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First report of *Termitomyces bulborhizus* holomorph from Goa, India

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ABSTRACT

This is the first report of *Termitomyces bulborhizus* T.Z. Wei, holomorph from Taleigao, Tiswadi and Latambarcem, Bicholim, Goa, India, along with its habit, habitat, sporodochial stage (anamorph), sporophores (teleomorph) and characters in pure cultures. *T. bulborhizus* is characterized by pale brown to darker, rugose pileus, having swollen stipe with bulbous base showing melanised, striate, rivulose surface. During the survey as many as 13 different developmental stages of the mushroom from primodia to mature sporophores were documented. Spherocysts with lipid globules were prominently detected in subhymenium. Sporodochial culture showed thin walled, simple septate hyphae. Basidiome context culture from the teleomorph showed typical conidia, globose sphaerocysts, aggregated hyphae, and hyphal knots with associated mycelia which take up intense staining with Ammonical Congo Red in addition to the presence of numerous Calcium oxalate mycoliths.

Keywords: Holomorph, anamorph, teleomorph, mutualistic system, *Termitomyces,* developmental stages

INTRODUCTION

The basidiomycetous genus *Termitomyces* is found in a symbiotic relation with termites belonging to subfamily *Macrotermitinae* (Sands, 1969; Heim, 1977; Dixon, 1983; Johjima *et al.*, 2006). The anamorphic stage of *Termitomyces* is maintained underground (hypogeal) inside the termite mound on fungus comb in the form of numerous small white spherules called as sporodochia, which in natural African fungal combs were described by Botha and Eicker (1991). During monsoon season sporophores, the teleomorphic stage of *Termitomyces* appears epigeally using a hypogeal basal pseudorrhiza. There are 86 taxa listed in Index Fungorum which may also include synonyms (www.indexfungorum.org/). *Termitomyces* spp. are distributed throughout tropical and subtropical areas in Africa and Asia (Rouland-Lefevre *et al.,* 2002; Johjima *et al.*, 2006). In India 8 new taxa were described which included *T. albidolaevis* Dhanch, Bhatt & Pant, *T. heimii* Natarajan, *T. indicus* Natarajan, *T. longiradicatus* Sathe & Daniel, *T. quilonensis* Sathe & Daniel, *T. pooensis* Sathe & Daniel, *T. radicatus* Natarajan, and *T. microcarpus* f. *santalensis* Heim later considered as *T. indicus* (Natarajan, 1979; Pegler and Vanchaecke, 1994; Tang *et al.*, 2005). *Termitomyces* species are reported from India includes *T. badius* Otieno, *T. clypeatus* Heim, *T. cylindricus* Heim, *T. eurrhizus* (Berk.) Heim, *T. heimii* Natarajan, *T. indicus* Natarajan, *T. indicus* var. *patialensis* Atri *et al.*, *T. mammiformis* Heim, *T. medius* Heim & Grasse, *T. microcarpus* (Berk. & Broome) Heim, *T. rabuorii* Otieno, *T. radicatus* Natarajan, *T. robustus* (Beeli) Heim, *T. sagittiformis* (Kalchbr & Cooke) Reid, *T. schimperi* (Pat.) Heim, *T. striatus* (Beeli) Heim, *T. tylerianus* var. *macrocarpa* Atri *et al.* and *T. umkowaan* (Cooke & Massee) Reid, etc. (Purkayastha and Chandra, 1975; Atri *et al.*, 2005; Karun and Sridhar, 2013). Two thirds of the *Termitomyces* species have been reported from six states of the Western Ghats known as biodiversity hotspot. According to Karun and Sridhar (2013), Kerala State possesses a total of 15 *Termitomyces* species, Karnataka 9 species, Tamil Nadu 2 Species and Maharashtra 3 species. *Termitomyces* species checklist from Goa includes 35 species (Kamat, 2016). In this paper we describe holomorph of *Termitomyces bulborhizus* from Goa, with taxonomic, cultural and developmental features for the first time from India.

