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The First Record of *Torula chromolaenae* on Dung Sample of *Equus kiang* from Ladakh, India

Krishnappa Kavyashree[1], Thimmappa Shivanandappa[2], and Gotravalli Ramanayaka Janardhana[*1]

[1]*Molecular Phytodiagnostic Laboratory, Department of Studies in Botany, University of Mysore, Manasagangotri, Mysuru - 570 006, Karnataka, India.*
[2]*Visiting Professor, School of Life Sciences, Pooja Bhagavat Memorial Mahajana Education Center, K. R. S. Road, Mysuru - 570 016, Karnataka, India.*
[*]*Corresponding author Email:grjbelur@gmail.com*

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ABSTRACT

An asexual hyphomycetes, *Torula chromolaenae* J., a new record from India, is being reported for the first time on a new substrate as a coprophilous inhabitant. The fungus was isolated from the dung sample of Tibetan wild ass (*Equus kiang)* endemic to the Tibetan plateau. The identity of the fungus was confirmed by both morphological and molecular approaches combining multi-locus phylogenetic (ITS, SSU, LSU) analysis.

Keywords: Coprophilous, Hyphomycetes, Herbivore dung, Tibetan wild ass, *Equus kiang*, Ladakh

INTRODUCTION

Coprophilous or fimicolous fungi, inhabiting the dung of animals, play a crucial role in recycling complex nutrients in the herbivore's dung by utilizing the undigested carbohydrates, hemicelluloses, and lignin, along with amino acids, vitamins, growth factors, and minerals, which aid in their colonization and growth (Dix and Webster, 1995). Over 1,200 fungi in ~260 genera belonging to Ascomycetes, Basidiomycetes, Zygomycetes, and Mitosporic fungi have been documented from the dung of various animals (Santiago *et al*., 2011).

The genus *Torula* was established by Persoon in 1795 with *T. herbarum* as the type species, describing *T. monilis* and *T. fructigena*. Later placed, the fungus to the genus *Torula* as *T. herbarum* (Ellis and Griffiths, 1975; Tian *et al*., 2023). The genus is habitual in aquatic and terrestrial habitats as a saprobe. As of July 2023, 540 specific epithets in Index Fungorum and only 21 specific epithets have molecular data in GenBank. *Torula chromolaenae* sp. nov., reported initially from Thailand on *Chromolaena odorata,* represents holotype MFLU 16-2819 (Li *et al*., 2017). During the enumeration of fungi inhabiting the dung of *Equus kiang* in the Ladakh region of India, *T. chromolaenae* was isolated from the Kiang dung substrate. This represents a new record for India and a first record on the new substrate.

MATERIALS AND METHODS

Isolation and morphological identification

Approximately 24 to 48 hours old dung samples of *Equus kiang* M. were collected from the Hawe (Hanle) village, Ladakh, India (32.79˚N, 79.00˚E) during the second week of July 2020. The collected dung samples were dried in shadow under laboratory conditions and stored at the ambient temperature. The surface sterilized dung samples were placed in a moist blotter chamber, incubated at 25±2°C for 2-8 weeks, and monitored for sufficient moisture content (Mohammed *et al*., 2017). The fungal fruiting bodies expressed on the dung samples were studied for macroscopic and microscopic features under the stereomicroscope (Discovery v20, Germany) and a bright field microscope (Carl Zeiss, Germany), respectively. The fruiting bodies expressed were carefully transferred onto the potato dextrose agar (PDA) and Malt Extract Agar (MEA) culture media, incubated at 25±2°C, and the colony characteristics were studied. The isolated fungus was identified morphologically (Li *et al*., 2017; Samarakoon *et al*., 2021) and confirmed by molecular methods. The pure culture of the fungus was deposited at the Microbial Type Culture Collection and Gene Bank, Institute of Microbiology (IMTECH), Chandigarh, India.

