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RESEARCH ARTICLE

Identification, isolation and *in vitro* culture of a wild strain of *Ganoderma australe* from Sikkim, India

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Abstract

Pure cultures of *Ganoderma* species were isolated from pristine forests of the eastern Himalayan region, Sikkim, India. Macroscopic, microscopic, and molecular analysis, including ITS sequencing confirmed the identity of the strain as *Ganoderma australe* (Fr.) Pat. Herein we report *Ganoderma australe,* a strain not previously reported from the eastern Himalayan region and establishment of its fructification in in-vitro condition. *Ganoderma australe* has been reported to possess various bioactive compounds, such as triterpenoids, polysaccharides, and phenolic compounds, which contribute to its potential medicinal properties. The pure culture *G. australe* strain isolated in the present study was successfully tested in two different substrate bases (maize and wheat) for spawn production. Fructification was observed in all the substrate bags containing different wood combinations sawdusts and rice straw as the main organic substrate supplemented with cornmeal and wheat bran. The present findings underscore the rich fungal biodiversity of Sikkim and also provide valuable insights into effective in vitro cultivation strategies of potential medicinal mushrooms.

Keywords: Sikkim Himalayas; Medicinal mushrooms; Pure culture; *Ganoderma australe*; spawn production; fructification

1. Introduction

Among the diverse array of medicinal mushrooms found in the world over, *Ganoderma* spp. have garnered considerable attention due to their therapeutic potential and long-standing use in traditional medicine, and ethno mycological uses especially in Asian countries, where they are known as "Lingzhi" in China and "Reishi" in Japan (Wu et al., 2020; Zhou and Dai, 2012). The bioactive compounds extracted from these fungi, includes triterpenoids, polysaccharides, and sterols, have been demonstrated to possess a range of therapeutic effects, such as immunomodulatory, anti-inflammatory, and anticancer activities (Hapuarachchi et al., 2015; Chen et al., 2012). Previous studies have revealed a remarkable diversity of *Ganoderma* species worldwide (Yuen and Hyde 2002; Richter et al., 2015). Up to now, 181 species are taxonomically accepted under the genus *Ganoderma*, making it one of the most species-rich genera in Ganodermataceae (Costa Rezende et al., 2020). The ability of these fungi to decompose lignocellulosic materials makes them integral to forest health and ecosystem stability (Schwarze et al., 2000). *Ganoderma* are ecologically indispensable as saprophytes, but some of them are pathogenic and can cause diseases in forest trees; they can cause white rot in hardwoods by decomposing lignin, cellulose, and related polysaccharides (Ding et al., 2020). *Ganoderma* fungus being essential decomposers is also crucial to the global carbon and nutrient cycles.

The biodiverse regions, such as Eastern Himalayas, India, represent a rich and largely untapped fungal resource with significant ecological and medicinal potential. The temperate and subtropical climates of the region support a good diversity of macrofungi including the genus *Ganoderma* (Wangdi et al., 2019; 2021). Pure cultures of *Ganoderma* spp. offer important taxonomic features and can be used in biotechnology and biomedicine (Badalyan et al., 2015). Successful cultivation of *Ganoderma* not only provides a reliable supply of fruiting bodies and mycelium for medicinal use but also offers economic opportunities for small-scale farmers and commercial producers while ensuring the conservation of these rare species. In recent times advances in cultivation techniques have led to improved yields and quality of *Ganoderma* products, making them more accessible for both research purposes and commercial applications (Stamets, 2000). Since there has been no previous attempt to isolation of *Ganoderma sp.* from Sikkim, the present study aims to explore the diversity, detailing methodologies for their isolation and cultivation, and discussing their ecological and medicinal significance, thereby contributing to the fields of mycology, ecology, and medicinal mushrooms.

2. Material and methods

2.1. *Study area and sample collection*

Sikkim is a small mountainous state of India, situated in Eastern Himalaya, between 27°04'46" and 28°07'48" North latitudes and 88°00'58" and 88°55'25" East longitudes in terms of GPS coordinates. Spanning approximately 7096 km, it has an elevation ranging from 300 to 8585 meters (Figure 1). Opportunistic and random samplings were carried out between April to July 2021-2022. The habitat and morphological characteristics of the macrofungi were noted and photographed. Fresh specimens were collected with great care without any damage using sterile tools, and debris was removed using a soft brush and placed in sterile plastic bags, labeled with the collection date and location, and brought to the laboratory for further analysis and culture isolation.