MATERIALS AND METHODS

Collection of natural anamorphic state: The surveys for the collection of *Termitomyces* spp. were carried out during 2001-2014 in North Goa District, Goa in permitted non-forest areas. Termite mounds were identified and ventilator dome was sliced opened to expose the fungus combs (**Fig. 1a, b**). Soil thermometer was used to determine the external and internal temperature of the mound. Fungus comb samples with sporodochial masses were collected in sterile polythene bags and further processed in the laboratory same day, to isolate the sporodochia.

Collection and identification of teleomorph: *Termitomyces bulborhizus* primodia and sporophore specimens along with different developmental stages were collected in monsoon season from termite mounds previously sampled for sporodochia located in Tiswadi, Goa (**Fig. 1c, d**). Additional samples were collected from Bicholim, Goa. These specimens were identified using descriptions and keys (Wei *et al.,* 2004; Sawhasan *et al.*, 2011; Daniels *et al.*, 2015). The dried herbarium specimen is preserved in Goa University Mushroom Herbarium (GUMH).

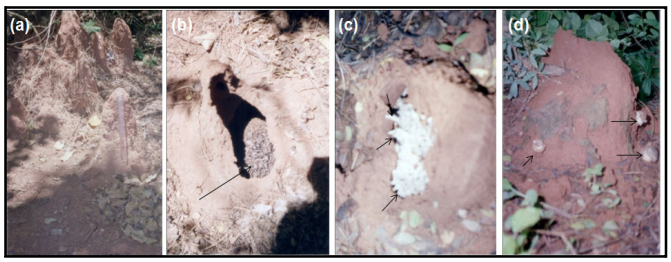

Fig. 1. Sampling site of *T. bulborhizus*. (**a**) Termite mound possibly of *Odontotermes* sp., (**b**) Exposed fungus garden with intact comb below the ventilator, (**c**) A cluster of primodia, (**d**) *T. bulborhizus* mature sporophores.

Micromorphology of anamorph and microanatomy of teleomorph: Mature single sporodochia were identified under steromicroscope and mounted in lactophenol cotton blue. Microanatomical sections of sporophores were studied using samples from Taleigao collection. Cryostatic microtome sections of various anatomically distinct regions such as primodia, umbo, gill, stipe and pseudorrhiza were mounted in plain lactophenol, lactophenol cotton blue, and stained in Congo red and mounted in 1% glycerol. Lipids were detected using Sudan IV.

Culture of anamorph and teleomorph: Pure mycelial culture of anamorphic comb sporodochia was obtained by sampling steromicroscopically and by aseptically removing healthy intact sporodochia using flame sterilized and cooled needle and placing it on the surface of 2% (w/v) Malt Extract Agar (MEA)(Hi Media) plates containing 0.2 mg/ml streptopenicillin to retard bacterial growth. Whereas teleomorph pure culture was obtained from the context by tissue culture method as described by (Stametes and Chilton, 1983). Aseptically 0.5 cm explants of pileus context were inoculated on 2% MEA along with incorporation of 0.01mg/ml of Nalidixic acid and Neomycin. Isolation plates were incubated in an incubator at 28°C in the dark for up to 3 weeks. Pure culture was obtained by hyphal tip technique and the purity was determined microscopically. Working cultures were maintained on fresh 2% MEA plates without antibiotics and deposited in Goa University Fungal Culture Collection (WFCC Reg. no. 946). Colony growth characters and micromorphology of isolates were recorded up to 14 days (Botha and Eicker, 1991). Slides from colony representing growth regions of interest were prepared with Ammoniacal Congo Red (ACR) and Lactophenol cotton blue stains and documented using Nikon Eclipse E200 microscope. Images were captured at different magnifications using Nikon DS-fi2 camera and NIS elements microscope imaging software.

RESULTS

Two locations yielded *T. bulborhizus* -Taleigao habitat location was fruitful in yielding samples of anamorph and different stages of teleomorph while the second location was not examined for anamorph.