Molecular identification and phylogenetic analysis

The total genomic DNA was extracted from 50mg of mycelium growing on PDA by CTAB method (Zhang *et al*., 2010). Three gene regions, internal transcribed spacer (ITS), partial 28S large subunit rDNA (LSU), and partial 18S small subunit rDNA (SSU) were amplified with the primer pairs ITS1/ITS4 (White *et al*., 1990), LROR/LR5 (Cubeta *et al*., 1991; Vilgalys and Hester, 1990) and NS1/NS4 (White *et al*., 1990) respectively. The Polymerase Chain Reaction (PCR) was performed in a 25µl reaction mixture containing 1µl of DNA template, 5µl of My Taq DNA buffer (Himedia), 0.3µl of Taq DNA Polymerase (Himedia), 1µl of each forward and reverse primers and 16.7µl nuclease-free water. The thermal cycler (PeqLab, Germany) was set at the following conditions: initialization for 3 min. at 95°C, 35 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, extension 72°C for 45 s, and a final extension for 03 min at 72°C. Amplified PCR products were

sequenced at Barcode Biosciences, Bangalore. Obtained sequences were subjected to the BLASTn search using GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The isolate was identified to the species level based on the nucleotide sequence homology. The representative sequences were deposited in the GenBank database, NCBI. The representative sequences of the fungal isolate and other closely related sequences were retrieved from GenBank and aligned by the Muscle program. Phylogenetic analysis employing the Maximum-likelihood (ML) method using Molecular Evolutionary Genetics Analysis (MEGA11) software was conducted for LUS, SSU, and ITS datasets (Tamura *et al*., 2021). Further, the multiple sequence alignment of all three gene datasets was concatenated, and the phylogenetic analysis of concatenated sequences was performed using the ML method with 1000 bootstrapping using MEGA11.

RESULTS

Molecular identification and Phylogenetic analysis

The blast homology search results of ITS, LSU, and SSU sequence data of the isolate showed similarity to the reference sequence retrieved from GenBank; (ITS= 98.55%, LSU= 99.76%, and SSU= 99.88%) of *T. chromolaenae* (KUMCC 16-0036) (Li *et al*., 2017). The phylogenetic analysis of combined LSU, SSU, and ITS DNA sequences data matrix using MEGA11 with the automatically selected suitable preferences represents the well-supported clades with the reference strains of *T. chromolaenae* including *T. masonii, T. mackenziei*, and *T. herbarum* **(Figure 1)**.

