Pak. J. Bot., 44: 413-416, Special Issue March 2012.

TRICHOLOMOPSIS FLAMMULA MÉTROD EX HOLEC (BASIDIOMYCOTA, AGARICALES)-AN ADDITION TO THE MUSHROOM FLORA OF PAKISTAN BASED ON MOLECULAR IDENTIFICATION

ABDUL RAZAQ*, A. N. KHALID AND S. ILYAS

Department of Botany, University of the Punjab, Lahore.
Corresponding author's e-mail: ectomycorrhiza@gmail.com

Abstract

A species of *Tricholomopsis* was collected from Himalayan moist temperate forest of Pakistan and identified on the basis of morphological and molecular characterization. The target region of rDNA (ITS1 5.8S ITS2) was amplified using universal fungus primers ITS1 and ITS4. Its rDNA sequence, when aligned in GenBank by performing BLAST, matches 100% with *Tricholomopsis flammula*. The rDNA sequence of this species forms a distinct clade from the rest of species of the same genus. This species is being reported first time from Pakistan.

Introduction

Tricholomopsis flammula Métrod ex Holec is a lignicolous fungus found on decaying logs of coniferous trees. It is a colourful mushroom with sulphur yellow pileus covered with fine red-violet scales, glabrous and sulphur yellow stipe, yellow lamellae and large clavate cheilocystidia (Holec, 2009). This rare fungus resembles a well known *T. rutilan* (Schaeff. Fr.) Singer (Holec & Kolařík, 2010). This is probably the reason why some mycologists believe it as a variety of *T. rutilans* (Boekhout & Noordeloos 1999) or may be variety of other similar fungus species *T. decora* (Fr.: Fr.) Singer (Breitenbach & Kränzlin 1991). However, it can easily be distinguished by its red-voilet scales, presence of pleurocystida and significant dissimilarity in ITS-rDNA sequences. The sequence analysis of rDNA of *T. flammula* clarified its taxonomic position as it forms a distinct clade from both other species in phylogenetic tree (Holec & Kolařík, 2010).

From Pakistan, *T. decora* and *T. rutilans* have been reported but the information about their morpho-anatomical descriptions and where these have been deposited is missing (Murakami, 1993, Ahmad *et al*., 1997). *Tricholomopsis flammula* has been collected from two geographically and ecologically different localities of Himalayan moist temperate forests of Pakistan. Both collections of this species were described and illustrated morpho-anatomically and molecularly.

Material and method

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 lucida.

Dried specimen was ground in liquid nitrogen and placed in 2% CTAB buffer and DNA was extracted (Porebski *et al*., 1997). 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 (white *et al*., 1990). PCR was performed in 25 µL reaction volume following the protocol given by Gardes & Bruns (1993). 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. (Macrogen, Korea). 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 & 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 (Table 1). The sequence alignments were performed using Clustal W and Clustal X 1.83 software.

Table 1. Sequences used for data analysis and phylogenetic tree construction.

Sr. No.	Species Name	Origin	Collector	GenBank
1.	*Tricholomopsis decora*	Czech Republic	Holec and Kolarik, 2009	FN554890
2.	*Tricholomopsis decora*	USA	Hughes, 2003	AY329597
3.	*Tricholomopsis flammula*	Czech Republic	Holec and Kolarik, 2009	FN554892
4.	*Tricholomopsis flammula*	Czech Republic	Holec and Kolarik, 2009	FN554893
5.	*Tricholomopsis flammula*	Czech Republic	Holec and Kolarik, 2009	FN554894
6.	*Tricholomopsis flammula*	Czech Republic	Holec and Kolarik, 2009	FN554896
7.	*Tricholomopsis flammula*	Czech Republic	Holec and Kolarik, 2009	FN554897
8.	*Tricholomopsis flammula*	Pakistan	Razaq and Khalid, 2011	FR822742
9.	*Tricholomopsis rutilans*	Czech Republic	Holec and Kolarik, 2009	FN554895
10.	*Tricholomopsis rutilans*	Canada	Denis. *et al*., 2007	EF530929

Results

Tricholomopsis flammula Métrod ex Holec, Journal of the National Museum (Prague), Natural History Series 178: 8 (2009)

Morpho-anatomical characterization

Pileus: 30– 43 mm wide, pale yellow color with red-violet scales all over the cap, concentrated on disc, hemispheric to convex, obtuse, rough, viscid when wet (Fig. 1 A-F). *Context* thin to moderately thick, light yellow, unchanging when bruised or cut; *margins* smooth, incurved; yellow to deep yellow. *Lamellae* adnate, close, entire, off white to pale color; *Lamellulae* varied in length, equally distributed between each pair of lamellae.

