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# Identification of puffball found in Chota Nagpur plateau through Molecular characterization

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#### Abstract

Food security is one of the issues for growing population. The search for alternative food resources points towards rich protein content food – mushrooms. Certain puffballs are also consumed similar to mushrooms. Popularly known as Rugda or Putu in areas of Chota Nagpur plateau. (Srivastava and Soreng, 2012) Many binomial names are given on the basis of morphological characters.

This current study is to find accurate and reliable method in the field of identification. The puffball was collected from forest located Khunti district. Washed and sterilized. Then the sample was treated with liquid nitrogen before Genomic DNA isolation through standard protocol of Doyle and Doyle (Doyle and Doyle 1987), Primer for ITS (Internal transcribed spacer) region was designed and then amplified by PCR, Amplicons were then processed for sequencing for Sanger method. Resultant sequence was searched for similarities in BLAST. The Molecular characterization process was found to be more reliable.

Keywords: puffball, rugda, molecular characterisation, Chota Nagpur, mushroom

# Introduction

Chota Nagpur plateau covers the area of Jharkhand, particular districts of Bihar, West Bengal, Chhattisgarh and Odisha. This plateau is rich in rocks and minerals with dense tropical forest area hidden with lots of unknown flora, fauna and dwelt tribal communities. It receives a good rainfall from 1200 mm to 1500 mm. July and August are the peak month of rainfall. This forest covers with trees like Shorea robusta (Sal), Butea monosperma, Madhuca indica (Mahua), Terminalia tomentosa (Asan), Millettia pinnata (Karanz) etc. (Kumar and Saikia 2020)<sup>[7]</sup>. The tribal communities use the forest products in their culture and day to day habits. The important forest products derived from different tree species are lac, fibres, floss, medicine etc. They use forest resources to make different things such as bamboo basket, broom from grass varieties and toothbrush of Millettia pinnata and Shorea robusta, dona and pattal from Shorea robusta leaves, make handcrafts from wood, liquor from mahua flower etc. Apart from plant and animal this forest constitutes non timber products like mushroom. The edible mushroom which are found in Chota Nagarpur includes Macrolepiota procera (Bada Khukhri), Termitomyces clypeatus (Namak Khukhri), Lentinula sp. (Bansh Khukhri), Volvariella sp. (Pagla Khukhri), T. albuminosa, T. heimii (Chirko, Bada khukhri, Patiyari), Boletus edulis (Jamun khukri), Calocybe sp. (Milky mushroom), etc. (Srivastava and Soreng 2014)<sup>[18]</sup> (Panda and Tayung 2015)<sup>[10]</sup> (Dutta and Acharya).

In monsoon tribal people of includes delicious dishes made form wild edible mushrooms and puffballs. (Verma et al., 2019)<sup>[20]</sup>. Different communities of different area of Chota Nagar plateau utilize as food and medicine. One such fascinating puffball popular among the local people as Rugda or as Putu. It is symbiotic associated fungus with roots of tree Shorea robusta. Many botanists have failed to culture this puffball in synthetic media. (Srivastava and Soreng 2012) [17, 19] It is grown in naturally decomposed leaf litter of Shorea robusta. It captures attention of local people due its meat like texture and high protein content. It is harvested manually and time consuming procedure to find it just beneath the soil surface. This puffball can found in clusters and solitary fruiting bodies according to varieties. It is rich bioactive compounds which give rise to profitable market of Medicine. (Pavithra et al., 2017) <sup>[11]</sup>. These are consumed but identified with different names as Lycoperdon, Geastrum, Scleroderma and so on. These synonyms are given based on morphological characters. It was considered as *Geastrum hygromertricus* belonging to gasteromycete (Morgan 1889)<sup>[9]</sup> and its physiological nature of hygroscopic and exoperidium sprouting into star shaped. (Ellis and Ellis 1990, Pegler, et al., 1995)<sup>[6, 12]</sup>. Later it was replaced as Boletales member Astraeus hygrometricus. (Miller and Miller 1988, Dring 1973)<sup>[8, 3]</sup>.