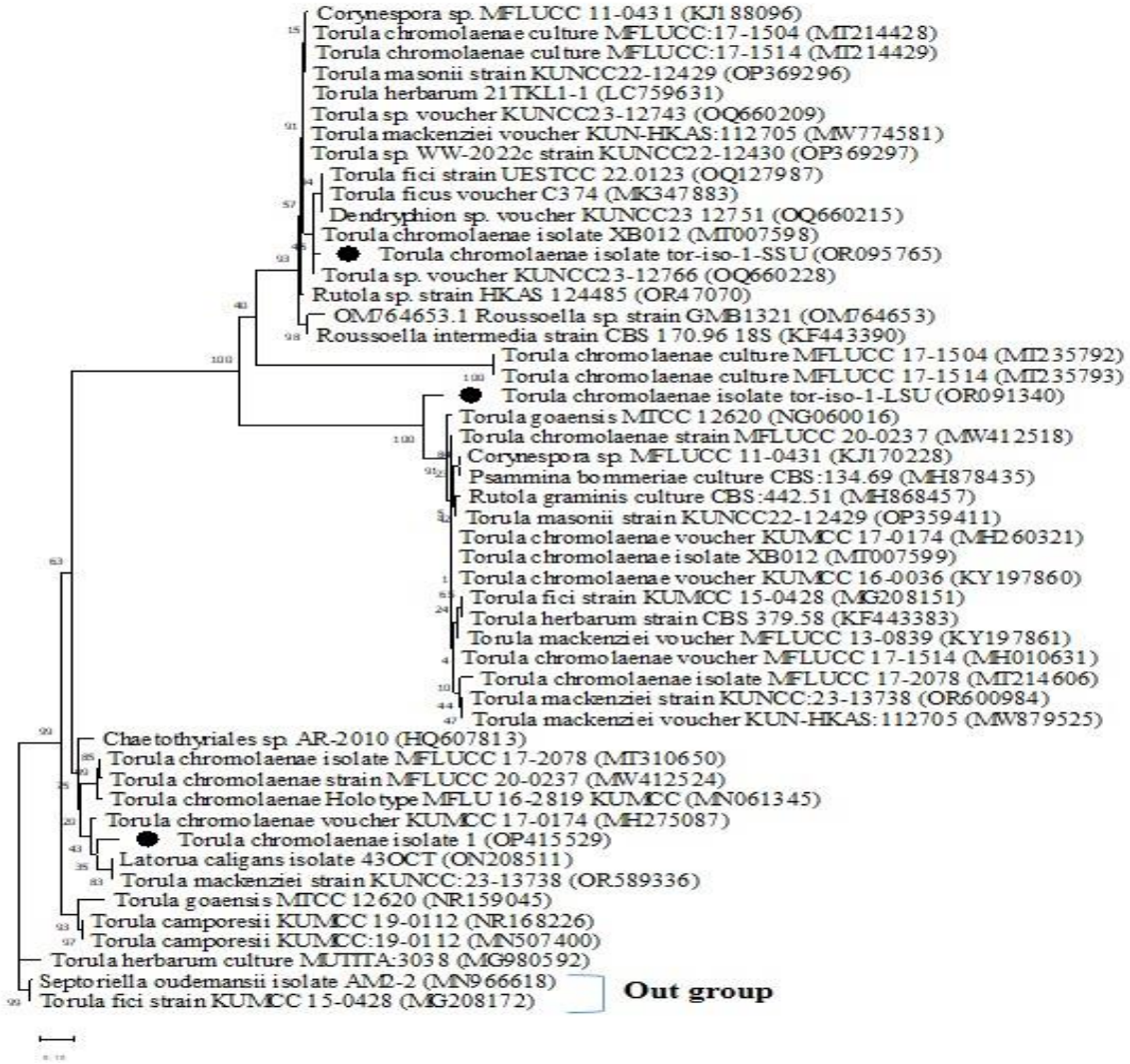

Figure 1: Phylogenetic tree constructed from maximum likelihood analysis based on combined ITS, LSU, and SSU sequence data of *T. chromolaenae* isolated from *Equus kiang* dung sample and reference sequences (NCBI-GenBank) using MEGA11 Software (Tamura *et al*., 2021) with 1000 bootstrap replicated. ITS, LSU, and SSU data of *T. chromolaenae* generated in the present study are represented in a black bullet.

The separate phylogeny of ITS, LSU, and SSU datasets inferred by using the Maximum Likelihood method and Tamure-Nei model (Tamura and Nei, 1993) involved 16, 17, and 17 nucleotide sequences, respectively, with the highest log likelihood (-2670.58), (-2634.72), (-3602.69) and a total of 981, 1381 and 1744 positions in the final datasets respectively. The percentage of trees in which the associated taxa clustered is shown above the branches. Initial tree(s) for the heuristic search were obtained automatically by applying the Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site using the 1000 bootstrap method **(Figure 2, 3, and 4).**