Stipe: 34–78 × 8–12 mm, cream to pale yellow in color, sometimes light brown, smooth, cylindrical, without swollen base. *Basidiospores* 6–7 × 3–4 µm, ovoid to ellipsoidal, elliptical-phasioliform, smooth, thin walled, inamyloid. *Basidia* 23–3 × 4.5–6 µm, 4-sterigmata, clavate to narrowly clavate, hyaline to pale yellow in KOH. *Pleurocystida* 31.5–55 × 7–11 µm, generally clavate to cylindrical, fusiform, thin walled, hyaline. *Cheilocysitidia* 40–74 × 17–28 µm, sterile clusters, very abundant, variable in size and shape, generally clavate to sub–clavate hyaline to pale yellow to brownish in KOH.

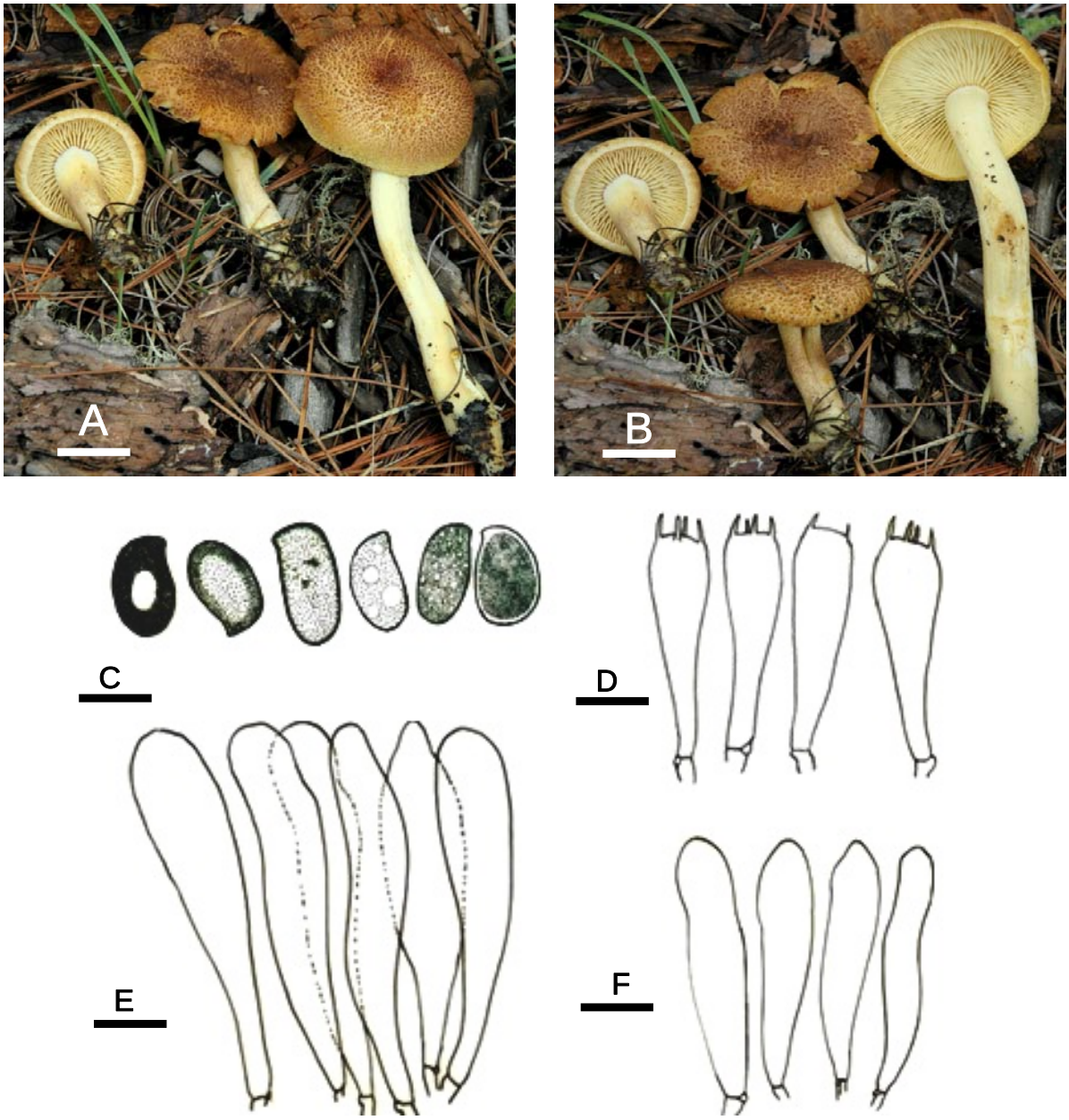

Fig.1. A-F. *Tricholomopsis flammula.* A-B. Basidiocarps. C. Basidiospores. D. Basidia. E. Cheilocystidia. F. Pleurocystidia Scale bar for A & B = 0.5cm, for C = 4.0µm, for D & E = 9 µm. The scale is mostly used in decimal values, e.g. 1 cm, 1 µm, 10 µm etc.

Material examined: Pakistan: Gilgit-Baltistan, Fairy Meadows (34.400570°S 150.891738°E), Pine forest of Himalayan moist temperate region, on decaying logs of coniferous tree at 3306 m a.s.l., on decaying logs of coniferous tree, 21st July, 2010, A Rrazaq, AN Khalid & S Ilyas, SR-16 (Herbarium # LAH 21071016), GenBank accession # FR822742.

Pakistan: Khyber Pakhtonkhaw, Mahnsehra, Ballakot, Nadi Bangla (34° 33' 0" N, 73° 21' 0"E), mixed forest of Himalayan moist temperate region, on decaying logs of coniferous tree, 13th August, 2011, A Rrazaq & AN Khalid, NB-44 (Herbarium # LAH 13081144).

