

# Ramaria barenthalensis a new record from western Himalayas, Azad Jammu and Kashmir, Pakistan

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## Abstract

The genus *Ramaria* is globally distributed, inhabiting different substrates. In the present study, samples of *R. barenthalensis* were collected during a fungal survey from Neelum valley AJK, Pakistan. Identification was carried out through combined morpho-anatomical and molecular data from nrITS region, which confirmed the identification as *R. barenthalensis*. The taxon is a new record for fungi of AJK, Pakistan.

## Keywords

Coral fungi, ITS, Mushrooms of Kashmir, Neelum valley, nrDNA, *Ramaria*

## Introduction

*Ramaria* species are cosmopolitan in distribution and grow on living and dead hard-wood, tree trunks, partially decomposed organic matter, and under conifers as mycorrhizal species (Marr and Stuntz 1973; Kuo 2009; Dorjey et al. 2016). They are commonly known as coraloid fungi, due to colored and much branched basidiocarps. They grow in very diverse habitats and form a mycelial mat in soil beneath the sporocarps (Kumar and Gautam 2017). This genus comprises more than 200 species distributed

worldwide (Ghosh et al. 2021). *Ramaria flava* (Schaeff.) Quél. and *R. stricta* (Pers.) Quél., are common edible coraloid fungi (Krupodorova and Sevindik 2020). *Ramaria* species have been identified through scanning electron microscopy and molecular techniques by Martin et al. (2020). Internal transcribed spacer (ITS) restriction length polymorphism was observed among *Ramaria* species and used as a diagnostic tool for characterization (Nouhra et al. 2005). The regions of Azad Jammu & Kashmir are enriched in terms of macro-fungal diversity.

*Ramaria abietina* (Pers.) Quél., *R. aurea* (Schaeff.) Quél., *R. apiculata* (Fr.) Donk, *R. botrytis* (Pers.) Bourdot, *R. flava* (Schaeff.) Quél., *R. flavescensoides* Hanif & Khalid, *R. formosa* (Pers.) Quél., *R. pallida* (Schaeff.) Ricken, *R. soluta* (P. Karst.) Corner and *R. stricta* (Pers.) Quél. have previously been reported from Pakistan (Ahmad et al. 1997; Khalid 2022). Previously, only *Ramaria stricta* (Pers.) Quél. has been reported from AJK, based on morpho-anatomical characters (Gardezi 2005). In current study, *R. barenthalensis* is reported as new record from Neelum valley and AJK, Pakistan to increase the knowledge about its distribution and phylogeny.