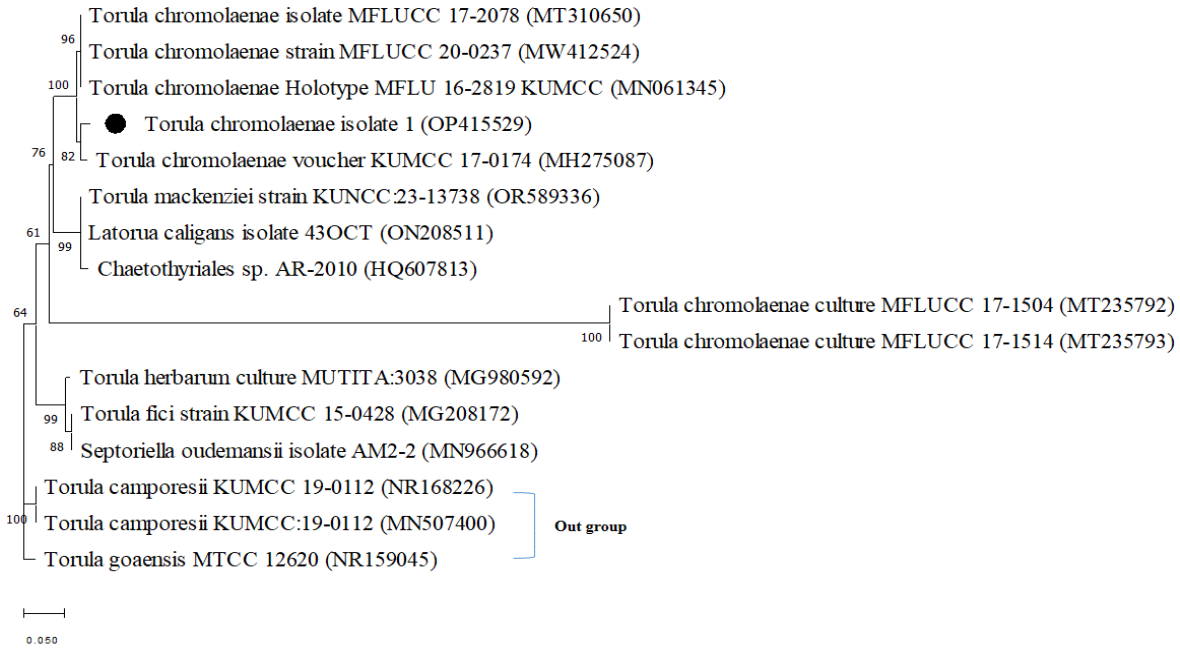

Figure 2: Phylogenetic tree constructed from maximum likelihood analysis with 1000 bootstrap replicated of ITS sequence data of *T. chromolaenae* isolated from *Equus kiang* dung sample and reference sequences (NCBI-GenBank) using MEGA11 Software (Tamura *et al*., 2021).

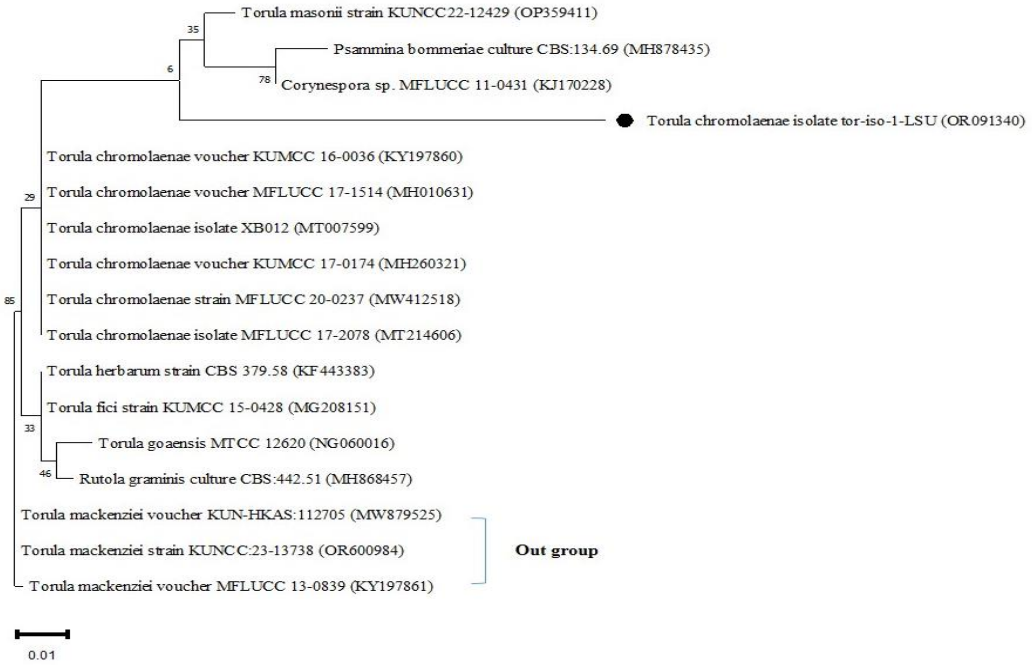

Figure 3: Phylogenetic tree constructed from maximum likelihood analysis with 1000 bootstrap replicated of LSU sequence data of *T. chromolaenae* isolated from *Equus kiang* dung sample and reference sequences (NCBI-GenBank) using MEGA11 Software (Tamura *et al*., 2021).