Molecular characterization and sequence analysis: Target region of genomic DNA was amplified generating a fragment approximately 570bp 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 *T. flammula* (GenBank accession # FN554897, FN554896). With other closely related species it shows percentage similarity 92.9% and 79.7% with *T. rutilans* (FN554892) and 2 sequences of *T. decora* (FN554890, FN554891), respectively (Table 2). Nucleotide sequence of Pakistani collection, *T. flammula* (SR-16) was submitted to EMBL database and is available in GenBank accession # FR822742.

Table 2. rDNA sequence bases percentage similarity of *Tricholomopsis flammula* (FR822742) with other species.

	Percent identity									
		1	2	3	4	5	6	7	8	
Divergence	1		100.0	91.3	92.9	79.7	79.7	67.0	100.0	1 FN554896
	2	0.0		91.3	92.9	79.7	79.7	67.0	100.0	2 FN554897
	3	5.1	5.1		95.1	83.7	83.7	66.0	91.3	3 EF530929
	4	6.3	6.3	2.5		81.5	81.5	67.0	92.9	4 FN554895
	5	15.5	15.5	14.0	14.0		100.0	64.1	79.7	5 FN554890
	6	15.5	15.5	14.0	14.0	0.0		64.1	79.7	6 FN554891
	7	37.2	37.2	35.9	37.0	35.5	35.5		67.0	7 FJ660941
	8	0.0	0.0	5.1	6.3	15.5	15.5	37.2		8 FR822742
		1	2	3	4	5	6	7	8	

RFLP (Restriction fragment length polymorphism) characterization of Tricholomopsis flammula (FR822742): Two sequences of *Tricholomopsis flammula* (GenBank accession # FN554897, FN554896) one sequence of *T. rutilans* (FN554892) and 2 sequences of *T. decora* (FN554890, FN554891) were used for this study. AluI, HinfI, MboI, ECoRI & TaqI generated specific restriction pattern with different species of *Tricholomopsis* genus. RFLP data generated is given in the Table 3.

Table 3. RFLP results of four restriction enzymes for different species of *Tricholomopsis*.

