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Taxonomic and molecular identification of *Verpa bohemica*: A newly explored fungi from Rajouri (J&K), India

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Abstract
A species of mushroom, *Verpa bohemica* was collected from lower Shivalik range of moist temperate Conifer forest of Rajouri and identified on the basis of morphological and molecular characterization. Universal fungus primers (ITS1 and ITS4) were used in amplification process of target region of rDNA (ITS1 5.8S I). Bioinformatics approach was followed for its molecular identification. Its rDNA sequence, when aligned in GenBank by performing BLAST, matches 100% with *Verpa bohemica*. The rDNA sequence of this species forms a distinct clade from the rest of species of the same genus. This species is being reported and explored first time from Rajouri Dist. of Jammu & Kashmir, India.

Keywords: *Verpa bohemica*, Rajouri Dist., rDNA sequence, molecular identification, phylogenetic analysis.

INTRODUCTION

Verpa bohemica is a species of fungus in the Morchellaceae family. It is commonly known as the early morel (or early false morel) or the wrinkled thimble-cap, it is one of several species known informally as a "false morel". The mushroom has a pale yellow or brown thimble-shaped cap, 2 to 4 cm (0.8 to 1.6 in) in diameter and 2 to 5 cm (0.8 to 2.0 in) long including a surface wrinkled and ribbed with brain-like convolutions. The cap hangs from the top was of a lighter-colored, brittle stem and measured up to 12 cm (4.7 in) long and 1 to 2.5 cm (0.4 to 1.0 in) thick. Microscopically, the mushroom is distinguished by its large spores, typically of 60–80 x 15–18 µm sized, and the presence of only two spores per ascus. In the field, this mushroom species was reliably distinguished from the true morels on the basis of cap attachment: *V. bohemica* has a cap that hangs (free from the stem) completely. Although, this widely considered edible, mushroom is generally not advised for consumption due to various reports of poisoning in susceptible individuals. Poisoning symptoms include gastrointestinal upset and lack of muscular coordination. *V. bohemica* is originally found in northern part of North America, Europe, and Asia. It fruits in early spring, growing on the ground in woods following the snowmelt, before the appearance of "true morels" (genus *Morchella*). The synonym *Ptychoverpa bohemica* is often used by European mycologists. Morels are difficult to study in nature due to their habit of sudden fruiting and their high variability with regard to ascocarp phenotype, habitat, and trophic state [1]. They are generally classified morphologically into black and yellow species, but the ascocarps within each species also differ in color nuances, shape, size, and ridge arrangement [2], complicating the task of systematizing them according to morphological traits. Globally, morels are found in a wide range of habitats and environmental conditions [3-10] and display different trophic stages [11]. With regard to seasonality, however, morels show less variability. All over the world, the different species of morels emerge in particular locations, generally in the spring, although several reports have described finding morels in the summer [12] or autumn [13], which vanish after a few weeks [2, 3, 10-14]. In the present investigation, a new species of mushroom (fungi), *Verpa bohemica* was collected from lower Shivalik range of moist temperate Conifer forest of Rajouri Dist. (J&K), India and was identified on the basis of morphological and molecular characterization. This is a new kind of species explored recently in Rajouri Dist. of J&K, India which adds a chapter in diversity of mushrooms. There were no previous records available regarding the taxonomic investigation, molecular identification and elucidation of phylogenetic relationships of *Verpa bohemica* in India.

a). Verpa bohemica b).Morphology of *V. bohemica*

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MATERIALS AND METHODS

Taxonomic Identification

Fresh material was characterized morphologically in the field while the dried specimen was analyzed microscopically. Sections of basidiomata were prepared and observed under the light microscope equipped with camera.

Molecular Identification and Elucidation of Phylogenetic Relationship

The methodology was adopted [15] with some modifications for molecular identification and elucidation of phylogenetic relationship of the species. Dried specimen was ground in liquid nitrogen and placed in 2% CTAB buffer and DNA was extracted [16]. Genomic material was suspended in nuclease free water and stored at -20°C. ITS regions of rDNA were amplified using universal primer pair ITS1 and ITS 4 [17]. PCR was performed in 25 µl reaction volume following the protocol as prescribed [18]. PCR product of the ITS amplified region containing ITS-1, 5.8 and ITS-2 was directly sequenced in both directions using the same pair of amplification primers. The sequence data was assembled and analyzed. Nucleotide sequence comparisons were performed with Basic Local Alignment Search Tool (BLAST) network services using National Center for Biotechnology Information (NCBI), USA database. Molecular identification up to species level was done and species designated based on similarity with best aligned sequence in BLAST search. Sequence obtained was analyzed for restriction pattern for selected enzymes (AluI, ECoRI, HinfI, MboI and TaqI) using Sequencher 4.10.1 software. For further phylogenetic analysis and alignment of sequence, closely related sequences were selected and extracted from GenBank database. The sequence alignments were performed using Clustal W and Clustal X 1.83 software.

RESULTS

Taxonomic Identification

The cap of this fungus (known technically as an apothecium) was found to be 2 to 4 cm (0.8 to 1.6 in) in diameter by 2 to 5 cm (0.8 to 2.0 in) long, with a conical or bell shape. It appears to be folded into longitudinal ridges that often fuse together (anastomose) in a vein-like network. The cap was attached to the stem at the top only hanging from the top of the stipe, with the lobed edge free from the stem and varies in color from yellowish-brown to reddish-brown while the underside of the cap was pale. The stem was 6 to 12 cm (2.4 to 4.7 in) long by 1 to 2.5 cm (0.4 to 1.0 in) thick, cream-white in color, and tapers upward so that the stem was thicker at the base than at the top. The stem appeared loosely stuffed with cottony hyphae (Figure 1). The spore deposition was yellow, and the flesh was white. The spores of *V. bohemica* were huge, typically measuring 60–80 by 15–18 µm. The spores were elliptical, smooth, sometimes curved, and appear hyaline (translucent) to yellowish (Figure 2). The spores, which were two (more rarely three) per ascus are characteristic for this species. The asci appeared smooth and elliptical, appeared 275–350 µm in length and 16–23 µm in width (Figure 3). The specimen of the species characterized was deposited in National Centre of Fungal Taxonomy (NCFT), New Delhi, India for future reference. The specimen is deposited in NCFT, New Delhi, India vide accession no. NCFT-5975.12.