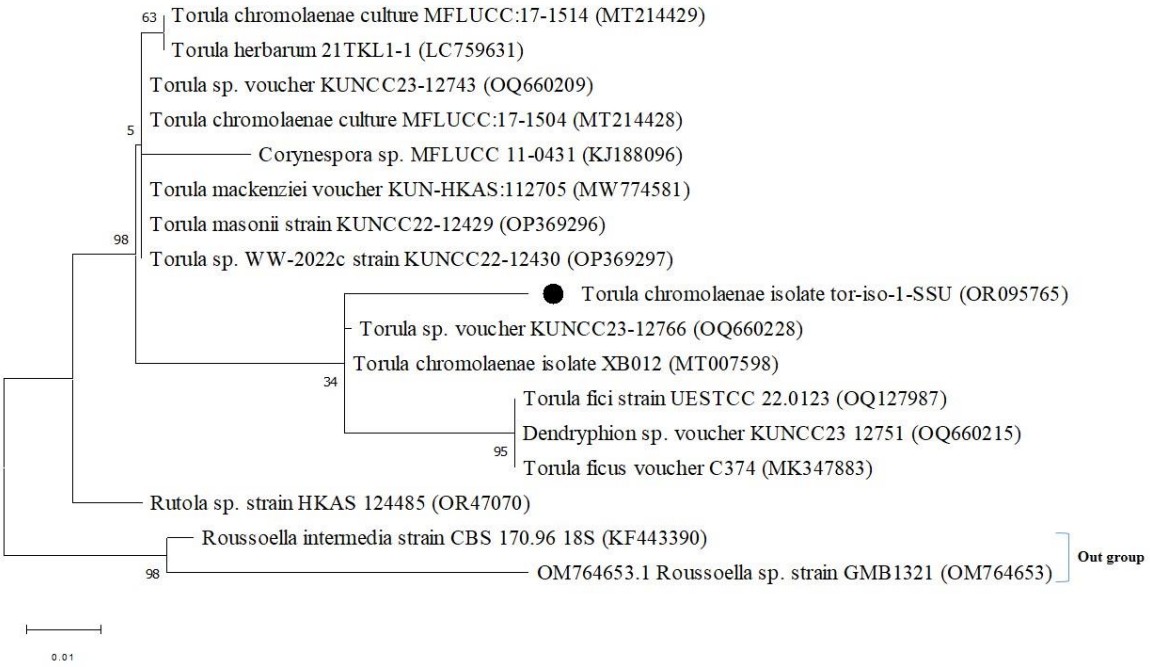

Figure 4: Phylogenetic tree constructed from maximum likelihood analysis with 1000 bootstrap replicated of SSU sequence data of *T. chromolaenae* isolated from *Equus kiang* dung sample and reference sequences (NCBI-GenBank) using MEGA11 Software (Tamura *et al*., 2021).

Morphology

Cultural characteristics

The colonies of *T. chromolaenae* appeared after six weeks of incubation. Colonies were shiny, dark brownish to black, dusty, and effuse thread-like on the substratum **(Figure 5A)**. Mycelium was slightly immersed, septate, narrow, sparingly branched, smooth, pale brown hyphae. The growth rate of the colonies was considerably slow. There is slightly more rapid mycelial growth on the Malt Extract Agar (MEA) than on the Potato Dextrose Agar (PDA) medium. Conidia germinated on PDA agar medium within 24 hours and attained a 3 cm colony diameter after 20 days of incubation at 25°C±2°C. Mycelium superficial and effused on PDA, appeared hairy to velvety, with regular margin, greyish white to grey at the periphery, olive green to golden brown shades at the center of the colony with whitish hairy mycelial clumps with golden yellow watery droplets. On the reverse, pale pink to brownish at the periphery and blackish grey at the center of the colony with distinctly marked cracks **(Figure 5B and 5C)**. Discretely formed conidia were observed after 48 days of incubation **(Figure 6A).**

Taxonomy

Torula chromolaenae J.F. Li, Phookamsak, A. Mapook and K.D. Hyde, *Mycological Progress*, **16(4)**:454 (2017).

Index Fungorum Number: 819536, **MycoBank Number**: 819536, and **Facesoffungi number**: FOF02713.