Termite mound located at Taleigao was 30 cm in height and was present under *Pithecellobium dulce* and *Calotropis gigantia*. Temperature outside the mound was 36°C whereas internal temperature was 33°C. The fungus comb was soft, spongy, brownish to grayish black with strong oily odour and displayed numerous galleries comprised of finely divided plant litter. The surface was fully covered with white sporodochia. *T. bulborhizus* teleomorph (**Fig. 2a-d**) showed 13 developmental growth stages from primordia to fully mature sporophore (**Fig. 2e: I-XIII**). At stage I conical white initials were seen which at stage II turn out to be pointed and melanised at the tip. Stage III was represented by elongated primordia with immature perforatorium (pre pseudorrhiza stage). Pre bulbous stage was seen at stage IV. Later at stage V small bulbous stage exhibited melanised perforatorium. Stage VI represented the transient phase from hypogeal to epigeal elongated bulbous teleomorph. Stage VII showed bulbous stipe with developing pileus. Later stages including

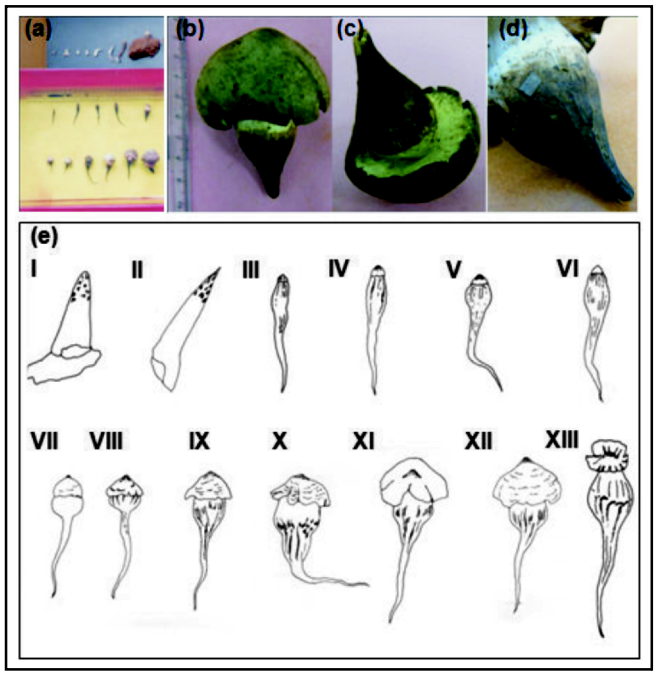

Fig. 2. *Termitomyces bulborhizus* teleomorph. **(a)** Developmental stages of *T. bulborhizus,* (**b**) teleomorph, **(c)** Inverted basidiome showing thick incurved margin and free lamellae, **(d)** Rivulose stipe surface composed of dark melanized striations, **(e)** Bivelanogiocarpic developmental **(I-XIII)** stages. Epigeal : (**I**) Primordia with basal disc stage 1 (**II**) Primordia stage 2 (**III**) Epigeal pseudorrhizal stage (**IV**) Transient pseudorrhizal stage 1 (**V**) Transient pseudorrhizal stage 2 with bulbous stipe (**VI**) transient stage 3. Hypogeal: (**VII**) Teleomorph stage 1 (**VIII**) Teleomorph stage 2 (**IX**) Teleomorph stage 3 (**X**) Teleomorph stage 4 (**XI**) Teleomorph stage 5 (**XII**) Teleomorph stage 6 (**XIII**) Stage of fully developed teleomorph

stage VIII showed enlargement of the bulbous region of teleomorph and the pileus which appeared rugose with incurved margin at stage IX. Stage X showed rugose pileus with irregular splitting of the margin and with rivulose melanised striations on the bulbous region. Enlargement of the pileus was seen at stage XI and XII. Mature teleomorph was complete in all respects seen at stage XIII with fully opened pileus having thick reflexed margin.

TAXONOMY (TELEOMORPH)

Termitomyces bulborhizus T.Z. Wei, *Mycol. Res.* **108**: 1458-1462, 2004 (**Figs. 2-4**)

Pileus 3 - 6 cm in diam., typically triangular or convex, with a broadly round or blunt pointed perforatorium; surface charcoal black at the centre, elsewhere pale brown to brown and rugose; margin thick, uniformly incurved, up to 3-4 mm, often splitting irregularly. Lamellae free, white, 8 mm wide, densely crowded, with unequal lamellulae. Stipe 4.5 cm long above the ground, white to gray, 0.8-6 cm thick, enlarged to 3-9 cm in diam. at the ground level and usually abruptly forming a prominent globose bulb below the ground, solid, robust, fibrous; surface white above and dark brown on the bulb with black visible hyphal strands, finally almost tapering and becoming pyramidal or conical in shape. Partial veil absent.