Species	Fragment length	AluI	HinfI	MboI	TaqI	ECoRI
T .flammula (FN554897)	475bp	150,325	8,123,156,188	13,62,115,116,169	59,112,135,169	Non-cutter
T .flammula (FN554896)	475bp	150,325	8,123,156,188	13,62,115,116,169	59,112,135,169	Non-cutter
T .flammula SR-16 (FR822742)	475bp	150,325	8,123,156,188	13,62,115,116,169	59,112,135,169	Non-cutter
T .rutilans (FN554895)	465bp	38,144,282	8,120,150,186	13,40,62,69,114,166	59,60,73,106,166	Non-cutter
T .decora (FN554890)	584bp	87,196,301	8,41,121,142,272	62,111,150,261	59,130,395	Non-cutter
T .decora (FN554891)	584bp	87,196,301	8,41,121,142,272	62,111,150,261	59,130,395	Non-cutter

Alu I cleaved once and generated 2 fragments of all isolates of *Tricholomopsis flammula* while *T. rutilans* and *T. decora* produced three fragments. Hinf I was the second restriction enzyme which showed same number of fragments for all species but fragment lengths for each species are different. RFLP pattern analyzed using MboI enzyme indicated that *T. flammula* isolates produced five fragments while six in *T. rutilans* and number of restriction sites reduced to three in *T. decora* as percentage similarity decreased from *T. flammula.* Number of restriction sites for Taq I further decreased in all species as compared to MboI. Four fragments were generated from two isolates of *T. flammula* while five and three fragments were noted in *T. rutilans* and *T. decora* respectively. ECoR I Could not cut any isolate of all species of *Tricholomopsis*.

Phylogenetic analysis: The sequence of SR-16 (FR822742) grouped in a clade formed by *Tricholomopsis flammula*, a sister clade to *T. rutilans* sequences. *T. decora* formed a separate clade from both of them showing more genetic variability. One isolate of *T. flammula* (FN554893) showed some intraspecific variability as well.

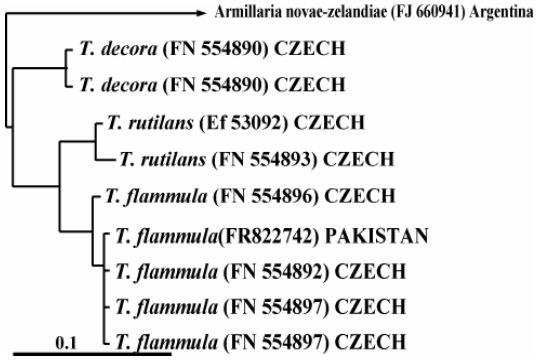

Fig. 3. Phylogenetic placement of Pakistani *T. flammula* (FR822742) inferred from ITS regions along with 5.8S part of rDNA. Maximum-likelihood analysis tree is presented.

Discussion

Tricholomopsis flammula collected from Pakistan differs morpho-anatomically from *T. decora* and *T. rutilans*. The basidiomata of *T. flammula* are smaller in size and slender from *T. rutlilans* and the occurrence of central black scales on pileus are also key feature of *T. decora* which are different from red-violet scales of *T. flammula* (Holec, 2009, Smith, 1960). Ellipsoid spores of *T. flammula* also differ from subglobose to broadly ellipsoid spores of *T. rutilans* reported from Pakistan (Murakami, 1993). The sizes of basidiospores, basidia and pleurocystidia fall in the ranges given by Holec (2009) and Smith (1960). Pleurocystidia are absent to scarcely present in *T. rutilans* and *T. decora* (Holec, 2009). Morpho-anatomical 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 (Gao & Yang, 2010). The sequence of ITS-rDNA regions of Asian *Tricholomopsis flammua* (FR822742) matches 100% with European *Tricholomopsis flammua* (FN554896, FN554897). Molecularly, *Tricholomopsis flammula* (FR822742) shows similarity below cut value with its closely related taxa. *Tricholomopsis rutilans* (FN554895) and *T. decora* (FN554890, FN554891) match 92.7% and 79.7%, respectively with *Tricholomopsis flammula* (FR822742).

Fingerprinting or ribotyping are rather quicker tools for species identification using different restriction enzymes without sequencing a gene if a RFLP-database is available (Gomes *et al*., 2002, Gardes & Bruns, 1996). Restriction fragment length polymorphism (RFLP) pattern of *Tricholomopsis flammula* (SR-16, FR822742) and other isolates showed a species specific pattern of restriction sites (Table. 3) and this exactly matches with that of *T. flammula*. Number of restriction sites of *T. rutilans* and *T. decora* are different from *T. flammula*. ECoR I remained non-cutter for *T. flammula* (SR-16, FR822742) and results matche with our specimen of all *Tricholomopsis* species Table. 3.