Saprobic on *Equus kiang* dung sample. Colonies discrete on the dung sample, black and powdery. Mycelium immersed in the substrate composed of septate, thick-walled, narrow, sparingly branched, smooth, and pale brown hyphae. **Conidiophores** 7.5–10–12.5µm long × 2.5–5 µm wide ($\bar{x}$ = 10 × 4 µm, n = 10), macronematous, mononematous, unbranched, straight or flexuous, solitary, pale brown, smooth, thick-walled, consisting of one-cell, determinate without apical branches, ellipsoid to subglobose, arising from hypha. **Conidiogenous cells** 5-7.5 µm long × 5.7–5 µm wide ($\bar{x}$=5 × 5 µm, n = 10), polyblastic, rarely monoblastic, terminal or sometimes integrated, spherical, smooth, distal fertile part thin-walled pale brown, proximal sterile part dark brown, and thick-walled, light brown to brown, paler at apex, smooth **(Figure 6B and 6C). Conidia** 12.5–15–17.5 µm long × 5–7.5 µm wide ($\bar{x}$= 14.25 × 6.1 µm, n = 20) catenated, acrogenous, simple, phragmosporous, brown to dark brown, smooth, 2-3 transversely septate, rounded at both ends, slightly constricted at some septa, chiefly subspherical. Conidial secession schizolytic **(Figure 6D)**.

Sexual state: Undetermined.

Sample examined: Hawe (Hanle) village, Ladakh, India (32.79˚N, 79.00˚E), isolated from *Equus kiang* (Tibetan wild ass) dung samples, 15 July 2020. Collected by K. Kavyashree, T. Shivanandappa, and G. R. Janardhana, Culture: MTCC13409.

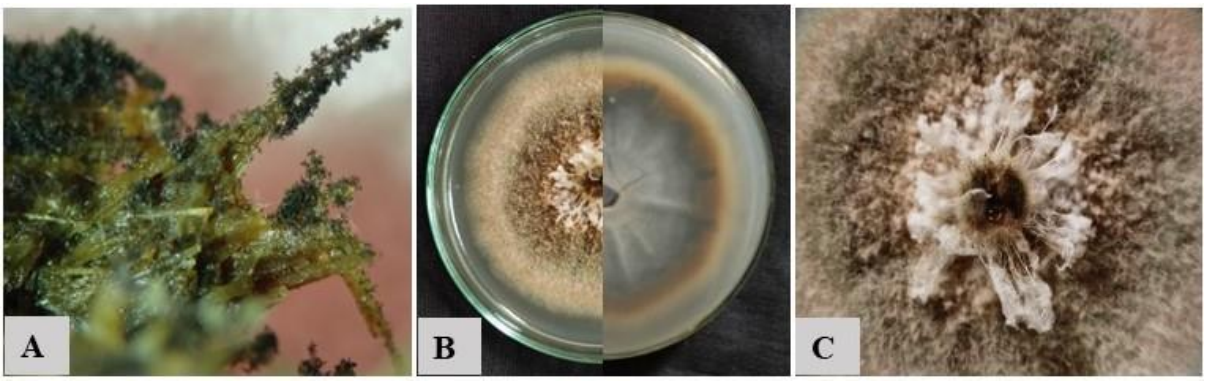

Figure 5: *Torula chromolaenae*. A, Conidiophores on the dung sample; B, Obverse and reverse view of the colony on PDA; C, Closer view of the colony showing whitish hairy mycelial clumps.

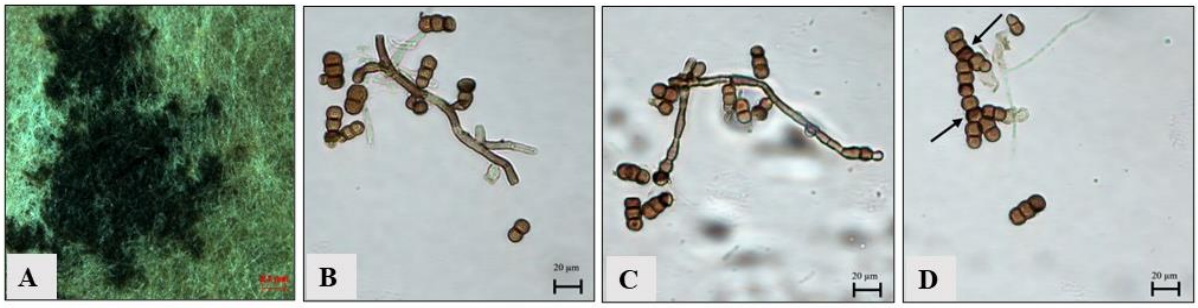

Figure 6: *Torula chromolaenae*. A, Conidiophores on PDA culture medium; B and C, Conidiophores with conidiogenous cells; D, Catenated conidia with polyblastic conidiogenous cells. Scale bars: A= 0.2 µm; B-D= 20 µm.

