* fruiting body: A – Mature fruiting body; B – Calyptra and gleba; C – SEM of basidiospores; D – Developing immature egg; E – Egg dissected to show internal features. Scale bars: A = 3 cm, B = 1 cm, C = 20 µm, D = 1 cm, E = 1 cm.

data should stimulate further study of *I. galericulata* and the genus *Itajahya*.

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