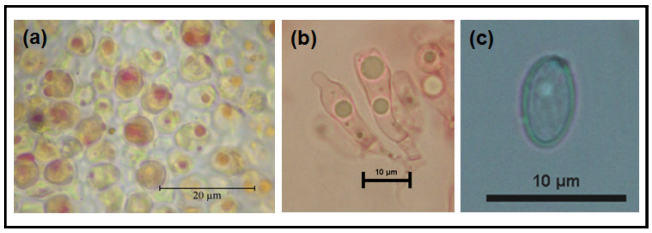

Fig. 3. Microscopic characters of *Termitomyces bulborhizus* teleomorph. (**a**) Isodiametric oleiferous spherocysts in subhymenium, (**b**) Cystidia and bisporic basidia with lipids globules, (**c**) Ellipsoidal basidiospores with smooth wall and membranous exosporium.

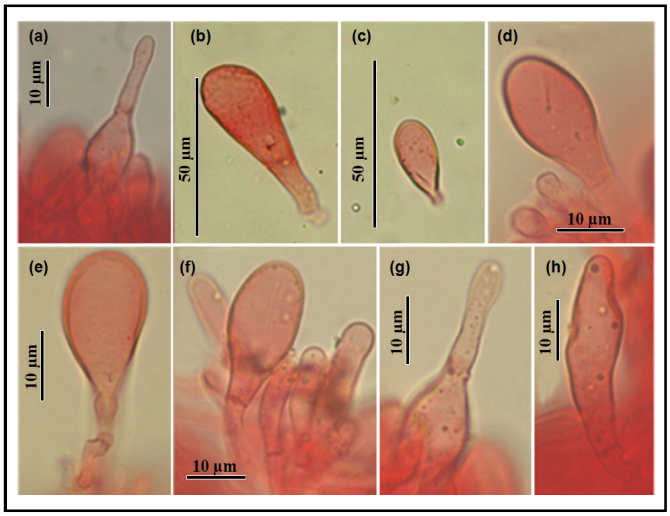

Fig. 4. Cystidial polymorphy in *Termitomyces bulborhizus*. (**a**) Lecithiform, (**b-c**) Clavate, (**d-e**) Spheropedunculate, (**f**) Fusiforms and Spheropedunculate, (**g**) Lecithiform, (**h**) Ventricose.

Pseudorrhiza small, subradicate, uniformily black, melanized, rough and solid, up to 1-5 cm long, 0.5-2 cm broad, starts narrowing immediately below the bulb. Pseudorrhiza with thin walled melanized hyphal cells. Basal disc small, indistinguishable. Context up to 2 cm thick, white, comprising of repent, thin-walled 2.5-8 µm broad hyphae, inflating to 35 µm in diam. Spore deposit pink. Subhymenial layer 10-25 µm wide with globular, isodiametric sphaerocyst cells distinct with Sudan IV positive lipid content (**Fig. 3a**). Basidia 16.5-27 x 4-9 µm, clavate, granulated bearing two sterigmata (**Fig. 3b**). Basidiospores 5-8.5 x 3.5-6 µm, ovoid to ellipsoid, subhyaline, thin-smooth walled with membranous episporium (**Fig. 3c**). Hymenophoral trama regular, 50-80 µm wide, composed of 3.5-20 µm broad hyaline hyphae. Cheilocystidia clavate to pyriform, 19-60 x 12-34 µm in size, thin-walled. Pleurocystidia 19-78 x 10-32 µm, polymorphic, thin walled (**Fig. 4**).

Specimens examined: TAL/TB-1/2001. Taleigao, Tiswadi, Goa, India. May-August 2001, collected by Nandkumar M. Kamat and Harshala S. Gad (GUMH-TAL-/TB-1/2001) . LB/1/2014. Latambarcem, Bicholim. July 2014, collected by Ankita Mopkar, (GUMH-LB/1/2014).