Known hosts and distribution: *Chromolaena odorata* in Thailand (Li *et al*., 2017 and Mapook *et al*., 2020), *Pandanus tectorius* in China (Tibpromma *et al*., 2018), *Clematis fulvicoma* in Thailand (Phukhamsakda *et al*., 2020), and *Musa* sp. In Thailand (Samarakoon *et al*., 2021).

GenBank accession numbers: Obtained sequences of the present work- ITS: (OP415529); LSU: (OR091340); SSU: (OR095765).

DISCUSSION

Herbivore dung is a rich source of numerous organic nutrients, influencing the colonization and growth of diverse fungi. India has reported the occurrence of some of the common fungal genera like *Chaetomium* spp., *Ascobolus* spp., *Pilobolus* spp., *Podospora* spp., and many other beneficial fungi from the dung samples of different animals (Mukerji, 1968; Thind and Sengal, 1971). A few of the novel species of hyphomycetous group viz., *Sympodina coprophila, Adhogamina ruchira, Bahupaathra samala,* and *Anngulimaya sundara*; *Beejasmuha samala* and *Sutravarana samala* have also been reported from India (Subramanian and Lodha, 1964; Subramanian and Chandrashekara, 1977).

In the present study, the dung samples of Tibetan wild ass, the only equid living on the Tibetan plateau and largest among the seven *Equus* species, were studied for the associated coprophilous microfungi for the first time. Detailed microscopic examination supports that *Torula chromolaenae* isolated using a moist chamber method similar to the fungal holotype (MFLU 16-2819) with slight differences in conidiophores, conidiogenous cells, and conidial size. The isolate from the present study exhibited slightly longer conidiophores comparatively with that of the holotype (7.5-10-12.5µm long vs. 5-6.3µm long); conidiogenous cells ($\bar{x}$=5 × 5 µm, n = 10 vs $\bar{x}$=4.7× 5.4 µm, n = 10) and conidia ($\bar{x}$= 14.25 × 6.1 µm, n = 20 vs $\bar{x}$= 14.5 × 4.3 µm, n = 20). The cultural characteristics were almost similar to the isolate (MFLU 20-0698) from the dead leaf vein of *Musa* sp. (Samarakoon *et al*., 2021). *Equus kiang* is native to the Tibetan Plateau. It has adapted to live in high-altitude habitats, distinguished by cold temperatures ranging between 12 - 14˚C during the day, which falls below 0˚C at night. The isolate is the first collection of herbivore dung samples from India. So far, the species have been reported from four different plant hosts viz., *Chromolaena odorata, Pandanus tectorius, Clematis fulvicoma,* and *Musa* sp. (Li *et al*., 2017; Mapook *et al*., 2020; Tibpromma *et al*., 2018; Phukhamsakda *et al*., 2020; Samarakoon *et al*., 2021). The documentation of new fungal records from new habitats and hosts helps us to know the fungal evolution, host range, host shift, speciation, and how fungi adapt to frequently changing environments (Hyde *et al*., 2020). Further studies

on the bioprospecting of the isolate may aid in finding the beneficial aspects of *T. chromolaenae*.

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REFERENCES

Cubeta, M.A., Echandi, E., Abernethy, T., *et al*., 1991. Characterization of anastomosis groups of binucleate *Rhizoctonia* species using restriction analysis of an amplified ribosomal RNA gene. *Phytopathology*, **81(11)**:1395-1400.

Dix, N.J. and Webster, J. 1995. Fungal Ecology. Chapman and Hall, London.

Ellis, D.H. and Griffiths, D.A. 1975. The fine structure of conidial development in the genus *Torula*. I. *T herbarum* (Pers.) Link ex S. F. Gray and *T. herbarum* f. *quaternella* Sacc. *Canadian Journal of Microbiology*, **21(11)**:1661-1675; doi: 10.113 9/m75-2440.