2.2. Morphological characterization and identification

Macroscopic and microscopic observations were carefully recorded, laying the foundation for further analysis and identification. Macro-morphological characters such as the type of basidiocarp (laccate or

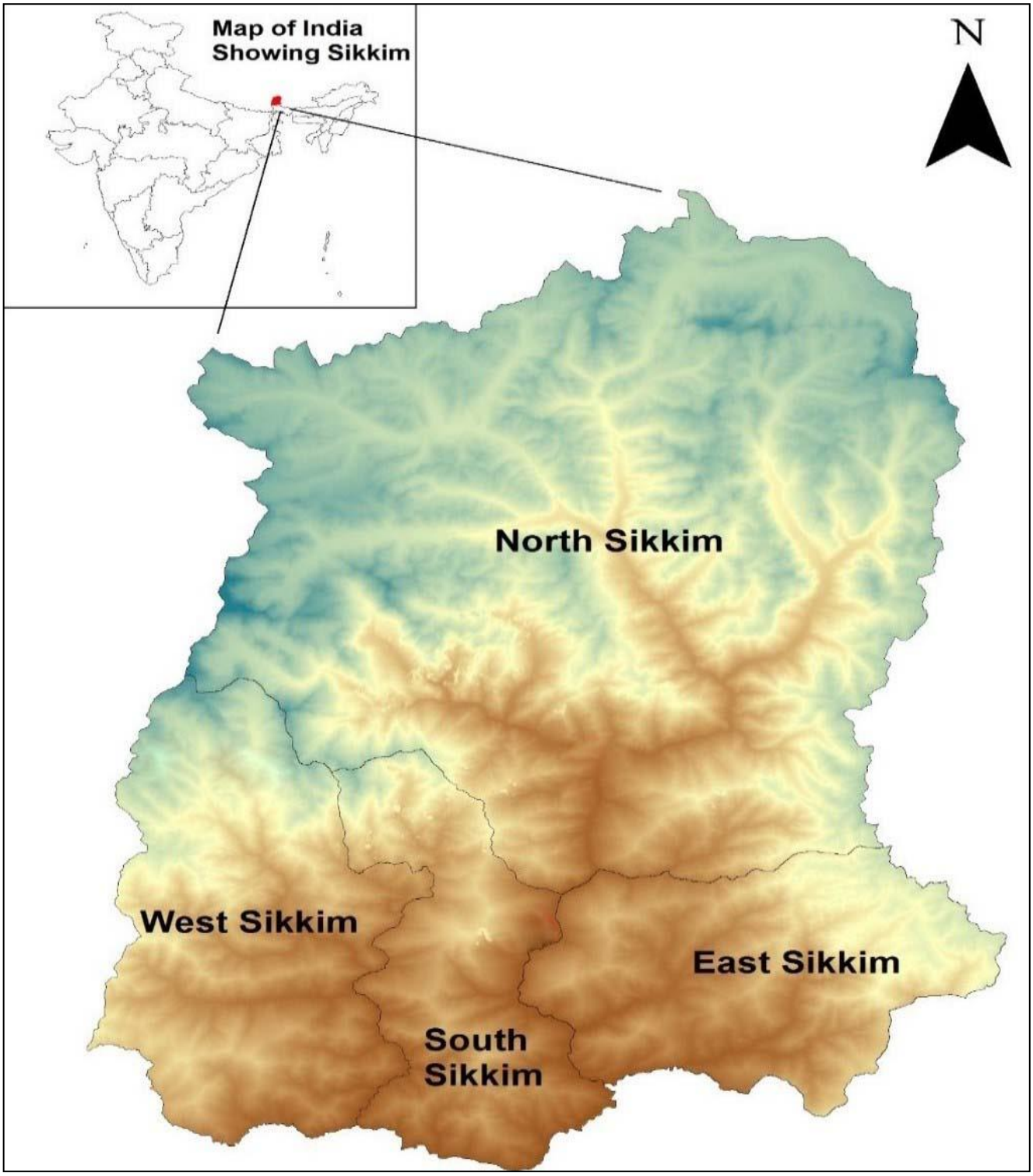

Figure 1. Map of study site - Sikkim Himalaya, India.