## Materials and methods

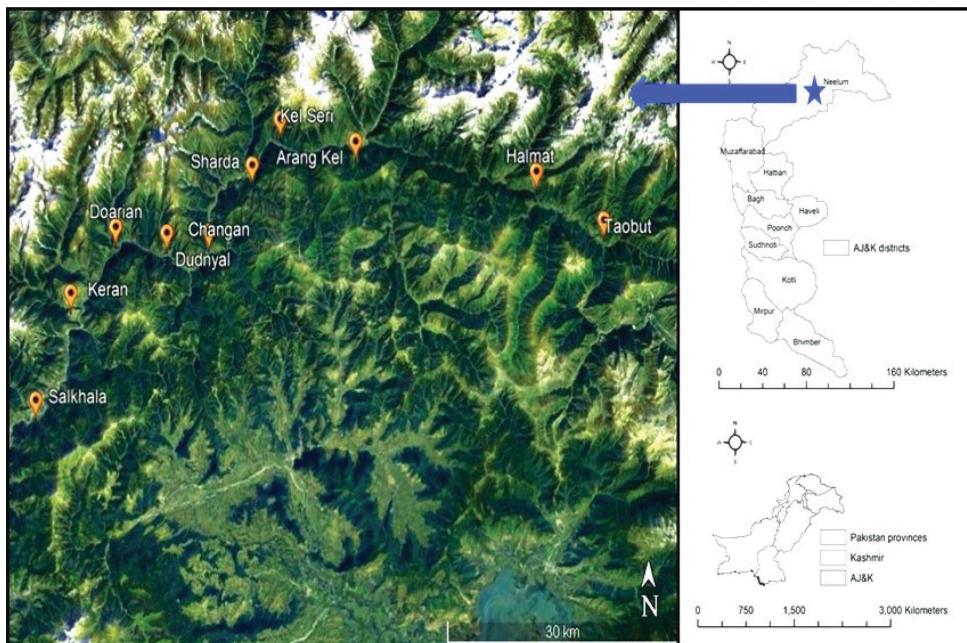
### Sampling sites description

The study area state of Azad Jammu and Kashmir lies between longitude 73–75°E and latitude 33–36°N and comprises an area of 13,297 km<sup>2</sup> (Khan 2008). Neelum valley is the largest district of AJK having an area of 13,297 km<sup>2</sup>. The climate is temperate (Average -2.0 °C) with very cold winter, moderate summers (Average temp. 37.0 °C) and average rainfall 165 cm, annually. This area is mostly hilly, and covered with dense forests of conifers. Soil is loamy to sandy loam, helping in better growth of forests. The main vegetation of the area includes *Pinus wallichiana* A.B.Jacks., *Abies pindrow* (Royle ex D.Don) Royle, *Cedrus deodara* (Roxb.) G.Don, *Aesculus indica* (Wall. ex Cambess.) Hook. and shrubs like *Viburnum grandiflorum* Wall. ex DC., *Indigofera heterantha* Wall. ex Brandis and *Betula jacquemontii* Spach.

### Collection and micro-morphological characterization

Fresh basidiomata were collected from selected sites in Neelum valley, AJK, Pakistan, during 2019–2020 through consecutive field surveys (Fig. 1). Photography with proper tags and field notes of collected specimens were made during field visits in fresh conditions. Specimens were air-dried dried and preserved in polythene zipper bags for further studies. All studied specimens were deposited in the LAH Herbarium of Institute of Botany, University of the Punjab, Lahore.

Slides were mounted in 5% KOH (w/v) and 1% Congo red to study anatomical features, examined by using a light microscope (MX4300H, Japan) at 100× magnification: size and shape of basidiospores, basidia, cystidia and other structures. For



**Figure 1.** Map of collection site Neelum valley, AJK, Pakistan.

basidiospores and other structures at least 50 measurements were made and fungal specimen names with authorities were retrieved from Index Fungorum (<http://www.indexfungorum.org>).

### DNA extraction, sequencing and phylogenetic analysis

DNA was extracted from dried samples using a modified CTAB method as proposed by Bruns (1995). Amplification of the ITS nrDNA region was carried out using universal primer sequences (ITS1/ITS4), ITS1F (5'-CTT GGT CAT TTA GAG GAA GTA A-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (White et al. 1990; Gardes and Bruns 1993). PCR products were sent to TsingKe, China for sequencing. The newly generated sequence was deposited in GenBank under accession number ON209680.

Consensus sequence was generated using the molecular tool BioEdit ver. 7.2.5 (Hall 1999). Most similar sequences were retrieved from NCBI using BLAST (<https://www.ncbi.nlm.nih.gov/guide/>) for the construction of a phylogenetic tree. Online MUSCLE tool (<https://www.ebi.ac.uk/Tools/msa/muscle>) was used for alignment of all the sequences. Maximum likelihood analysis was performed on CIPRES (Miller et al. 2010) using the RAxML-HPC2 with 1000 bootstrap replicates (Stamatakis 2014). Figtree ver. 1.4.2. software was used for phylogenetic tree visualization and exported to Adobe illustrator for final editing.

