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Re-investigation of *Amanita flavipes*: Identification and Characterization Based on rDNA-ITS Sequence, Morphology and Anatomy

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ABSTRACT: Swat valley is Himalayan moist temperate forest of Pakistan. It is also called mini Switzerland of Pakistan. This area has diverse flora. A wide diversity of mushrooms is present over here but number of species explored is very small. In the present study, a species of Amanita was collected during field tour and identified on the basis of morpho-anatomical and molecular markers which confirmed its identification as Amanita flavipes. BLAST analysis of this species has been re-investigated from new locality swat and is being reported first time from this area.

Keywords: Ellipsoidal spores, Miandam, Phylogenetic, section Validae, Amanita flavipes.

INTRODUCTION

Genus Amanita Pers. (Amanitaceae; Agaricales) has more than 500 species which are distributed worldwide (Kirk et al., 2008; Menolli et al., 2009; Tulloss, 2009; Wartchow et al., 2009; Justo et al., 2010; Wartchow & Gamboa-Trujillo, 2012; Cho et al., 2015; Hosen et al., 2015; Tang et al., 2015; Wartchow & Cortez, 2016). Whereas, most of the regional species of the genus are still unknown (Bas, 2000; Tulloss, 2005). Ectomycorrhizal symbiosis is the most important character of the genus. Except some species, all are found to be associated with trees belonging to different types of genera and play a vital part in ecosystems (Yang, 1997, 2000; Wolfe, 2012). Till now, 18 *Amanita* Species are identified in Pakistan (Ahmad et al. 1997; Tulloss et al. 2001). Other novel species of *Amanita* have also been reported, but due to a lack of critical data necessary for species description their identity remains unknown to the mycological community (Ahmad et al., 1997; Tulloss et al., 2001). Many taxa of the genus have been reported as comestibles (Buyck, 1994; Montoya-Esquivel, 1997; Tulloss & Bhandary, 1992).

Family Amanitaceae is characterized by white

spore deposit, presence of universal veil around volva and sometimes in the form of warts on cap. Sometimes partial veil is present which later becomes a ring around stalk; known as annulus. Members of this family have free lamellae. Spores of family Amanitaceae are glubose to ellipsoidal, thin walled; amyloid or non amaloyed and their gill trama is bilateral. During field work in Miandam, Swat valley, fruiting body of a species of genus Amanita was collected and characterized on anatomical and molecular basis.

MATERIALS AND METHODS

Collection, Anatomic and Morphological Characterization

The *Amanita flavipes* was collected, labeled and photographed from Maindam, Swat valley. Fresh sample was morphologically studied and its morphological features i.e. colour, length and width of all parts was noted. It was dried and submitted to Fungarium, GC University, Lahore.

From dried mushroom, small pieces of Pileus, gills, cap and stipe were taken separately and sections through gills, stipe, and pileus were made with the help of sharp blade. One to two drops of 1% KOH were used for making microscopic slides and observed under the light microscope to observe basidia, spores, cystidia, stipitipellis and pelipellis. Measurements were taken with the help of micrometer.

Molecular Analyses

For molecular identification and characterization, DNA from *Amanita flavipes* was extracted by modified CTAB method following Gardes & Bruns (1993).

Polymerase Chain Reaction (PCR) was carried out following Gardes & Bruns (1993), using ITSI and ITS4 primers to amplify the nuclear ribosomal internal transcribed spacer region. The amplified product was sent to Macrogen, Korea for sequencing.

BLAST Analysis of ITS sequences:

The sequence obtained was edited manually, submitted to BLAST and used to query the nucleotide collections using default setting in GenBank.

Phylogenetic Analysis:

For phylogenetic analysis, rDNA its sequences downloaded from GenBank and *A. flavipes* were aligned. Neighbor joining method was used to construct the phylotree. Total 100 sequences were used for making phylogram.

RESULTS

Amanita flavipes G.F. Atk (Fig. 1)

Morphological Characterization:

Basidiomata; 8 cm long and pedunclate, Pileus; 3.5 cm wide, golden yellow, conical shaped with smooth margins. Reminants of partial veil on cap. Hymenium; Lamellae free, crowded and white. Stipe; 6 cm long, hard, fibrillose, yellowish and centrally attached. Volva present with ringed bulb. Veil present, detaches from the margins as cap open up but remain attached to the stipe giving the appearance of a ring.