non-laccate and stipitate, sessile, or dimidiate), length and width, concentric zones, margin shape (lobed, rounded/acute), margin colour (brown, white, reddish, etc.) were recorded. The cap with pore side down is placed on clean black and white paper; the fruit body was covered with clean paper and left overnight. Basidiospores obtained from spore prints of each specimen were examined under a microscope by mounting them in a drop of lactophenol drop at 100× magnification. Spore colour, spore surface, tube colour, pore size, and the shape was recorded. The morphological characters recorded were compared with keys and taxonomic literature specific to *Ganoderma* spp. (Ryvarden 2000; Smith and Sivasithamparam, 2003b; Gottlieb and Wright, 2000; Lincoff, 1982; Lodge et al., 2004; Kornerup and Wanscher, 1978). A voucher specimen was deposited at the Mycology and Plant Pathology, Department of Botany, NBBGC Tadong.

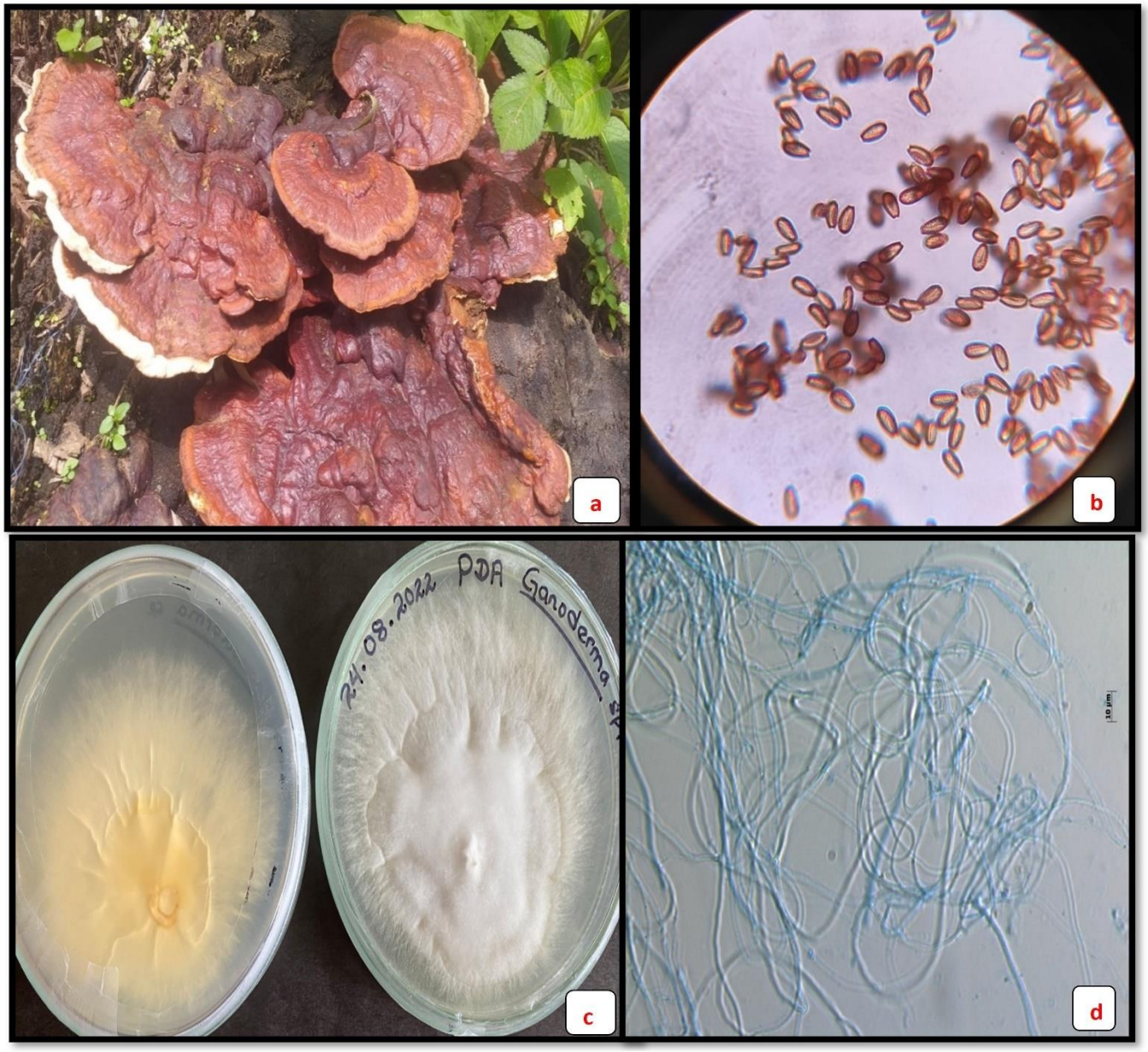

Figure 2. *Ganoderma* species macroscopic and microscopic features (**a-d**); **a**. *Ganoderma australe* basidiocarp, **b**. Spore print of basiodiocarp, **c**. Pure culture of *G. australe* after 10 days of incubation, **d**. Hyphae of *G. australe*.

Table 1. Fruiting initiation comparison in different substrate combination.

Compost	Mycelium establishment	Primordia head initiation	Basidiocarps formation
Substrate 1- Alder sawdust + Cornmeal	10-15 days	1.5 months	2-4 months
Substrate 2- Alder sawdust + Wheatbran	10-15 days	1.5 months	2-4 months
Substrate 3- Oak sawdust + Cornmeal	10-15 days	1.5 months	2-3 months
Substrate 4- Oak sawdust + Wheatbran	10-15 days	1.5 months	2-4 months
Substrate 5- Pine sawdust + Cornmeal + Rice Straw	10-15 days	1 month	2-3 months
Substrate 6- Pine sawdust + Wheatbran + Rice Straw	10-15 days	1 month	2-3 months

2.3. *Isolation of pure mycelium culture*