## Results

### Taxonomy

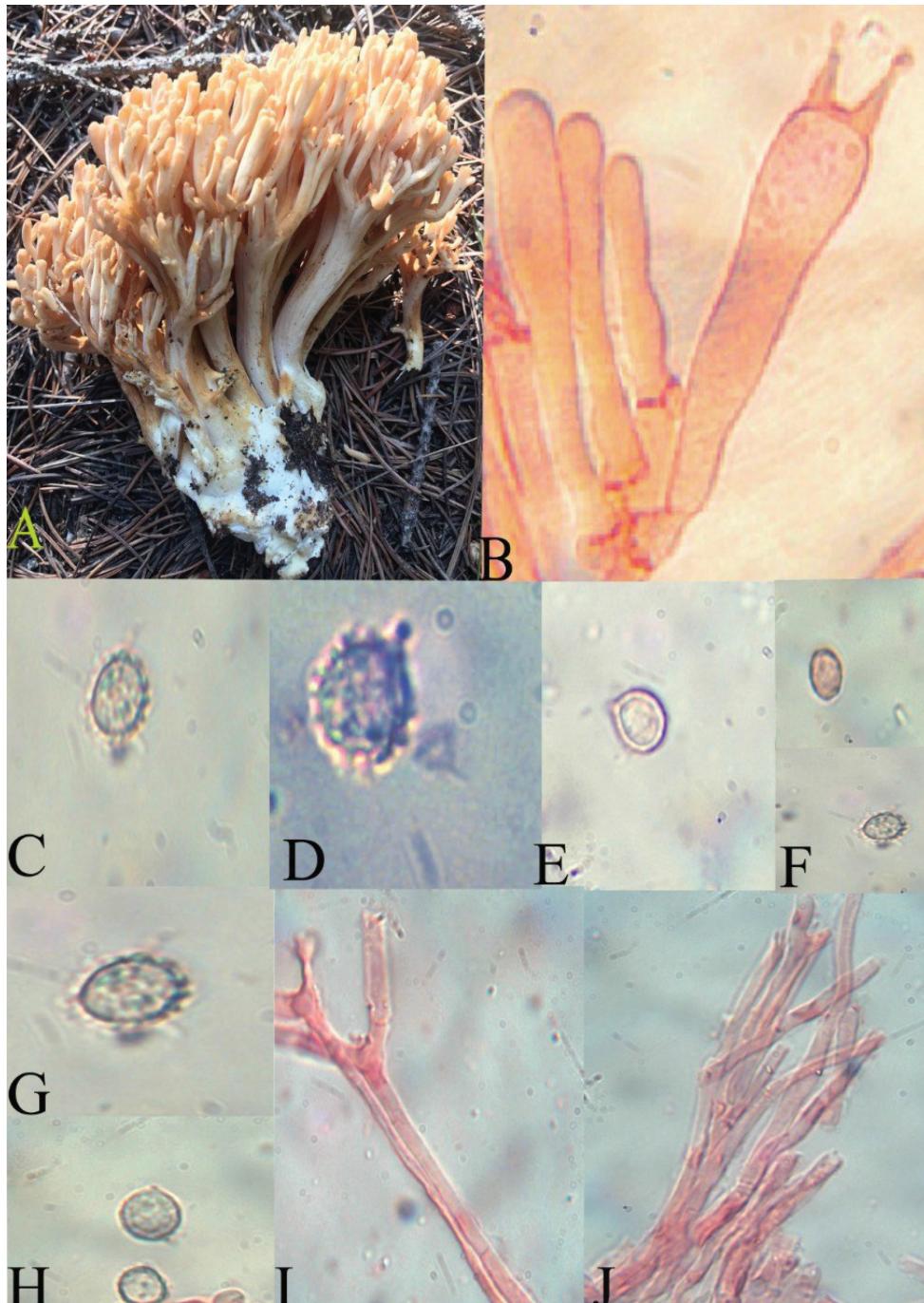
**Ramaria barenthalensis** Franchi & M.Marchetti, Riv. Micol. 61: 199 (2019)

Figure 2

**Description.** **Basidiomata** 5–12 cm in height, 4–9 cm wide with a well-developed branched mycelium. **Branches** vertically oriented, furcated, elongated to flattened, smooth, light brown when young and dark brown at maturity. **Base** variable, sometimes reduced, and well-developed, whitish to brown. **Flesh** whitish to light brown and soft. **Odor** is not distinctive and pleasant. **Basidiospores** [50/5/2] 6.5–9 × 3–4.6 µm, elliptical to ovoid, roughened. **Basidia** 45–59 × 7.5–9 µm, sterigmate. **Cheilocystidia** 46–62 × 7–8.5 µm, elongated. Clamp-connections are present. Trama hyphae are thick-walled with clamps. Hyphae in basal tomentum are smooth, with a tapering base (Fig. 2). Comparisons in morpho-anatomical characteristics with other described species of *Ramaria* are also given (Table 2).