Micromorpholgy of sporodochial anamorph

Comb sporodochia monilioid, thin walled, smooth, ellipsoid to broadly cylindrical with truncate to slightly obtuse ends; conidial chains repeatedly branched sympodially. Conidial chains develop from undifferentiated conidiophore hyphae with the same diameter as the conidia. Deeply stained granular protoplasm was present within. Holoarthic conidia produced in basipetal succession. Conidia spread all over after separation of the transverse septa. Conidiogenous hyphae, terminal and determinate. Globular cyst thick walled, inflated cells situated at the periphery of sporodochia, consisting of (i) basal obovate, ellipsoid cell with truncate end, deeply staining contents and a slightly thickened cell wall. (ii) an apical globose to subglobose cell with a thick wall, bipolar staining and granular protoplasm. The micromorphological details are shown in (**Fig. 5a**).

Microanatomy of teleomorph

Bivelangiocarpic development from primodia to bulbous stages could be easily discerned. Sporophore primordia represent the first hypogeal stage which arises from the differentiated sporodochia. Primordia comprises of mosaic of short hyphal strands. Hyphae short, simple, thin walled, up to

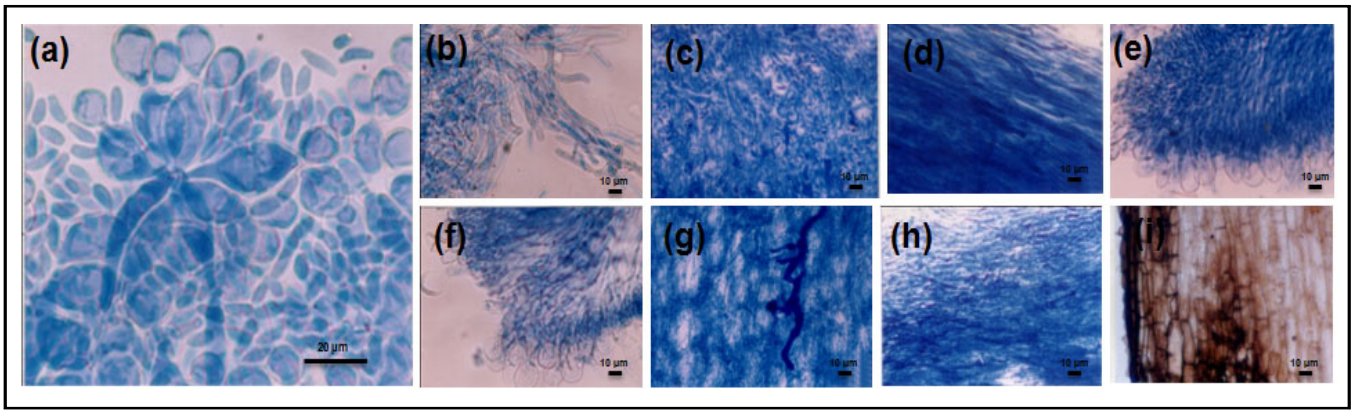

Fig. 5. Micromorphology of comb sporodochia and microanatomical features of various developmental stages of teleomorph. **(a)** sporodochia, **(b)** T.S. passing through primordium, **(c)** L.S. passing through umbo, **(d)** L.S. pileal context tissue, **(e)** L.S. showing hymenophoral trama, **(f)** L.S. of lamella showing cystidia and basidia, **(g)** L.S. of pileus showing laticifers darkly stained, **(h)** L.S. of stipe, **(i)** L.S. of melanized pseudorrhiza

1µm wide, hyaline, unbranched and inflated (**Fig. 5b**). The primordia further differentiates to give rise to pseudorrhizal transient stage, which further develops into the epigeal portion of the sporophore comprising of pileus, stipe and pseudorrhiza. The pileus comprises of the prominent umbo. Umbonal tissue comprises of short hyphal cells which are compactly arranged with darkly stained laticifers (**Fig. 5c**). Pileal context comprises of thin walled, elongated hyphae having uniform diameter and darkly stained cyanophilic laticiferous cells criss-crossing the hyphae (**Fig. 5d**). Hymenophoral trama divergent, comprising of interwoven thin walled hyphae. The edges of hymenophoral trama are lined by polymorphic cheilocystidia and clavate basidia (**Fig. 5e-f**). Stipe with simple, thin walled, hyaline hyphae, showing presence of laticifers (**Fig. 5g-h**). The wall layers of pseudorrhizal region shows darkly melanized hyphal cells (**Fig. 5i**).