A standard tissue culture technique of Stamets (2000) was followed to isolate pure mycelial culture. *Ganoderma* basidiocarps freshly collected from the wild were thoroughly rinsed with water to remove dirt and dust; surface sterilised with 70% alcohol, again rinsed with sterile water, and blotted dry with tissue paper. Bits of internal tissues (0.5–1 cm) of basidiocarp were taken with the help of sterilised forceps and then surface sterilised with 3% sodium hypochlorite solution for 2-3 minutes before rinsing in sterile distilled water 3 times, after which the bits were dried on sterile Whatman filter papers under aseptic conditions in the laminar flow inoculation chamber. Placed the explants on the sterilised Potato Dextrose Agar (PDA) amended with chloramphenicol in the petri plates and incubated at 27±2°C for 7-10 days. Actively growing mycelia were transferred to fresh PDA media and incubated at 27°C for 10–15 days for pure

culture isolation. Morphological features of the isolate (shape, colour, and texture of the colony) and the growth rate of the mycelium were recorded.

2.4. *Molecular identification and preservation of culture isolates*

Genomic DNA obtained from Ganoderma culture isolates (MF-4) was used for molecular identification; the ITS-rDNA partial gene was successfully amplified using primers ITS4 and ITS5. The sequencing PCR was set up with the ABI-BigDye® Terminatorv 3.1 Cycle Sequencing Kit. The sequence data was aligned with publicly available sequences and analyzed to reach the identity of the culture isolate (Altschul, 1990). Mushroom spawn production requires the preservation of strains and genetic traits of axenic cultures. The pure culture discs were made with a sterile cork borer (about 3 mm). Culture discs were placed in 10 mL screw cap tubes with paraffin liquid labelled with the strain Id following the methods of Johnson and Martin (1992) and Nakasone et al., (2004). The tubes were subsequently stored in the NBBGC's Myco-patho laboratory at Tadong, Gangtok, Sikkim, at 4°C for short-term preservation. Molecular identification was carried out at the National Fungal Culture Collection of India (NFCCI), Pune, Maharashtra. For long-term preservation, the pure culture was deposited at the Aghakar Research Institute's culture collection centre in Pune, India, with the accession number (MF-4-5389).