**Table 1.** ITS dataset (Fig. 3) used in the current study on genus *Ramaria*. Our newly generated sequence shown in boldface.

Species	Strain	Location	Substrate	GenBank Accession Number
<i>Ramaria</i> sp.	SD125.4	USA	Fir forest	DQ365646
<i>Ramaria</i> sp.	OSC 81622	USA	Fir forest	EU652343
<i>R. flavobrunneosens</i>	AY102864	USA	Fir forest	AY102864
<i>R. barenthalensis</i>	AMB17386	Spain	mixed forest	MK493039
<i>R. barenthalensis</i>	AMB17381	Spain	<i>Abies</i> and <i>Picea</i> forest	MK493038
<b><i>R. barenthalensis</i></b>	<b>T40</b>	<b>Kashmir</b>	<b>Dead hardwood of conifers</b>	<b>ON209680</b>
<i>R. edwinii</i>	ALV11173	Spain	<i>Abies</i> and <i>Picea</i>	MK493034
<i>Ramaria</i> sp.	RAM2	Srinagar J&K	<i>Abies</i> forest	MH930937
<i>Ramaria</i> sp.	OSC 73311	USA	<i>Abies</i> and <i>Picea</i> mix wood forest	KP658154
<i>Ramaria</i> sp.	OSC 144044	USA	<i>Abies</i> and <i>Picea</i> mix wood forest	KF206335
<i>Ramaria</i> sp.	MHM312	Mexico	NA	EU569259
<i>Ramaria</i> sp.	NVE 367	Amazonia	NA	KF937356
<i>Ramaria</i> sp.	OSC 115803	USA	NA	KP658139
<i>Ramaria</i> sp.	DB215-08/5	USA	NA	KT968608
<i>R. flava</i>	AMB n. 17484	Italy	<i>Abies</i> and <i>Picea</i>	MK581224
<i>R. flava</i>	AMB 17393	Italy	<i>Abies</i> and <i>Picea</i>	MK493035
<i>R. flava</i>	AMB n. 17481	Italy	Mix forest type	MK557953
<i>R. flava</i>	MA-Fungi 48072	Spain	<i>Abies</i> and <i>Picea</i>	AJ408367
<i>R. flava</i>	ZT Myc 55613	Italy	NA	KY626146
<i>R. pseudoflava</i>	AMB 17390	Italy	NA	MK493045
<i>R. pseudoflava</i>	AMB 17392	Italy	NA	MK493046
<i>R. pseudoflava</i>	AMB 17391	Italy	NA	MK493044
<i>R. flava</i>	MA-Fungi 48061	Spain	<i>Abies</i> and <i>Picea</i>	AJ408364
<i>Ramaria</i> sp.	OSC 61837	USA	Mix forest	DQ365602
<i>Ramaria</i> sp.	OSC 134657	USA	Mix forest	JX310403
<i>R. magnipes</i>	WTU-F-063057	USA	Mix forest	MK169351
<i>R. magnipes</i>	WTU 063057	Italy	Fir forest	MK493040
<i>R. gracilis</i>	OSC 134659	USA	<i>Abies</i> and <i>Picea</i> mix wood forest	JX310399
<i>R. gracilis</i>	OSC 112168	USA	<i>Abies</i> and <i>Picea</i> mix wood forest	KY354745
<i>Hysterangium crassirahis</i>	OSC 4860	USA	<i>Abies</i> and <i>Picea</i> mix wood forest	MN809540



**Figure 2.** **A** Basidiomata of *Ramaria barenthalensis* (TS-40) **B** basidia and basidiole **C-H** basidiospores **I** clamp connections **J** trama hyphae. Scale bars: 3.6 cm (**A**); 12  $\mu\text{m}$  (**B**); 6.7  $\mu\text{m}$  (**C-H**); 13  $\mu\text{m}$  (**I**); 12.8  $\mu\text{m}$  (**J**).