Cultural state of anamorph

Colony white, comprised of slow, dense growth mode with thin walled, hyaline, profusely branched hyphae, having simple septa, with deeply staining granular protoplasm in young rapidly growing leader hyphal tips (**Fig. 6a**).

Morphology of teleomorph culture (Fig. 6b-i)

Growth characters: Growth is slow, colony diameter 12-15 mm after 3 weeks of growth. Advancing zone bayed, farinaceous, appressed for 3-5 mm then raised. Mycelium mat creamy white, moderately soft forming granular stroma, covered with numerous globose, cottony, white 0.1-0.3 mm broad sporodochia.

Hyphal characters: Hyphae in the advancing zone thin-walled, sympodially branched with simple septa. Aerial mycelial hyphae as in advancing zone 2-5 µm wide. Conidiophores monilioid, thin walled, 10-38 x 8-13 µm. Conidia cylindrical to broadly ellipsoid or reniform, produced in an acropetal manner, cyanophilic. Hyphae are highly constricted at septate regions and prominently somatogamous.

Submerged mycelium: Hyphae in advancing zone with many hyphal twist and knots. Mycelia cords are also seen with extracellular polysaccharides. Contain large ungerminated conidia scattered throughout the agar and of variable shapes, either single or in clusters.

Termitomyces bulborhizus culture is slow growing, creamy white with granular stroma and many sporodochia at older region along with farinaceous advancing zone. Microscopically culture shows extracellular polysaccharides production indicated by regions darkly stained with ACR and also found to produce numerous Calcium Oxalate mycoliths.

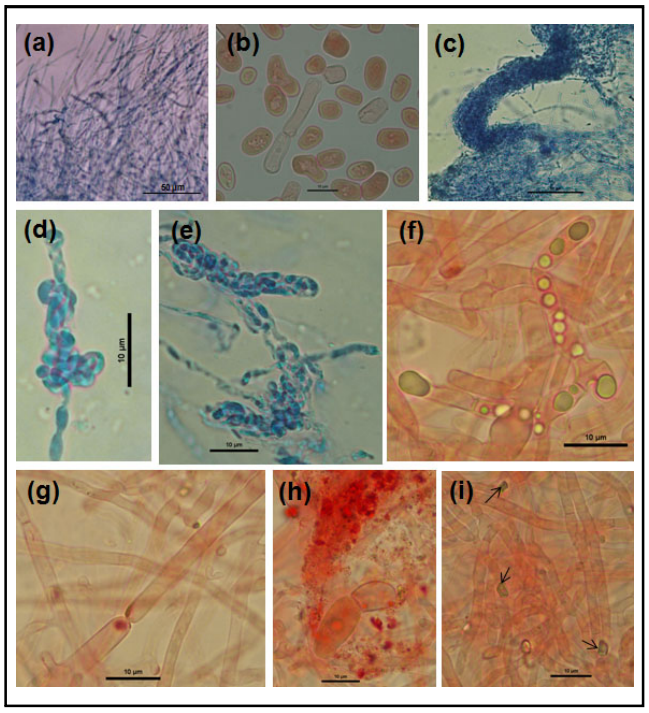

Fig. 6. Micromorphological details of sporodochial (anamorph) and sporophore (teleomorph) pure cultures. **(a)** sporodochial thin walled hyphae, **(b)** Conidia often single or in clusters showing ACR stained granulated protoplasm, **(c)** Mycelial cord showing high degree of differentiation, **(d-e)** Complex hyphal knots, **(f)** Oleiferous hypha with distinct lipid globules, **(g)** Hyphal tip fusion, **(h)** Extracellular polysaccharide darkly stained with ACR **(i)** Calcium oxalate mycoliths.