2.5. *Spawn Preparation*

Three different substrates were used, viz., wheat, paddy, and maize grain, for spawning to investigate the possible growth of an isolated culture of *Ganoderma* strain. The grains were washed 2-3 times and soaked in water overnight. Following that, the water was drained and the substrate was boiled for 20-30 minutes to soften grains and disinfect before being dried on sterile sheets for half an hour or two to reduce the moisture content. Finally, 10 g of calcium sulphate ($CaCO_3$) was combined with all the substrates respectively, to maintain at pH 7 and for clump-free substrates. Sterilised conical flasks were three-fourths filled with substrates, the flask's mouth was closed with non-absorbent cotton plugs, and thereafter autoclaved twice at 121°C for 15 minutes at 15 psi. After sterilisation, the flasks were allowed to cool for 1 hour, then with the help of a pre-sterilized cork borer, 5 mm discs of mycelial mat from pure cultures along with a fraction of PDA were inoculated into the different substrate grains in sterile conditions and incubated in the dark for 15–20 days at a temperature 23°C – 24°C. After plugging, the containers were shaken to distribute the mycelium fragments. The spawn bottles were examined for late contaminants during storage in the refrigerator for three weeks at regular intervals (Borah et al., 2019; Nwanze et al., 2005). The prepared spawn was used for inoculating different solid-state substrate combinations for fruiting development.

2.6. *Compost preparation*

In the present study, rice straw, sawdust, and cornmeal and wheatbran were the main compost substrates used with six different combinations, viz; 1. Alder sawdust (250g) + Cornmeal (100g), 2. Alder sawdust (250g) + Wheatbran (100g), 3. Oak sawdust (250g) + Cornmeal (100g), 4. Oak sawdust (250g) + Wheatbran (100g); 5. Pine sawdust (250g) + Cornmeal (100g) + Rice Straw (100g), 6. Pine sawdust (250g) + Wheatbran (100g) + Rice Straw (100g) to test optimum fruiting. All the substrates were added with 10 gm calcium sulphate ($CaSO_4$) and calcium chloride ($CaCO_3$) respectively. The substrates were soaked overnight, drained the water, and boiled for half an hour and then dried for half an hour at room temperature. The different compost combinations were then placed into the plastic bags, half filled. The openings of bags are plugged with non-absorbent cotton and then sterilized for 1 hour at 121°C (15 b psl) in an autoclave and allowed to cool for another 1 hour. All the compost mixtures were inoculated with spawns and incubated at 27°C in the dark in sterilized conditions until the colonization of substrate is achieved. Once the desired primordial initiation is achieved under weak light conditions, the bags were kept in a ventilated chamber for fruiting development at room temperature (20°-25°C). The methods of Borah et al (2019) and Stamets (2000) were followed with slight modifications.

3. Results

3.1. *Morphological characterization*

The polypore mushroom specimens, phenotypically similar to *Ganoderma* spp., were collected from temperate forest hardwood stumps from Geyzing, west Sikkim, Latitude 27.29° North and longitude 88.24° East, on June 2022. Basidiome annual, sessile but very indistinct, short, thick lateral stipe present, contracted base, laccate when fresh, becoming dull and cracked with age. Pilus woody, 5-17 cm in width, up to 2 cm thick at the base; upper surface is brown to reddish brown; zonation on the surface is typically fan-shaped or semicircular; plano-convex; applanate; margin is soft, slightly lobate, and concolorous with the pileus. The lower surface is white cream-coloured, spore print is brown; pore surface turns light brown with maturity. Pores circular 4–6 per mm, context up to 0.5 cm thick, dry, woody, brown. Basidiospores are mostly broadly ellipsoid elongated; they are truncated, reddish-brown, and have a double-walled inner endosporium. Their length ranges from 7 to 12 µm, and their width is 5 to 6 µm (Figure 2 b). Based on the above characterization, the specimens were identified as *Ganoderma* sp. (Figure 2 a).