**Table 2.** Morphoanatomical comparisons of different *Ramaria* species with *R. barenthalensis*.

	<i>Ramaria abietina</i>	<i>R. subaustraliaca</i>	<i>R. stricta</i>	<i>R. flava</i>	<i>R. flaccida</i>	<i>R. flavescens</i>	<i>R. apiculata</i>	<i>R. formosa</i>	<i>R. barenthalensis</i>
<b>Basidiomata</b>	Basidiomata up to 12 cm high, 4–8 cm in width, erect, branched, tips pointed with glabrous surface.	Basidiomata have densely crowded branches and sometimes with a fused base. Orange to brownish.	Mature fruit bodies branched, 4–12 cm high, 4–8 cm wide. Branches with tapered and pointed ends, mostly erect and slender.	Basidiomata 10.5–20 cm in length with a width of 7–15 cm having numerous densely crowded branches.	Mycelium, multi branched, slender and less or more weak.	Basidiomata branched and large up to 18.8 cm; clamps are present.	Basidiomata much branched 4–9 cm tall, fan shaped, rounded, tips.	Basidiomata up to 20 cm high and multi branched mycelium.	5–12 cm high, 4–9 cm wide with a well-developed branched mycelium. Vertically oriented, furcate, elongated to flattened, smooth.
<b>Color</b>	Light pink to pinkish buff, pale yellow to light brown tips, darkened when bruised.	A persistent light brown to light yellow color from early stage of development to final stage of maturity.	Surface glabrous, pinkish-buff, tips of branches pale-yellow, buff brown when bruised.	Brown to light yellow more dark to ochraceous with age.	tips of branches tan to gold, no color change on bruising.	Branches yellowish-brown when young, pale to pinkish brown at maturity.	Branches Brown-light to yellow brown.	Branches Light brown. The flesh is white to yellowish.	Light brown when young and dark brown at maturity. Clamp connections present.
<b>odor</b>	Slightly aromatic and bitter			Aromatic	Pleasant	Pleasant	Not known	Pleasant	Distinctive
<b>Sipe</b>	Sometime less developed and reduced. Up to 1–2 cm in length, 0.5–1.5 cm wide, color changes to light-brown on bruising.	1–2.5 cm in length, 0.5–1.5 cm in width, sometimes absent. When present, pallid and tomentose with a tough context, light-brown on bruising.	Reduced and sometime developed up to 2 cm, flesh whitish to light brown.	Sipe, 50–80 mm long with a width of 40–50 mm. Base of sipe, whitish, pale-yellow to reddish brown on bruising.	Medium sipe 1.5–2 cm high and whitish.	Sipe large, 2.5–6.6 cm with a width of 1.5–2 cm, whitish.	Stipe large, 1.5–4 cm with a width of 1–1.5 cm, whitish.	Stipe large, 2–4.5 cm with a width of 1.5–18 cm, light brown-whitish.	Stipe variable in length and width, sometimes reduced to well-developed. Flesh whitish to brown.
<b>Spores</b>	6.5–9.5 µm in diameter, ellipsoidal, slightly warted.	Spores 7–8.5 µm, ellipsoidal.	Elliptical, rough, rusty-yellowish 7.5–10.5 × 3.5–4.5 µm, clamp connections present.	pale elliptical and roughened, 10.5–17.5 × 4–6.5 µm.	Pale elliptical, less to more roughened, 9–14.5 × 3–4.5 µm.	Thick walled, minute and rough outer surface, cyanophilous warts, ellipsoid, tips squared, 7–10 × 3.5–5 µm.	less to more roughened, with cyanophilous warts, ellipsoidal with a squared tip: 7–10 × 4–5 µm.	slightly roughened, with cyanophilous warts ellipsoidal and squared tip; 7.5–9 × 3–3.5 µm.	6.5–9 × 3–4.6 µm, elliptical to ovoid, roughened.
<b>Edibility</b>	Edible	Edible	Edible on choice	Edible	Edible on choice sometimes laxative	Edible	Not known	Not known	Edible when young
<b>Taste</b>	Pleasant	Taste mild to pleasant	bitter	Taste mild to pleasant	Pleasant	Pleasant	Taste mild to pleasant	Bitter/mild	Pleasant