Anatomical Characterization:

Basidia: Clavate to subclavate, guttulated, 12-15 \times 2-4 μm wide and 2-4 sterigmate,

attached with cellular mass. Cystidia: $16-17 \times 2-4 \mu m$. Cheliocystidia present; thin walled, bifid, clavate, ventricose rostrate, and subulate in shape, frequent to abundant, arising from hymenium along with basidia, attached with cellular mass, Pleurocystidia absent. Basidiospores: , smooth, globose, sub-globose to broadly ellipsoidal, rarely elongated, thick walled, densely and prominently verucose. Hyphae of Stipe: $1-12 \mu m$, septate, unbranched and clamp-less. Hyphae Cap is about 2-4 μm in diameter, non -septate, branched and clampless.



Fig. 1 *Amanita flavipes* **A.** Basidiomata **B.** Hymeneal part showing basidia and cystidia along with cellular mass **C.** Basidia **D**.Chelocystidia **E**.Basidiospores **F.** Hyphae of cap **G.** Hyphae of stipe Scale bars: **A.** 1.36cm **C.** 2.52µm **D.** 3.04µm **E.** 2.50µm **F.** 1.8µm **G.** 0.75µm

Molecular characterization:

Sequencing of ITS subunit of *Amanita* sp. resulted in consensus sequence of 468 base pairs. From BLAST analysis *Amanita* species matched 100% with *Amanita flavipes* FJ375327.1 and 100% query coverage.

DNA sequence of Amanita flavipes from ITS region:

TGTGCACGTCTTTTGCTGCTTGCTTCATTCTCTTTTCCACCTGTGCACTCTTTGTAGA-CACTCGGGATGGGAGAGAGGGTTGGCATTGATGGTGGACTCTCTTGATATTGAAAAGTC TGGGTGTTTATGTATTTTTTGACATACACGGTTGAATGTCTATAGAATGAAATGTAGGCT TTTGTCAGCCTTTAAATGATAAAATACAACTTTCAACAACGGATCTCTTGGCTCTCGCAT CGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCA TCGAATCTTTGAACGCATCTTGCGCTCCTTGGTATTCCGAGGAGCATGCCTGTTTGAGT GTCATTAAATATCTCAAAAAGTTTGTGCTTTTTTGGCATAAGATTTTTTGGACATTGGGA GTTGCCGGCTGCTGATAAAAAGTGGGTGGGCTCTTCTGAAAAGCATTAGTTGAGGA





Fig. 2 Phylogram based on rDNA-ITS sequences representing phylogenetic relatedness inferred from Maximum Likelihood with other species in genus *Amanita*. Sequence generated from Pakistani collection is marked.

Phylogenetic Analysis

rDNA-ITS amplified sequence of Amanita flavipes resulted in 630 fragment. The amplified sequences were BLAST searched for more similar alignments in GenBank using BLAST. All 100 most similar alignments were used for making phylogenetic tree. A. flavipes clustered with similar species and sharing 100% of its genetic characters with no genetic divergence. This confirms its place in section Validae of genus Amanita.

DISCUSSION

Family Amanitaceae is characterized by white spore deposit, presence of universal veil around volva and sometimes in the form of warts on cap. Sometimes partial veil is present which later becomes a ring around stalk; known as annulus. Members of this family have free lamellae. Spores of family Amanitaceae are glubose to ellipsoidal, thin walled; amyloid or non amaloyed and their gill trama is bilateral (David et al., 2009). There are 600 species of family Amanitaceae distributed worldwide (Kirk et al., 2008). There are 14 species of Amanita including Amanita flavipes present in Pakistan (Ahmad et al., 1997). A. flavipes shares the most important features of section Validae like having a persistent partial veil, bulbous stipe and friable universal veil (Fig 1). This species seems very close to A. flavocinia as indicated by phylogram (Fig 2). Both species have similar shaped pileus and stipe, both have remnants on cap and volva. A little difference exists in the color of cap from the margins and center which is intense orange from center and in the color of warts present on cap (Kirk et al., 2008). The color difference may be due to environmental factor on the basis of color we

cannot separate it. This species has been collected and described first time from Miandam forest of Swat Valley.

CONCLUSION

Amanita flavipes has been re-investigated from new locality in Swat. This species was identified and characterized on the basis of morpho-anatomical and molecular markers. The diversity of mushrooms in Swat Valley seems poorly reported that needs to be addressed to seriously. Present study will also emphasize on the usage of molecular markers along with morpho-anatomical features.

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