3.2. *Culture characterization and Molecular identification*

With the overlapping features and the existence of different morphs for the same taxa, morphology-based taxonomy can occasionally fail to resolve species effectively (Hyde et al., 2016). Through the tissue culture method, isolation of a pure culture of *Ganoderma* species collected from Sikkim's temperate forest was established on PDA medium. The isolates MF-4 showed white mycelia colonies fast growing within 3-4 days of incubation, and the texture and density are floccose to velvety white. As it matures, the density of mycelia becomes creamy white and leathery, and colonies grow radially (8.80 mm/day), with fine rays at the margins. The mycelia covered the petri plates 90 mm after 10 days of inoculation. Colonies were continuous and dense in the centre, concentric ring; reverse white initially turns pale yellowish with age. Optimal growth is observed at temperatures ranging from 25°C to 27°C, usually forming visible colonies within a week (Figure 2 c). Hyphae thick and thin, hyaline, smooth- walled, branched, septate, anastomoses, and hyphal loops present, 1.52–3.48 µm wide (Figure 2 d). The molecular identification of *Ganoderma* species of Sikkim was confirmed through ITS-rDNA sequencing. The results of ITS-rDNA partial gene sequence and NCBI GenBank BLASTn search confirmed the identification *Ganoderma* species from Sikkim as species complex of the *G. australe* with 99.66% sequence similarity with LC084747.1 *Ganoderma australe* (Fr.) Pat. isolate 20-1 and 99.66% with LC084736.1 *Ganoderma australe* isolate: 27-1.

Ack Code- 3494, ITS Sequence of *G. australe*

GATCATTATCGAGTTTTGACTGGGTTGTAGCTGGCCTTCCGAGGC
ATCGTGCACGCCCTGCTCATCCACTCTACACCTGTGCACTTACTGT
GGGTTATGGATYGCGTGTAAAAGCGCGTCCGTGCCTGCGTCTTAC
CACAAACACTATAAAAGTATCAGAATGTGTATTGCGATGTAACGC
ATCTATATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGA
TGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATT
CAGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATT
CCGAGGAGCATGCCTGTTTGAGTGTCATGAAATCTTCAACCTACA
AGCTTTTTAATAATGGCTTGTTAGGCTTGGACTTGGAGGCTTGTC
GGTCTTTATTGATCGGCTCCTCTYAAACGCATTAGCTTGGTTCCTT
TGCGGATCGGCTGTCGGTGTGATAATGTCTACGCCGCGACCGTGA
CGCGTTTGGCGAGCTTCTAATCGTCTCGCTTTTGGGACAACTTTTA
TGACCTCTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAG
CATATCA

3.4. *Spawn production and fruiting trials*

Figure 3. In vitro cultivation of *G. australe* (**a-d**); **a**-*G. australe* spawn, **b**- Substrate compost preparation for fruiting, **c**- Different substrate combination in polybag, mycelium establishment and primodia head stage, **d.** Fruiting bodies and brown spore mass.

Spawn production is a crucial step in the cultivation process; in the present study maize grains substrates was found to be the best substrate for spawn production since the whole substrate was covered with mycelium within 10 days of incubation. In wheat grains substrate mycelium establishment was visible in 10–15 weeks. The paddy grain substrate did not support good mycelium growth. The optimal conditions for mycelia growth on spawn were 25–30 °C (Figure 3 a).

The result of developmental stages of basidiocarp or fructifications in all the different compost is depicted in (Table 1, Figure 3 b-d). Overall, it took on an average 2-4 months from mycelium running to fructifications in all the compost substrate combinations. The moisture content in substrates was maintained at 60% to 70% level with good air ventilation with indirect light to stimulate fruiting initiation and also to prevent CO_2 buildup, which could inhibit fruiting. Currently, the methods adopted for commercial production uses a variety of substrates, which mainly include the wood log, short basswood segment, tree stump, sawdust bag, and agricultural by-products, such as cotton seed husk, straw, and corn cob, in the cultivation of *Ganoderma* (Zhou, 2017; Stamets, 2000). Luangharn et al., (2017) used Para rubber sawdust with organic and inorganic additives as a standard cultivation substrate in Thailand for the cultivation of *G. australe*. However, regarding the choice of what kind of method or raw materials to use, the basic process for the production of fruiting bodies is roughly the same.