Hyde, K.D., Silva, N.I., Jeewon, R., *et al*., 2020. AJOM new records and collections of fungi: 1-100. *Asian Journal of Mycology*, **3(1):**22-294; doi: 10.5943/ajom/3/1/3.

Li, J.F., Phookamsak, R., Jeewon, R., *et al*., 2017. Molecular taxonomy and morphological characterization reveal new species and new host records of *Torula* species (Torulaceae, Pleosporales). *Mycological Progress*, **16**:447-461; doi: 10.1007/s11557-017-1292-2.

Mapook, A., Hyde, K.D., McKenzie, E.H., *et al*., 2020. Taxonomic and phylogenetic contributions to fungi associated with the invasive weed *Chromolaena odorata* (Siam weed). *Fungal Diversity*, **101**:1-175; doi: 10.1007/s13225-020-00444-8.

Mohammed, N., Shinkafi, S.A., Enagi, M.Y. 2017. Isolation of coprophilous mycoflora from different dung types in some local government areas of Niger state, Nigeria. *American Journal of Life Sciences,* **5**:24-29.

Mukerji, K.G. 1968. Fungi of Delhi-IV. A new species of *Piptocephalis* from India. *Mycologia,* **60**:326-330; doi: 10.1080/ 00275514.1968.12018573.

Phukhamsakda, C., Mckenzie, E.H., Phillips, A.J., *et al*., 2020. Microfungi associated with *Clematis* (Ranunculaceae) with an integrated approach to delimiting species boundaries. *Fungal Diversity*, **102**: 1-203; doi: 10.1007/s13225-020-00448-4.

Samarakoon, B.C., Phookamsak, R., Karunarathna, S.C., *et al*., 2021. New host and geographic records of five pleosporalean hyphomycetes associated with *Musa* spp. (Banana). *Studies in Fungi*, **6(1)**:92-115; doi: 10.5943/sif/6/1/5.

Santiago, A.L.C.M.D.A., Trufem, S.F.B., Malosso, E., *et al*., 2011. Zygomycetes from herbivore dung in the ecological reserve of Dois Irmãos, Northeast Brazil. *Brazilian Journal of Microbiology*, **42**:89-95; doi: 10.1590/S1517-83822011000100012.

Subramanian, C.V. and Lodha, B.C. 1964. Four new coprophilous Hyphomycetes. *Antonie van Leeuwenhoek*. **30**:317-330; doi: 10.1007/ BF02046737.

Subramanian, C.V. and Chandrashekara, K.V. 1977. *Beejasamuha* and *Sutravarana*, two new genera of coprophilous hyphomycetes. *Canadian Journal of Botany*, **55(3)**:245-253; doi: 10.1139/b77-034.

Tamura, K. and Nei, M. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution,* **10**:512-526.

Tamura, K., Stecher, G., Kumar, S. 2021. MEGA11: molecular evolutionary genetics analysis version 11. *Molecular Biology and Evolution*, **38(7)**:3022-3027; doi: 10.1093 /molbev/msab120.

Thind, K.S. and Sengal, H.S. 1971. The Pezizales of India-XI. *Indian Journal of Mycology and Plant Pathology*, **1**:36-48.

Tian, W., Su, P., Chen, Y., *et al,* 2023. Four New Species of *Torula* (Torulaceae, Pleosporales) from Sichuan, China. *Journal of Fungi*, **9(2)**:150; doi: 10.3390/jof9020150.

Tibpromma, S., Hyde, K.D., McKenzie, E.H., *et al*., 2018. Fungal diversity notes 840–928: micro-fungi associated with Pandanaceae. *Fungal Diversity*, **93**:1-160.

Vilgalys, R. and Hester, M. 1990. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *Journal of Bacteriology*, **172(8)**:4238-4246; doi: 10. 1128/jb.172.8.4238-4246.1990.

White, T.J., Bruns, T., Lee, S.J.W.T., *et al*., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and applications*, **18(1)**:315-322.

Zhang, Y.J., Zhang, S., Liu, X.Z., *et al.*, 2010. A simple method of genomic DNA extraction suitable for analysis of bulk fungal strains. *Letters in Applied Microbiology*, **51**:114-118; doi: 10.1111/j.1472765X.2010.02867.x.