	<i>Ramaria abietina</i>	<i>R. subaurantiaca</i>	<i>R. stritula</i>	<i>R. flava</i>	<i>R. flaccida</i>	<i>R. flavesans</i>	<i>R. apiculata</i>	<i>R. formosa</i>	<i>R. barenthalensis</i>
<b>Ecology</b>	Solitary and gregarious, on hardwood and tree trunks of conifers.	Solitary and in small groups on hardwood and logs, soil and decomposed organic matter.	Growing in groups, under conifers and mixed vegetation, on dead wood and on ground.	Solitary and gregarious. Fruiting season varies from late summer to autumn.	Grow on the ground. In mixed forest and hardwoods. Late autumn.	Solitary to gregarious, on decomposed hardwood in association with rotten conifer needles.	Gregarious, on soil with decomposed organic matter and conifer needles.	Gregarious, on soil with decomposed organic matter and conifer needles.	Gregarious and sometime alone, on fallen tree turns of coniferous and other mixed vegetation during late summer.
<b>References</b>	(Petersen 1969; Sultan et al. 1997)	(Petersen 1989; Ahmad et al. 1997)	(Petersen 1969; Petersen and Olexia 1967; Petersen 1969; Ahmad et al. 1997)	(Ahmad et al. 1997)	(Ahmad et al. 1997)	(Ahmad et al. 1997)	(Ahmad et al. 1997)	(Kuo 2005)	Present study
Basidiomata of different species of <i>Ramaria</i>									
	<i>Ramaria abietina</i>	<i>R. subaurantiaca</i>	<i>R. stritula</i>	<i>R. flava</i>	<i>R. flaccida</i>	<i>R. flavesans</i>	<i>R. apiculata</i>	<i>R. formosa</i>	<i>R. barenthalensis</i>

**Ecology.** In mixed coniferous forest, associated with *Abies pindrow* and *Betula jacquemontii*, solitary or scattered and gregarious, common in the temperate zone of AJK, Pakistan.

**Material examined.** PAKISTAN, Neelum valley (Azad Jammu and Kashmir) collected from a mixed coniferous forest on decayed hardwood, in late summers, 1524 m a.s.l, August 2020, Tariq Saiff Ullah, TS-40.