Fig 1. Stems of *V. bohemica* with soft cotton appeared tissues

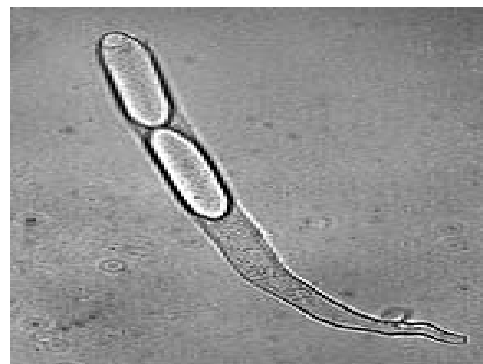

Fig 2. Elliptical spores of *V. bohemica*

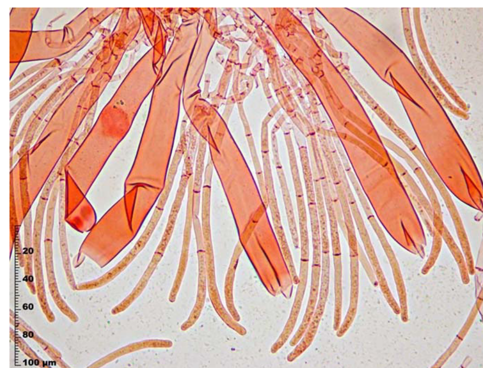

Fig 3. Asci of *V. bohemica* with elliptical appearance

Molecular Identification and Elucidation of Phylogenetic Relationship

The phylogenetic relationship of *Verpa bohemica* was analysed. Analyses using ITS sequences displayed a monophyletic behavior of the family Morchellaceae as well as *Verpa* genus.

Target region of genomic DNA isolated from *Verpa bohemica* was amplified generating a fragment approximately 570 bp consisting of internal transcribed spacers (ITS) regions and 5.8S region of rDNA. Results of sequencing with same pair of primers, nucleotide sequence comparisons were performed with BLAST network services using National Center for Biotechnology Information (NCBI), USA database and it matches 100% with *V. bohemica* (GenBank accession AJ698471 and U42672). With other closely related species it shows percentage similarity 99% with *Disciotis venosa* AJ698472 (Figure 4). Nucleotide sequence of collection identified was submitted to EMBL database and is available in GenBank accession.

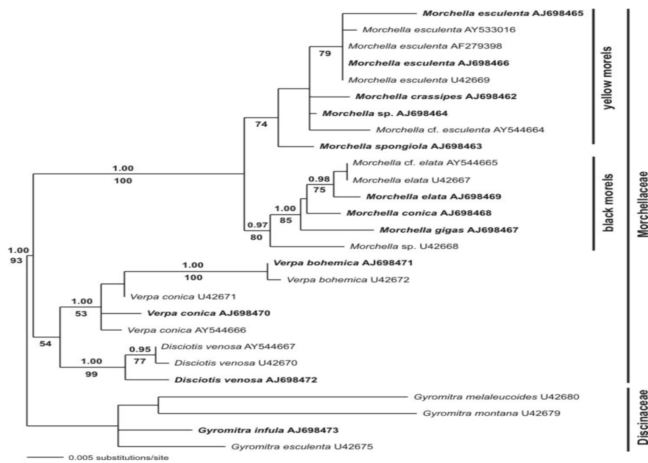

Fig 4. Phylogenetic relationship of *Verpa bohemica* (Identified from Rajouri Dist., J&K, India) inferred from ITS regions along with 5.8S part of rDNA.

DISCUSSION

In the present investigation, a new isolate of *Verpa bohemica* was collected first time ever from Rajouri Dist. of Jammu & Kashmir, India. The species was taxonomically identified and its phylogenetic relationship with gene sequences information available in databases was analysed with bioinformatics tools. The species differs morpho-anatomically from *Verpa conica* and other species of Morchellaceae family. The stem was thick, cream-white in color and tapers upward so that the stem was thicker at the base than at the top. The stem appeared loosely stuffed with cottony hyphae [19]. The spore deposition was yellow, and the flesh was white. The spores of *V. bohemica* were huge, typically measuring 60–80 by 15–18 μm. The spores were elliptical, smooth, sometimes curved, and appear hyaline (translucent) to yellowish [20]. The spores, were two (more rarely three) per ascus are characteristic for this species [21]. The asci appeared smooth and elliptical, appeared 275–350 μm in length and 16–23 μm in width [22]. Taxonomic identification is further supported by molecular characterization. The species identification depends upon the similarity of the known and unknown sequences in GenBank; the similarity more than 97% is not erected as a new species [23]. The sequence of ITS-rDNA regions of our taxonomically identified *Verpa bohemica* matches 100% with German *Verpa bohemica* (AJ698471) and Poland *Verpa bohemica* (U42672). Our explored species showed 99% similarity with *Disciotis venosa* AJ698472. Fingerprinting or ribotyping are rather quicker tools for species identification using different restriction enzymes without sequencing a gene if a RFLP-database is available [24, 25].

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