Modern method in molecular based techniques is followed in this study. Molecular tools provide more accurate methods for identification than the few characters afforded by traditional morphological features. (Rajaratnam, *et al.*, 2012) <sup>[16]</sup>. Molecular markers, PCR (Polymerase Chain Reaction) and non-PCR based are widely used for mushroom identification, characterization and phylogenetic studies. These techniques are quick and reliable to establish the identities of wild mushroom collections.

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#### Materials and methods Collection and survey

The Rugda was collected form forest located between village Jurdag and Jaltanda of Khunti district apart of study area Chota Nagpur. The Sample was photographed and survey was conducted with the tribal people belonging to around villages during collection.



Fig 1: Map of study area- (a) Chota Nagpur Plateau (b) Khunti District

# **DNA** isolation and Purification

Puffball sample were brought to lab cleansed with tap water distilled for removing muddy content in outer surface. Then it was cleansed with 70 % of alcohol. Sample were treated with Liquid nitrogen and crushed in motor pestle making fine powder. Powdered sample were mixed with CTAB and Centrifuged as per Doyle and Doyle protocol. (Doyle and Doyle 1987)<sup>[4]</sup>. The genomic DNA was checked by agarose gel electrophoresis in 1.5 % agarose gel. The obtained DNA content was purified by RNase treatment at 37°C for 60 minutes followed by Phenol- Chloroform-isomylalcohol (25:24:1 ratio). Centrifugation at 12,000 rpm for 10 min of purified supernatant with equal volume of 70% ethanol. The resultant DNA pellet were air dried and suspended to ice box after dissolving in 1X TE buffer. (Aamir, *et al.*, 2015)<sup>[1]</sup>.

#### Amplification

PCR reactions was performed for amplification with Forward Primer 5'-ACT GAA CCT TAT CAT TTA GAG- 3' and Reverse Primer 5'- AAG TCC ACT GAA CCT TAT CAT- 3' in a 50 µl reaction volume with 30 ng template DNA and ran on programme with initial denaturation at 94°C for 5 minutes, denaturation at 94°C for 30 seconds, annealing at 60°C for 40 seconds, extension at 72°C for 1 minute and final extension at 72°C for 2 minutes. The amplicons were eluted and Agarose gel run with Ethidium Bromide stain for 90 minutes 50V and photographed

# **ITS sequence analysis**

ITS (Internal transcribed spacer) amplicons containing were processed to Sequencing by Sanger Sequencing method in

Bunshi Bioscience Pvt Ltd. The resultant ITS Sequence was obtained and analysed by finding the similarities in BLAST. (Altschul *et al.*, 1997)<sup>[2]</sup>.

# **Results and Discussion**

Rugda showed great ethnomycological and commercial utilisation among the tribal and local people of Chota Nagpur. It is added as favourite dish in cuisine during monsoon season. Its meat like texture and mushroom like taste it is called as "vegetarian meat". It is recommended for diabetic condition and good food supplements. The fruiting body is cooked with mustard seed oil, and used to heal burns and it act as a haemostatic agent. (Panda and Tayung, 2015)<sup>[10]</sup> It is expensive due to it manual harvesting and rare occurrence. Many experiments have failed to culture the fruiting bodies which catch attraction to the scientist for the research. This puffball is harvested in first session of rainy season around late may month to early September month just beneath the humus created by leaf litters of Shorea robusta. The villager burnt the leaf litters just before the start of monsoon which is belief to give good yield of puffball. The photograph of Habitat and morphological structure were recorded (Fig 2) The sample collected was processed for DNA isolated and ITS was amplified by PCR for sequencing (Fig 3). The resulted sequence (Table 1) and identified as Astraeus asiaticus by BLAST analysing through similarities with NCBI GENEBANK database (Fig 4). ITS sequence was deposited in NCBI GENBANK and other species of Astraeus

ITS sequence deposition were documented (Table 2).