DISCUSSION

This is the first report of *T. bulborhizus* from India and also first work on the holomorph of any termitophyllous mushroom from India. Distribution and characteristics of *T.*

Table 1: Comparison of Indian collection of *T. bulborhizus* with the collections of this species documented from elsewhere

Name of spp.	Pileus diameter (cm)	Stipe length (cm)	Bulbous base diam. (cm)	Pseudorrhiza length (cm)	Cheilocystidia	Pleurocystidia	Basidia	Spores	Location & Distribution	Reference
Termitomyces bulborhizus T.Z. Wei	9.2-21	5-9 x 6-8	8.7-14.5	1-4	Not determined	Not determined	16-25 x 4-9 µm	5-8.5 x 3.5-6 µm	Thailand- Sai Yok district, Kanchanaburi province	Sawhasan *et al.*, 2011
	4-12	(4-)7-11 x 0.5-2	2-3.5	5-20	27-42 x (11-) 13-24 µm	28-56 x (10-)11-20(-35) µm	18-24 (-27) x 6-8.5 µm	(5.5-)6-7(-8) x (3-)3.8-4.4(-5) µm	Niger-Tillabery	Daniels *et al.*, 2015
	(5-) 10-22	3-13 x 0.8-6	3-9	80	19 - 60 x 12-34 µm	19-78 x 10-32 µm	17.5-27 x 5.5-9 µm	6-9 x 4-6 µm	China- Miyi, Chengdu, Sichuan province; Kunming, Yunnan province; Xishuangbanna Tropical Botanical Garden (CAS), Mengla	Wei *et al.*, 2004
	3-6	4.5	3-9	1-5	19-60 x 12-34 µm	19-78 x 10-32 µm	16.5-27 x 4-9 µm	5-8.5 x 3.5-6 µm	India- Latambarcem, Bicholim Goa	Present study

bulborhizus recorded in other countries has been tabulated in **table 1**. It can be seen that sporphores in Goa are smaller with inconspicuous pseudorrhiza. Pleurocystidia and cheilocystidia are identical and well conserved in sporophores from both India and China whereas basidia as well as spore morphology of sporophore in Goa is quite identical to those reported from Thailand. Reyes *et al*. (2016) has also reported *T. bulborhizus* from Luzon, Philippines based on molecular identification but it is not accompanied by details of species characters.

T. bulborhizus reported from Goa is found to be a distinct Indian ecotype , an intermediate species as indicated in **table 1**. The micromorphological details were found to be similar to those reported by Batra and Batra (1966) and Botha and Eicker (1991) in other *Termitomyces* species examined by them. The microscopic characters are also similar in branching pattern of conidial chains (conidiogenous hyphae), conidium ontogeny and presence of large inflated globular cysts with thickened refractive walls situated at the periphery of sporodochia. Interestingly *T. bulborhizus* was found to be oleiferous as the cells close to the hymenium showed lipid storage in globular form. Lipid contents have also been reported in different *Termitomyces* species such as *T. heimii* (Abd Malek *et al*., 2012), *T. clypeatus*, *T. letestui* and *T. microcarpus* (Woldegiorgis *et al*., 2015; Ogwok *et al*., 2017). These storage depots of lipids within the carpophores context tissue may play an important nutritional role in *Termitomyce*s during periods of stress. These stored lipids being hydrophobic, may also be required to produce hydrophobicity in the fertile tissue so as to withstand heavy rains and prevent rapid decomposition of fertile tissue before the normal discharge of basidiospores. It has not escaped our notice that the speciation event and present distribution of *T. bulborhizus* in Niger, India, China, Thailand may be linked to plate tectonics and continental drift since India separated from Madagascar and Africa about 89-91 Mya (Vérard *et al.,* 2017). We consider the Niger, African species as ancestral, the Indian ecotype as intermediate and specimens from China and Thailand as well developed and distinct. However, detailed molecular phylogenetic studies would be required to solve the mystery behind the sparse fruiting, peculiar habit, unique morphology and relatively rare occurrence, distribution and oleiferous nature of *T. bulborhizus*. We predict that exhaustive surveys may reveal good distribution of *T. bulborhizus* in relatively unexplored or poorly explored regions and specially on lateritic plateaus in the Western Ghats as also in northeastern states of India. Finally the olieferous nature of the mushroom indicates its potential in biotechnology for tapping its edible lipid reservoir.

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