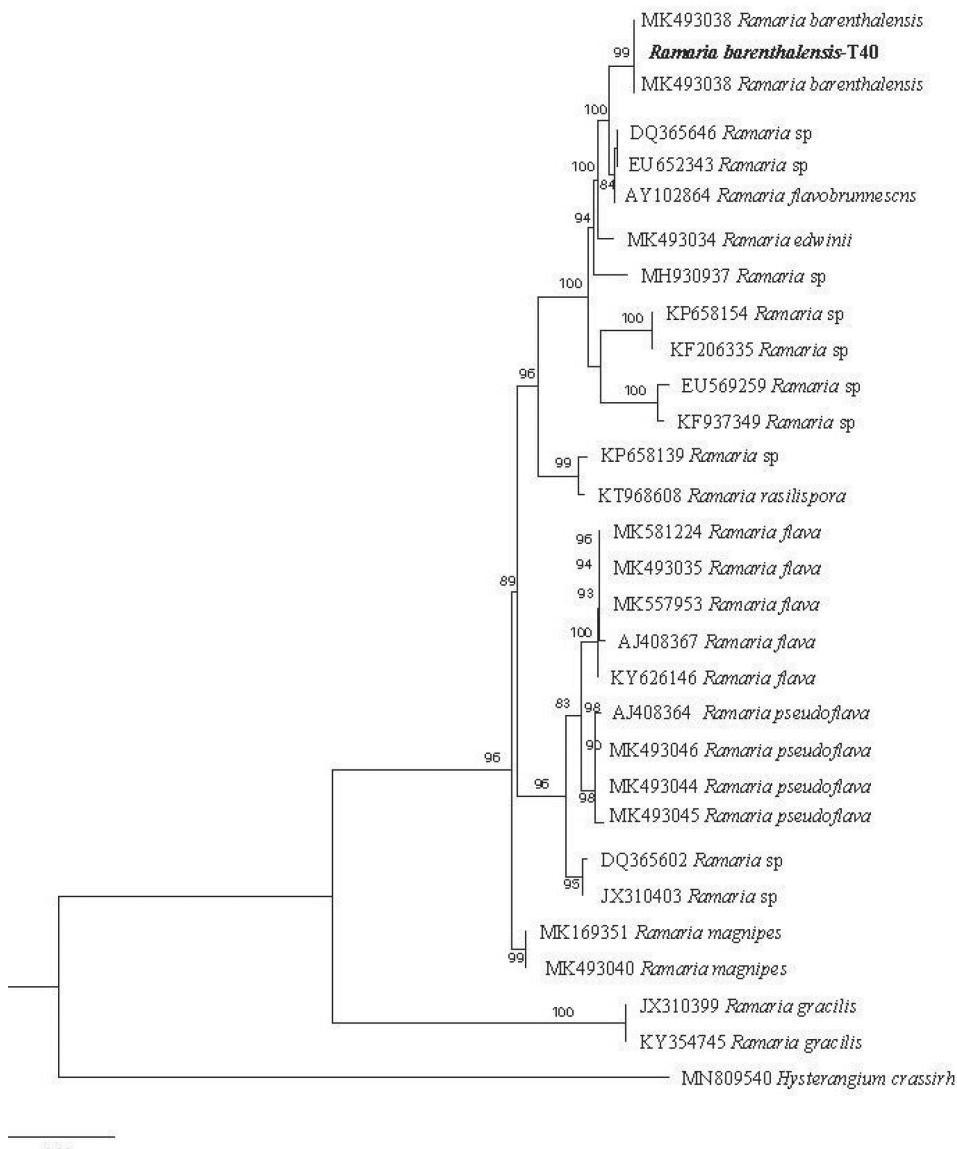
## Phylogeny

The newly generated ITS sequence of Pakistani *Ramaria* (T40) specimen, yielded a fragment of 702 base pairs. In initial BLAST search results, our sequence showed 99.33% similarity with *R. barenthalensis* MK493039, with 98% query cover, and 0.0 E value. The phylogram comprised 30 sequences and *Hysterangium crassirh* Zeller & C.W. Dodge was chosen as an out-group taxon. The final aligned dataset consist of 421 conserved sites, 256 were variable, 167 were informative and 86 were singletons. Our sequence *Ramaria barenthalensis* (T40) grouped with *R. barenthalensis* (MK493038 and MK493029) with strong bootstrap value (Fig. 3).

## Discussion

In present study, a specimen of genus *Ramaria* was studied on the basis of morpho-anatomical and molecular approach. Our study is consistent with the original specimen description given by Franchi and Marchetti (2019). In phylogram, our specimen grouped in the same clade with *R. barenthalensis* (MK493038 and MK493039) with a strong bootstrap value.

The analyzed sample of *R. barenthalensis* (T-40) has similar morphological features, but with slight differences in basidiomata to other previously described species of *Ramaria* (Agerer et al. 1996). Few species of genus *Ramaria*: *R. apiculata*, *R. flava* (Schaeff.) Quél., *R. flavescens* (Schaeff.) R.H.Petersen, *R. flavescensoides*, *R. formosa*, *R. pallida* (Schaeff.) Ricken, and *R. subaurantiaca* Corner, have been reported from the regions of Jammu and Kashmir, in India and Pakistan based on morphological features (Ahmad et al. 1997; Nasim et al. 2008; Sharma et al. 2015; Hanif et al. 2019). Morpho-anatomical features are insufficient to identify a mushroom species. Fifteen species of *Ramaria* were identified and characterized by Martin et al. (2020) using combined morphological and molecular data. In conclusion, *R. barenthalensis* is recorded as a new coralloid fungus to the state of Jammu and Kashmir, Pakistan based on morpho-anatomical and molecular characterization. This fungal species could be used as a source of food and medicines after biochemical characterization.



**Figure 3.** Molecular Phylogenetic tree of *Ramaria barenthalensis* (ON209680) by maximum likelihood method based on ITS sequences. The sequence generated in this study represents in bold.

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