Phylogenetically *T. flammula* (SR-16) formed a position within the clade clearly formed by *T. flammula* isolates. *Tricholomopsis rutilans* formed sister clade to *T. flammula* isolates, which mean both are close relatives of each other while *T. decoa* formed a very distinct and different clade from rest of both species showing its far away relationship. *T. flammula* (FN554893) formed somewhat different branch from the rest of isolates. Intraspecific genetic variability may exist but it is negligible (Holec & Kolařík, 2010). Using morpho-anatomical and molecular characters, our specimen has been proved to be *Tricholomopsis flammula* well distinguishable from both *T. rutilans* and *T. decora*.

Acknowledgments

This work was financially supported by Higher education commission (HEC) of Pakistan under the "Indigenous Ph.D. Fellowship Scheme 5000 Phase IV". We deeply acknowledge Dr. Jan Holec, National Museum/Národní museum, 193 00 Praha 9, Czech Republic for confirming our results and Miss Nousheen Yousaf Department of Botany, University of the Punjab, Lahore for helping in anatomical work.

References

Ahmad, S., S.H. Iqbal and A.N. Khalid. 1997. *Fungi of Pakistan*. Sultan Ahmad Mycological Society of Pakistan, depatmrnt of Botany, university of the Punjab, Quaid-e-Azam campus, Lahore. pp. 248.

Boekhout, T. and M.E. Noordelooos. 1999. Tricholomopsis. In: *Flora agaricinaneerlandica*. (Eds.): C. Bas, T.W. Kuyper, M.E. Noordeloos, E.C. Vellinga. Vol. 4. Balkema, Rotterdam, pp. 151-152.

Breitenbach, J. and F. Kränzlin. 1991. Pilze der Schweiz. Band 3. Röhrlinge und Blätterpilze 1. Teil.–Luzern: Verlag Mykologia, pp. 364.

Gao, O. and Z.L. Yang. 2010. Ectomycorrhizal fungi associated with 2 species of Kobresia in an alpine meadow in the eastern Himalaya. *Mycorrhiza*, 20: 281-287.

Gardes, M. and Bruns, T.D. 1996. ITS-RFLP matching for identification of fungi. *Methods in Molecular Biology*, 50: 177-186.

Gardes, M. and T.D. Bruns. 1993. ITS primers with enhanced specificity of basidiomycetes: application to the identification of mycorrhizae and rusts. *Molecular Ecology,* 2: 113-118.

Gomes, E.A., Kasuya, M.C.M., de Barros, E.G., Borges, A.C. and Araujo, E.F. 2002. Polymorphism in the internal transcribed spacer (ITS) of the ribosomal DNA of 26 isolates of ectomycorrhizal fungi. *Genetics and Molecular Biology*, 25(4): 477-483.

Holec, J., 2009. Valid publication of the name Tricholomopsis flammula (Fungi, Basidiomycota, Tricholomataceae), a species clearly separated from *T. rutilans*. *Journal of National Museum (Prague), Natural History Series*, 178(3):7-13.

Holec, J. and M. Kolařík. 2010. Tricholomopsis flammula (Basidiomycota, Agaricales) - molecular taxonomy, delimitation, variability and ecology. *Mycolgical Progress*, 1(10): 93-99.

Murakami,Y. 1993. Larger fungi from northern Pakistan In: *Cryptogamic flora of Pakistan*. (Eds.): T. Nakaike and S. Malik). Vol. 2, Natural Science Museum, Tokyo.

Porebski, S., Bailey, L.G. Baum, B.R. 1997. Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. *Plant Molecular Biology Report,* 15(1): 8–15.

Smith, A.H. 1960. Tricholomopsis (Agaricales) in the Western Hemisphere, *Brittonia,* 12: 41-70.

White, T.J., T.D. Bruns and L.J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR protocols: A guide to methods and applications*, (Eds.): M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White. Academic Press, New York. Pp. 315-322.

(Received for publication 12 February 2012)