Fig 2: Rugda (Astraeus asiaticus) (a) Habitat (b) fruiting body (c) Morphological Structure



Fig 3: Agarose Gel documentation. M- Ladder a. Amplified ITS of Sample

BLAST <sup>®</sup> » <u>blastn suite</u> » results for RID-7VJ2TB79016											
Your search is limited to records that exclude: uncultured/environmental sample sequences											
Job Title	Nucleotide Sequence										
RID	7VJ2TB79016 Search expires on 03-28 17:33 pm										
Program	BLASTN										
Database	nt										
Query ID	Icl Query_47031										
Description	None										
Molecule type	dna										
Query Length	Length 970										
Descriptions											
Description		Max Score	Total Score	Query Cover	E value	Per. Ident	Accession				
Astraeus asiaticus genes for ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence, strain: Aswan29		841	841	66%	0.0	90.28%	AB507404.1				
Astraeus asiatic rRNA gene, 285 strain ASTRAE-	us 18S rRNA gene, 5.8S s rRNA gene, ITS1 and ITS2, 64	841	841	66%	0.0	90.28%	AJ629400.1				
Astraeus asiatic rRNA gene, 28S strain ASTRAE-	us 18S rRNA gene, 5.8S s rRNA gene, ITS1 and ITS2, 54	837	837	66%	0.0	90.36%	AJ629397.1				

Fig 4: BLAST analysis of Resultant ITS sequence

Table 1: ITS sequence of Rugda Sample

Sample	Resulted ITS Sequence						
Rugda	CAAGCCCTTAGAAGAAAAGGACACACGCCTAGAAGTAGCCTAGACTAAACTTGGCGCATGTAAAAGTTGGAAGCATG						
	AGACACGTCAATCCAAGCACTTCCAGCCCGCGACGATCACTACGACGTCGAACAGGCCGTGCCATGCAAAGGACTTGC						
	ACCCACTGCTAATACATTTAAGGAGAGCCGACATCCCAAAGCAAAGGGGTCGCCCGCAAACTCCCAAAGTCCAAAAA						
	CCAAGCTCCGACAAGGCAAAGCTAGGATTTTGAGATTTCGATGACACTCAAACAGGCATGCTCCTCGGAATACCAAGG						
	AGCGCAAGGTGCGTTCAAAGATTCGATGATTCACGGAAAATCTGCAATTCACATTACTTATCGCAATTCGCTGCGTTCT						
	TCATCGATGCGAGAGCCAAGAGATCCATTGCTGAAAGTTATATTTATT						
	CTGCCAAGTTTAATAAAATTATACCTTAGTCCGCCCAAAAAAAA						
	AAGGAATAGAAAAGGTTTCGAAGTTCCAAAGCCAAACCTTCCAAAAACTTCACCTTCCCCCAAAAGGCCACCACCACC						
	CTCCCAAAATCCAGAACCCCCCTACCCCCCTCCGCCTTT						

Table 2: Data of Other Species of Rugda reported in NCBI GENEBANK around the Chota Nagpur Plateau

S. No.	Species of	pecies of NCBI		Publication	Author	Sompling Area
	Astraeus	Acession No.	(bp)	month and year	Autior	Samping Alea
1	A. hygrometricus	AJ629895	452 bp	June 2008	Phosri C.	West Bengal
2	A. odoratus	KJ847767	272 bp	MAY-2016	Martin, M.P.	Sahibganj, Jharkhand
3	A. odoratus	MN262679	834 bp	AUG-2019	Singh, G., Lal, S., Vishal, V. and Munda, S.	Jharkhand
4	A. asiaticus	MN257431	793 bp	AUG-2019	Lal, S., Singh, G., Vishal, V. and Munda, S.	Jharkhand
5	A. asiaticus	MT611066	664 bp	JUN-2020	Topno, KR and Srivastava, AK.	Khunti, Jharkhand

# Conclusion

Molecular based techniques were found to be more reliable. ITS sequence obtained by this puffball revealed as *Astraeus asiatica*. The GENEBANK database review that there three species of *Astraeus* are found in Chota Nagpur forest. These species are also reported in NCBI GENEBANK with the accession no. MN262679 and KJ847767 Astraeus orodotus Accession No. AJ629895 for Astraeus hygrometricus and MN257431 and MT611066 for Astraeus asiatica have been reported (Table 3) Species *A. odoratus, A. asiaticus* and *A. sirindhorniae* are reported from Thailand. (Phosri *et al.* 2004, 2007, 2014) <sup>[13, 14, 15]</sup>. This study concluded that ITS Sequencing for the further accurate identification of other species which can establish profitable food and supplement market.

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