4. Discussion

In general, it is difficult and subjective to identify Ganoderma species solely based on morphological evidence, as their phenotypic traits are sensitive to extrinsic factors, such as illumination, ventilation, and humidity (Hapuarachchi et al., 2019a). Due to the variability of Ganoderma species on the hosts, it is evident that molecular identification must be supported by the isolation of pure culture. Moreover, the bioactive compounds are extractable from either the *Ganoderma* mycelium or fruiting body and represent important components of the expanding *Ganoderma* biotechnology industry (Paterson, 2006). Therefore, in the present study, the molecular

identification of the *Ganoderma* species of Sikkim was confirmed through ITS-rDNA sequencing from a pure mycelia culture isolate. The results of ITS-rDNA partial gene sequence and NCBI GenBank BLASTn search confirmed the identification *Ganoderma* species from Sikkim as species complex of the *G. australe*. This study reports a new record of *Ganoderma australe* (Fr.*)* Pat from Sikkim, eastern Himalayan region.

Ganoderma australe is a cosmopolitan white rot fungus of tropical regions (Ryvarden and Johansen, 1980; Yamashita et al., 2009). The species is recognized to be a species complex comparable to that of the Ganoderma *lucidum* complex (Martinez et al., 1991). Previously, this species was only known from New Zealand (Buchanan and Wilkie, 1995). Recently, a specimen of *G. australe was* isolated from Thailand by Luangharn et al., (2017), southern India by Kaliyaperumal and Kalaichelvan (2008); and Taiwan by Wang et al., (2020). Chakraborty and Shivekumar (2021), Wu, et al., (2020) reported potential bioactive compounds from *G. australe*. Albino Smania, et al., (2007) isolated australic acid and the new methyl australate from the Brazilian fungus *Ganoderma australe*. Methyl australate and its corresponding acid were shown to be active against fungi and Gram-positive bacteria, the methyl ester being also active against Gram-negative bacteria. Considering the potential of this species, an attempt was made to culture the wild strain of *G. australe* for the purpose of preservation of culture and fructification.

In the present study, *G. australe* pure culture was successfully isolated from the wild, followed by spawn production in two different substrate bases (maize and wheat). Currently, the methods adopted for commercial production uses a variety of substrates; mainly include the wood log, short basswood segments, tree stumps, sawdust bag, and agricultural waste by-products, such as cotton seed husk, straw, and corn cob, in the cultivation of fruit bodies (Erkel, 2009; Zhou, 2017; Stamets, 2000). Luangharn et al., (2017) used Para rubber sawdust with organic and inorganic additives as a standard cultivation substrate in Thailand for the cultivation of *G. australe*. Generally, wood-inhabiting mushrooms can be grown successfully in a variety of lignocellulosic substrates, including straw, sawdust, and rice husk (Thawthong, 2014). Different woods sawdust and rice straw were used as the main organic substrate supplemented with cornmeal and wheat bran; fructification was observed in all the substrate combination bags in the present experiments.

5. Conclusion

Identification, characterization and cultivation of *Ganoderma australe* (Fr.) Pat as a previously unrecorded species in the region further emphasizes the need for continued exploration and conservation efforts in the eastern Himalaya region. Moreover, *Ganoderma australe* has been reported to possess various bioactive compounds, such as triterpenoids, polysaccharides, and phenolic compounds, which contribute to its potential medicinal properties. Isolation of pure culture and sustainable cultivation practices are essential to protect the natural habitats while enabling the utilization of *Ganoderma* for scientific and commercial purposes. The outcomes of this study have significant implications for various stakeholders, including the pharmaceutical industry, traditional medicine practitioners, and conservationists. Exploring their ethnomycological functions offer more hints about their potential in various fields, while their ecological activities as wood-decaying fungi can lead to additional applications in mycoremediation.

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Author's contribution
LPW: Research design and concepts, supervision, manuscript drafting.
AL: Field survey, lab work.

Declaration of Conflict of interest
Authors have no conflict of interest.

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